

# Interactions of Surfactant Protein D with Bacterial Lipopolysaccharides.

## Surfactant Protein D is an *Escherichia Coli*-Binding Protein in Bronchoalveolar Lavage

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### Abstract

Surfactant protein D (SP-D) is a collagenous glycoprotein that is secreted into the pulmonary airspaces by alveolar type II and nonciliated bronchiolar cells. SP-D exhibits  $\text{Ca}^{++}$ -dependent carbohydrate binding in vitro and is structurally related to the collagenous C-type lectins, including serum conglutinin, serum mannose-binding proteins, and surfactant protein A. Preliminary studies showed calcium- and saccharide-dependent binding of fluorescein-conjugated or radioiodinated SP-D to a variety of microorganisms, including Gram-negative bacteria and fungi. A laboratory strain of *Escherichia coli* (Y1088) was chosen to further examine the mechanism(s) of binding. Binding of SP-D to Y1088 was time dependent, saturable, and inhibited by cold SP-D or competing saccharides; Scatchard analysis gave a  $K_d$  of  $2 \times 10^{-11}$  M. At higher concentrations, SP-D also caused  $\text{Ca}^{++}$ -dependent agglutination of Y1088 that was inhibited by  $\alpha$ -glucosyl-containing saccharides, antisera to the carbohydrate-binding domain of SP-D, or Y1088 LPS. Lectin blots showed specific binding of  $^{125}\text{I}$ -SP-D to Y1088 LPS, as well as LPS from other several strains of enteric Gram-negative bacteria. Immunogold studies demonstrated strong and uniform surface labeling of the bacteria. Rat and human bronchoalveolar lavage (BAL) caused  $\text{Ca}^{++}$ -dependent agglutination of *E. coli* that was dose dependent and inhibited by competing saccharides or anti-SP-D. SP-D was selectively and efficiently adsorbed from rat BAL by incubation with *E. coli*, and incubation of *E. coli* with radiolabeled rat type II cell medium revealed that SP-D is the major *E. coli*-binding protein secreted by freshly isolated cells in culture. We suggest that SP-D plays important roles in the lung's defense against Gram-negative bacteria. (*J. Clin. Invest.* 1992. 90:97-106.) Key words: lung • collagen • lectin • agglutinin • Gram-negative bacteria • lipopolysaccharides

### Introduction

Surfactant protein D (SP-D)<sup>1</sup> is a collagenous glycoprotein that is synthesized and secreted by lung epithelial cells (1-3). Structural studies of rat and human SP-D have shown that the protein is assembled as multimers of disulfide-bonded trimers com-

posed of 12 apparently identical 43-kD subunits. Each chain consists of a short amino-terminal noncollagenous domain, a relatively long uninterrupted and glycosylated collagenous domain, and a nonglycosylated carboxy-terminal carbohydrate-binding domain (4). The carboxy-terminal domain reveals high sequence homology with the mammalian C-type lectins, including bovine serum conglutinin, the serum mannose-binding proteins (MBPs), and pulmonary surfactant protein A (SP-A) (4-6).

The possible functions of the C-type lectins are diverse; however, several members of the family, particularly the collagenous lectins, have been implicated in antimicrobial host defense. For example, conglutinin, a bovine serum lectin, binds to C3bi and can agglutinate complement-coated particles, including complement-fixed *Escherichia coli* (7-9). Serum levels of conglutinin have been shown to decrease in cattle in association with infection (10); and conglutinin protects mice in experimental challenges with *Salmonella typhimurium* and *E. coli* (11). Human serum MBP is an acute-phase reactant and opsonin (12, 13) that has been shown to activate complement via the classical pathway (14). In addition, MBP agglutinates *E. coli* and elicits bactericidal activity via a C4-dependent mechanism (15). SP-A, a lung-specific C-type lectin, is believed to play important roles in the structural reorganization and metabolism of surfactant. However, recent studies have shown that preincubation of macrophages with SP-A enhances the surface phagocytosis of *Staphylococcus aureus* (16) and that substrates coated with SP-A enhance FcR- and CR1-mediated phagocytosis (17).

Continuous exposure of the respiratory tract to inhaled bacteria, fungi, and viruses necessitates efficient mechanisms of antimicrobial defense. In addition to physical mechanisms (e.g., mucociliary clearance), there are two or more levels of defense (18). The first involves the action of locally synthesized proteins such as secretory immunoglobulins and complement, as well as resident inflammatory cells. When these surveillance mechanisms fail to maintain pulmonary sterility, augmenting humoral and cellular immune mechanisms are called into play. As a first step toward testing our hypothesis that SP-D plays important role(s) in lung host surveillance, we examined the binding of SP-D to various microorganisms and carried out an in-depth study of the binding and agglutination of selected strains of Gram-negative bacteria.

### Methods

**Materials.** All sugars, FITC (isomer I), colloidal gold-labeled protein A (10 nm), lipid A, and several LPS preparations were from Sigma Chemical Co. (St. Louis, MO). Bolton-Hunter reagent was purchased from Amersham Corp. (Arlington Heights, IL).

**Bacteria.** Clinical isolates (see Table I) were kindly provided by Drs. Soman Abraham and Steven Moser (Department of Pathology, Washington University, St. Louis, MO). The bacteria were grown overnight at 37°C in Luria-Bertani broth and washed three times with Tris-buf-

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1. Abbreviations used in this paper: BAL, bronchoalveolar lavage; HBS, HEPES-buffered saline; MBP, mannose-binding protein; SPA and SP-D, surfactant proteins A and D, respectively; TBS, Tris-buffered saline.

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ferred saline, pH 7.4 (TBS) before use. The bacterial concentration in colony-forming units per milliliter was determined from the turbidity at 700 nm by comparing the values with standard curves derived from diluted samples of known number of bacteria. *E. coli* strains Y1088, Y1089, and Y1090 were purchased from American Type Culture Collection (Rockville, MD) (19).

**SP-D and antisera.** Rat SP-D was isolated from the 10,000 g supernatant of lavage from rats with silica-induced lipoproteinosis by sequential affinity chromatography on maltosyl-agarose and gel filtration chromatography (3, 20). Human SP-D was purified from EDTA extracts of the 10,000 g pellet of therapeutic lavage from a patient with alveolar proteinosis (4). The SP-D was pure by the criteria of SDS-PAGE and silver staining. Antisera to SP-D or SP-D-derived peptides were prepared in rabbits and characterized by ELISA and immunoblotting as previously described (2). In particular, the antibodies showed no cross-reaction with SP-A or immunoglobulin by Western blot.

**Type II pneumocyte isolation and metabolic labeling.** Rat type II cells were isolated and metabolically labeled with tritiated L-proline within 2 h after isolation as previously described (1).

**FITC conjugation reactions.** Conjugation of SP-D with FITC was performed using a dialysis technique (21). Briefly, purified SP-D was adjusted to 25  $\mu\text{g}/\text{ml}$  in 25 mM  $\text{Na}_2\text{CO}_3$ - $\text{NaHCO}_3$ , pH 9.0, and dialyzed in the dark against 10 vol of FITC solution (0.1 mg/ml) for 48 h at 4°C. The retentate was dialyzed against Hepes-buffered saline, pH 7.5 (HBS) until no fluorescein was detectable in dialysate. Fluorescein labeling of bacteria was performed by mixing  $\sim 10^9$  bacteria with 5 ml of 25 mM  $\text{Na}_2\text{CO}_3$ - $\text{NaHCO}_3$ , pH 9.0, containing 5 mg FITC. The bacterial suspension was incubated with gentle agitation in the dark for 24 h at 4°C. The bacteria were collected by centrifugation for 5 min at 3000 g and were repeatedly washed with HBS until the supernatant was free of unconjugated fluorescein.

**Fluorometric binding and microscopic agglutination assays.** For initial screening of SP-D binding to bacteria, equal volumes of bacterial suspension ( $\approx 10^7$  cells/ml) and FITC-SP-D (10  $\mu\text{g}/\text{ml}$ ) were mixed on a glass slide and immediately examined by fluorescence microscopy. Control incubations were performed in calcium-free buffer or in the presence of 100 mM maltose. For some experiments, FITC-conjugated bacteria were mixed with unlabeled SP-D and examined for evidence of bacterial agglutination.

**Spectrophotometric assay of bacterial agglutination.** The time course of macroscopic bacterial agglutination was monitored using a spectrophotometric sedimentation assay modified from Ericson et al. (22). Stock solutions of bacteria were prepared in HBS ( $\text{OD}_{700 \text{ nm}} = 1.0$ ). A 3-ml suspension of bacteria was then adjusted to a final calcium concentration of 2 mM, and SP-D was added in the presence or absence of various inhibitors. The change in  $\text{OD}_{700}$  was then monitored continuously for  $\geq 2$  h. Antisera (preadsorbed with Y1088 to remove natural antibody) were preincubated with SP-D or bronchoalveolar lavage (BAL) for 30 min at room temperature before the addition of bacteria.

**Radioiodination of SP-D.**  $^{125}\text{I}$ -SP-D was prepared using  $^{125}\text{I}$ -Bolton Hunter reagent (3). Briefly, 10  $\mu\text{g}$  of SP-D was dissolved in 100  $\mu\text{l}$  0.1 M sodium borate, pH 8.5, and was reacted with Bolton Hunter reagent in the presence of 30 mM maltose and 2 mM  $\text{CaCl}_2$  at 4°C for 2 h. The free iodine and  $^{125}\text{I}$ -SP-D were separated using a Bio-Gel P2 column (0.7  $\times$  11 cm, Bio-Rad Laboratories, Richmond, CA) equilibrated with HBS containing 10 mM EDTA and 0.1% Triton X-100. Active SP-D was concentrated by maltosyl-agarose affinity chromatography after readdition of calcium (3). The specific activity of the protein was  $\sim 1 \times 10^6$  cpm/ $\mu\text{g}$ .

**Binding of  $^{125}\text{I}$ -SP-D to bacteria.** The binding of  $^{125}\text{I}$ -SP-D to *E. coli* was carried out in a 1.5-ml microfuge tube containing a 200- $\mu\text{l}$  suspension of bacteria ( $5 \times 10^7$  cells/ml in HBS, 2 mM  $\text{CaCl}_2$ ) and 100  $\mu\text{l}$   $^{125}\text{I}$ -SP-D (50–500 ng). The mixture was gently agitated on a shaker for various periods of time at room temperature, and the reaction was terminated by centrifugation at 8,000 g for 5 min in a microfuge. The pellets were rapidly resuspended with HBS containing 2 mM  $\text{CaCl}_2$ , transferred to new microfuge tubes, washed twice at 4°C, and counted

in a gamma counter. Control reactions were performed in the presence of 100 mM maltose or a 50-fold excess of unlabeled SP-D.

**Immunoelectron microscopy.** Suspensions of bacteria were directly applied to Formvar-coated nickel grids (Ernest Fullam, Inc., Latham, NY). After blocking nonspecific binding sites with 0.5% (wt/vol) BSA, the bacteria-coated grids were incubated with 1  $\mu\text{g}/\text{ml}$  SP-D and were washed before sequential incubations with rabbit anti-rat SP-D (1:100) and gold-conjugated protein A (1:30). All incubations were for 20 min at room temperature. Washed grids were stained with phosphotungstic acid and viewed by transmission electron microscopy.

**Preparation of outer membranes and LPSs.** Outer membrane fractions of Y1088 were prepared according to Dougan (23). Briefly, cell pellets were incubated with lysozyme to digest the cell wall, and outer membranes were precipitated with Sarkosyl (International Biotechnologies, Inc., New Haven, CT). For the agglutination-inhibition studies, LPS was isolated by the phenol-water method (24). Bacteria were extracted with 45% (vol/vol) aqueous phenol at 65°C, and LPS was obtained from the water layer. However, for most electrophoretic and lectin blotting studies, the proteinase K digestion method was used (25). Overnight cultures of bacteria were washed three times with PBS and bacterial pellets were heated for 10 min at 100°C in 100  $\mu\text{l}$  of SDS-PAGE sample buffer (pH 6.8) containing 50 mM DTT and 10% glycerol. Proteinase K was added to a final concentration of 6  $\mu\text{g}/\text{ml}$  and samples were incubated for 60 min at 60°C before electrophoresis.

**SDS-PAGE and lectin blotting.** Samples of LPS (5  $\mu\text{g}$ ) or outer membrane (10  $\mu\text{g}$ ) were resolved by SDS-PAGE on duplicate 15% acrylamide slab gels containing deionized 4 M urea. One gel was stained by the periodate-silver method (26). Samples on the second gel were electrophoretically transferred to nitrocellulose for lectin blotting. Nonspecific binding sites were blocked by washing with 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5, containing 10 mg/ml BSA and 0.1% (vol/vol) Tween 20 (TBS) for 1 h at room temperature. The membranes were then incubated overnight at room temperature in the above buffer containing  $5 \times 10^5$  cpm/ml  $^{125}\text{I}$ -SP-D, in the absence or presence of competing sugars. After extensive washing with TBS, bound SP-D was visualized by autoradiography.

## Results

**Screening for bacterial binding and agglutination.** Several clinical isolates and laboratory strains of bacteria were tested for their ability to bind to radiiodinated (or FITC-conjugated) rat SP-D in the absence of complement. Three patterns of interaction of SP-D and bacteria were observed under the conditions of assay (Table I). Some bacteria bound to SP-D in the absence of competing saccharides and were macroscopically agglutinated and sedimented under unit gravity in the presence of 0.1–5  $\mu\text{g}/\text{ml}$  of SP-D (Figs. 1 and 2). This group included three laboratory “rough” strains of *E. coli* (i.e., Y1088, Y1089, Y1090). Other bacteria bound to SP-D but showed only microscopic agglutination under the conditions of assay. Some bacteria, such as *S. aureus*, showed no detectable binding or agglutination. Y1088, an agglutinating strain of *E. coli*, was chosen as a model for in-depth studies of binding and agglutination.

**Binding of  $^{125}\text{I}$ -SP-D to *E. coli* Y1088.** The time course, saturability, specificity, and reversibility of binding were examined by incubating subagglutinating concentrations of  $^{125}\text{I}$ -SP-D with *E. coli*. Binding to *E. coli* was rapid and efficient;  $> 50\%$  of the maximal binding occurred in the first 5 min and maximal binding ( $> 95\%$  of added  $^{125}\text{I}$ -SP-D) was achieved by 20 min (Fig. 3). When increasing amounts of  $^{125}\text{I}$ -SP-D were added to a fixed number of bacteria, there was a dose-dependent increase in binding (Fig. 4, inset). No precipitation of  $^{125}\text{I}$ -SP-D was observed in the absence of *E. coli* under the conditions of assay.

Table I. Binding and Bacterial Agglutination

Bacterial strains	FITC-SP-D binding		Bacterial agglutination	
	No maltose	+ Maltose	FITC-SP-D	Spectrophotometer
<b>Laboratory strains</b>				
<i>E. coli</i> (Y1088)	+	-	+	+
<i>E. coli</i> (Y1089)	+	-	+	+
<i>E. coli</i> (Y1090)	+	-	+	+
<b>Clinical strains</b>				
<i>E. coli</i> (C15)	+	-	+	-
<i>E. coli</i> (44785)	+	-	+	-
<i>E. coli</i> (97379)	+	-	+	-
<i>E. aerogenes</i>	+	-	+	-
<i>Salmonella paratyphi</i>	+	-	+	-
<i>Klebsiella pneumoniae</i>	+	-	+	-
<i>Staphylococcus aureus</i>	-	-	-	-

Scatchard analysis gave a straight line, consistent with a single class of high affinity binding sites (Fig. 4). The  $K_d$  for the binding of SP-D to *E. coli* was  $2 \times 10^{-11}$  M, with  $> 1,700$  predicted binding sites/cell, assuming that native SP-D is as-

sembled as a dodecamer (516 kD). Binding to *E. coli* was inhibited by the addition of a 100-fold excess of unlabeled SP-D but not BSA (data not shown).

To assess the carbohydrate specificity of SP-D binding, assays were performed in the presence of increasing concentrations of various sugars (Fig. 5). Glucose and maltose were the most effective inhibitors of SP-D binding, fucose and mannose were less effective, while *N*-acetylglucosamine did not inhibit. The saccharide inhibition profile was virtually identical to that observed for SP-D binding to maltosyl-BSA (3) but different from that of previously characterized *E. coli* lectins (see below).

**Electron microscopy.** Transmission electron microscopic and immunogold studies were performed to characterize the surface structure of *E. coli* Y1088 and to visualize the site(s) of SP-D binding. There was dense and relatively uniform gold labeling of the bacterial cell surface (Fig. 6). No binding was observed in control incubations performed without SP-D or in the presence of maltose.

**Bacterial agglutination.** Macroscopic aggregation and precipitation were observed within 45–60 min after the addition of SP-D to suspensions of Y1088 (Fig. 2). Aggregation was  $Ca^{++}$ -dependent and completely inhibited by the presence of 100 mM maltose. Although some strains of Gram-negative bacteria can spontaneously agglutinate, there was no significant agglutination in the absence of added SP-D.

A spectrophotometric assay was used to further examine the aggregation of *E. coli* Y1088 (Fig. 7 A). Incubation of a

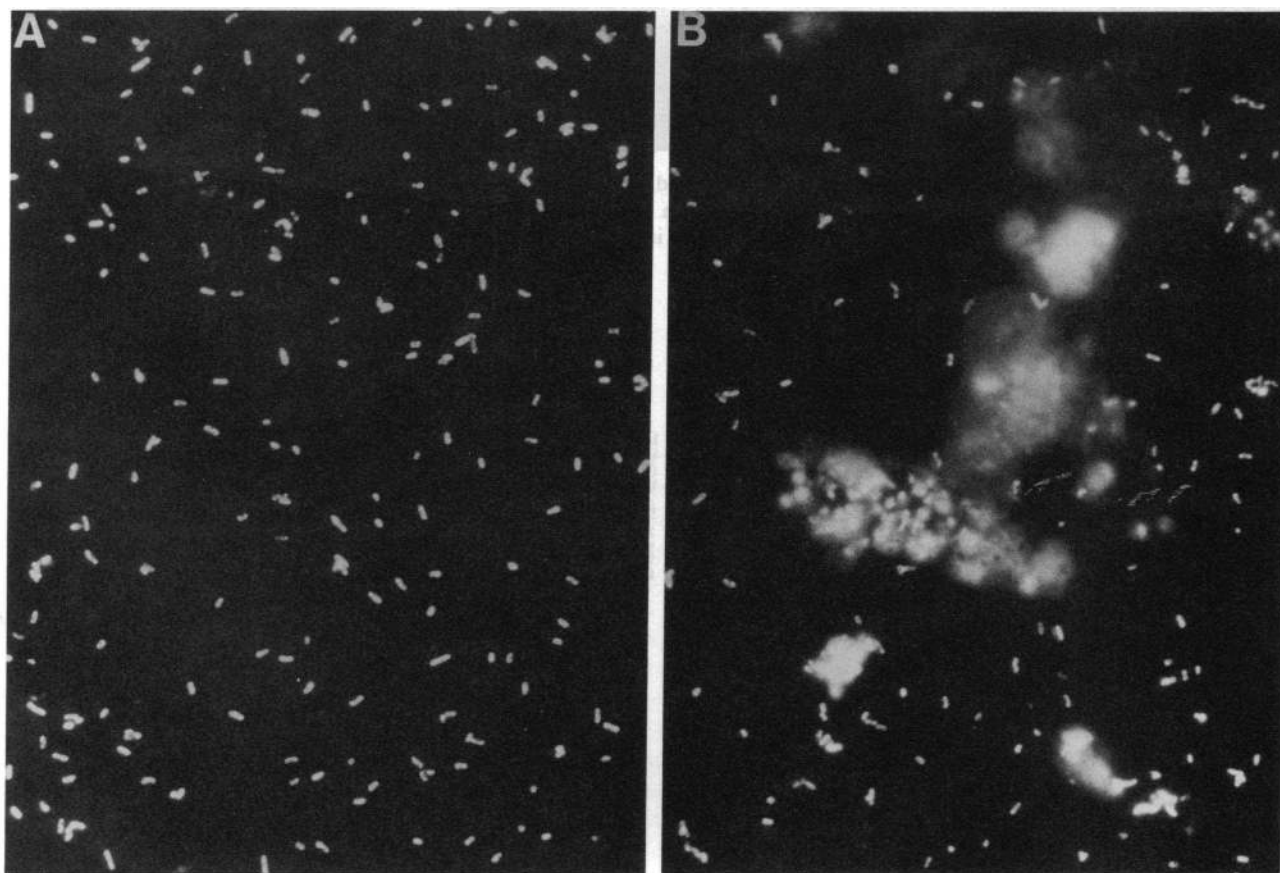


Figure 1. Fluorescence micrograph of FITC-labeled *E. coli* Y1088 after incubation with SP-D. (A) FITC-labeled *E. coli* Y1088 without treatment of SP-D. (B) FITC-labeled *E. coli* Y1088 ( $1 \times 10^7$  cells/ml) were incubated with an equal volume of SP-D ( $10 \mu\text{g/ml}$ ) in TBS containing 1 mM  $CaCl_2$  for 1 h at room temperature.

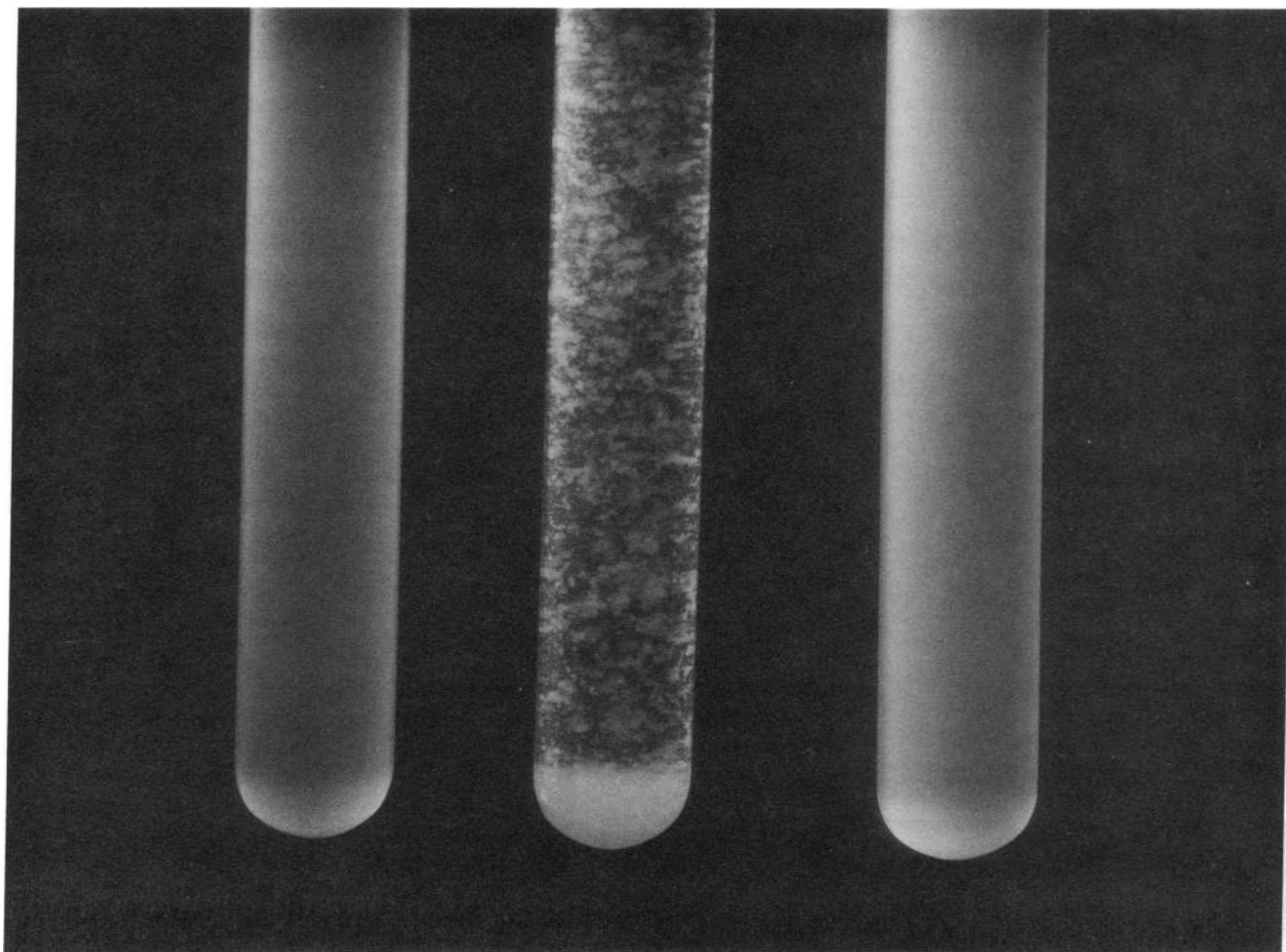


Figure 2. Macroscopic aggregation of *E. coli* by SP-D. (Left tube) Untreated suspension of *E. coli* Y1088. (Middle tube) Bacteria incubated for 45 min with 1  $\mu\text{g/ml}$  rat SP-D. (Right tube) Bacteria incubated for 45 min with 1  $\mu\text{g/ml}$  SP-D in the presence of 100 mM maltose. Similar inhibition of aggregation was observed when incubations with SP-D were performed in the absence of added calcium or in the presence of 10 mM EDTA.

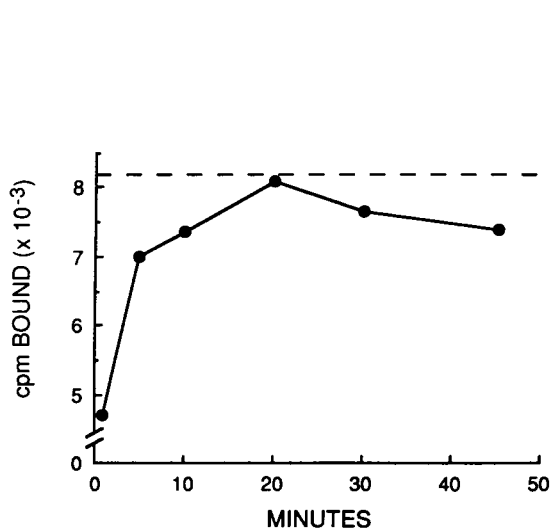


Figure 3. Time-dependent binding of SP-D to *E. coli*. 200- $\mu\text{l}$  aliquots of *E. coli* Y1088 suspension ( $5 \times 10^7$  cells/ml) were incubated at room temperature for various times with 100  $\mu\text{l}$  of  $^{125}\text{I}$ -SP-D (8,200 cpm or  $\approx 8$  ng SP-D). Bacterial pellets were collected by centrifugation, washed, and the bound radioactivity was quantified with a gamma counter.

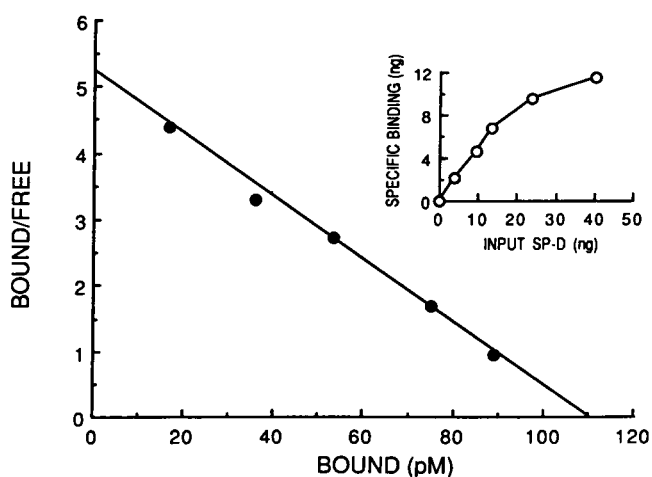
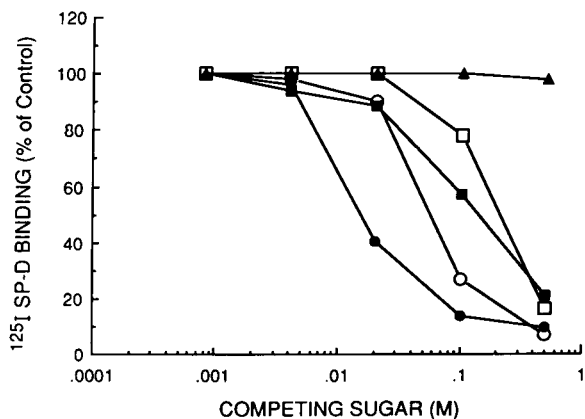


Figure 4. Scatchard analysis of SP-D binding to *E. coli*. (Inset) Concentration-dependent binding of SP-D to *E. coli*. Bacteria ( $1 \times 10^8$ /ml) were incubated for 1 h at room temperature with increasing concentrations of  $^{125}\text{I}$ -SP-D in a total volume of 500  $\mu\text{l}$ . Bacterial pellets were collected, washed, and the bound radioactivity was quantified in a gamma counter. Results were corrected for nonspecific binding by subtracting radioactivity bound in the presence of excess cold SP-D. Approximately 60–70% of binding was inhibited by cold SP-D.



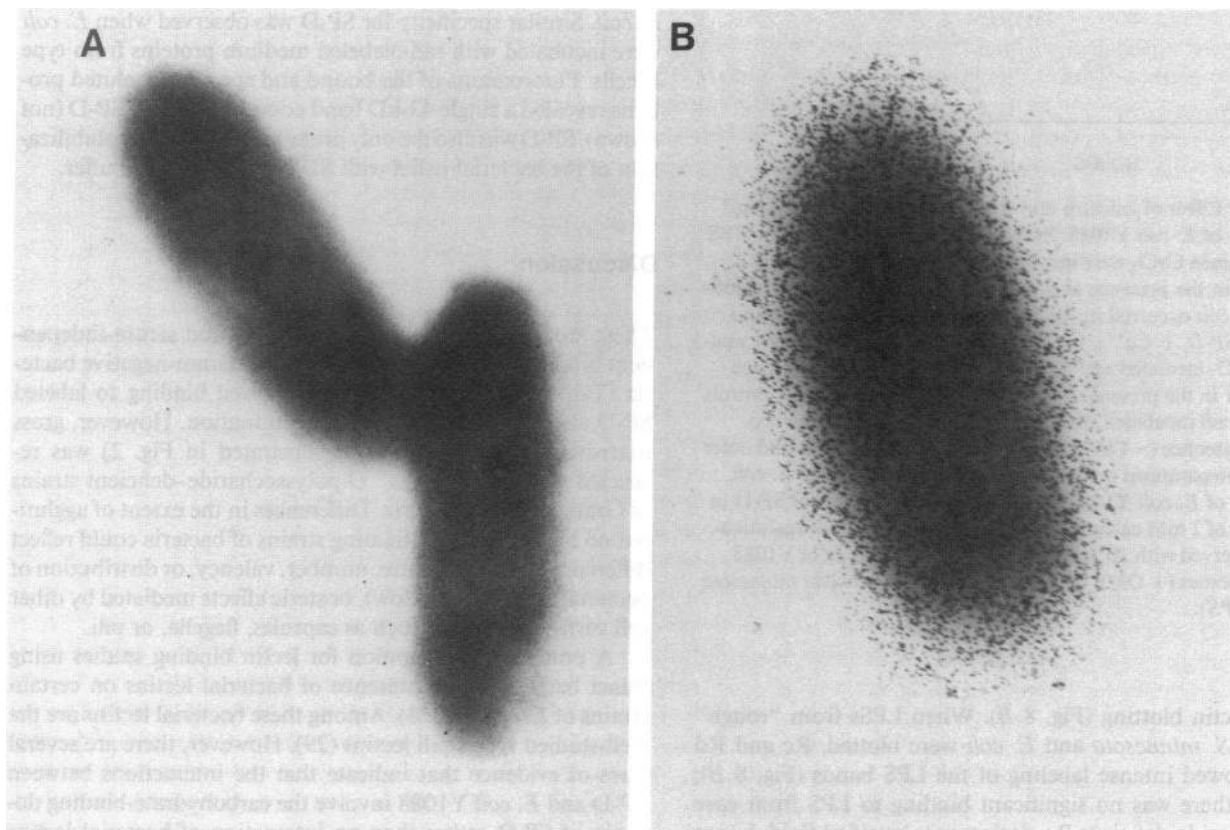
**Figure 5.** Inhibition of SP-D binding to *E. coli* Y1088 by selected sugars. *E. coli* Y1088 ( $1 \times 10^7$ /ml) were incubated with  $^{125}\text{I}$ -SP-D ( $\approx 3000$  cpm) and competing sugars at various concentrations for 1 h at room temperature. The bacterial pellets were collected by centrifugation, washed, and the radioactivity was counted in a gamma counter. GlcNAc ( $\blacktriangle$ ), mannose ( $\blacksquare$ ), fucose ( $\square$ ), glucose ( $\circ$ ), maltose ( $\bullet$ ).

bacterial suspension in the presence of rat (or human) SP-D and 2 mM  $\text{CaCl}_2$  showed a stable or transiently increased  $\text{OD}_{700\text{ nm}}$  (attributed to microaggregation), followed by a gradual decrease, with a more rapid decrease (reflecting sedimentation) after 30–60 min of incubation. No visible agglutination was seen in the initial phases, whereas coarse aggregates were

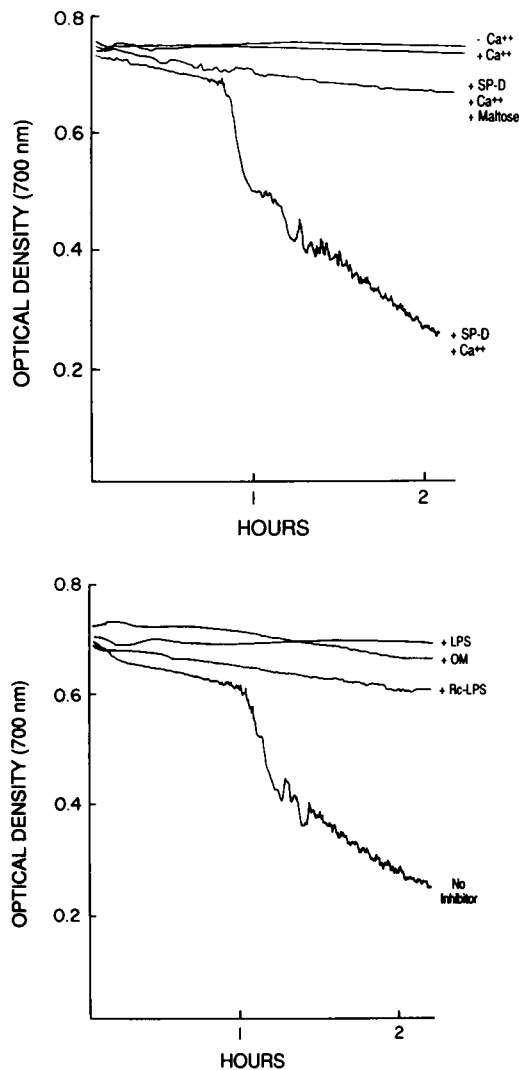
visible to the naked eye during the period of rapid decrease. The aggregation of *E. coli* by SP-D was dose dependent, and increasing the amount of SP-D reduced the latent period before the rapid decrease in optical density (data not shown). No aggregation was observed in the absence of SP-D or calcium (not shown) or in the presence of 50 mM glucose or maltose (Fig. 7 A). Aggregation was also inhibited by microgram amounts of outer membrane or purified LPS isolated from Y1088 (Fig. 7 B). Notably, agglutination was also inhibited by LPS from *Salmonella minnesota* R5, an Rc strain with an incomplete core polysaccharide containing heptose and terminal glucose (see below). Commercial preparations of LPS from a smooth strains of *E. coli* showed only partial inhibition in concentrations as high 3 mg/ml (not shown).

**Lectin blots of bacterial LPSs.** Y1088 LPS showed broad and heterogeneous bands (average molecular mass of  $\sim 10$  kD) similar in size and appearance to the major bands of commercial preparations of LPS from several “rough” (Rc and Rd) strains of Gram-negative bacteria deficient in terminal O-polysaccharides (Fig. 8 A). Lectin blotting demonstrated specific binding of  $^{125}\text{I}$ -SP-D to purified Y1088 LPS and to LPSs associated with Y1088 outer membranes (Fig. 8 A). No binding was observed when incubations were performed in the presence of 100 mM maltose or EDTA (data not shown). In addition, there was no detectable binding of SP-D to components in other subcellular fractions.

To further define the mechanism(s) of binding, LPSs from selected mutant strains and other types of bacteria were exam-



**Figure 6.** Immunogold localization of SP-D binding sites on *E. coli* Y1088. Cells and reagents were prepared as described in Methods. (A) Control cells incubated in the absence of rat SP-D. (B) Cells incubated with SP-D followed by rabbit anti-rat SP-D and colloidal gold-protein A. Final magnification,  $\times 37,000$ .



**Figure 7.** (A) Effect of calcium and maltose on the SP-D-mediated agglutination of *E. coli* Y1088. Suspensions of *E. coli* Y1088 in HBS containing 2 mM  $\text{CaCl}_2$  were incubated with (+ SP-D) or without 1  $\mu\text{g}/\text{ml}$  SP-D in the presence or absence of 100 mM maltose. Agglutination of *E. coli* occurred in the presence of purified rat SP-D and calcium (+ SP-D, +  $\text{Ca}^{++}$ ), but not in the presence of 100 mM maltose. No SP-D-mediated agglutination was observed in the absence of calcium or in the presence of 10 mM EDTA (not shown). Controls included *E. coli* incubated without SP-D in HBS in the presence (+  $\text{Ca}^{++}$ ) or absence (-  $\text{Ca}^{++}$ ) of calcium. (B) Effect of LPSs and outer membrane preparations on SP-D-induced agglutination of *E. coli*. Suspensions of *E. coli* Y1088 were incubated with 1  $\mu\text{g}/\text{ml}$  SP-D in the presence of 2 mM calcium (no inhibitor). Inhibition of agglutination was observed with 10  $\mu\text{g}$  Y1088 LPS (+ LPS), 50  $\mu\text{g}$  of Y1088 outer membranes (+ OM), or 10  $\mu\text{g}$  LPS from *Salmonella minnesota* R5 (+ Rc-LPS).

ined by lectin blotting (Fig. 8 B). When LPSs from "rough" strains of *S. minnesota* and *E. coli* were blotted, Rc and Rd strains showed intense labeling of the LPS bands (Fig. 8 B); however, there was no significant binding to LPS from core oligosaccharide-deficient Re strains or to purified lipid A (not shown). There was also no detectable binding to an Ra strain, presumed to contain a complete core oligosaccharide, and comparatively weak labeling of a lower molecular weight subpopu-

lation of LPS from two clinical isolates of "smooth" *E. coli* expressing terminal O-antigen polysaccharides. Binding was also observed for commercial preparations of LPS isolated from *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Salmonella typhi* (not shown).

**Binding of BAL proteins to *E. coli*.** Bacterial agglutination was also observed when the *E. coli* were incubated with rat (Fig. 9 A) or human (Fig. 9 B) BAL. However, no aggregation was observed using SP-D-deficient BAL prepared by previous adsorption with maltosyl-agarose (Fig. 9 A and B). The bacterial aggregation by BAL was calcium and dose dependent and was abolished when incubations were performed in the presence of maltose, polyclonal antisera specific for SP-D, or the collagenase-resistant carboxy-terminal carbohydrate-binding domain. Normal rabbit sera and immune sera to other protein antigens showed no inhibition of agglutination.

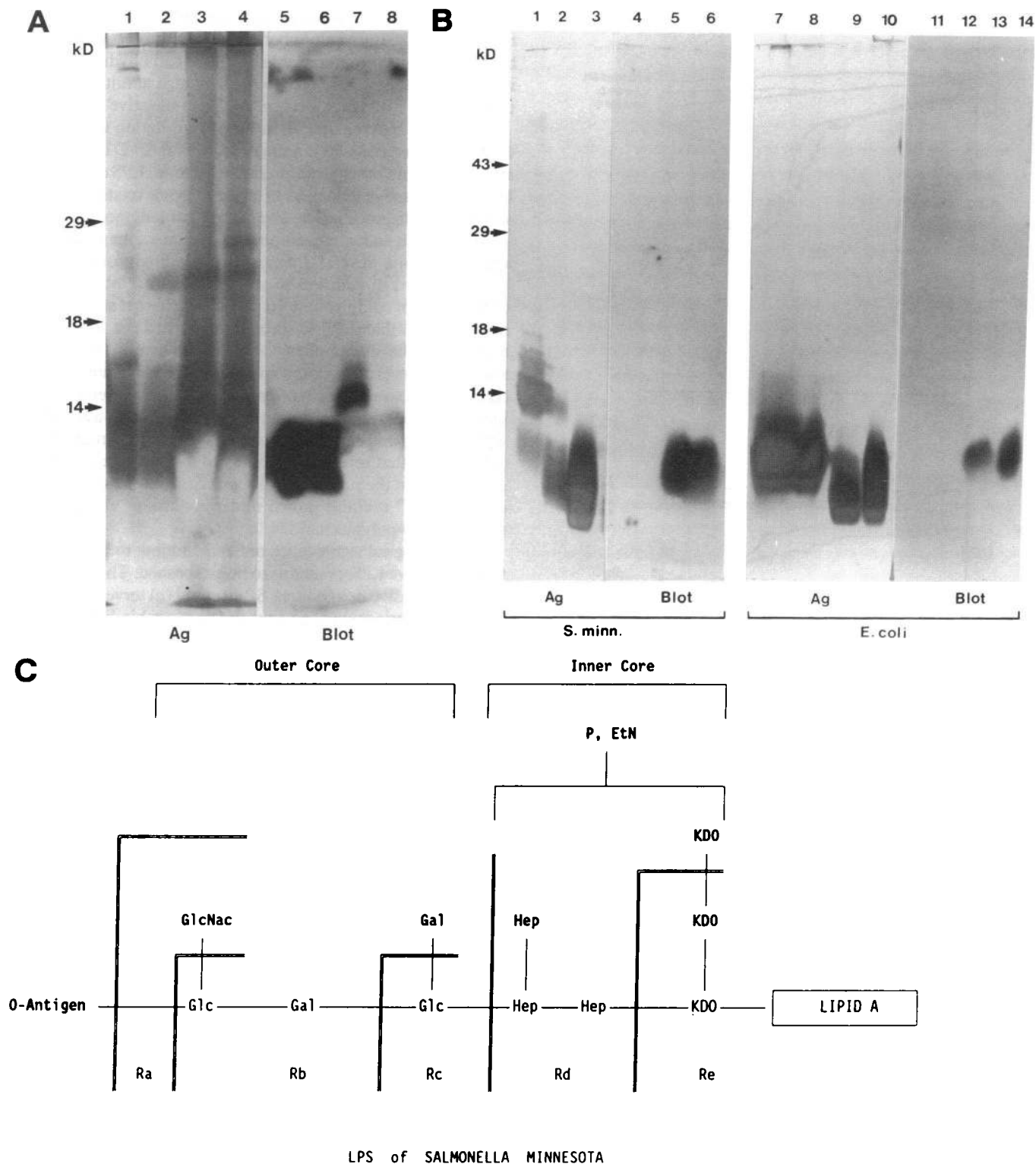
To further confirm specific binding of BAL SP-D to *E. coli*, bacteria were incubated with the 10,000 g supernatant of rat BAL. After incubation, the bacteria were collected, washed by centrifugation, and eluted with competing sugars. Proteins in the bound and unbound fractions were resolved by SDS-PAGE and visualized by silver staining. *E. coli* Y1088 selectively and efficiently adsorbed SP-D from the lavage (Fig. 10, lanes 1 and 2), and the bound SP-D was selectively eluted by competing sugars, including glucose and maltose, but not TBS, lactose, or N-acetylglucosamine (Fig. 10, lanes 4-7). Washes with TBS also failed to elute SP-D (Fig. 10, lane 3). Identification of SP-D was confirmed by immunoblotting with antibodies to rat SP-D (not shown).

**Binding of  $^3\text{H}$ -proline-labeled proteins from Type II cells to *E. coli*.** Similar specificity for SP-D was observed when *E. coli* were incubated with radiolabeled medium proteins from type II cells. Fluorograms of the bound and specifically eluted proteins revealed a single 43-kD band comigrating with SP-D (not shown). SP-D was also the only protein detected after solubilization of the bacterial pellet with SDS-PAGE sample buffer.

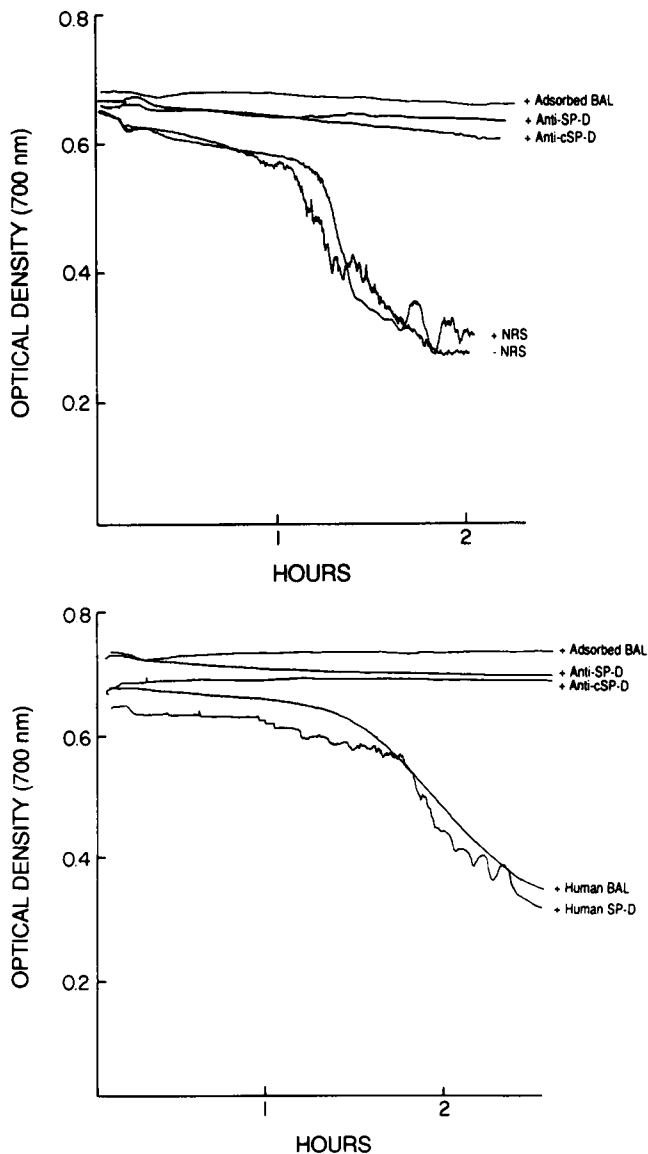
## Discussion

These studies demonstrate complement- and serum-independent binding of SP-D to several types of Gram-negative bacteria (Table I). All the strains that showed binding to labeled SP-D also showed microscopic agglutination. However, gross macroscopic agglutination (as illustrated in Fig. 2) was restricted to certain "rough" O-polysaccharide-deficient strains of Gram-negative bacteria. Differences in the extent of agglutination by various agglutinating strains of bacteria could reflect differences in the structure, number, valency, or distribution of bacterial ligands (see below), or steric effects mediated by other cell surface structures such as capsules, flagella, or pili.

A potential complication for lectin binding studies using intact bacteria is the presence of bacterial lectins on certain strains of *E. coli* (27, 28). Among these bacterial lectins are the well-studied type I pili lectins (29). However, there are several lines of evidence that indicate that the interactions between SP-D and *E. coli* Y1088 involve the carbohydrate-binding domain of SP-D rather than an interaction of bacterial lectins with N- or O-linked sugars on SP-D, which are associated with the amino-terminal collagen domain. First, the binding of SP-D to *E. coli* Y1088 is calcium dependent, consistent with the



**Figure 8.** Lectin blots. Duplicate samples of bacterial outer membranes and/or LPS were resolved by SDS-PAGE as described in Methods. One set of samples was silver stained (Ag) for protein and carbohydrate (25); the other set was transferred to nitrocellulose before lectin blotting (B) with radiolabeled rat SP-D in the absence or presence of maltose. (A) Binding of  $^{125}\text{I}$ -SP-D to LPS from *E. coli* Y1088 and two clinical isolates of *E. coli* (smooth strains) in the absence of maltose. Lanes 1 and 5, outer membrane from Y1088; lanes 2 and 6, Y1088 outer membrane treated with proteinase K; lanes 3 and 7, proteinase K digest of *E. coli* 44785; lanes 4 and 8, proteinase K digest of *E. coli* 97379. (B) Binding of  $^{125}\text{I}$ -SP-D to LPS from selected strains of *Salmonella minnesota* (*S. minn.*) and *E. coli* in the absence of maltose. Lanes 1 and 4, *S. minnesota* (smooth strain); lanes 2 and 5, *S. minnesota* R5 (Rc strain); lanes 3 and 6, *S. minnesota* R7 (Rd strain); lanes 7 and 11, *E. coli* 0111:B4 (smooth strain); lanes 8 and 12, *E. coli* EH100 (Ra strain); lanes 9 and 13, *E. coli* J5 (Rc strain); lanes 10 and 14, F583 (Rd strain). (C) Schematic diagram of "rough" LPS of *S. minnesota* (Ra to Re). P = phosphate, Etn = ethanolamine, Hep = L-glycero-D-mannoheptose, KDO = 3-deoxy-D-mannoctulosinic acid. Adapted from Brade et al. (39).



**Figure 9.** Agglutination of *E. coli* by SP-D in rat and human BAL. (A) Two-milliliter aliquots of *E. coli* Y1088 suspension (absorbance 1.0 at 700 nm) were incubated with 1-ml aliquots of rat BAL or with 3 ml of rat BAL depleted of SP-D by a maltosyl-agarose affinity chromatography. The final volume of each reaction mixture was adjusted to 3 ml using HBS containing 2 mM calcium. Some incubations were performed in the presence of normal rabbit sera (NRS, 1:60) or identical dilutions of antisera to rat SP-D (+ *Anti-SP-D*) or the collagenase-resistant domain of human SP-D (+ *Anti-cSP-D*). (B) Parallel studies were performed with aliquots of BAL from normal human volunteers and using purified human SP-D (1  $\mu\text{g}/\text{ml}$  final concentration).

known binding properties of SP-D, and unlike the calcium-independent binding of type I pili. Second, the observed sugar specificity (glucose > mannose) is consistent with SP-D and different from that of the major *E. coli* lectins, which are effectively inhibited by mannosides but not glucosides (30). Third, the aggregation of Y1088 by SP-D or BAL was specifically inhibited by antisera to the nonglycosylated carboxy-terminal lectin domain of SP-D. Finally, specific ligands (i.e., LPSs) were identified on the surface of agglutinating organisms (see

below). Although we cannot exclude the participation of bacterial lectins in the interactions between SP-D and certain SP-D-binding bacteria, the data establish that the carbohydrate-binding domains of SP-D are able to mediate high affinity interactions of SP-D with certain strains of Gram-negative bacteria.

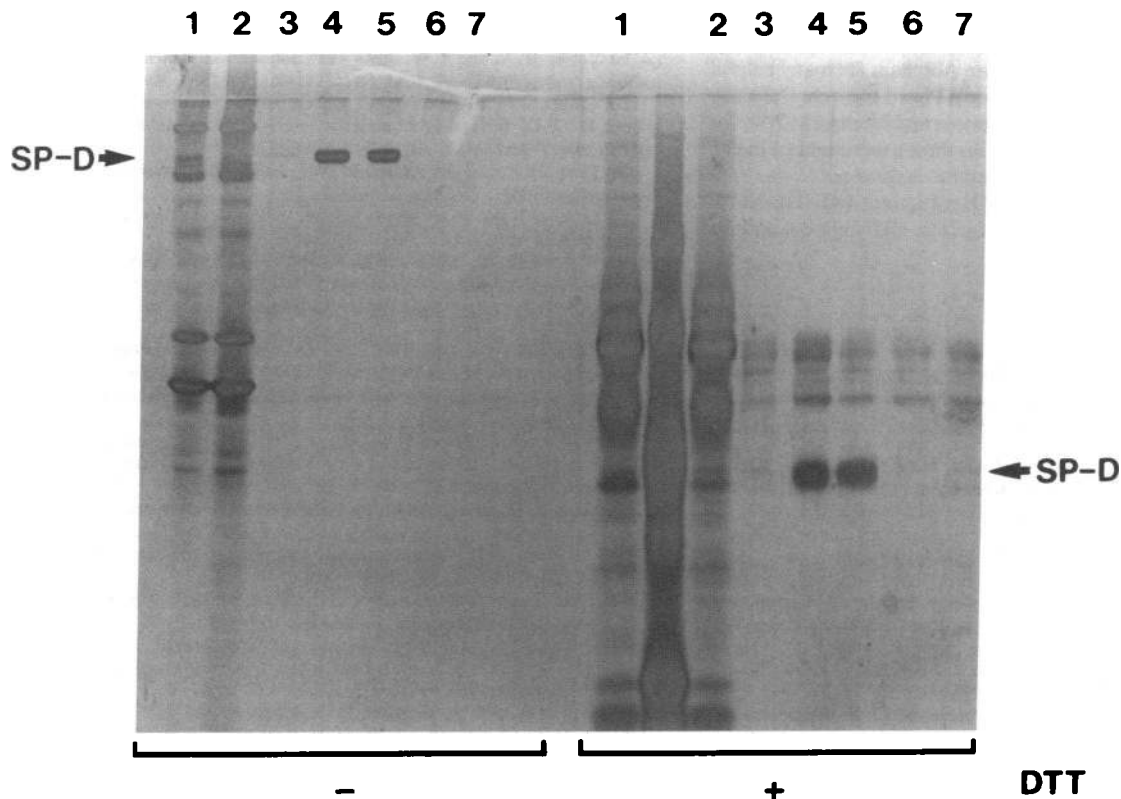
The lectin blotting and agglutination-inhibition studies indicate that bacterial LPS is a major ligand for SP-D on *E. coli*, as well as certain other Gram-negative bacteria. LPS consists of lipid A (the active principal of endotoxin), a relatively conserved core oligosaccharide, and a terminal polysaccharide of variable length and composition that comprises the O-specific antigen domain. Several different LPS-binding proteins have been identified in addition to specific immunoglobulins and components of complement. In particular, there is strong indirect evidence that LPS is recognized by at least one other member of the mammalian C-type lectin family. Kawasaki and coworkers (15) have shown that serum MBP can bind to certain rough strains of *E. coli*, presumably via *N*-acetyl-glucosamine and/or *L*-glycero-*D*-manno-heptose residues associated with core oligosaccharides. In other studies, Kuhlman et al. (13) showed that serum MBP binds to mannose-rich *O*-polysaccharides on the surface of wild-type *Salmonella montevideo* or to "rough" mutants grown under conditions allowing expression of *O*-polysaccharides.

The biological significance of SP-D binding to bacteria (or Gram-negative LPSs) remains to be established. There are several nonexclusive possibilities. SP-D could (a) serve as a bridge between bacteria and phagocytes and enhance binding, uptake, and killing; (b) inhibit the binding of organisms to respiratory epithelium; (c) facilitate the physical clearance of bacteria (see below); or (d) interfere with bacterial proliferation. SP-D could also contribute to the inactivation or clearance of soluble LPS (endotoxin) released at sites of colonization or infection by Gram-negative bacteria. With regard to the first possibility, preliminary experiments have demonstrated both saccharide-dependent and -independent binding to alveolar macrophages in vitro and in situ.

SP-D-mediated agglutination, or interactions with airway mucoproteins, could facilitate the mechanical removal of bacteria via the mucociliary escalator. The agglutination of bacteria has been extensively studied in the context of oral microbes and saliva (22, 31), and there appear to be several types of salivary proteins that can mediate bacterial agglutination. Among these is a group of nonimmunoglobulin, nonmucous salivary agglutinins with conglutinin-like activity (32, 33). Interestingly, immunostaining of rat salivary gland with anti-SP-D demonstrates the presence of cross-reactive material in ductal epithelial cells (Crouch et al., unpublished data).

The ability of SP-D to agglutinate bacteria is consistent with the existence of two or more functional carbohydrate-binding sites per SP-D molecule. Multiple binding sites were suggested by previous neoglycoprotein binding studies (3) and are also consistent with protein biochemical studies that indicate that SP-D is assembled as tetramers of trimers, similar to bovine conglutinin (2, 4). Although there are differences in apparent carbohydrate specificity (3), both proteins can also mediate complement-dependent erythrocyte agglutination (conglutination) reactions and agglutination of zymosan (Crouch et al., unpublished data). On the other hand, we cannot entirely exclude the possibility that interactions between SP-D molecules are favored by conformational changes that may occur when SP-D binds to bacteria.





**Figure 10.** Binding of BAL proteins to *E. coli*. *E. coli* Y1088 were washed by centrifugation with HBS containing 1% BSA and 10 mM maltose, and were rewashed in the absence of maltose. 25- $\mu$ l bacterial pellets were then incubated with 5 ml of rat BAL for 1 h at room temperature in the presence of 2 mM calcium and washed extensively with calcium-containing buffer. Equal aliquots of washed bacterial suspension were then transferred to microfuge tubes and the cell pellets were incubated with TBS or TBS containing various competing sugars (10 mM final). Aliquots of rat BAL, *E. coli*-adsorbed BAL, and bacterial eluates were examined by SDS-PAGE and silver staining in the absence (*left*) and presence (*right*) of sulfhydryl reduction. Lane 1, total rat BAL; lane 2, equivalent volume of *E. coli*-adsorbed BAL; lane 3, TBS eluate of BAL-treated *E. coli*; lane 4, glucose eluate; lane 5, maltose eluate; lane 6, lactose eluate; and lane 7, *N*-acetyl-glucosamine eluate. The positions of unreduced (*left*) and reduced (*right*) SP-D are indicated. Identification of SP-D was confirmed by immunoblotting (not shown).

The efficient labeling of LPS from Rc and Rd mutants (but not from Re mutants or lipid A) and the efficient inhibition of Y1088 agglutination by Rc-LPS strongly implicate core terminal glucose and/or heptose residues in SP-D binding to LPS. Although our previous studies have shown that SP-D preferentially interacts with  $\alpha$ -glucosyl-containing saccharides (3), preliminary studies indicate that binding to Y1088 can also be inhibited with mannoheptulose. Furthermore, the relatively poor labeling of LPS from Ra mutants or smooth strains and the limited ability of "smooth" LPS to inhibit agglutination suggest that terminal polysaccharides can "mask" binding sites associated with the core oligosaccharide, similar to the masking of core immunologic epitopes by *O*-specific antigens.

The ability of SP-D to interact with species of LPSs that are deficient in terminal *O*-polysaccharide is particularly interesting. The majority of clinical Gram-negative isolates and isolates of Gram-negative bacteria from the gut of healthy humans express *O*-polysaccharides and demonstrate a "smooth" phenotype under the usual conditions of culture (34). Enteric bacteria that lack *O*-specific polysaccharides are usually avirulent and are rapidly cleared from the systemic circulation, presumably secondary to the concerted action of complement and high molecular weight serum lectins, such as MBP (13, 15). On

the other hand, many nonenteric Gram-negative organisms known to colonize the upper aerodigestive tract do not express *O*-specific polysaccharides (35). Furthermore, there is growing evidence that the expression of *O*-polysaccharide by enteric organisms can be modulated by the growth state of the bacteria (35), as well as other environmental variables (36) that could result in expression of a "rough" phenotype under appropriate conditions in vivo. For example, isolates of *Pseudomonas aeruginosa* from patients with chronic lung infections in the setting of cystic fibrosis are typically deficient in *O*-polysaccharides (37, 38). The ability of SP-D to recognize exposed core residues could also be advantageous given relative conservation of this domain among various Gram-negative organisms.

Our data indicate that SP-D is a major *E. coli*-binding protein and agglutinin in the soluble phase of alveolar and airway lining material of rat and human lung. The large size of multimeric circulating host defense lectins, such as MBP and conglutinin, is expected to limit their diffusion into the otherwise healthy lung and necessitate local production of one or more functionally related molecules. Based on these studies we hypothesize that SP-D plays a critical role in lung surveillance, contributing to the lung's front-line defense against inhaled microorganisms.

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