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Pulmonary Collectins Play Distinct Roles in Host Defense against *Mycobacterium avium*

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Pulmonary collectins, surfactant protein A (SP-A) and surfactant protein D (SP-D), play important roles in the innate immunity of the lung. *Mycobacterium avium* is one of the well-known opportunistic pathogens that can replicate within macrophages. We examined the effects of pulmonary collectins in host defense against *M. avium* infection achieved via direct interaction between bacteria and collectins. Although both pulmonary collectins bound to *M. avium* in a Ca²⁺-dependent manner, these collectins revealed distinct ligand-binding specificity and biological activities. SP-A and SP-D bound to a methoxy group containing lipid and lipoarabinomannan, respectively. Binding of SP-D but not SP-A resulted in agglutination of *M. avium*. A chimeric protein with the carbohydrate recognition domain of SP-D, which chimera revealed a bouquet-like arrangement similar to SP-A, also agglutinated *M. avium*. The ligand specificity of the carbohydrate recognition domain of SP-D seems to be necessary for agglutination activity. The binding of SP-A strongly inhibited the growth of *M. avium* in culture media. Although pulmonary collectins did not increase membrane permeability of *M. avium*, they attenuated the metabolic rate of the bacteria. Observations under a scanning electron microscope revealed that SP-A almost completely covers bacterial surfaces, whereas SP-D binds to certain areas like scattered dots. These observations suggest that a distinct binding pattern of collectins correlates with the difference of their biological activities. Furthermore, the number of bacteria phagocytosed by macrophages was significantly increased in the presence of SP-D. These data indicate that pulmonary collectins play critical roles in host defense against *M. avium*. The Journal of Immunology, 2011, 187: 2586–2594.

Pulmonary surfactant is a mixture of lipids and proteins that covers alveolar surfaces and keeps alveoli from collapsing. Four specific surfactant proteins have been described. Hydrophobic surfactant proteins B and C play critical roles in biophysical functions of surfactant (1). Hydrophilic surfactant proteins A and D (SP-A and SP-D), pulmonary collectins, belong to the C-type lectin superfamily and have been implicated in the regulation of pulmonary host defense and inflammation (2–4). Engineered genetic defects in the pulmonary collectins of mice have demonstrated the important functions of these collectins in protecting the lung from microbial infections and inflammation (5, 6). The structure of pulmonary collectins is characterized by four domains that consist of an N terminus involved in interchain disulfide bonding, a collagen-like domain, a coiled coil domain, and a carbohydrate recognition domain (CRD) (7). SP-A forms a bouquet-like structure consisting of six trimeric subunits. SP-D exhibits a cruciform structure consisting of four trimeric subunits. This structural difference causes distinct biological functions. For example, these collectins modulate LPS-induced inflammatory cell responses by direct interaction with TLR4 and MD-2 in different manners. SP-A inhibits TNF-α secretion elicited by the smooth serotype of LPS, whereas it enhances TNF-α secretion stimulated by rough LPS (8, 9). In contrast, SP-D inhibits TNF-α secretion elicited by both smooth and rough LPS (10).

The mycobacteria *Mycobacterium avium* present ubiquitously in the environment, such as in water, soil, and house dust. Like other microbes, *M. avium* can be phagocytosed by host macrophages, but the bacteria can escape from digestion and replicate within macrophages. Although *M. avium* is less virulent than *Mycobacterium tuberculosis*, it is one of the well-known opportunistic pathogens associated with AIDS (11). Different from *M. tuberculosis* infection, *M. avium* often causes chronic infection, and it is very difficult to manage this infection completely because of its multidrug resistance. Understanding of the molecular mechanisms involved in host defense against *M. avium* will contribute to establishing a practical way to manage the infection.

The pulmonary collectins SP-A and SP-D bind *M. tuberculosis* but affect phagocytosis of the bacteria by macrophages in a manner different from each other. SP-A directly interacts with macrophages and enhances phagocytosis of *M. tuberculosis* through the mannose receptor by upregulation of receptor activity and localization on the cell surface (12, 13). In contrast, SP-D exhibits inhibitory effects on phagocytosis of *M. tuberculosis* by macro-
phages (14, 15). We have reported that pulmonary collectins also bind to *M. avium* and enhance phagocytosis of the bacteria by macrophages (16). Although important functions of pulmonary collectins on phagocytosis have been reported, the effects of direct interactions between pulmonary collectins and *M. avium* on the bacterium itself are poorly understood. In this report, we examined the direct effects of pulmonary collectins, SP-A and SP-D bound to *M. avium* through interaction with a methoxy group containing lipid and lipoparinimonnan (LAM), respectively. Binding of collectins suppressed the growth of *M. avium* in the growth medium by covering the bacterial surface. Binding of SP-D resulted in agglutination of the bacteria. Differences of ligand specificity and oligomeric organization seemed to correlate with distinct immune functions of pulmonary collectins. These data indicate that pulmonary collectins play important roles in preventing the dissemination of bacteria in the lung.

**Materials and Methods**

**Materials**

Mycobroth and 7H11-C agar plate were from Kyokuto Pharmaceutical (Tokyo, Japan). Glycopeptidolipid invariant lipid core from *M. avium* serotype 4 and LAM from *M. avium* serotype 4 were from Nacalai Tesque (Kyoto, Japan). Trehalose 6,6′-dimycolate was from Sigma. HPTLC silica gel 60 plate was from Merck.

**Collectins**

The 1.13-kb cDNA for human SP-A1 and the 1.181-kb cDNA for human SP-D were inserted into pE14 plasmid vectors, and recombinant human SP-A and SP-D were expressed in CHO-K1 cells using the glutamine synthetase gene amplification system and purified using a mannose-Sepharose 6B column, as described previously (17, 18).

Chimeric protein with SP-A and SP-D (A/D chimera), consisting of Glu–Pro(1–46) of SP-A and Asp–Val(47–80) of SP-D, was prepared as described previously (10). The physical forms of the recombinant collectins were observed by electron microscopy with rotary shadow (10). SP-A and A/D chimera appeared to form a typical bouquet-like arrangement. SP-D appeared to form a cruciform dodecamer or multimerized oligomer consisting of SP-D molecules associated at their N terminus.

Collagenase-resistant fragment (CRF) of SP-A was prepared as described previously (9, 19). SP-A was incubated with collagenase III from *Clostridium histolyticum* (Advance Biofactures Corporation) at 37°C for 22 h. CRF was isolated by gel filtration using Superose 6 10/300 GL (GE Healthcare). The N-terminal sequence of the purified CRF determined by protein sequencer (Applied Biosystems) was Gly–Pro–Pro and Gly–Leu–Pro–Ala, indicating that the N terminus region and the collagenous domain of SP-A (Glu–Arg(50) or Pro(77)) are removed and the CRF starts at position 78 of SP-A.

We used recombinant human collectins in all experiments except those of Fig. 1D and 1E in which native human collectins were used. Native human SP-A and SP-D were purified from bronchoalveolar lavage fluid of an alveolar proteinosis patient as described previously (20, 21).

**Preparation of bacteria**

Clinically isolated *M. avium* from sputum of an infected patient was a kind gift of Dr. Kazunori Tsunematsu (Hokkaido Prefecture Tomakomai Hospital, Tomakomai, Japan). The bacteria were cultured in Mycobroth at 37°C for 10 days. The bacteria were washed and suspended with 0.9% NaCl. The concentration of bacterial suspension was determined by measuring absorbance at 600 nm. For preparing UV-killed bacteria, the cultured medium was UV-irradiated for 5 min. The bacteria were then washed and suspended with PBS. The suspension was subdivided into small volumes in tubes and stored at −80°C. The thawed bacterial stocks were used in the assays. Once the stocks were thawed, the remaining bacteria were discarded to avoid the freeze–thaw cycle. For FITC-labeling, *M. avium* (4.0×10^7 CFU) in 1.0 ml 0.1 M Na2CO3, pH 9, was mixed and incubated in the dark with 0.02 mg aminoreactive FITC (Invitrogen) in 2 μl DMSO. After incubation, the labeled bacteria were washed three times and suspended with PBS.

**Binding of collectins to M. avium and bacterial lipids**

The suspension of UV-killed bacteria (1.0×10^8 CFU in 50 μl H2O/well) was put into microtiter wells (Immulon 1B; Thermo) and dried. After washing the wells three times with PBS containing 0.1% (v/v) Triton X-100 (washing buffer), nonspecific binding was blocked with 5 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 2% (w/v) fatty acid-free BSA. Collectins in 5 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, 2 mM CaCl2, and 2% (w/v) fatty acid-free BSA (binding buffer) or 5 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, 2 mM EDTA, and 2% (w/v) fatty acid-free BSA were added into wells and incubated at 37°C for 1 h. The wells were washed three times with a washing buffer, followed by incubation with an anti–SP-A or anti–SP-D polyclonal Ab in the binding buffer at 37°C for 1 h. After washing three times with the washing buffer, HRP-conjugated goat anti-rabbit IgG was added and again incubated at 37°C for 1 h. Peroxidase reaction was finally performed using o-phenylenediamine as a substrate. The binding of SP-A or SP-D to *M. avium* was detected by measuring absorbance at 492 nm. In some experiments, bacterial lipids suspended in 50 μl H2O were put onto microtiter wells and dried. Binding of SP-A to coated lipids was examined as described above.

**Surface plasmon resonance analysis**

Sonicated LAM (0.1 mg/ml in running buffer) was immobilized on an HPA sensor chip of the BIAcore 3000 system (BIAcore, Uppsala, Sweden), according to the manufacturer’s specifications. For the running buffer, 5 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 2 mM CaCl2 was used. SP-D was injected at a flow rate of 30 μl/min. At the end of each cycle, the surface of the sensor chip was regenerated by injecting 5 mM EDTA. SP-A binding of the immobilized LAM was obtained by using various collectins. Binding of SP-D (5–80 nM) were analyzed by the BIAevaluation program.

**Partition of M. avium cell wall constituents into organic phase and aqueous phase**

Cell wall constituents of *M. avium* were partitioned using the method of Bligh and Dyer (22). The resulting organic and aqueous phases were dried completely and resuspended with water. After sonication using a probe sonicator, the samples were coated onto the microtiter well, and binding of SP-A to coated constituents was examined as described earlier.

**Purification of the ligand for SP-A**

Total bacterial lipids were extracted with CHCl3/CH3OH (2:1, v/v) and treated with equal volume of 0.6 N NaOH in CH3OH at 30°C for 3 h to decompose phospholipids. After neutralization with HCl, 2 volumes of CHCl3 and 0.67 volumes of H2O were added. Organic solvent phase was recovered by centrifugation and dried under N2 gas. Alkaline-resistant lipids were further washed with CHCl3/CH3OH/H2O (4:2:1, v/v) and suspended in a small volume of CHCl3. The lipids were applied to a silica gel 60 spherical 40–50 μm; Kanto Chemical Co., Tokyo, Japan) column equilibrated with CHCl3 and eluted sequentially with 0, 10, 20, 30, and 40% CH3OH in CHCl3. Eluted lipids were monitored by TLC developed with CHCl3/CH3OH/H2O (90:10:0.5, v/v), and fractions containing the SP-A ligand were pooled. Pooled lipids were separated on a TLC plate developed with CHCl3/CH3OH/H2O (0:5:8:1, v/v), and fractions containing the SP-A ligand was scraped out and purified from the plate.

**SP-A blotting**

Bacterial lipids were separated on a TLC plate. After drying, the plate was incubated with 50 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, 2 mM CaCl2, and 2% (w/v) fatty acid-free BSA (blocking buffer) at 4°C for 16 h. The plate was then incubated with 5 μg/ml SP-A in a blocking buffer at room temperature for 2 h. After washing with 50 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, 2 mM CaCl2, and 2% (w/v) fatty acid-free BSA (washing buffer), the plate was incubated with an anti–SP-A polyclonal Ab in the blocking buffer at room temperature for 1 h. The plate was washed with the washing buffer, followed by incubation with HRP-conjugated goat anti-rabbit IgG in the blocking buffer at room temperature for 1 h. The plate was visualized by using a chemiluminescence reagent (Super Signal; Pierce) according to the manufacturer’s instructions.

1H-nuclear magnetic resonance analysis

1H-nuclear magnetic resonance (1H-NMR; 500 MHz) spectra of the SP-A ligand were recorded in CDC13 at room temperature on a Bruker AMX-500 spectrometer, and chemical shifts were measured from tetramethylsilane as an internal standard.
Agglutination assay

FITC-labeled M. avium were incubated with collectins in 5 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, 2 mM CaCl₂, and 2% (w/v) fatty acid-free BSA at 37°C for 4 h. After incubation, the bacteria were observed under fluorescence microscopy. In some experiments, 2 mM EDTA was added to the assay buffer instead of CaCl₂.

Growth inhibition assay

Mycobroth was supplemented with 2 mM CaCl₂ and used as an assay medium. M. avium (1000 CFU) were cultured in assay media at 37°C for 5 d in the presence or absence of collectins. On days 0, 1, 3, and 5, the aliquot of the culture was withdrawn and spread onto a 7H11-C agar plate. The plate was incubated at 37°C, and the colony number on the plate was counted.

Membrane permeability assay

M. avium in 5 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 2 mM CaCl₂ were incubated with 50 μg/ml pulmonary collectins at 37°C for 1 h. After incubation, bacterial membrane permeability was examined using Live/Dead BacLight bacterial viability kit (Molecular Probes). The kit constituted a two-color fluorescence stains, SYTO9 and propidium iodide. SYTO9 stains all the bacteria in a population, whereas propidium iodide only stains bacteria with damaged membranes. Thus, with an appropriate mixture of these stains, bacterial membrane permeability could be assessed by the green/red fluorescence ratio. M. avium killed at 70°C for 20 min were used as a standard for dead bacteria. Melittin (Sigma), cytolytic venom from honey bee, was used as a positive control.

Scanning electron microscopy

M. avium were fixed with 2.5% glutaraldehyde in 0.1 M PBS (pH 7.3). After several rinses with PBS, the cells were postfixed in 1% osmium tetroxide at 4°C for 3 h and then rinsed thoroughly with distilled water, dehydrated by graded ethanol, and freeze-dried. The specimens were sputter-coated with platinum and observed with a scanning electron microscope (S-4300; Hitachi, Tokyo, Japan) operating at 10 kV.

Metabolic radiolabeling assay

M. avium (1000 CFU) were cultured in Mycobroth supplemented with 2 mM CaCl₂ and 1 μCi/ml [1,2-14C]sodium acetate at 37°C for 3 d. The cultures were harvested by centrifugation at 8000 × g and suspended in PBS containing 0.2% Triton X-100, and the radioactivity was quantified by liquid scintillation spectrometry.

Isolation of monocyte-derived macrophages

Human monocyte-derived macrophages (MDM) were isolated from peripheral blood obtained from healthy volunteers (Hokkaido Red Cross Blood Center, Sapporo, Japan). Mononuclear cells were isolated from heparinized blood on Ficoll gradients and purified by adherence. The monocytes were collected and cultured in RPMI 1640 containing 10% heat-inactivated FCS and 20 nM human GM-CSF (Peprotech, Rocky Hill, NJ) for 5 d.

Phagocytosis of M. avium by MDM

MDM seeded onto 24-well plates were infected with M. avium in 200 μl glucose-free RPMI 1640 containing 1 mg/ml fatty acid-free BSA in the absence or presence of 50 μg/ml pulmonary collectins for 1 h at 37°C. Postinfection, the cells were incubated with 50 μg/ml gentamicin in the same medium for 15 min at 37°C to kill bacteria existing outside the cells. The cells were then washed three times with the same medium and lysed with distilled water. The resulting cell lysate was plated onto a 7H11-C agar plate. The plate was incubated at 37°C, and the colony number on the plate was counted.

Results

Pulmonary collectins bind M. avium

In a previous study, we demonstrated the binding of pulmonary collectins to M. avium using proteins purified from broncho-alveolar lavage fluids of individuals with alveolar proteinosis and as well as proteins from rat (16). In the current study, we used recombinant SP-A and SP-D produced by CHO cells. Additionally, we used live or UV-killed bacteria instead of heat-killed bacteria to prevent the destruction of the cell wall structure through heating. We first confirmed the binding of the recombinant collectins to M. avium coated onto microtiter wells. Both SP-A and SP-D bound to M. avium in a concentration-dependent manner. Moreover, the bindings were inhibited in the buffer containing EDTA instead of CaCl₂ (Fig. 1A, 1B). LAM, the major cell wall-associated lipoglycan of M. avium, has been identified as a ligand for SP-D (16). The addition of LAM to an assay buffer attenuated the binding of SP-D to M. avium in a dose-dependent manner. In contrast, the binding of SP-A was not affected by LAM, even at 1 μg/ml (Fig. 1C). We performed the same experiments using native human collectins to compare the abilities to bind M. avium with those of recombinant collectins. Native collectins also bind M. avium in a dose-dependent manner (Fig. 1D). The results are shown in Figure 1.
were subjected to 1H-NMR analysis. We could not obtain a clear spectrum of spot II due to the poor yield (data not shown). A spectrum of spot I revealed that the molecule contained −(CH$_2$)$_n$CH$_3$, −COCH$_2$CH$_2$−, −OCH$_3$, −(RO)CHCH$_2$−, and −(RO)CHCHX− (R refers to any atom, and X represents any group except hydrogen). Furthermore, the characteristic signals around δ 3.5 and 3.8 ppm were obtained (Fig. 3). Although we could not identify the complete structure of the SP-A ligand, these data suggest that the molecule is a methoxy group containing fatty acyl or fatty acid.

Next, we determined the binding parameters of SP-D with LAM using a surface plasmon resonance sensor (Fig. 1F). The passage of SP-D at various concentrations over LAM immobilized on a sensor chip yielded an association rate constant of $k_a = 1.03 \times 10^7$ M$^{-1}$ s$^{-1}$ and a dissociation rate constant of $k_d = 2.28 \times 10^{-4}$ s$^{-1}$, for a consequent dissociation constant of $K_D (k_d/k_a) = 2.2 \times 10^{-9}$ M. The injection of 5 mM EDTA resulted in complete dissociation of SP-D, indicating that SP-D interacts with LAM in a Ca$^{2+}$-dependent manner.

Purification of ligands for SP-A from M. avium

We tried to identify the ligand for SP-A that exists on the surface of M. avium. Cell wall constituents of M. avium were partitioned into organic and aqueous phases and coated onto microtiter wells. As shown in Fig. 2A, SP-A bound to organic-phase soluble constituents at almost the same degree as the bacteria itself. In contrast, no SP-A binding was detected in the wells coated with aqueous-phase soluble constituents. The surface of M. avium is rich in lipid such as glycopeptidolipid, trehalose 6,6'-dimycolate, and LAM. We determined the abilities of these well-known mycobacterial lipids to attenuate the binding of SP-A. Addition of organic-phase soluble constituents significantly inhibited the binding of SP-A to M. avium (Fig. 2B). In contrast, glycopeptidolipid, trehalose 6,6'-dimycolate, and LAM revealed little effects on SP-A binding. These data prompted us to purify the organic solvent-soluble ligands for SP-A. During the purification, the ligands turned out to be resistant to alkaline treatment. Because SP-A binds to phospholipids such as phosphatidylcholine and sphingomyelin (23, 24), we decided to use alkaline-treated total lipids. Under these conditions, phospholipids were degraded by alkaline treatment. The lipids were separated into six fractions through silica gel column chromatography. Each fraction was resolved on TLC plates, and lipids were visualized with iodide vapor (Fig. 2C, left panel) or subjected to SP-A blotting (Fig. 2C, right panel). Of the six fractions, fraction II and fraction V contained spots to which SP-A binds. Because no spot was detected in blotting without SP-A, the spots detected in SP-A blotting were not the results of nonspecific binding of the anti–SP-A polyclonal Ab (Fig. 2C, right panel). To characterize the binding of SP-A in detail, aliquots of the two fractions were coated onto microtiter wells. Consistent with the results obtained in Fig. 1A, binding of SP-A to fraction II was calcium dependent, whereas binding of SP-A to fraction V was calcium independent (Fig. 2D). Furthermore, addition of fraction II but not fraction V into the assay buffer inhibited the binding of SP-A to M. avium coated onto microtiter wells in a dose-dependent manner (Fig. 2E). These data suggest that fraction II contained ligands for SP-A exposed on the bacterial surface. Two spots bound to SP-A in Fig. 2C were scraped out and purified from the TLC plate. The lipids isolated were developed on the TLC plate and visualized with an iodide vapor and were analyzed by SP-A blotting (Fig. 2F). The results demonstrate that spots I and II are SP-A ligands. To characterize the chemical structures of ligands for SP-A, the purified ligands were subjected to 1H-NMR analysis. We could not obtain a clear spectrum of spot II due to the poor yield (data not shown). A spectrum of spot I revealed that the molecule contained −(CH$_2$)$_n$CH$_3$, −COCH$_2$CH$_2$−, −OCH$_3$, −(RO)CHCH$_2$−, and −(RO)CHCHX− (R refers to any atom, and X represents any group except hydrogen). Furthermore, the characteristic signals around δ 3.5 and 3.8 ppm were obtained (Fig. 3). Although we could not identify the complete structure of the SP-A ligand, these data suggest that the molecule is a methoxy group containing fatty acyl or fatty acid.
**FIGURE 3.** ¹H-NMR spectrum of spot I.

**SP-D agglutinates M. avium**

We next determined the biological functions of pulmonary collectins in innate immunity against M. avium. One of the well-known functions of host defense lectins is agglutination of microbes. FITC-labeled M. avium were incubated with collectins and observed under a fluorescent microscope (Fig. 4). SP-A did not agglutinate M. avium, even at 10 μg/ml (Fig. 4B). In contrast, SP-D strongly agglutinated M. avium in the presence of Ca²⁺ (Fig. 4C). The agglutination activity of SP-D was completely diminished in the buffer containing EDTA (Fig. 4D). Addition of LAM into the assay buffer inhibited agglutination activity (Fig. 4E). These data are consistent with the results of binding analysis in Fig. 1B, 1C, and 1F and indicate that interaction of SP-D with LAM is important to agglutinate M. avium. We further determined the agglutination activity using A/D chimera, in which the N-terminal region and the collagenous domain of SP-D were replaced with those of SP-A (10). The A/D chimera protein possesses the CRD of SP-D, but it exhibits a bouquet-like arrangement similar to SP-A. The chimera protein agglutinated M. avium, but a much higher concentration of the protein was required to agglutinate the bacteria compared with that required for SP-D to agglutinate the bacteria (Fig. 4C, 4F, 4G). These data indicate that lectin property of SP-D is necessary for agglutination activity and that the cruciform structure of SP-D is more suitable than the bouquet-like arrangement to agglutinate M. avium.

**Pulmonary collectins inhibit the growth of M. avium**

We examined whether pulmonary collectins affect the growth of M. avium in the growth media (Fig. 5). Cultures were started at 2000 CFU/ml, and there was no significant difference among the samples on day 0 (Fig. 5A, immediately after the cultures were started). SP-A dose-dependently inhibited the growth of M. avium on days 1, 3, and 5 (Fig. 5B–D). In particular, the growth of the bacteria cultured with 50 μg/ml SP-A was suppressed to <10% of control throughout the observation (4.7% on day 3, and 9.0% on day 5). SP-D also inhibited the growth of the bacteria, although its effects were feeble and not dose dependent. Because both SP-A and SP-D exist simultaneously in the lung, we further examined the inhibitory effect in the presence of both collectins (Fig. 5E). The addition of both SP-A and SP-D resulted in a synergistic effect on growth inhibition of M. avium.

To examine the molecular basis of the inhibitory effect of SP-A, we used CRF produced from SP-A by collagenase treatment and anti–SP-A mAbs (Fig. 5F). CRF did not appear to inhibit the growth of M. avium. The inhibitory effect was quite weak compared with that of nontreated SP-A. These data indicate that integrity of the collagenous domain of SP-A is critical for growth inhibition. mAbs PE10 and PC6, whose epitopes lie within the region Thr¹⁸⁴–Gly¹⁹⁴, inhibit cell surface receptor binding of SP-A and block the inhibitor activity of SP-A on surfactant lipid secretion from alveolar type II cells (19, 25, 26). However, the addition of these Abs did not affect the inhibitory effects of SP-A on the growth of M. avium. These data suggest that the SP-A region of Thr¹⁸⁴–Gly¹⁹⁴ is not responsible for growth inhibition.

**Pulmonary collectins cover the surface of M. avium and attenuate metabolic incorporation of [1,2-¹⁴C]sodium acetate into the bacteria**

It is reported that pulmonary collectins increase membrane permeability and inhibit the growth of Escherichia coli (27). We examined the effects of pulmonary collectins on permeabilization of M. avium membrane using two-color fluorescence stains, SYTO9 and propidium iodide (Fig. 6). Pulmonary collectins did not increase membrane permeability even at high concentration of 50 μg/ml, whereas 60% of the bacteria were permeabilized when...
incubated with 100 μM melittin. To analyze further the effects of SP-A and SP-D on \textit{M. avium} growth, we examined \textit{M. avium} grown in the presence of collectins using a scanning electron microscope. Fig. 7 shows the representative specimens. Most of the bacteria were almost completely covered with SP-A, and the bacterial surfaces were slightly visible (Fig. 7C, 7D). These tucked bacteria may be inhibited to grow, and some of the bacteria showed an unusual globular shape like cocci (Fig. 7E, 7F). In contrast, SP-D caused a large aggregate of the bacteria, but the majority of the bacteria were not covered with the protein (Fig. 7G, 7H). \textit{M. avium} seems to be able to grow in these large aggregates. Indeed, the dividing bacteria were observed in these aggregates (Fig. 7H, arrowhead). Consistent with the feebleness of the growth inhibition by SP-D, some of the agglutinated bacteria were covered with SP-D as in the case with SP-A (Fig. 7D). These data clearly indicate that pulmonary collectins inhibit the growth of \textit{M. avium} by surrounding the bacterial surface. These results prompted us to hypothesize that SP-A inhibits the incorporation of nutrition by forming a physical barrier around the bacterial surface. Thus, we examined the metabolic rate of the bacteria by quantifying the incorporation of [1,2-\textsuperscript{14}C]sodium acetate. During the growth of the \textit{Mycobacterium}, \textsuperscript{14}C provided as [1,2-\textsuperscript{14}C]sodium acetate is incorporated into cell wall lipids (28). As expected, SP-A dramatically decreased \textsuperscript{14}C incorporation into the bacteria (Fig. 8).
SP-D facilitates uptake of M. avium by human macrophages

We next determined the ability of pulmonary collectins to modulate phagocytosis of macrophages. MDM from human peripheral blood were infected with M. avium in the presence or absence of collectins. Postinfection, the number of phagocytosed bacteria in the cell lysate was assessed as CFU (Fig. 9). The number of phagocytosed bacteria was significantly increased in the presence of SP-D, whereas SP-A revealed little effect. Although the mechanisms underlying the enhanced phagocytosis remain unclear, these data suggest that SP-D plays an important role in clearance of M. avium by macrophages.

Discussion

In the current study, we examined the effects of direct interaction between pulmonary collectins and M. avium. Pulmonary collectins bind M. avium in a Ca2+-dependent manner (Fig. 1A, 1B). The binding of SP-D to M. avium was inhibited by the addition of LAM into the binding buffer (Fig. 1C). Surface plasmon resonance analysis showed that SP-D bound to LAM with a high affinity of  \( K_d = 2.2 \times 10^{-9} \) M (Fig. 1F). These data clearly indicate that SP-D binds to M. avium via interaction with LAM. We also tried to identify the ligand for SP-A. Although the principal activity of SP-A is to bind saccharides as a host defense lectin, it also binds to lipids such as dipalmitoylphosphatidylcholine and disaturated phosphatidylglycerol (23, 24). We found that SP-A bound organic solvent-soluble constituents of M. avium (Fig. 2).

\(^1\)H-NMR analysis showed that one of the ligands (spot I in Fig. 2F) was a methoxy group containing lipid (Fig. 3). The spot I revealed a yellow-gold color by orcinol/H\(_2\)SO\(_4\) staining (data not shown). Together with the \(^1\)H-NMR spectrum, these data indicate that this lipid contains no saccharide. The surface of M. avium is rich in lipids containing glycolipids, waxes, fatty acids, mycolic acids, and glycopeptidolipids (29–31). The \(^1\)H-NMR spectrum obtained in Fig. 3 contained characteristic signals that differ from that of known mycobacterial lipids. We could not conclude whether these characteristic signals indicate the existence of unknown mycobacterial lipids or modification of known molecules during purification. Although we could not identify the complete structure of the ligand, our data suggest that SP-A recognizes surface-exposed lipid.

Pulmonary collectins bind a variety of ligands in various pathogens (3, 32). Many ligands include a lipid moiety such as LPSs of Gram-negative bacteria, lipoteichoic acid of Gram-positive bacteria, and membrane lipids of Mycoplasma. Cell surface glycoproteins of fungi, yeasts, and viruses are also recognized by pulmonary collectins. Among these ligands, the binding of collectins to LPS are well characterized. SP-A binds rough LPS and lipid A but not smooth LPS (8, 33). SP-D binds Re LPS through the neck domain and Rc LPS, core oligosaccharide of LPS, and selective mannose-rich smooth LPS through the CRD domain (10, 34, 35). As analysis of the crystal structure of SP-A reveals the extensive hydrophobic surface, it is suggested that the different characteristics of the protein structures are responsible for those of binding specificity between pulmonary collectins (36). SP-A binds lipid ligand on M. avium containing no saccharide, whereas SP-D binds the mannose-rich glycolipid LAM. Our findings further indicate that ligands for SP-A are more hydrophobic than those of SP-D.

The binding of SP-D but not SP-A to M. avium resulted in agglutination of the bacteria (Fig. 4A–C). We further determined agglutination activity using the A/D chimera protein with a notice on considerable structural differences between SP-A and SP-D. We hypothesized that the cruciform structure of SP-D is necessary for agglutination activity. However, as a result, the A/D chimera protein agglutinated M. avium, although a higher concentration is required than that of SP-D (Fig. 4F, 4G). These data indicate that the cruciform structure of SP-D is important to express its full activity of agglutinating bacteria, but the cruciform structure is not necessary for agglutination activity. These data also indicate that the ligand specificity of CRD of SP-D is necessary for agglutination activity. In binding analysis, the binding of SP-A and SP-D to M. avium revealed an unsaturable and a saturable curve, respectively (Fig. 1A, 1B). These data suggest that the binding sites for SP-A existing on the surface of M. avium are much more abundant than those for SP-D. This idea was strongly supported by scanning electron microscope analysis. SP-A covered almost all the surface of M. avium, whereas SP-D bound to certain areas like scattered dots (Fig. 7). The CRD of SP-A may be occupied with ligands on a bacterium and could not bridge one bacterium to another.

Previous studies have shown that pulmonary collectins inhibit the growth of Gram-negative bacteria, including E. coli, Klebsiella pneumoniae, Enterobacter aerogenes, and Legionella pneumophila by increasing membrane permeability. The inhibitory effects of collectins on E. coli and L. pneumophila growth are independent of the bacterial aggregation caused by collectins (17, 27). Pulmonary collectins also exhibited inhibitory effects on the growth of M. avium (Fig. 5). Despite SP-A revealing negligible agglutination activity, SP-A strongly inhibited the growth of M. avium. Furthermore, M. avium seemed to be able to grow in the large aggregate (Fig. 7H, arrowhead). Thus, it is suggestive that the effect on growth inhibition of M. avium was not dependent on...
the agglutination activity. Inhibitory effect of CRF on the growth of *M. avium* was much weaker than that of SP-A (Fig. 5F). The binding of CRF to *M. avium* coated onto a microtiter well was also much weaker than that of SP-A; the absorbance at 492 nm for CRF binding was 2.3 ± 1.8% (mean ± SD, n = 3) of that for SP-A binding. This is consistent with the result described in a previous study indicating the importance of the collagenous domain in the interaction of SP-A with phospholipids (23). Although the complete structure of lipid ligand on *M. avium* remains to be clarified, the collagenous domain and the oligomeric structure dependent upon the collagen tail are critical for the lipid-binding activity of SP-A. We examined the effects of collectins on membrane permeability based on a fluorescence dye influx into the permeabilized bacteria. When *M. avium* were incubated with 50 μg/ml SP-A or SP-D, >90% of the bacteria were still viable (Fig. 6). To examine whether collectins affect the surface structure of bacteria, we analyzed the *M. avium* under scanning electron microscope. Surprisingly, *M. avium* cultured in the presence of SP-A were covered with the protein. Moreover, bacteria with an abnormal globular shape like a coccus were observed in some specimens (Fig. 7E, 7F). SP-A also inhibits the growth of *Mycoplasma pneumoniae* by affecting its metabolic activity through an unknown mechanism (24). SP-A dramatically decreased the amount of 14C incorporated into bacteria (Fig. 8). Our observations infer that SP-A inhibits the growth of *M. avium* and *Mycoplasma pneumoniae* by the same mechanism.

Recently, immunological roles of clot formation have been clarified using transglutaminase-knockdown *Drosophila* (37). The mortalities of the transglutaminase-knockdown flies after infection by certain pathogens were significantly increased. Notably, like *M. avium* cultured in the presence of SP-A (Fig. 7C–F), pathogens in the hemolymph of wild-type larvae were sequestered by proteins within the clot. Fascinatingly, the major proteins that bound to the surface of pathogens were hemerxemins, whose homolog in cockroach revealed lectin-like activity (38). Surrounding the bacterial surface by lectins to sequester pathogens might be a primitive and important host defense mechanism that was conserved through protostome to deuterostome.

Phagocytosis of *M. avium* by macrophages was significantly enhanced in the presence of SP-D. In contrast, SP-A slightly increased the number of phagocytosed bacteria (Fig. 9). In the previous report, we demonstrated that both SP-A and SP-D significantly enhance phagocytosis (16). The bacteria used in previous experiments were killed by heat treatment. It is possible that destruction of surface structure by heat treatment influenced the results, which are different from those of the current study. Although the mechanisms underlying the enhanced phagocytosis remain unclear, these data suggest that SP-D plays an important role in clearance of *M. avium* by macrophages.

It is difficult to discuss the exact concentration of collectins in vivo. Because SP-A and SP-D are constituents of a pulmonary surfactant that exists in the epithelial lining fluid of the alveolus, no one can determine their concentrations directly. However, we can estimate their concentrations according to previous studies, which calculate the concentrations based on the recovery of the proteins in bronchoalveolar lavage fluids (39–45). The calculated concentration of SP-A ranges from 180 to 1800 μg/ml, and the SP-D concentration was calculated as ~63 μg/ml. Although their concentrations appear to vary in a diseased state, the concentrations of collectins used in our study are within the best estimates of the physiological ranges.

In conclusion, pulmonary collectins directly interact with *M. avium* in a Ca2+-dependent manner. SP-A and SP-D recognize different lipid ligands that exist on the surface of *M. avium*. SP-A strongly inhibited the growth of *M. avium*, whereas SP-D agglutinated the bacteria. Different ligand specificity contributes to the functional difference. These data indicate that pulmonary collectins play important roles in the first-line defense against *M. avium* by preventing the expansion of infection. Our observations provide a new insight into the innate immune mechanisms by host defense lectins.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


