Immunogenicity of Surfactant and Its Implications for Replacement Therapy*

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Introduction

The etiology and pathogenesis of neonatal and adult respiratory distress syndromes (RDS) differ widely but share the characteristic inability of the lungs to adequately oxygenate the blood. Neonatal RDS occurs in premature infants deficient in surfactant (SRF) [1]. SRF deficiency causes alveoli to collapse on expiration, resulting in progressive respiratory failure. Because neonatal RDS directly reflects deficiency of surfactant, various investigators have tried therapeutic administration of heterologous, artificial, and homologous surfactants [2–4]. These preparations generally lower surface tension *in vitro* and in animal models, and are reported to be efficacious in infants with RDS.

Adult RDS is more complex. It is a final common pathway for pulmonary insults of many different origins, ranging from traumatic to infectious. Although ARDS occurs in the presence of adequate SRF production, treatment with SRF is being tried therapeutically in an attempt to improve pulmonary function.

Our therapeutic trials of neonatal RDS were designed using human surfactant derived from amniotic fluid. As lipid-protein mixtures are generally highly immunogenic, we decided to study potential immunologically mediated complications of surfactant administration.

Subsequently, trials of Curosurf (porcine surfactant) as treatment for ARDS were initiated. For the same reasons, we felt it was important to determine whether patients so treated showed any manifestations of immunologic reactivity to surfactant or of immunogically mediated tissue damage.

One of the most potentially damaging aspects of immune reactivity to surfactant is production of circulating immune complexes between surfactant and anti-surfactant antibodies. Such immune complexes might mediate tissue damage such is seen in other diseases (e.g., post-streptococcal glomerulon-ephritis). We therefore developed an assay to detect immune complexes between surfactant and anti-surfactant antibodies [5].

Another important potential for immunologically mediated damage in these circumstances would be the ability of antibodies to surfactant to inactivate surfactant functionally. Accordingly, we examined whether anti-surfactant antibodies inhibit surfactant function in vitro.

Methods

Antibodies to Surfactant. Rabbits were immunized with the same human surfactant preparations used to treat infants with RDS. Prebleed and immune sera were collected from these animals, and absorbed exhaustively in solid phase with serum proteins from normal human blood.

Antibodies to porcine surfactant are currently being prepared. The data discussed here for patients with ARDS treated with porcine surfactant (Curosurf, the kind gift of Dr. Bengt Robertson) reflect the use of anti-human surfactant antiserum for immune complex assays.

The specificity of these antisera were determined by several methods. Using Western blotting procedures, we found that anti-SRF antisera do not bind to normal human serum proteins. These sera recognize SRF-containing lamellar bodies in human type II alveolar pneumocyte carcinoma line, A549 [5]. By immunoprecipitation and autoradiography of ^{125}I-labeled surfactant preparations, we found that these antisera bound surfactant species of 35 kd, 18 kd, and 5–7 kd (D. Strayer, et al., submitted for publication).

ELISA for Surfactant–Anti-Surfactant Immune Complexes. This assay is illustrated in Fig. 1. Immune complexes containing surfactant in complex with antibody to surfactant bind to plastic plates coated with rabbit anti-surfactant antibody via immune recognition of the surfactant portion of this immune complex. The human anti-SRF portion of the immune complex is then detected using antibody to human immunoglobulin [5].

Plasma samples from infants in surfactant treated and control groups are incubated with the anti-surfactant coated plates. Plates are then washed. Enzyme-conjugated heavy chain-specific anti-human IgG was added. Originally, horseradish peroxidase-conjugated secondary antibody was used. More recently, we have used alkaline phosphatase conjugated antibody. This secondary antibody recognizes that human IgG present bound by virtue of the human IgG being part of an immune complex with surfactant which is in turn bound to the immobilized rabbit anti-SRF antibody. Binding of the enzyme-linked anti-IgG antibody is visualized by adding substrate for the enzyme in question and reading the result on an automatic ELISA reader (Dynatech). Data are recorded as absorbance at 405 nm (alkaline phosphatase) or 490 nm (horseradish peroxidase, HPO) [6].

Data from assays using plates coated with anti-SRF are compared to identical assays in which plates are coated with albumin alone. Absorbance values from the latter are substracted from the former to yield the specific absorbance readings for the sample in question.
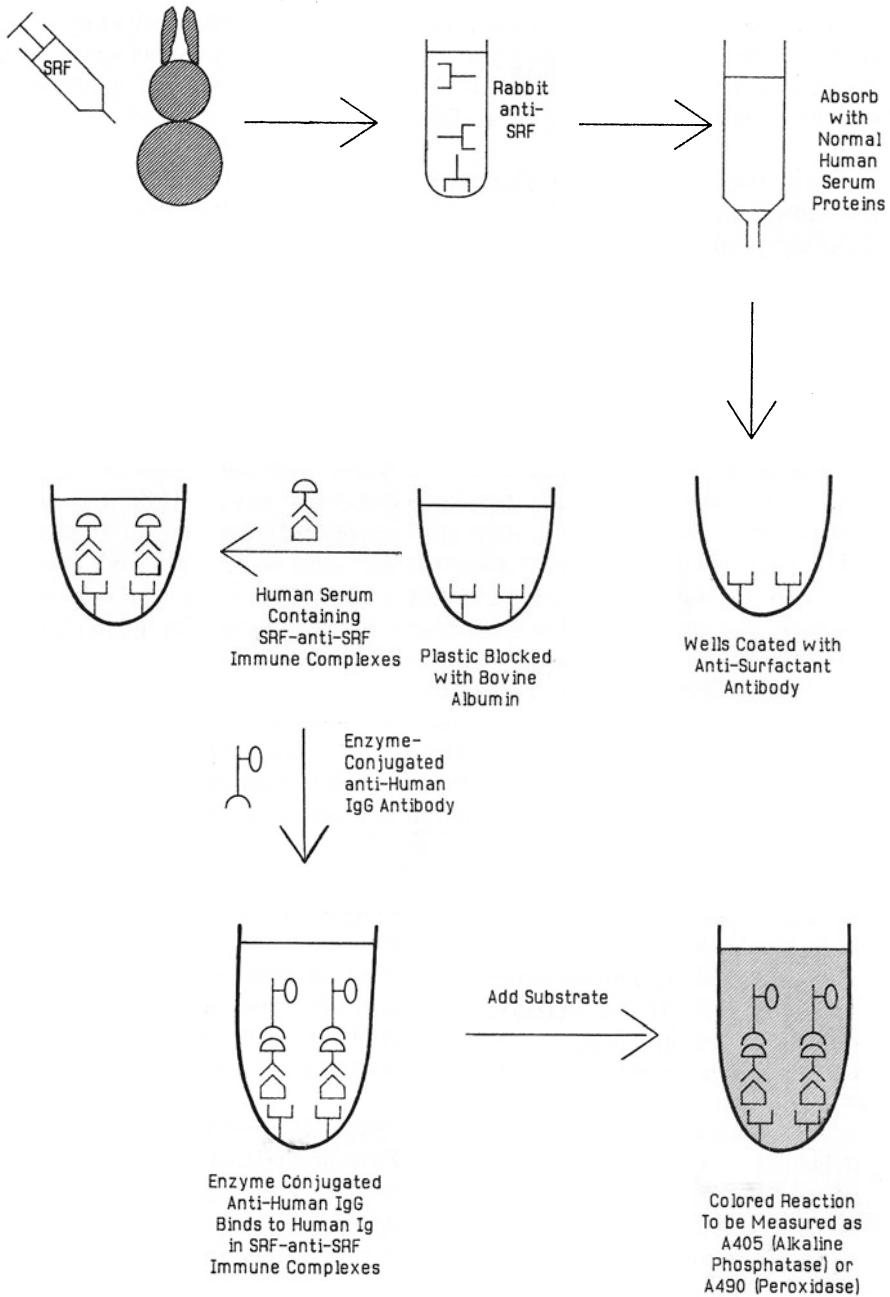
Fig. 1. The assay for surfactant-anti-surfactant immune complexes in the plasma of babies with neonatal RDS. Plastic plates are first coated with rabbit anti-SRF antibody and then bovine albumin to block nonspecific plastic binding sites for protein. The plasma sample to be tested is then added. A secondary antibody, enzyme-conjugated (either alkaline phosphatase or horseradish peroxidase) antibody directed to human IgG, follows. Finally, the substrate for the enzyme is added and the reaction is visualized at 405 nm (alkaline phosphatase) or 490 nm (horseradish peroxidase)

The only human IgG present on the plate would be that bound by virtue of being complexed with surfactant. Thus, this assay specifically detects circulating surfactant-anti-surfactant immune complexes. The specificity and sensitivity of this assay have been discussed [5].

In Vitro Assays of Surfactant Function. Surfactant isolated from amniotic fluid was slowly thawed from –90° C and mixed 1 : 1 (v : v) with control sera or antibody preparations using a Vortex mixer for 30 sec. These combinations were thereafter incubated for 30 min at 37° C with shaking. The final concentration of surfactant was 4 mg phospholipid/ml. The total protein concentration was 60–130 µg/ml.

Surface activity is measured using a pulsating bubble surfactometer, as described by Enhorning [7]. This surfactometer (Surfactometer International, Toronto, Ontario, Canada) measures the pressure gradient across air-liquid interphase of a bubble formed in a 37° C water-enclosed sample chamber. The bubble size was viewed through a microscopic optic, while the pressure changes were recorded on a strip chart recorder calibrated for 0 and –2 cm H_2O. The bubble pulsates between a maximal (0.55 mm) and a minimal (0.45 mm) radius at a rate of 20 cycles/min at 37° C in a water-saturated chamber. The surface tension is calculated by the laws of Young and La Place, and expressed as dynes/cm. In the present experiments, a bubble was formed to a maximal radius and 15 sec later pulsation was started. A continuous tracing of the pressure differences was made for 5 min. Calculations of the surface tensions were made 15 sec after the bubble formation. We calculated the surface tension at minimum radius (γ min) additionally after 5 min of pulsation. In addition, the time required to reach minimum surface tension was determined.

Patient Data. Adults and infants whose plasma samples were tested for surfactant-anti-surfactant immune complexes had clinical and roentgenographic evidence of RDS. The technique of surfactant administration to infants has been described [4]. Neonates received a single dose of 60 mg SRF/kg body weight by intratracheal instillation no later than 10 hours after birth. Requirements for inclusion in the study were a need for $FiO_2 > 0.6$ and $PaO_2 \leq 60$ mm Hg.

In addition, we monitored neonates with RDS serum complement levels (CH50, C3, C3a, and C1q binding). Daily analysis of urine for blood and protein were made by conventional techniques. Each patient was checked for skin lesions and rashes. Clinically indicated roentgenography was performed.

In all cases, samples were assessed by one of this group (DDS) in a blind fashion. Interpretations rendered reflect analysis of data without prior knowledge of clinical status, course, or treatment modalities. The relevant clinical data were supplied by others of this group (TAM, MH, RS) following such analysis.

Results

Immune Complexes in the Blood of Neonates with RDS. The results of our assays for circulating SRF-anti-SRF immune complexes in neonatal RDS have been compiled [5]. For illustrative purposes, Fig. 2 shows typical results for patients with RDS treated conventionally and with SRF replacement. Comparable analyses from a premature infant with no RDS are included for comparison. With only occasional exceptions, all infants with RDS showed evidence of circulating SRF-anti-SRF immune complexes. In these illustrations absorbance (ordinate) is shown as a measure of concentration of immune complexes, as a function of time after birth. Levels of immune complexes varied between children. In some, absorbance values not greatly higher than those observed at birth were noted. In others, considerable increases in absorbance values were found [5].
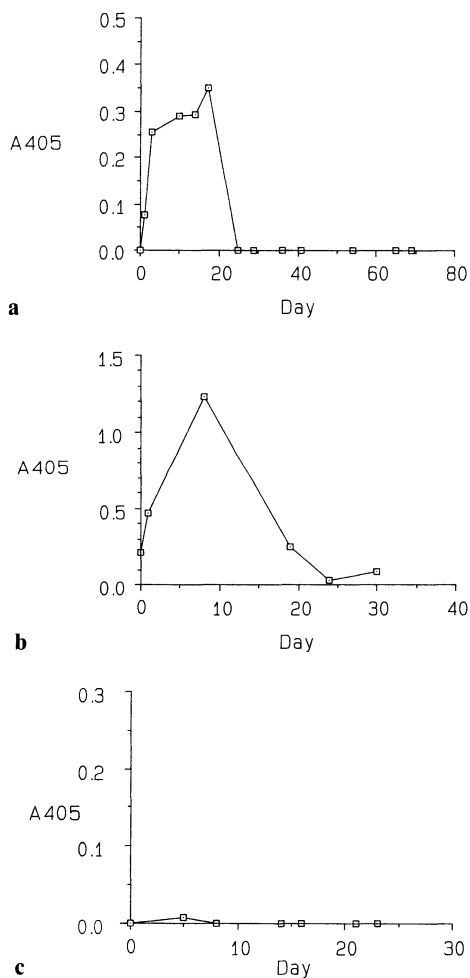
Fig. 2a–c. Appearance of SRF-anti-SRF immune complexes in plasma as a function of time for three representative patients. Absorbance is reported as relative absorbance, which is equal to the $A_{405\ or\ 490}$ at the time in question/$A_{405\ or\ 490}$ at birth. Infants were followed for a variable time after birth. **a** Analysis of plasma for an infant with RDS treated by conventional means. **b** An infant with RDS treated with surfactant. **c** A preterm infant without RDS. The procedure for performing the assays for immune complexes is described in Fig. 1

Levels of circulating immune complexes peaked at different times. They reached maximum height as early as 2–3 days in some infants, while others took as long as 2–3 weeks. Analyses of plasma from nearly all infants showed early peaks in immune complex concentrations. A subsequent decline usually followed. Considerable variation was noted between infants in all parameters. Sera from some infants showed very sharp peaks followed by comparably sharp declines. For other children, decreases in immune complex concentrations were much more gradual.

Clinical Data and Courses for Infants with Neonatal RDS. Clinical data for some of these infants are summarized elsewhere [5]. Detailed clinical histories are available on request. We have to date tested over 40 infants with neonatal RDS, both with and without SRF therapy. SRF administration produced rapid improvement in pulmonary function All infants with RDS weighed less than 1470 g at birth (range 850–1460 g), and ranged in gestational age from 27–32 weeks. All survived their episodes of RDS, though many had complications such as patent ductus arteriosus, intraventricular hemorrhage, and pulmonary interstitial emphysema.

Serum complement levels were normal in all infants. There was no evidence of hematuria, proteinuria, or rash at any time during observation.

Adults with RDS. Serum samples from nine adult patients with ARDS were examined for evidence of immune complexes between SRF and anti-SRF antibodies. Of these patients, three had received porcine surfactant (Curosurf). The remainder received conventional therapy. These serum samples were tested for immune complexes between human surfactant and antibodies to human surfactant. The extent to which porcine surfactant crossreacts immunologically with human surfactant is currently under study.

Sera from none of the three surfactant treated patients, but 2 of the 6 conventionally treated patients showed evidence of SRF-anti-SRF immune complexes over the course of their illnesses. Results from these examinations are preliminary, and much clinical information needs yet to be evaluated.

Inhibition of Surfactant Activity by Polyclonal Antibody. Rabbit polyclonal antiserum to surfactant has been raised and analyzed [5]. The reactivity of this antiserum to surfactant has been confirmed by Western blotting and immunofluorescence examination of stained lung tissues [5]. We tested this antibody for its ability to inhibit surfactant activity as measured by a pulsating

Fig. 3a–c. Effects of rabbit anti-surfactant antibodies on the functional activity of human ▶ alveolar surfactant. Digested and undigested antibody preparations were added to SRF at 4 mg/ml SRF (see text). Surface tension was measured at 1 **a** and 5 **b** min, and differences between the surfactant alone groups and those groups receiving antibody or normal serum preparations are shown. Adsorption time **c** is the time from initial bubble formation at maximum radius until there is adsorption to equilibrium surface tension prior to instituting pulsations. All differences between anti-SRF-treated preparations and those containing normal serum are statistically significant at the level of $P < 0.05$. $\Delta\gamma$ values are in dynes/cm and equal γ antibody-γcontrol
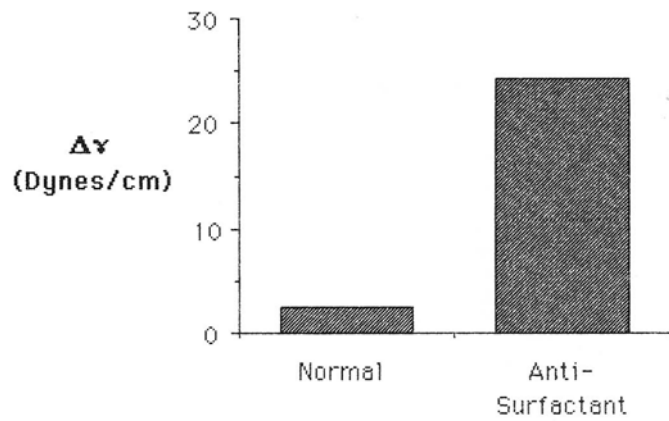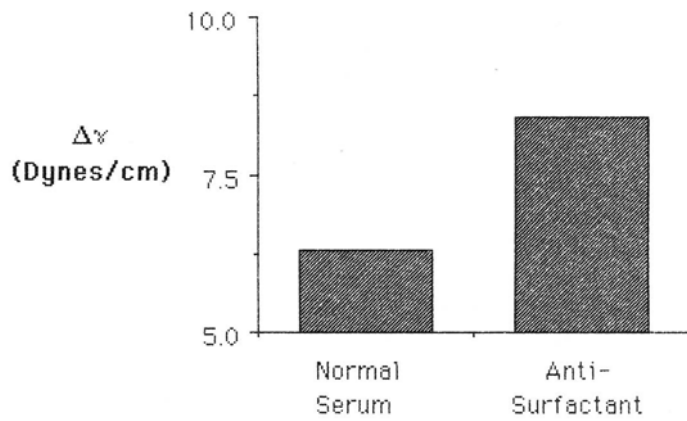
a Serum

b Serum
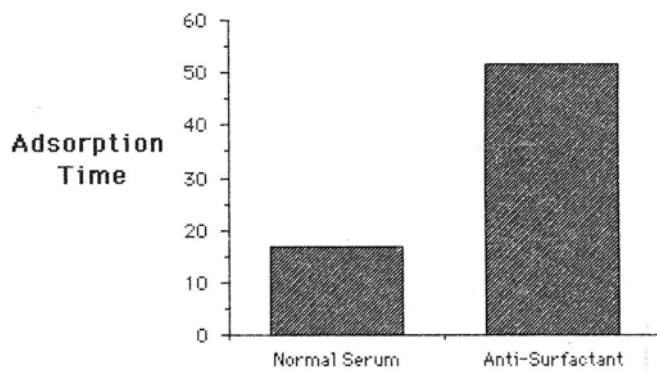
c Serum

bubble surfactometer (Fig. 3). This antiserum clearly decreased surface activity more than did control preparations of normal rabbit serum as measured by c_{min} and time required to reach minimum surface tension.

In additional experiments (not shown), we asked whether this inhibition reflected the ability of antibody to agglutinate surfactant, or rather the ability of antibody to bind sites on surfactant molecules responsible for intermolecular interactions between surfactant species. We found that bivalent antibody (F[ab]$_2$ fragments) to surfactant inhibits biophysical activity. Monovalent antibody preparations (F[ab] fragments) inhibit surface activity no more than comparable preparations of normal rabbit serum (D. S. Strayer, submitted for publication). We conclude that most if not all of the functional inhibitory activity in these polyclonal antibody preparations reflects the ability of polyclonal antibodies to agglutinate surfactant.

Therefore, we have repeatedly detected circulating immune complexes between SRF and anti-SRF antibodies in neonates with RDS. These immune complexes develop over time, and then usually disappear. They are present only in babies with RDS, regardless of whether or not the infants were treated with homologous surfactant. We have not yet found evidence of tissue injury mediated by these immune complexes. Preliminary data suggest that some adult patients with RDS may also develop circulating SRF-anti-SRF immune complexes.

Discussion

Type II alveolar pneumocytes synthesize pulmonary surfactant and secrete it into alveolar spaces and terminal air ducts. Surfactant may be metabolized and partially recycled locally [8], or transported proximally to be expectorated or swallowed. Thus, surfactant normally avoids contact with the immune system.

Recent studies have suggested that administered surfactant phospholipids may reach the systemic circulation (A. Jobe, this volume). These studies, however, did not examine the fate of surfactant protein.

That surfactant is sheltered from the body's immune system suggests that immunologic tolerance to pulmonary surfactant might not be well developed. We hypothesized that SRF might therefore resemble materials such as keratins and gastrointestinal mucins. These substances are excreted from the body and under normal circumstances do not contact the immune system. Some of these are highly immunogenic [9, 10].

Pulmonary surfactants are protein-lipid combinations of variable molecular size. Protein and lipid may be covalently or noncovalently bound to each other. As generally prepared, they are marginally water soluble. As mixtures of hydrophobic and hydrophilic phases surfactants may act as their own adjuvants. One would therefore expect pulmonary surfactant preparations to be highly immunogenic. Surfactant's immunogenicity has been demonstrated by the ease with which we have been able to elicit antibodies to human surfactant in animals, using immunization protocols both with and without additional adjuvants (5, and D. S. Strayer, et al., submitted for publication).

When the integrity of the alveolar wall is interrupted, surfactant could then be released into the circulation. Such circumstances could accompany any pulmonary injury. Environmental toxic agents, viruses, ionizing radiation, or other disruptive insults could breach the alveolar lining enough to permit surfactant to leak into the blood. Pulmonary damage in RDS includes increases in pulmonary capillary permeability and pulmonary edema [11]. Autoimmunization by pulmonary surfactant could account for the presence of immune complexes in plasma from patients with RDS. In many infants, the time course of appearance of immune complexes suggests primary immunization. This sequence of events parallels that of endogenous antikeratin antibodies in patients with extensive burns [10], and is not unlike the appearance of antibodies to myocardial antigens reported in patients with ischemic and other myocardial diseases [12–14, however see 15 and 16].

The immune system appears to mature selectively. Responsiveness to certain antigens predates the appearance of responsiveness to others [17].

Immunoglobulins are first detected in humans at about 10 weeks of gestation [18]. Human feti produce IgM antibody to a variety of antigens. Such antibodies are used to diagnose prenatal infections [19–21]. The human cell-mediated immune system also appears to be substantially mature *in utero* [22–25]. Preterm infants are therefore sufficiently immunocompetent to recognize surfactant as an antigen and elaborate anti-surfactant antibodies.

In addition, autoimmunization may be part of the development of tolerance to autoantigens. According to the network theory of immune regulation [26], a cascade of mutually interacting antibodies follows immunization. Initial antibodies recognize the antigen in question. Subsequent antibodies, called anti-idiotypic antibodies, recognize antigen binding regions (idiotypes) of the first set of antibodies. Anti-idiotypic antibodies thereby regulate clonal expression and levels of the initial antibodies [27, 28]. Some investigators suggest that tolerance to self antigens involves first producing antibodies to these "self" antigens. Primary autoantibodies could then elicit anti-idiotypic [anti-(anti-self)] secondary antibodies, eliminating or modulating anti-self clones [29]. Anti-surfactant antibodies in immune complexes in infants with RDS may reflect transient autoresponsiveness before development of immunologic tolerance.

Anti-SRF antibodies in our infants could also be of maternal origin. Surfactant absorbed from amniotic fluid might elicit an immune response in pregnant mothers. IgG is actively transported across the human placenta. Resulting IgG anti-SRF could then cross into the fetal circulation. Its combination with surfactant which, due to RDS, is released into the neonatal circulation could account for the observed immune complexes. This type of mechanism could explain the very rapid appearance of immune complexes in some infants. Others have noted maternal anti-SRF antibody in amniotic fluid (W. Taeusch, personal communication).

None of these hypotheses necessarily excludes others. In each patient, various mechanisms may contribute differently to SRF-anti-SRF immune complex levels as detected in individuals with RDS.

Other investigators who have treated patients with xenogeneic surfactants

have sought but not found circulating anti-surfactant antibodies (J. Whitsett, personal communication). We have not seen the experimental protocols used by these investigators, reviewed their assay procedures or tested their patients' samples in our assays. We also do not know at which time(s) they drew patients' sera for analysis. Thus, we cannot yet explain the differences between their findings and ours. Although, as we understand these experiments, they have been designed to detect circulating free antibody to surfactant. We have found that infants with RDS have circulating immune complexes which form at times of antigen excess or near equivalence between antigen and antibody. Immune complex formation thus precedes detectable free antibody. Our data and those of our colleagues are thus not necessarily contradictory. If most anti-SRF antibody in SRF-anti-SRF immune complexes was of maternal origin, it is possible that immune complexes account for most or all of the original maternal anti-SRF; so that free antibody could not be detected. In addition, if the pulmonary circulation remains leaky, circulating free antibody might be immediately bound by the large pool of antigen (alveolar surfactant) in the lungs.

The data shown here are illustrative of many studies on infants with neonatal RDS. We have consistently found that premature infants without pulmonary disease do not have detectable circulating SRF-anti-SRF immune complexes (5, and D. S. Strayer, et al., in preparation). We find such complexes in plasma samples from patients with RDS regardless of treatment.

We have as yet found no evidence of immune complex-mediated damage. All infants were examined for evidence of complement activation and skin and kidney damage, but none was found. Such absence of evident circulating immune complex-mediated tissue injury would be similar to the studies done in men with vasectomies. These men develop antibodies to sperm antigens and have circulating antigen-antibody complexes in their blood. Nonetheless, investigators have found no evidence of immunologically mediated tissue injury in these patients [30, 31].

We report here preliminary data suggesting that a small number of patients with ARDS may also show evidence of circulating SRF-anti-SRF immune complexes. Our data are too preliminary to permit separation of ARDS patients who develop SRF-anti-SRF immune complexes on the basis of treatment regimen(s). These studies are preliminary in nature and undertaken prospectively to evaluate the potential immunogenicity of surfactant in adult patients with pulmonary disease. Unlike neonatal RDS, adult RDS is a composite of diseases of many different etiologies that may develop similar respiratory distress syndromes. This diversity of pathogeneses and etiologies suggests that in some cases anti-surfactant immunologic reactivity and inhibition of surfactant function may play a role in the development of RDS. This possibility is strengthened by those data showing that bivalent anti-SRF antibody inactivates surfactant functionally.

The suggestion of SRF-anti-SRF immune complexes in ARDS patients is based on evaluation of a small number of patients. Additional patient accrual and data correlation are needed before these findings can be substantiated. Nevertheless, these preliminary indications, together with experimental

observations on the effects of anti-SRF on SRF function, suggest that immunologically mediated mechanisms may enhance respiratory dysfunction in some cases of ARDS.

The nature of the antigens in surfactant is an important question. To treat neonatal RDS, we use a heterogeneous combination of human pulmonary surfactant species, including 34 kd apoprotein, 18 and 9 kd proteins, and 5–7 kd lipoprotein. Preparations used by others generally involve xenogeneic surfactants which may be predominantly one molecular species or another. The antigenicity of these different molecular species should not be in doubt. Our rabbit polyclonal antibody reacts with all these species as determined by radioimmunoprecipitation. In addition, we have raised murine monoclonal antibodies to human surfactant. Most of these monoclonal antibodies recognize the 5–7 kd proteolipid species (D. S. Strayer, et al., submitted for publication). The latter is of comparable size to such highly immunogenic proteins as insulin [32]. Thus, although it is smaller than the other surfactant molecules, the 5–7 kd lipoprotein is large enough to act as an independent antigen. All known molecular forms of alveolar surfactant are therefore potentially immunogenic.

We have sought to determine whether human alveolar surfactant is immunogenic as it is used in therapy of adult and neonatal respiratory distress syndromes (RDS). We have developed an enzyme linked immunosorbant assay (ELISA) to detect specific immune complexes between surfactant and antibodies directed to surfactant. Plasma samples from neonates with RDS were analyzed for such immune complexes. Premature infants with RDS were divided into two groups: one group to receive surfactant replacement and one to receive conventional therapy. Infants were also examined for clinical and serological evidence of immune complex-mediated tissue damage. Almost all infants with RDS, whether or not they had received surfactant therapy, showed evidence of circulating surfactant-anti-surfactant immune complexes. Plasma samples from premature babies without RDS showed no such immune complexes. These immune complexes generally appeared early in postnatal life. Their concentrations then peaked, and diminished afterwards. We have found no evidence of complement activation or clinically evident organ damage attributable to these immune complexes.

Sera from a small number of patients with adult RDS were similarly examined for SRF-anti-SRF immune complexes. About one-third of these patients showed such immune complexes. In addition, we found that antibodies directed against surfactant can inhibit surfactant function *in vitro*.

Thus, circulating immune complexes between surfactant and antibodies to surfactant are common in neonatal RDS and may be found in adult RDS, though less commonly. To date, they seem to cause no tissue injury in neonatal RDS. However as anti-surfactant antibodies are capable of inhibiting surfactant functionally, RDS both in neonates and adults may be potentiated by these antibodies. As both human and heterologous surfactants are now used in treating RDS, the pathogenic potential of immunologic reactivity to surfactant cannot be ignored.

In conclusion, we have shown that human surfactant is immunogenic and that circulating surfactant-anti-surfactant immune complexes are detectable in

the plasma from infants and in adults with RDS. We found these immune complexes regardless of whether SRF was used in the individual treatment regimen. These immune complexes do not yet appear to cause disease in the short term. Long term effects, if any, are unknown. Indications for surfactant replacement therapy in neonatal RDS are clear. Trials of surfactant are just beginning in adult RDS. In all such situations, potential for side effects must be balanced against therapeutic efficacy and the gravity of the disease. Our data indicate that surfactants, particularly heterologous surfactants, are potent immunogens. One cannot assume that the use of homologous or heterologous surfactants in patients with RDS will always be immunologically innocuous.

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