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Lung surfactant apoprotein SP-A (26–36 kDa) binds with high affinity to isolated alveolar type II cells

(receptors/epithelial cells/lipoproteins)

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ABSTRACT **Pulmonary surfactant is synthesized and secreted by alveolar type II cells. These cells recycle surfactant lipids by an internalization process that is enhanced *in vitro* by the surfactant proteins with molecular masses of 26–36 kDa (SP-A). SP-A also inhibits the secretion of lipid by type II cells. These results suggest that SP-A may play a role in feedback regulation of surfactant pool size and are consistent with the hypothesis that the type II cell surface has receptors for SP-A. The goal of this study is to characterize the binding of radioiodinated SP-A to isolated rat type II cells. Binding of SP-A to type II cells at 4°C has a $K_{1/2}$ of approximately 5×10^{-10} M, is saturable, and is inhibited by excess unlabeled SP-A. Binding is dependent on calcium and is reduced by heat treatment of SP-A. The binding of a proteolytic fragment of SP-A that is produced by collagenase treatment is reduced by excess unlabeled SP-A. The binding of the fragment to macrophages and lung fibroblasts is not inhibited by excess unlabeled SP-A. Trypsinization of the type II cell surface reduces the binding of both intact SP-A and the collagenase-resistant fragment. These results show that SP-A binds to type II cells with high affinity and suggest that these cells have receptors that recognize the carboxyl-terminal domain of SP-A.**

Lung surfactant is synthesized by the alveolar type II cell and stored intracellularly in lamellar bodies until it is secreted into the alveolar air space. Once in the alveoli, the secreted lamellar body contents can form tubular myelin, which is thought to be a precursor to the monomolecular surface film (for review, see ref. 1). Several studies suggest that alveolar surfactant can be internalized by type II cells, incorporated into lamellar bodies, and eventually resecreted (2–5). It has been calculated that between 25 and 95% of the alveolar surfactant enters this "recycling" pathway (6, 7).

Results from recent studies suggest that the major surfactant protein group of 26- to 36-kDa SP-A (previously referred to as SP 26–36) can enhance the uptake of lipids by type II cells (5, 8, 9). SP-A has also been shown to inhibit the secretion of phosphatidylcholine by type II cells in primary culture (10–12). These results suggest that SP-A may play an important role in regulating alveolar surfactant pool size by balancing secretion and reuptake. These results also suggest that SP-A may interact with receptors on type II cells.

The goal of the work reported here was to characterize the interaction of SP-A with isolated alveolar type II cells. A preliminary report of this work has appeared in abstract form (13). While this manuscript was in preparation, Kuroki *et al.* (14) reported that isolated type II cells that have been maintained in primary culture bind SP-A with high affinity.

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MATERIALS AND METHODS

Materials. Agarose, Bio-Gel P-6DG, and Enzymobeads were obtained from Bio-Rad. Sephacryl S-200 and heparin-Sepharose CL-6B were obtained from Pharmacia. Elastase was obtained from Cooper Biomedical. Collagenase was purchased from Worthington. Media and serum were obtained from the University of California Cell Culture Facility. $Na^{125}I$ was obtained from Amersham. All other enzymes and chemicals were obtained from Sigma.

Isolation, Iodination, and Analysis of SP-A and Its Collagenase-Resistant Fragment (CRF). SP-A was isolated from rat lung lavage and the lavage fluid of a patient with alveolar proteinosis as described in detail (9). CRF was isolated from human SP-A because only small amounts of rat SP-A can be obtained by lung lavage (8–10 μg per lung). We found no qualitative differences between binding of rat SP-A and human SP-A (i.e., binding of both was saturable, required calcium, and was inhibited by excess unlabeled SP-A). CRF was obtained by digesting human SP-A with collagenase from *Clostridium histolyticum*. Non-specific protease contaminants were removed from the collagenase by chromatography over heparin-Sepharose CL-6B and Sephacryl S-200 as described (15). SP-A was incubated with collagenase in 5 mM Tris·HCl/5 mM $CaCl_2$, pH 7.40 at 37°C for 16 hr. After digestion the sample was centrifuged (100,000 × *g*, 60 min, SW 40 rotor), the supernatant was applied to a Sephacryl S-200 column, and material was eluted in 5 mM Tris·HCl/100 mM NaCl, pH 7.40 to separate CRF from intact SP-A. Protein was measured by the method of Lowry *et al.* (16).

SP-A and CRF were iodinated using Bio-Rad Enzymobeads according to the manufacturer's directions. Rat or human SP-A (75–300 μg) in 5 mM Tris·HCl (pH 7.40) or human CRF in 5 mM Tris·HCl/154 mM NaCl, pH 7.40 was incubated at room temperature with 0.5–1 mCi $Na^{125}I$ for 15–20 min (1 Ci = 37 GBq). Free $Na^{125}I$ was separated from ^{125}I-labeled protein on 8-ml columns of Bio-Gel P-6DG that had been pre-eluted with 20–50 μg of the appropriate protein. Fractions that contained radioactivity greater than 88% precipitable by trichloroacetic acid were combined and then stored at 4°C. Specific activities ranged from 0.2 to 2 μCi/μg. Radiolabeled SP-A was used within 2–3 weeks after the iodination; storage for longer periods of time reduced binding of protein to cells. Radiolabeled CRF was used within 1–2 weeks.

$NaDodSO_4$/PAGE was performed on slab gels as described (8). All samples were reduced with 50 mM dithiothreitol. Gels were stained with Coomassie blue, dried, and exposed to Kodak diagnostic film X-OMAT AR in a Kodak X-OMATIC cassette with intensifying screens at −70°C.

Abbreviations: SP-A, surfactant proteins of 26–36 kDa; CRF, collagenase-resistant fragment of SP-A.

The molecular size of SP-A was determined by gel filtration chromatography. Samples (0.1 ml) containing 1–10 μg were applied to a 1.9 × 90 cm, 4% agarose column and eluted with 5 mM Tris·HCl/154 mM NaCl, pH 7.40 at room temperature at a flow rate of 8 ml/hr. Fractions of 2.5 ml were collected and analyzed for radioactivity. The elution profiles of the standards blue dextran, thyroglobulin, apoferritin, catalase, and albumin were monitored by measuring absorbance at 280 nm.

Isolation and Primary Culture of Lung Cells. Type II cells (17) and macrophages (9) were isolated by described methods. Type II cells and macrophages were either used on the day of isolation (freshly isolated cells) or they were placed in primary culture. For primary culture, the cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal bovine serum and gentamicin (50 μg/ml). Cells were plated in either 96-well plates at 5×10^5 cells per 0.25 ml per well or in 24-well plates at 10^6 cells per 0.5 ml per well. The cells were incubated for 16–18 hr in an atmosphere of 10% CO_2/90% air.

Fibroblasts were prepared from rat lung tissue. Lungs from two rats were minced finely and incubated with occasional agitation in 20 ml of Dulbecco's phosphate-buffered saline containing 0.2% trypsin (type III, from bovine pancreas) and 0.006% DNase I at 37°C for 40 min, after which time 25 ml of phosphate-buffered saline (4°C, pH 7.40) containing 0.1% trypsin inhibitor (type II-S from soybean) was added to the minced lung tissue. The digest was centrifuged at 140 × *g* for 10 min and the pellet was resuspended in 20 ml of DMEM containing 4.5 g of glucose/liter, 10% fetal bovine serum, and gentamicin (50 μg/ml). The cell suspension was filtered through two layers of surgical gauze and the cells were then cultured at 1–2 × 10^6 cells per 100-mm tissue culture dish. Cells were allowed to grow to confluence and passaged onto 75-cm^2 tissue culture plates. Cells were routinely used between passages 5 and 15.

On the day before the binding assay was performed, fibroblasts were removed from flasks by trypsin treatment and were resuspended in plating medium and plated in 96- or 24-well plates as described above.

Trypsin Treatment of Type II Cells. For some studies, freshly isolated type II cells were suspended in Dulbecco's phosphate-buffered saline containing 0.025% trypsin (from bovine pancreas, type III) and incubated with gentle agitation at room temperature for 10 min, after which time fetal calf serum or trypsin inhibitor (from soybean, type II-S) was added to a final concentration of 20% or 0.05%, respectively. Cells and medium were separated by centrifugation and cells were resuspended in 1 ml of the appropriate medium containing radiolabeled protein. Control cells were treated in an identical fashion except that trypsin was not included in the incubation medium.

Binding Assays with Freshly Isolated Cells. From 2 to 2.5 × 10^6 cells were resuspended at 4°C in a custom-formulated medium containing modified Krebs improved salts (18) (1.6 mM K_2HPO_4/5.4 mM KCl/1.29 mM $MgCl_2 \cdot 6H_2O$/112 mM NaCl/4 mM $NaHCO_3$/15.7 mM sodium phosphate, pH 7.40), 13 mM glucose, 25 mM Hepes, 2 mM sodium pyruvate, and Eagle's minimal essential medium (binding medium). This medium contains a low concentration of added calcium (2 μM as calcium pantothenate). Bovine serum albumin (0.1%, fraction V) was included in the binding medium because albumin reduced adherence of SP-A to microcentrifuge tubes and tissue culture plastic ware but did not alter the binding of SP-A to lung cells. Cells were incubated in a microcentrifuge tube and mixed every 7–10 min. At the end of the incubation period, cells and medium were separated by centrifugation at 140 × *g* for 10 min in a Beckman model 12 Microfuge in horizontal tube holders in a cold room. The medium was removed and the cells were gently resuspended in a small volume of medium, transferred to a fresh tube, and then washed and collected by centrifugation twice. The final cell pellet was resuspended in 0.5 ml of medium; an aliquot was removed for determination of cell number (in duplicate) in a Neubauer counting chamber.

Samples without cells were carried through the procedure concurrently and the amount of radioactivity that sedimented or remained associated with the centrifuge tubes in the absence of cells was determined and was subtracted from the samples with cells. This correction increased with increasing protein concentration and was generally 5–20%.

Binding Assays with Adherent Cells. Because only small amounts of rat SP-A can be obtained by lung lavage, a microassay with adherent cells in primary culture was developed to assess the potential inhibitory role of excess unlabeled SP-A. (The advantage of this assay is that fewer cells and smaller volumes of medium can be used than are required for the binding assays using cells in suspension. It was necessary to use a minimum of 2×10^6 cells in the binding assays with freshly isolated cells to minimize losses during washing and centrifugation.) After the cells had been allowed to adhere for 16–18 hr, they were removed from the CO_2 incubator, placed in room air on ice, and washed four times with binding medium. Fresh binding medium containing 0.1% bovine serum albumin was added to the wells (0.2 ml per well of a 24-well plate or 0.1 ml per well of a 96-well plate). At the end of the incubation with radiolabeled protein, the cells were washed four times with binding medium and the cells were dissolved in 0.1 M NaOH and analyzed for radioactivity. Binding of SP-A to both freshly isolated type II cells and cells in primary culture required calcium, was inhibited by excess unlabeled SP-A, and was saturable.

RESULTS

Characterization of Radiolabeled SP-A and CRF. A representative autoradiogram of iodinated rat and human SP-A and human CRF is shown in Fig. 1. The molecular mass heterogeneity of these preparations is due in large part to their variable degrees of glycosylation (19).

We have shown (9) that SP-A enhances lipid uptake by freshly isolated type II cells. We tested whether iodinated SP-A retained this biologic property and found that iodinated SP-A also enhanced lipid uptake by freshly isolated type II cells. The radiolabeled protein could be immunoprecipitated with a polyclonal antibody (20). Binding properties of iodinated protein were compared to those of a mixture of nonlabeled and iodinated protein. The requirement for calcium for maximal binding to type II cells and the concentra-

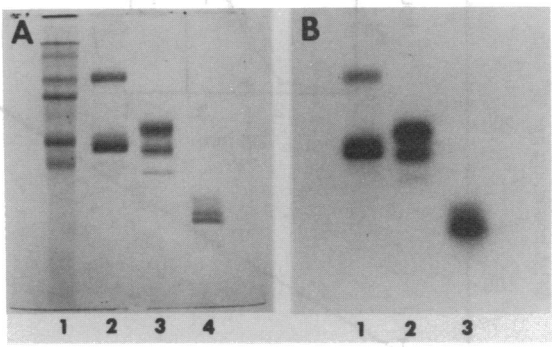

FIG. 1. $NaDodSO_4$/PAGE of purified ^{125}I-labeled surfactant proteins. (*A*) Gel stained with Coomassie blue. Lanes: 1, molecular mass standards (from top to bottom, 180 kDa, 116 kDa, 84 kDa, 58 kDa, 48.5 kDa, 36.5 kDa, and 26.6 kDa); 2, human SP-A; 3, rat SP-A; 4, human CRF. (*B*) Autoradiogram of the same gel. Lanes: 1, human SP-A; 2, rat SP-A; 3, human CRF.

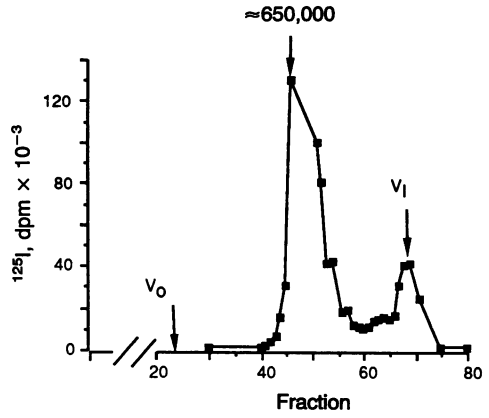

FIG. 2. Analysis of ^{125}I-labeled rat SP-A by gel filtration chromatography. The elution position is indicated for blue dextran by V_o (void volume), for thyroglobulin by ≈650,000, and for the included volume by V_I. Recovery of radioactivity was approximately 70%. This profile is representative of those obtained for four preparations.

tion at which binding reached a plateau with rat SP-A were similar with either preparation (data not shown).

Iodinated rat SP-A was sized by gel filtration chromatography (Fig. 2). Ninety percent of the recovered material was eluted with an approximate molecular mass of 650 kDa. Approximately 10% of the material was eluted with the included volume, was not precipitable with trichloroacetic acid, and was probably mostly free ^{125}I. Occasionally a very small amount (less than 1% of the recovered material) was eluted in the void volume. Similar results were obtained when the elution was performed at 4°C.

Binding of SP-A to Freshly Isolated Type II Cells. Binding of rat SP-A was rapid. We estimated that approximately 2 min elapsed between the addition of SP-A and the beginning of centrifugation, and thus our first time point was obtained at approximately 2 min. Binding reached maximal levels within 20 min (Fig. 3). The binding of SP-A increased rapidly at low concentrations and less rapidly at higher concentrations (Fig. 4). Specific high-affinity binding was calculated using the slope-peeling method described by Goldstein and Brown (21). An analysis of the binding as described by Klotz (22) (Fig. 4 *Inset*) reveals that half-maximal binding occurs at 5×10^{-10} M and that there are approximately 40,000 SP-A binding sites per cell. Estimates of specific binding of SP-A

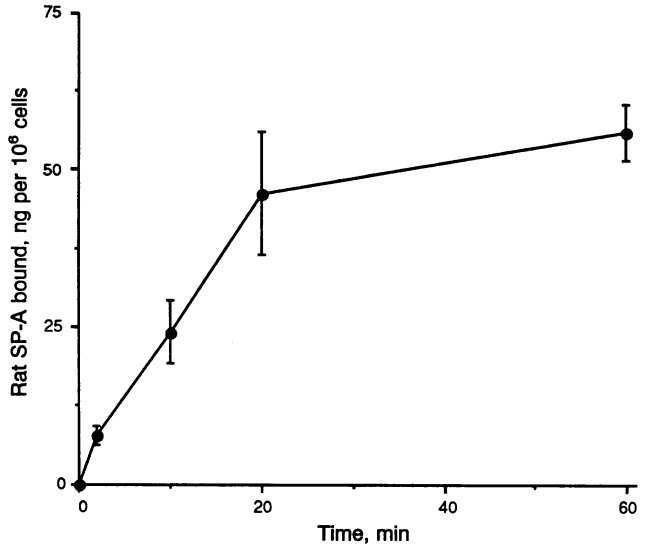

FIG. 3. Time course of binding of rat SP-A (1 μg/ml) to freshly isolated type II cells. Cells (2–2.5 × 10^6 cells per ml of binding medium) were incubated at 4°C. Cells were separated from the medium and washed by centrifugation. Data shown are mean ± SEM, n = four to six experiments.

to type II cells in primary culture were similar when calculated either by slope peeling or by measuring binding in the presence of excess unlabeled SP-A (data not shown).

Inhibitors of Binding to Type II Cells. Heating SP-A at 90°C for 3 min reduced binding by 75–80%. Binding was largely dependent on calcium since inclusion of EGTA in the medium reduced binding by approximately 70% (Table 1). Excess unlabeled SP-A reduced the binding of iodinated rat SP-A both to freshly isolated type II cells (data not shown) and to isolated type II cells in primary culture by approximately 74%. Collagen (type IV, from human placenta) reduced binding to a lesser extent. Freshly isolated type II cells that were pretreated with trypsin just prior to the binding assay bound less SP-A than control cells (50 ± 14% less than the non-trypsin-treated cells, n = 5).

Since SP-A is a lectin (23), the effects of carbohydrates and lectins that could potentially compete for binding sites were tested. Cells were preincubated with mannose, galactose, glucose, or *N*-acetylglucosamine (at 60 mM), mannan or

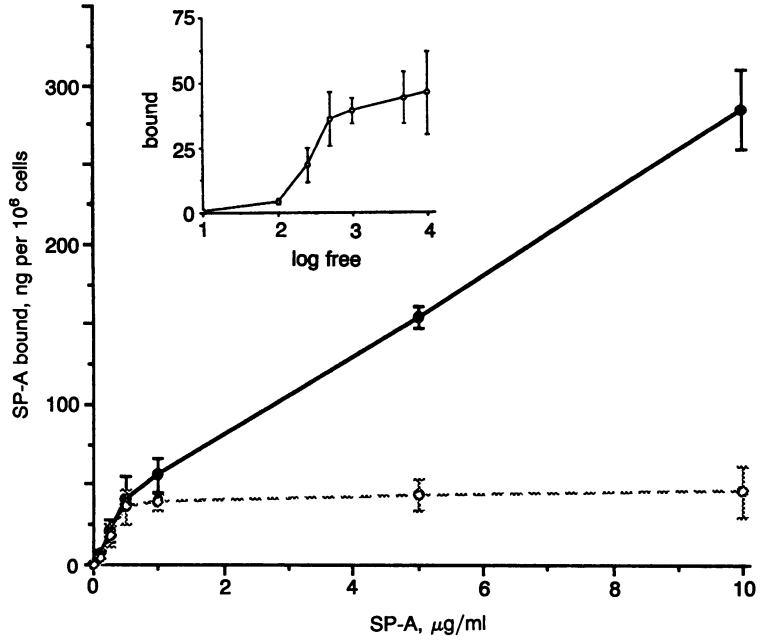

FIG. 4. Concentration-dependent binding of rat SP-A to freshly isolated type II cells. Incubation condition and analyses were as described in Fig. 3. Specific binding (○) was calculated by subtracting nonspecific binding (as estimated by curve peeling) from total binding (●). Data shown are mean ± SEM, n = 3. (*Inset*) Klotz plot of the binding data. The x axis is the log of the concentration free SP-A (ng/ml), and the y axis is the amount of specifically bound SP-A (ng per 10^6 cells).

Table 1. Inhibition of binding of SP-A to lung cells

Inhibitor	Radiolabeled SP-A bound, % of control		
	Type II cells	Macrophages	Fibroblasts
None	100 (4)	100 (4)	100 (7)
Excess unlabeled SP-A	26 ± 5 (5)	47 ± 14 (10)	38 ± 7 (11)
Collagen	59 ± 5 (3)	70 ± 5 (2)	40 ± 1 (2)
EGTA	32 ± 4 (11)	42 ± 9 (11)	79 ± 18 (10)
Heat-treated SP-A	22 ± 7 (3)		

Type II cells in primary culture were incubated at 4°C with radiolabeled SP-A (1 μg/ml) for 60 min alone or in the presence of the following inhibitors: excess unlabeled SP-A (50 μg/ml), collagen (100 μg/ml), or 0.2 mM EGTA. Heat-treated SP-A was warmed to 90°C for 3 min prior to addition to freshly isolated type II cells. Control (100%) binding is as follows: for type II cells, 766 ± 148 pg of SP-A per μg of cell protein; for macrophages, 952 ± 168 pg of SP-A per μg of cell protein; and for fibroblasts, 523 ± 92 pg of SP-A per μg of cell protein. Data shown are mean ± SEM. Numbers of experiments are in parentheses.

invertase (at 0.5 mg/ml), or the lectins *Maclura pomifera* or *concanavalin A* (at 100 μg/ml) for 30 min at 4°C prior to the addition of SP-A at 1 μg/ml. None of the sugars or lectins that were tested reduced binding.

Binding of SP-A to Other Lung Cells. SP-A also bound to alveolar macrophages and fibroblasts (Table 1). The binding to fibroblasts was only slightly inhibited by chelation of calcium, whereas the binding to macrophages was inhibited by approximately 58%. Binding to both macrophages and fibroblasts was reduced by an excess of unlabeled SP-A and by collagen.

Binding of CRF to Isolated Lung Cells. CRF bound to a greater extent to type II cells than to either fibroblasts or macrophages (Table 2). Excess unlabeled SP-A inhibited binding of CRF to type II cells but not to fibroblasts or macrophages. Binding of CRF to type II cells was reduced by approximately 60% by EGTA. Isolated type II cells that were pretreated with trypsin just prior to the binding assay bound less CRF (89 ± 4% less than the non-trypsin-treated cells, $n = 3$).

DISCUSSION

The goal of the present studies was to characterize the binding of SP-A to freshly isolated alveolar type II cells and to determine if the binding properties are characteristic of a high-affinity receptor. Binding of rat SP-A to isolated type II cells was saturable and largely dependent on calcium. Thus, the SP-A-dependent enhancement of lipid uptake (9), the inhibition of secretion (10–12), and the binding of SP-A to type II cells all require calcium. Binding of labeled SP-A was reduced by the presence of excess unlabeled SP-A and by heat treatment of the protein. Trypsin treatment of the type II cell surface reduced binding. The results suggest that type II cells have high-affinity binding sites for SP-A and are consistent with the possibility that type II cells have surface receptors for SP-A.

Meaningful interpretation of our estimates of affinity and receptor number requires that the radiolabeled SP-A accurately reflect the properties of native SP-A and that isolated type II cells are similar to those *in vivo*. We found that the radiolabeled SP-A retained its ability to enhance lipid uptake and to react with polyclonal antibody. Our estimate of the molecular mass of SP-A in solution agrees with that predicted from the study of Voss *et al.* (24) in which the macromolecular structure of SP-A was characterized by rotary shadowing and electron microscopy.

The validity of using isolated type II cells as accurate representatives of cells *in vivo* can be questioned. It is well established that type II cells do not maintain their differentiated state in primary culture and most do not divide (25, 26). For this reason and because we have demonstrated (9) that SP-A enhances lipid uptake in freshly isolated type II cells, we chose to characterize most extensively the binding to freshly isolated cells. However, it is possible that any preparation of type II cells may not be in a steady state with respect to the number of receptors on their surfaces. Estimates of affinity and receptor number are also complicated by the fact that SP-A can self-associate in solution (27). Therefore, these estimates should be interpreted cautiously. Our estimate of a $K_{1/2}$ of approximately 5×10^{-10} M is within the range of published values for other receptor–ligand interactions (e.g., refs. 28–31).

Our observation that SP-A binds with high affinity to freshly isolated type II cells agrees with the observation of Kuroki *et al.* (14) that type II cells maintained for 1 day in primary culture bind SP-A with high affinity. However, our actual estimates of $K_{1/2}$ and receptor number are different. We estimate that type II cells have approximately 40,000 receptors and an $K_{1/2}$ of 5×10^{-10} M. Kuroki *et al.* (14) report a similar $K_{1/2}$ (6.4×10^{-10} M) but a higher receptor number (135,000 receptors). One possible explanation for this difference is that receptors on freshly isolated type II cells may have been damaged by exposure to elastase during the isolation procedure and that the cells in culture have recovered. We found that treatment of freshly isolated type II cells again with elastase immediately before the binding assay was carried out only modestly reduced binding (15% of control) compared to trypsin. In addition Kuroki *et al.* (14) performed their binding assays at 37°C and our binding assays were done at 4°C. Another possible explanation is that our sources of SP-A differed. Kuroki *et al.* (14) treated rats with silica to enhance the SP-A content in lavage. SP-A isolated from silica-treated rats was reported to have a molecular mass of 1.6 MDa, which is approximately 2–3 times greater than the size of rat SP-A or recombinant human SP-A expressed by Chinese hamster ovary cells (27). Another major difference is that we found that trypsin treatment of the type II cell surface just prior to the binding assay resulted in a significant reduction in binding of both SP-A and CRF. In contrast, Kuroki *et al.* (14) were unable to demonstrate that protease treatment of the type II cells reduced binding. However, they

Table 2. Binding of CRF to isolated lung cells

Lung cells	CRF bound, pg of CRF per μg of cell protein	CRF bound in presence of SP-A, % of control
Type II cells	99 ± 19 (4)	47 ± 10 (9)
Macrophages	62 ± 16 (4)	99 ± 25 (7)
Fibroblasts	24 ± 7 (4)	131 ± 33 (7)

Cells in primary culture were incubated at 4°C with CRF (1 μg/ml) for 60 min alone or with unlabeled SP-A (50 μg/ml). Data shown are mean ± SEM. Binding in the presence of excess unlabeled SP-A is expressed as a percentage of the amount of binding that occurred in the absence of excess unlabeled SP-A (% of control). Numbers of experiments are in parentheses.

used 20 times less trypsin than we did, presumably because higher concentrations may have resulted in detachment of cells from the tissue culture plates.

Because SP-A has sequence homology with several mannose-binding proteins (32) and was shown to bind several sugars (23), we evaluated the effects of various potentially competing sugars and lectins on binding of SP-A. Since none of the substances reduced binding, it is possible that SP-A interacts with the type II cells independent of binding to carbohydrates. However, it is also possible that the carbohydrates we tested do not have high enough affinities to effectively compete for binding sites.

We observed that intact SP-A bound specifically (i.e., binding could be reduced by excess unlabeled SP-A) to type II cells, macrophages, and fibroblasts. Because SP-A contains a collagen-like domain (33) and because we have not yet been able to isolate a purified preparation of the collagen-like domain of SP-A, we tested the effects of commercially prepared collagen on binding of intact SP-A. Collagen reduced the binding of radiolabeled SP-A to all three cell types. Voss *et al.* (24) concluded that SP-A has a similar structure to that of complement component C1q, which also contains a collagen-like domain. Tenner *et al.* (34) observed that both C1q and SP-A enhance the phagocytosis by macrophages of sheep erythrocytes opsonized with IgG or with IgM and complement. Tenner and Cooper (31) have demonstrated that C1q binds to peripheral blood mononuclear cells by a receptor-mediated, ionic strength-dependent process and have suggested that it is the collagen-like portion of the C1q molecule that contains the receptor-binding site. These observations suggest that SP-A and C1q may interact with several types of cells through their collagen-like domains. (However, we have not excluded the possibility that a direct interaction between SP-A and collagen reduced SP-A binding to the cells.)

Human CRF (i.e., the non-collagen-like domain) bound to type II cells to a greater extent than to macrophages or fibroblasts. The binding of CRF to only type II cells was inhibited by excess unlabeled SP-A. We speculate that SP-A may interact with cells through a variety of mechanisms, including association with cell surface lipids. It has been shown that SP-A binds to lipids (35–37) and that lipid binding does not require calcium. The CRF prepared in our laboratory by collagenase treatment of human SP-A binds very poorly to lipid mixtures or to solvent-extracted surfactant lipids (S.H., unpublished observations). Ross *et al.* (19) also reported that a canine CRF bound weakly to lipid. Therefore, the CRF binding may reflect better the association of SP-A with nonlipid components of the cell surface. Our data are consistent with the possibility that type II cells may have receptors that recognize the carboxyl-terminal portion of SP-A. However, SP-A binds to several types of cells, perhaps through a variety of mechanisms, and possibly to more than one type of receptor. Additional studies are required to elucidate the detailed nature of the interaction of SP-A with cell surface receptors.

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