

# Serum SP-D is a marker of lung injury in rats

TIANLI PAN,<sup>1</sup> LARRY D. NIELSEN,<sup>1</sup> MARTIN J. ALLEN,<sup>1</sup> KATHLEEN M. SHANNON,<sup>2</sup>  
JOHN M. SHANNON,<sup>2</sup> MOISES SELMAN,<sup>3</sup> AND ROBERT. J. MASON<sup>1,4</sup>

<sup>1</sup>Department of Medicine, National Jewish Medical and Research Center, Denver 80206;

<sup>4</sup>Department of Medicine, University of Colorado Health Sciences Center, Denver, Colorado 80206;

<sup>2</sup>Pulmonary Cell Biology, University of Cincinnati, Cincinnati, Ohio 45229; and

<sup>3</sup>National Institute of Respiratory Disease, Mexico City DF14080, Mexico

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**Pan, Tianli, Larry D. Nielsen, Martin J. Allen, Kathleen M. Shannon, John M. Shannon, Moises Selman, and Robert. J. Mason.** Serum SP-D is a marker of lung injury in rats. *Am J Physiol Lung Cell Mol Physiol* 282: L824–L832, 2002. First published December 14, 2001; 10.1152/ajplung.00421.2000.—Pulmonary surfactant protein D (SP-D) is expressed in alveolar type II and bronchiolar epithelial cells and is secreted into alveoli and conducting airways. However, SP-D has also been measured in serum and is increased in patients with acute respiratory distress syndrome, pulmonary fibrosis, and alveolar proteinosis. To demonstrate that SP-D can be measured in rat serum, we instilled rats with keratinocyte growth factor, which produces type II cell hyperplasia and an increase in SP-D in bronchoalveolar lavage fluid (BALF). To evaluate serum SP-D as a biomarker of lung injury, we examined several injury models. In rats treated with 1 unit of bleomycin, serum SP-D was elevated on *days 3, 7, 14, and 28* after instillation, and SP-D mRNA was increased in focal areas as detected by *in situ* hybridization. However, there was no increase in whole lung SP-D mRNA when the expression was normalized to whole lung 18S rRNA. After instillation of 2 units of bleomycin, the serum levels of SP-D were higher, and SP-D was also increased in BALF and lung homogenates. In another model of subacute injury, serum SP-D was increased in rats treated with paraquat plus oxygen. Finally to evaluate acute lung injury, we instilled rats with HCl; SP-D was increased at 4 h after instillation. Our data indicate that serum SP-D may be a useful indicator of lung injury and type II cell hyperplasia in rats.

bleomycin injury; paraquat injury; HCl injury; keratinocyte growth factor; alveolar type II cells; surfactant protein D

A SERUM MARKER OF LUNG INJURY would be very useful for evaluating inhalational exposures and monitoring lung injury and treatment protocols without the need for biopsy or lavage. In acute respiratory distress syndrome (ARDS) several types of serum markers have been evaluated with some success (22, 27). Factors that are lung specific such as the surfactant proteins appear to be more promising than general markers of inflammation, which may arise from other organs in reaction to sepsis. Of the lung-specific markers, serum levels of surfactant protein (SP)-A, -B, and -D have been re-

ported to be increased in ARDS (10, 15). In addition, serum levels of SP-A and SP-D have found to be increased in individuals with pulmonary fibrosis and alveolar proteinosis (13, 22, 29). In patients with idiopathic pulmonary fibrosis and sarcoidosis, serum SP-D appears to be slightly more specific and sensitive than SP-A in terms of predicting the extent of parenchymal disease and survival (13).

SP-D is a collagenous glycoprotein and a member of the collectin family of calcium-dependent lectins that serve an important role in innate immunity (7, 24). Rat SP-D is a multimeric protein, most commonly consisting of 12 identical monomers, each of which has a molecular mass of 43 kDa. In the lung, SP-D is found in alveolar type II cells and bronchiolar cells but is not a component of tubular myelin or lamellar bodies. SP-D is thought to function primarily in host defense and binds to viruses, bacteria, fungi, and pneumocystis (7, 24). However, its precise physiological role is not known, and the phenotype of SP-D-deficient mice suggests that SP-D alters the catabolism of surface active material (5, 21). In humans, SP-D is primarily restricted to the lung, although there are reports of low-level expression in the prostate, pancreas, parotid gland, sweat glands, and other mucosal surfaces (23). In rats, SP-D is expressed in the lung and in the stomach and at low levels in other areas such as the middle ear and intestine (11). Although SP-D is a large molecule and might seem unlikely to enter the systemic circulation, SP-D has been shown to be in human serum by Western blotting (29).

The purposes of this study are to determine whether SP-D could be measured in rat serum, to define the time course for elevation during lung injury, and to compare serum levels to levels in lavage and whole lung. Our hypothesis is that serum SP-D will be a useful biomarker of lung injury in rodents as well as in humans.

## MATERIALS AND METHODS

**Materials.** Human recombinant keratinocyte growth factor (KGF) was a kind gift of Amgen (Thousand Oaks, CA) and

Address for reprint requests and other correspondence: R. J. Mason, Dept. of Medicine, National Jewish Medical & Research Center, 1400 Jackson St. -K625, Denver, CO 80206 (E-mail: masonb@njc.org).

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prepared in the diluent provided by Amgen. Saline was used as the vehicle control. Monoclonal antibodies IIE11 and VIF11 to rat SP-D were purchased from Bachem Bioscience (King of Prussia, PA).

**Animal instillations.** Pathogen-free adult male Fischer 344 rats (Harlan, Indianapolis, IN) weighing 180–250 g were used unless otherwise stated. The rats were anesthetized with 2 ml/kg of a solution of 0.5 ml ketamine (100 mg/ml; Aveco, Fort Dodge, IA), 0.5 ml xylazine (20 mg/ml; Mobay Animal Health Division, Shawnee, KS), and 1 ml normal saline. Once anesthetized, animals were weighed, positioned supine, and intubated orally with a 16-gauge intravascular Teflon catheter (Qick-Cath; Baxter, Deerfield, IL) under direct visualization with a fiberoptic light (Dolan-Jenner Industries, Lawrence, MA). The catheter was curved before intubation so that it could be directed toward the left bronchus. Intratracheal intubation was evaluated by assessing chest and abdominal movement with inflation of air. A very fine catheter (~9.5 cm long, which equals the distance from rat mouth to the left lung), connected to a Luer stub adapter, was curved similarly to the Teflon catheter so that drug or saline could be delivered to the left lung.

**KGF instillation.** Fischer 344 rats were instilled intrabronchially with 1 mg of KGF in 0.3 ml of the KGF diluent or 0.3 ml of saline into the left lung. Uninstilled rats served as controls. The rats were killed on *day 2* after KGF instillation, which is the time of maximal type II cell proliferation (34). Blood was collected by cardiac puncture, and serum was prepared and stored at  $-20^{\circ}\text{C}$  until used for SP-D measurement.

**Bleomycin.** Pathogen-free adult male Fischer 344 rats were anesthetized, and 1, 1.5, or 2 units of bleomycin sulfate (Pharmacia, Kalamazoo, MI) were dissolved in a volume of 0.5 ml of sterile saline (treatment group) or 0.5 ml of sterile saline (vehicle control) were instilled into the left lung. In all experiments there was also a group of uninstilled controls. The animals were killed on *days 1, 3, 7, 14, 21, and 28* after bleomycin instillation. At the time of death, serum, left lung, and lavage fluid from the left lung were collected.

**Paraquat plus oxygen model of pulmonary fibrosis.** These studies were performed in Mexico City in accordance with a protocol described previously (3). In brief, Wistar rats were housed continuously in clear Plexiglas living chambers flushed with either room air (controls) or oxygen kept at a concentration of 75%. Beginning on the first day of exposure to oxygen, animals received intraperitoneal injections of either sterile saline vehicle (controls) or paraquat (1,1'-dimethyl-4,4'-bipyridilium) in sterile saline at 2.5 mg/kg body wt. Injections were administered to all animals twice weekly for up to 4 wk, throughout which all animals remained in the exposure chambers with ad libitum access to water and standard rat chow. Uninjected rats were also used as controls. Rats were killed after 1, 2, 3, and 4 wk of treatment. Serum was collected for SP-D measurement.

**HCl instillation.** Fischer 344 rats were anesthetized, and 0.5 ml of HCl (0.1 N, pH 0.81) or saline was instilled into the left lung. The animals were killed 4 and 24 h after instillation. A separate group of uninstilled animals served as controls. Serum was collected for SP-D measurement. We excised the left lung en bloc, dissected it away from the heart and thymus, and gently removed any blood. The left lung was immediately weighed and then placed in a desiccating oven at  $65^{\circ}\text{C}$  for 48 h, at which point the dry weight of the left lung was measured.

**Bronchoalveolar lavage.** Immediately after death the right lung was ligated and the left lung was lavaged by intratracheal instillation of five aliquots of 5 ml of PBS. The lavage

fluid was centrifuged at 300 g for 10 min to remove the cells, and the supernatant was stored at  $-20^{\circ}\text{C}$ . The cell pellet was resuspended in 1 ml of PBS for total cell count. Differential cell counts were performed on cell pellets prepared by cyto-centrifugation (Shandon Southern Instruments, Pittsburgh, PA), and the cells were stained by hematoxylin and eosin. The percentage of each cell type was assessed by differential count of 400 cells. Total number of macrophages, polymorphonuclear cells, and lymphocytes was calculated by multiplying the total nucleated cells by the differential percentages of each cell type.

**Left lung homogenate.** After the lungs were perfused free of blood and harvested for lavage fluid, the left lung was placed in a tube and frozen rapidly in dry ice and ethanol and then stored in  $-70^{\circ}\text{C}$ . At the time of analysis, the tissue was placed in homogenizing buffer (50 mM Tris·HCl, pH 7.5, containing 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 2.5 mM *N*-ethylmaleimide) at a defined ratio of 1 g of lung tissue to 9 ml of homogenizing buffer. The lung tissue was homogenized on ice with a Polytron (Brinkman Instruments, Westbury, NY). The lung homogenate was sonicated on ice for 15 s three times, spun at 300 g for 5 min to sediment tissue debris, and then added to ELISA buffer, which contained 1% Triton X-100 and 4% defatted skim milk.

**Total protein assay.** Protein concentrations in lavage fluid and lung homogenate were measured by the bicinchoninic acid method (Pierce, Rockford, IL).

**Determination of SP-D levels by ELISA.** SP-D levels in lavage fluid, lung homogenate, and serum were determined by standard ELISA with antirecombinant SP-D polyclonal antibodies as reported previously (20, 26, 34). The recombinant rat SP-D expressed in Chinese hamster ovary cells, which was used as the standard, and the rabbit polyclonal IgG antibodies to rat SP-A and rat SP-D were the kind gifts of Dr. Dennis Voelker, Denver, CO. For validation of the ELISA for serum measurements, we used monoclonal antibodies to rat SP-D, which were designated IIE11 and biotinylated VIF11.

**Detection of rat serum SP-D by Western blot.** SP-D binds *Saccharomyces cerevisiae* in a calcium-dependent manner (2). Aliquots of rat serum from a commercial source (Pel-Freez, Rogers, AR), Fischer 344 control rat serum (not instilled), or serum from Fischer 344 rats instilled with 2 units of bleomycin 7 days earlier were dialyzed against Tris-buffered saline (TBS), pH 7.4, containing 1 mM EDTA. After dialysis the sera were adjusted to 5 mM  $\text{Ca}^{2+}$  with  $\text{CaCl}_2$ , and 1.1 ml of each was incubated with  $10^8$  paraformaldehyde-fixed *S. cerevisiae* cells for 2 h at  $25^{\circ}\text{C}$  by gentle shaking. After incubation the cells were washed three times with TBS, pH 7.4, containing 5 mM  $\text{CaCl}_2$ . The cells were then incubated twice with 60  $\mu\text{l}$  of TBS, pH 7.4, containing 5 mM  $\text{CaCl}_2$  and 50 mM inositol for 1 h at  $25^{\circ}\text{C}$  to remove bound SP-D. After the incubations the cells were centrifuged and the supernatants were removed, pooled, and analyzed for SP-D by immunoblot. The samples were placed in Laemmli buffer, boiled, and electrophoresed in 8–16% Novex gels before transfer and detection with a rabbit anti-rat SP-D IgG.

**Ribonuclease protection assay.** Animals were killed, and left lung was homogenized in 4 M guanidinium isothiocyanate with a Polytron and stored at  $-70^{\circ}\text{C}$  until further use. Total RNA was isolated by centrifugation for 18 h at 150,000 g through a cushion of 5.7 M CsCl. mRNA levels of SP-D were determined with a ribonuclease protection assay (RPA). Fragments of 245 bp for SP-D were isolated by polymerase chain reaction using full-length rat cDNAs as templates. The forward primers included a *Bam*HI restriction site added to the 5'-end and the backward primers included an *Eco*RI

restriction site added to the 5'-end to facilitate directional cloning into pGEM 4Z (Promega, Madison, WI). The primers for SP-D were 5'-CGGATCCCGGAAGAGCCTTTTGAGGATG-3', coding sense and corresponding to nucleotides 831–851, and 5'-GGAATTCACAGTTCTGTGCCCTCCATTG-3', coding antisense and corresponding to nucleotides 1,054–1,075. The probe transcribed from this clone identified a fragment of 245 bp.

The vectors were linearized with *Bam*HI, and radiolabeled antisense probes were transcribed in vitro using a commercially available kit (Promega) and  $\alpha$ -[<sup>32</sup>P]CTP (800 Ci/mmol; ICN). RNA probes were purified on an 8% polyacrylamide/7 M urea gel. An 18S rRNA probe, which was synthesized using pT7 RNA 18S template, T7 MEGA shortscript kit (both from Ambion, Austin, TX), and  $\alpha$ -[<sup>32</sup>P]CTP, was used as an internal standard for RNA quantitation. Ten micrograms of total cellular RNA were hybridized at 45°C for 24 h with  $6 \times 10^4$  counts/min (cpm) of the antisense probe of SP-D. Probe not protected by hybridization with target RNA was digested with a mixture of RNase A and T1. The protected RNA duplex fragments were precipitated, resuspended, and separated on an 8% polyacrylamide/7 M urea gel. The gel was dried and exposed to Hyper film (Amersham Life Science Products, Arlington Heights, IL) at -70°C. Radioactive counts were obtained from protected fragments by Image-Quant analysis (Molecular Dynamics, Sunnyvale, CA) and normalized to 18S rRNA.

**In situ hybridization.** In situ hybridization was performed essentially as described previously (9). Briefly, the left lung was washed free of blood with RNase-free PBS and inflated in freshly prepared 4% paraformaldehyde at 25 cmH<sub>2</sub>O for 2 h. The left lung was kept in 4% paraformaldehyde overnight at 4°C and transferred the next day to 70% ethanol for embedding in paraffin. Sections 4–6  $\mu$ m thick were dewaxed in xylene, rehydrated in a graded series of ethanol, and then refixed in 4% paraformaldehyde. The refixed sections were treated with 20  $\mu$ g/ml of proteinase K (Boehringer Mannheim, Indianapolis, IN) in 50 mM Tris-HCl (pH 8.0) and 5 mM EDTA for 5 min at room temperature. The sections were refixed in 4% paraformaldehyde and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min at room temperature. Sections were dehydrated through a graded series of ethanol and dried in air. Radiolabeled sense and antisense riboprobes were transcribed from a 1,265-bp cDNA for rat SP-D that was previously cloned into plasmid pGEM 4Z (Promega). Riboprobes were transcribed as previously described (9) except that [<sup>33</sup>P]UTP (NEN-DuPont) was substituted for [<sup>35</sup>S]UTP.

Sections were hybridized with  $1.5 \times 10^6$  cpm of either sense or antisense riboprobe in 15  $\mu$ l of hybridization solution (9) in humidified chambers for 18 h at 55°C. Hybridization with radiolabeled sense riboprobes was done as a control. Hybridized sections were washed in  $5 \times$  SSC at 55°C twice and then at high stringency in  $50 \times$  formamide,  $2 \times$  SSC at 65°C for 30 min. The sections were rinsed three times and treated with 20  $\mu$ g/ml of RNase A (Sigma) and 5 U/ml of RNase T1 (Boehringer Mannheim) for 30 min at 37°C. The slides were washed at high-stringency wash again, followed by successive washes in  $2 \times$  SSC and  $0.1 \times$  SSC. The sections were dehydrated, air dried, and then dipped in Kodak NTB-2 nuclear track emulsion. Autoradiograms were developed after 1–2 days for SP-D with Kodak D19 developer at 15°C and fixed with Kodak fixer. Sections were lightly counterstained with hematoxylin and viewed for photomicrography.

**Statistics.** All data are expressed as means  $\pm$  SE. Analysis of variance was used to test for overall significance among three or more means. Tukey's honestly significant difference

post hoc multiple comparison procedure was used to determine whether paired comparisons were different. Where appropriate, dependent and independent *t*-tests were also performed. A value of  $P < 0.05$  was considered statistically significant for all analyses. The SAS statistical package (SAS Institute, Cary, NC) was used for all analyses.

## RESULTS

**Serum SP-D in rats instilled with KGF.** The initial study was designed to determine whether SP-A or SP-D could be measured in rat serum and whether there were increases with type II cell hyperplasia. Instillation of KGF into the normal rat lung produces transient type II cell proliferation and an increase in levels of SP-A and SP-D in lavage (34). KGF increased serum SP-D from  $36 \pm 3$  ng/ml in uninstilled controls to  $132 \pm 8$  ng/ml in rats instilled with KGF (Fig. 1). In these same samples, we also measured serum SP-A, which was  $<5$  ng/ml in uninstilled controls, animals instilled with saline, and animals instilled with KGF. In addition, from previous experiments we knew that our rabbit polyclonal antibody to SP-D recognized SP-D but not SP-A, whereas our rabbit polyclonal antibody to SP-A also recognized some SP-D by Western blotting. Because of the low level of SP-A detected in rat serum and the concern about cross-reactivity, measurements of SP-A in rat serum were not evaluated further.

To confirm that the value measured by the ELISA, which used rabbit polyclonal antibodies to rat SP-D, was accurate, we used an ELISA based on a monoclonal antibody for detection to verify our results. Initially the monoclonal antibody VIF11 was used to capture SP-D on the microtiter plate and the biotinylated antibody IIE11 was used to detect SP-D (19). However,

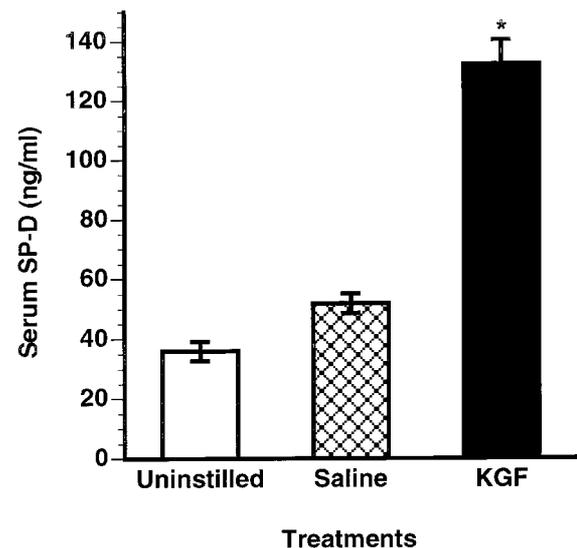


Fig. 1. Serum surfactant protein (SP)-D levels in rats treated with keratinocyte growth factor (KGF). Fischer 344 rats were instilled intrabronchially with KGF or sterile saline. Uninstilled rats served as controls. The rats were killed 2 days after KGF instillation, and serum was collected for SP-D measurement by ELISA. The results represent the means  $\pm$  SE from 3–6 animals/group. \* $P < 0.05$ , KGF group compared with uninstilled controls and saline groups.

the monoclonal capture antibody VIF11 was too insensitive to detect SP-D in serum. Therefore, the rabbit IgG antibody was used to bind SP-D to the plate and the monoclonal antibody VIF11 was used to detect SP-D. With this hybrid sandwich ELISA, measurement of SP-D in serum was within 10% of the values determined by the polyclonal ELISA. Hence, we concluded that SP-D was present in rat serum and that the rabbit polyclonal assay could be used to measure the concentration.

**SP-D levels after bleomycin instillation.** In our initial study we instilled 1.0 unit of bleomycin, which was designed to produce a moderate amount of fibrosis (8, 18). As shown in Fig. 2, there was a modest increase in serum SP-D, and the peak serum level occurred on day 7. To correlate with the serum levels, lung mRNA for SP-D was also determined in these animals. Left lung SP-D mRNA levels were measured by an RPA, and the expression was localized by in situ hybridization. Figure 3 shows SP-D mRNA levels from left lung treated with 1 unit/0.5 ml bleomycin compared with saline instillation or uninstilled controls. These data were normalized by 18S rRNA. There is no statistical significance between bleomycin treatment and saline or uninstilled controls. There was, however, a slight reduction in the ratio of SP-A, SP-B, and SP-C to 18S rRNA (data not shown). It should be noted that the left lung mRNA data are normalized to 18S rRNA, which would include RNA of inflammatory cells recruited to the injured lung. However, by in situ hybridization, there were focal areas of intense SP-D mRNA expression after bleomycin treatment (1 unit/0.5 ml, left lung) (Fig. 4). These areas of increased expression are not seen in the saline or uninstilled controls on days 3, 7, and 14. By immunocytochemistry there were also focal areas of intense staining for SP-D (data not shown). These areas were adjoining small airways and ap-

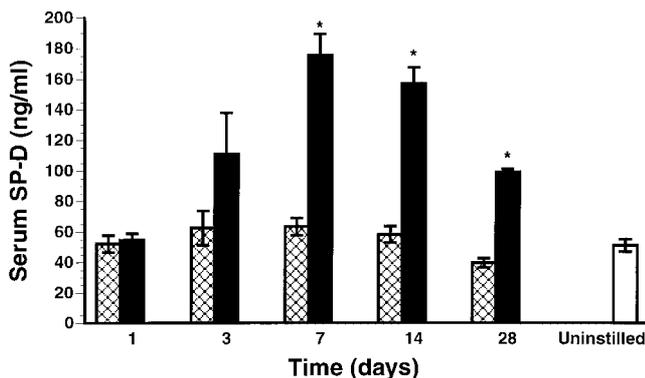


Fig. 2. Serum SP-D levels in rats treated with 1 unit of bleomycin. Fischer 344 rats were instilled intrabronchially into the left lung with 1 unit of bleomycin or saline. Uninstilled rats served as controls. Rats were killed on days 1, 3, 7, 14, and 28 after bleomycin treatment, and serum was collected for SP-D measurement by ELISA. Crosshatched bars, saline treatment; solid bars, bleomycin treatment; open bar, untreated controls. The results are the means  $\pm$  SE from 5–10 animals in each group. \* $P < 0.05$ , bleomycin-injured group compared with the saline group at the same time points or with uninstilled controls.

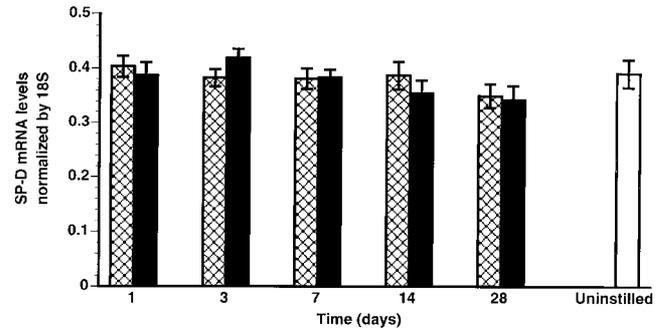


Fig. 3. SP-D mRNA levels. Fischer 344 rats were instilled intrabronchially with 1 unit of bleomycin or saline. The uninstilled rats served as controls. The rats were killed on days 1, 3, 7, 14, and 28 after bleomycin treatment, and the left lung was harvested for SP-D mRNA measurement by a ribonuclease protection assay. Cross-hatched bars, saline treatment; solid bars, bleomycin treatment; open bar, uninstilled controls. The results are the means  $\pm$  SE from 3–6 animals/group. There are no statistical significances between the different groups.

peared to be the same as the areas of increased expression of SP-D observed by in situ hybridization.

To compare the time course for relative changes in serum, lavage, and lung levels of SP-D, we used higher concentrations of bleomycin. We increased the dose of bleomycin to 1.5 units per left lung. However, there was only a modest increase in the serum concentration of SP-D on day 14 ( $214 \pm 28$  vs.  $58 \pm 4$  vs.  $56 \pm 13$  ng/ml, bleomycin vs. saline vs. uninstilled control, respectively; means  $\pm$  SE,  $n = 6$ ,  $P < 0.01$ ). Therefore, we increased the dosage of bleomycin further to 2 units instilled into left lung. SP-D levels in serum, bronchoalveolar lavage fluid (BALF), and lung homogenate for these experiments are shown in Fig. 5. Serum SP-D levels were much higher than those seen with 1.0 unit of bleomycin, but the pattern was similar to that observed with the lower dose. The highest concentration measured occurred on day 7 (Fig. 5C). SP-D levels in BALF (Fig. 5A) and lung homogenates (Fig. 5B) were also increased after bleomycin injury. Compared with changes in SP-D serum concentration, the peak of SP-D in BALF and lung homogenates occurred earlier, on day 3. SP-D in BALF returned to normal 21 days after bleomycin instillation, but SP-D in lung homogenate and serum remained elevated.

We used immunoblotting to demonstrate that the antigen recognized in rat serum that increased after bleomycin instillation was SP-D (Fig. 6). Equivalent volumes of normal rat serum and serum from rats that had been instilled with bleomycin 7 days previously were dialyzed and adsorbed with yeast (2), and the SP-D was eluted with 50 mM inositol. By immunoblotting, SP-D was identified in serum and the relative amount increased with bleomycin, similar to the results determined by ELISA.

**Serum SP-D levels in rats injured with paraquat and oxygen.** To determine if an increase in serum SP-D was seen in another form of subacute injury, we measured serum SP-D in rats injected with paraquat and exposed to oxygen (3, 28). For the paraquat experiments, serum

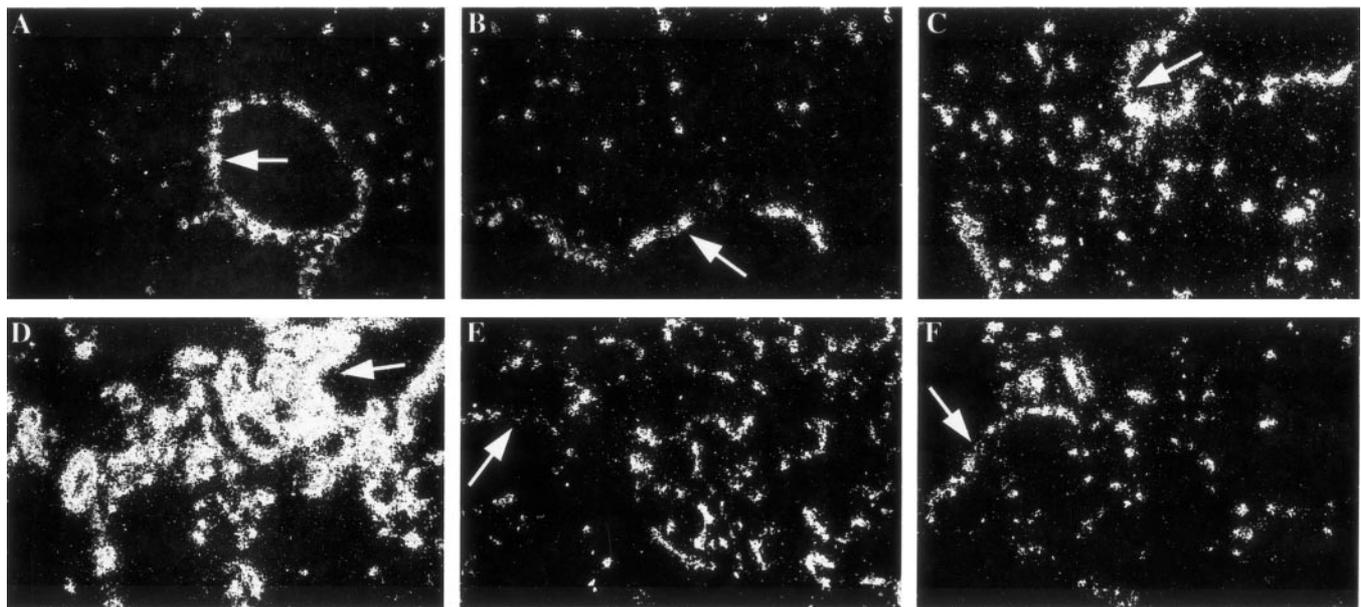


Fig. 4. In situ hybridization of SP-D after bleomycin. The rats were killed on *days 1 (B), 3 (C), 7 (D), 14 (E), and 28 (F)* after bleomycin instillation. A: an uninstilled control. The arrows point to small bronchioles. The left lung was washed free of blood with RNase-free PBS and fixed in 4% paraformaldehyde overnight at 4°C and transferred the next day to 70% ethanol for embedding in paraffin. In situ hybridization with rat antisense and sense SP-D probes were performed on tissue sections of the left lung. The sense probes were negative (data not shown).

SP-D was measured in untreated animals, animals injected with saline and exposed to ambient air, and animals injected with paraquat and exposed to 75% oxygen. This model of lung injury produces significant fibrosis and has been well defined (3, 28). As seen in Fig. 7, there was an increase in serum SP-D. In these studies, there were no differences in animals treated for 1, 2, 3, or 4 wk, and hence the data were pooled. In these experiments the mRNA for the surfactant proteins was also measured by a RPA and normalized to whole lung 18S rRNA. There was also no change in SP-D mRNA level in rats treated with paraquat and oxygen compared with control lungs when normalized to 18S rRNA (data not shown).

**Serum SP-D levels after HCl instillation.** The previous models are complicated because of the coexistence of type II hyperplasia and lung injury. To evaluate a model of an acute lung injury at a time point before type II cell proliferation could occur, we instilled rats with HCl, which is a reproducible means of producing acute lung injury (6, 33). In this model, HCl produces pulmonary edema as measured by an increase in the wet/dry lung weight (Fig. 8A) and an increase in serum SP-D 4 h after instillation (Fig. 8B). In lungs fixed with paraformaldehyde and instilled with agarose to prevent alveolar collapse, 4 h after instillation there is evidence of airway and alveolar injury but no marked inflammatory infiltrate or type II cell hyperplasia (17).

## DISCUSSION

Clinically, serum SP-D is a promising biomarker for acute lung injury and type II cell hyperplasia as seen in pulmonary fibrosis and other forms of interstitial lung disease (22, 29). Greene et al. (13) showed that serum

SP-D levels are increased in patients with pulmonary fibrosis and that the elevation of SP-D is related to the extent of the parenchymal lung disease and predicts survival. Serum SP-D is also increased in patients with ARDS (14). However, the exact time course, precise pathology at the time of the serum collection, sites of increased expression, and mechanism whereby SP-D is increased in serum are not known.

To investigate these problems further, we evaluated serum SP-D in rats treated with agents to produce type II cell hyperplasia, lung injury, or both. To produce transient type II cell hyperplasia without pulmonary fibrosis, we instilled KGF, which increased serum SP-D. After instillation of KGF there is a significant increase in SP-D recovered in lavage, an increased expression in airway epithelial cells as well as alveolar epithelial cells, and an increase in whole lung SP-D mRNA, whereas there is relatively little increase in lavage albumin levels (34). After instillation of an adenovirus expressing KGF, which produces extensive bronchial and alveolar epithelial proliferation, as well as more acute inflammations but no fibrosis, there is also an increase in serum SP-D and SP-D recovered in lavage fluid (25). To produce subacute lung injury and fibrosis we instilled rats with bleomycin. There was a dose-response relationship of the serum level of SP-D to the amount of bleomycin instilled. Although physiological assessment of lung injury was not done in these experiments, we previously reported that, at 7 days after instillation of 1 unit of bleomycin, there is an elevated wet/dry weight ratio of excised lungs, an increase in hydroxyproline, a reduction in total lung capacity, an increase in lavage cells and protein, and a reduction in disaturated phosphatidylcholine and

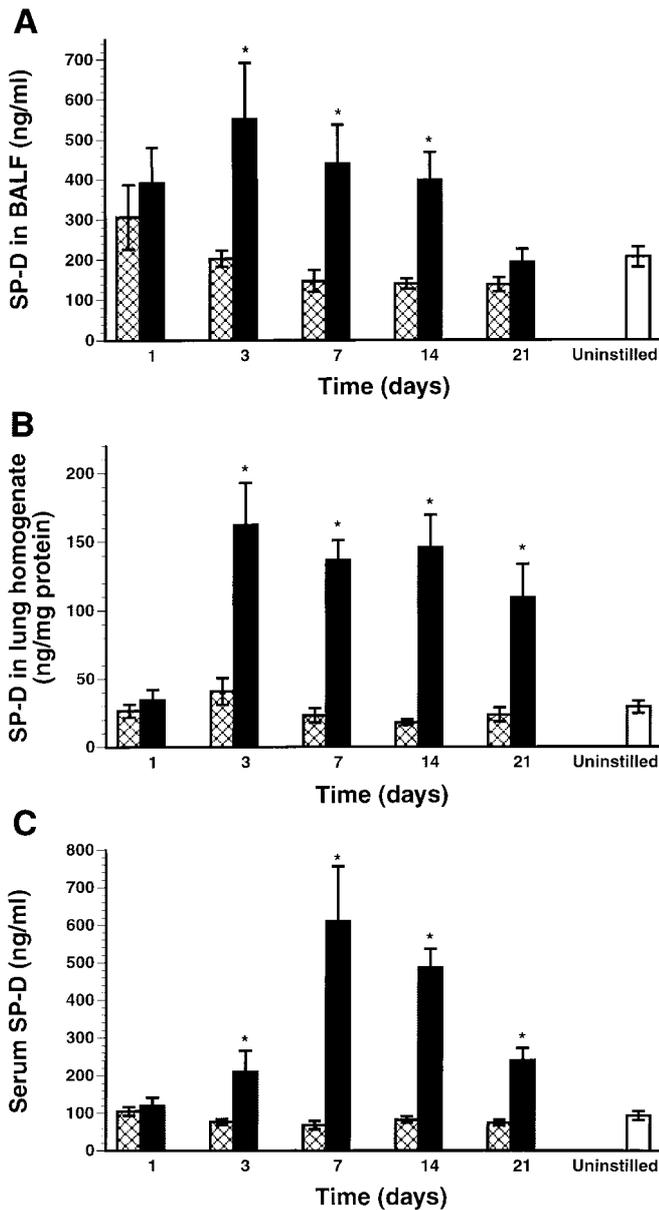


Fig. 5. SP-D in lavage fluids, lung homogenates, and serum of rats instilled with bleomycin. Fischer 344 rats were instilled intrabronchially with 2 units of bleomycin or saline into left lung. The uninstilled rats served as controls. The rats were killed on days 1, 3, 7, 14, and 21. A: SP-D in lavage. Bronchoalveolar lavage fluid (BALF) was harvested from the left lung and centrifuged to separate the supernatant from the cells. SP-D was measured by ELISA. B: SP-D in lung homogenate. The left lung was homogenized and processed as described in MATERIALS AND METHODS. C: serum SP-D. Serum was obtained at the time of death. Crosshatched bars, saline treatment; open bar, uninstilled controls; solid bars, bleomycin treatment. The results represent the means  $\pm$  SE from 6 animals/time point and treatment group. \* $P < 0.05$ , bleomycin-injured group compared with the same time in the saline group or to uninstilled controls.

phosphatidylglycerol in lavage lipids (18). We also evaluated serum SP-D in animals that are exposed to oxygen and injected with paraquat. In this form of subacute lung injury, we also demonstrated an increase in serum SP-D. The pathology and biochemical characterization of this model have been reported pre-

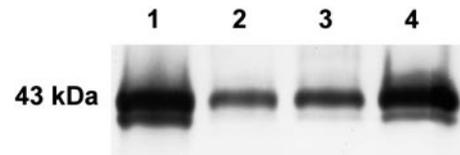


Fig. 6. Immunoblotting of SP-D in serum. SP-D was partially purified from serum by adsorption to *Saccharomyces cerevisiae*. SP-D was eluted with inositol and analyzed by immunoblotting. The samples were subjected to 8–16% SDS-PAGE under reducing and denaturing conditions, transferred to nitrocellulose, and stained for SP-D using polyclonal rabbit anti-rat SP-D antibody. The blot shows 100 ng of recombinant rat SP-D (lane 1), SP-D extracted from commercial rat serum (lane 2), SP-D extracted from control (not instilled) rat serum (lane 3), and SP-D extracted from serum of rats 7 days after bleomycin instillation (lane 4).

viously (3, 28). In addition, we previously reported increased serum SP-D in rats treated with amiodarone, which produces mild fibrosis (30). Finally, HCl was used as an agent to produce acute lung injury, and we demonstrated an increase in serum SP-D at 4 h after acid instillation. This was at a time before type II cell proliferation occurs. Hence, serum SP-D is elevated in models of type II cell proliferation without injury (KGF instillation), acute lung injury without type II cell proliferation (HCl instillation), and subacute lung injury with type II cell proliferation and fibrosis (bleomycin instillation or paraquat plus oxygen).

The precise mechanisms for the increase in the serum SP-D in acute lung injury or transient type II cell

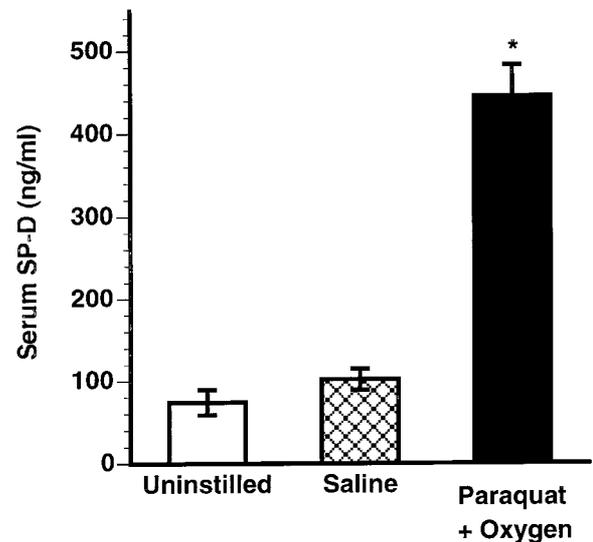
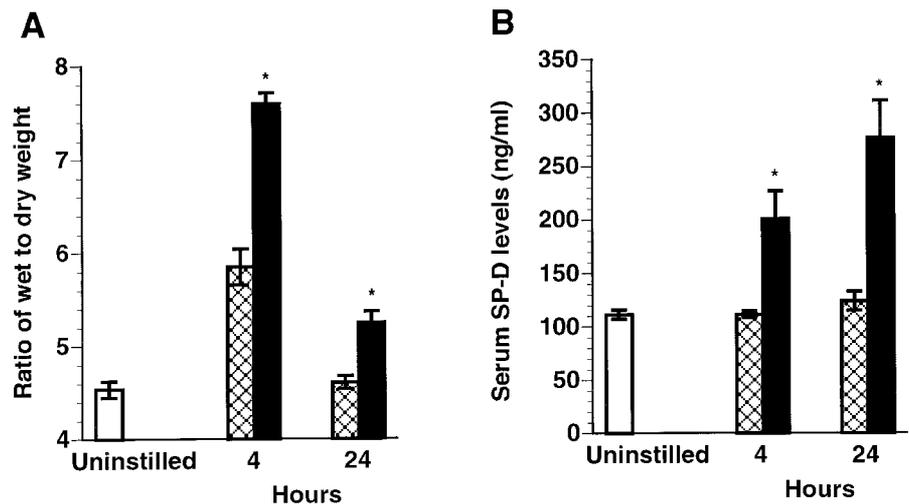


Fig. 7. Serum SP-D levels in rats injured with paraquat and oxygen. Wistar rats were injected intraperitoneally with paraquat twice weekly and exposed to 75% oxygen. Untreated rats and rats injected with saline and exposed to air served as controls. The rats were killed on wk 1, 2, 3, and 4 after the first injection of paraquat. However, the results for the treatment groups were pooled since there was no change in serum SP-D levels with time. Open bar, untreated controls; crosshatched bar, saline treatment and exposure to air; solid bar, animals injected with paraquat and exposed to 75% oxygen. The results represent the means  $\pm$  SE for 8 untreated rats, 11 injected with saline, and 19 injected with paraquat and exposed to oxygen. \* $P < 0.05$ , compared with the saline group or untreated control group.

Fig. 8. Ratio of wet to dry weight of the left lung and serum SP-D levels after HCl instillation. Fischer 344 rats were instilled intra-bronchially with HCl (0.5 ml, 0.1 N, pH 0.81) or saline (0.5 ml) into the left lung and were killed 4 and 24 h later. A separate group of uninstilled animals served as controls. **A**: the ratio of wet to dry weight of the left lung. **B**: the serum SP-D levels were determined by ELISA. Open bars, uninstilled controls; cross-hatched bars, saline instillation; solid bars, HCl instillation. The results are the means  $\pm$  SE from 5 animals/group. \* $P < 0.05$ , HCl group compared with the same time in saline group or control group.



hyperplasia have not been determined but are likely to be multiple. After KGF instillation there is an increase in SP-D in lavage fluid and an increase in the number of type II cells and airway cells that express SP-D. Hence, in this model the mechanism is likely to be complex, with increased production and an increased alveolar to interstitial gradient for diffusion. In addition, in proliferating epithelial cells there is likely a transient loss of apical-basolateral cell polarity (32). Hence, potentially there could also be some direct basolateral secretion. KGF probably does not greatly alter the epithelial barrier to protein flux based on protein measurements in lavage and other studies (4, 16, 34). After bleomycin instillation, there is focal increased expression of SP-D, an increase in lavage, and an increase in epithelial permeability. However, the left lung SP-D mRNA levels did not change compared with uninstilled controls or saline instillation, when the values were normalized by 18S rRNA. We suspect that the failure to detect an increase in SP-D mRNA after bleomycin is due to the increase of inflammatory cells, which do not express SP-D but do contain 18S rRNA. There were focal areas of increased expression, which could be seen by *in situ* hybridization or immunocytochemical staining for SP-D. There is also obvious inflammation and reduction of the epithelial barrier to protein flux. One of the problems with the bleomycin model is that the pathological changes are variable and patchy. It is difficult to assign a single pathological process to account for the increase in serum SP-D. Adamson and Bakowska (1) showed an increase in protein in lavage fluid after instillation of bleomycin, and radioactive albumin fluxes have also been used to demonstrate an increase in epithelial permeability after bleomycin (12, 31). In addition, SP-D is very similar to mannose-binding protein and probably is not rapidly cleared from the vascular compartment, although intravascular clearance studies have not been performed. Hence, we cannot absolutely exclude the possibility that the increased serum level is due to reduced intravascular clearance or a reduced volume of distribution. In experiments not shown, we studied the se-

cretion of SP-D *in vitro* and found apical secretion and very little basolateral secretion (unpublished observations). Hence, we believe that the reason for the increase in SP-D in serum after bleomycin is due to focal increased production, an increase in alveolar fluid SP-D, and an increased alveolar permeability. Serum levels of SP-D may also reflect type II cell hyperplasia and the mass of type II cells in the lung. In rats treated with KGF or a replication-deficient adenovirus expressing KGF, there is extensive type II cell hyperplasia, and serum SP-D levels rise and fall with the extent of type II cell hyperplasia. In addition, serum SP-D may also arise from airway epithelial cells, and expression in these cells is altered by KGF and lung inflammation. In rats, there is a high level of SP-D expression in nonciliated bronchiolar epithelial cells, and it is probable that the serum SP-D comes from both alveolar and bronchiolar epithelial cells.

It is possible but extremely unlikely that serum SP-D could arise from nonpulmonary sources. There is readily detectable SP-D in rat stomach and low levels of SP-D at other mucosal surfaces. In sepsis, it would be theoretically possible that SP-D in serum would arise in part from extrapulmonary sites. However, with instillation of bleomycin, HCl, and KGF, it is very unlikely that the SP-D arose from extrapulmonary sites.

The reason why there is little SP-A in rat serum is not known. In humans there are higher levels of SP-A in serum, and the level correlates with acute lung injury and pulmonary fibrosis (22). However, in rat serum we found very little SP-A by ELISA, but the low value varied with lung injury. We did not determine whether this low value was true SP-A or cross-reactivity of SP-D with our polyclonal antibody to SP-A. Because SP-A binds surfactant phospholipids avidly, SP-A may not readily leave the alveolar compartment. In the studies on human pulmonary fibrosis, there is a better correlation of the extent of parenchymal disease with serum SP-D than with serum SP-A (13). Hence, we focused on SP-D and did not examine rat serum SP-A levels further.

Although surfactant proteins have been measured by ELISA in serum previously, there have been concerns that the antibodies might be cross-reacting with some other serum antigen that coincidentally increases after lung injury. However, SP-D has been measured in human serum by monoclonal sandwich ELISAs and demonstrated in serum by Western blotting. The mean values measured in human volunteers are 43–97 ng/ml, depending on the study group and presumably genetic background of the individuals, and are similar to serum values in rats (13). Although we used a polyclonal ELISA for our measurements in rat serum, we confirmed these measurements with a monoclonal antibody for detection in parallel experiments. We also isolated SP-D from rat serum and demonstrated that the antigen had the same molecular mass as SP-D and demonstrated an increase after bleomycin treatment. There was some variation in the absolute levels of SP-D in control animals based on the batch of antigen and antibody used in the ELISA. Nevertheless, the serum concentration of SP-D in uninstilled rats was  $66 \pm 24$  ng/ml (mean  $\pm$  SD,  $n = 17$ ), which is similar to the values found in humans.

In our studies we used agents that produced fairly extensive lung injury, i.e., bleomycin, paraquat plus oxygen, or acid instillation or diffuse type II cell hyperplasia without severe injury, e.g., KGF. We suspect that the serum values reflect both alveolar epithelial leak and focal production. We do not know whether serum SP-D will be a marker of less severe injury. Potentially, SP-D may be an important marker for the effects of mild inflammation in the lung, such as with air pollution or animal models of asthma. Because of high-level expression in Clara cells and their prominence in rodent lungs, SP-D should be evaluated as a biomarker of airway disease in rodents. Expression of SP-D in airways of rodents is quite different from humans, where conducting airways have relatively little SP-D by immunocytochemistry (24).

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