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Immunogenicity of surfactant. II. Porcine and bovine surfactants

D. S. STRAYER*, M. HALLMAN† & T. A. MERRITT‡ *Department of Pathology and Laboratory Medicine, University of Texas Health Science Center, Houston, TX, USA, †Department of Pediatrics, University of Helsinki, Helsinki, Finland, and ‡Department of Pediatrics, University of California, La Jolla, CA, USA

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SUMMARY

Protein-containing surfactants of human and animal origin are being used increasingly to treat neonatal and adult respiratory distress syndromes. This trend led us to examine the antigenicity of two important preparations of animal surfactant, cow lung surfactant extract (CLSE) and a porcine surfactant preparation, Curosurf. We describe here 15 monoclonal antibodies against Curosurf and four against CLSE. Antibodies were studied by Western blot analysis to determine their ability to recognize protein components of their respective surfactant preparations. They were also tested for their ability to inactivate surfactant *in vitro*, assayed using the pulsating bubble surfactometer. Several antibodies directed against CLSE or Curosurf functionally inactivate the surfactant to which they were raised. We determined the degree of immunologic cross-reactivity between antibodies directed to CLSE and Curosurf against the other surfactant and also against human surfactant, both by Western blot and by examining functional inactivation *in vitro*. Antibodies to these animal surfactants that are commonly used therapeutically may inactivate the specific animal surfactant to which they were raised, as well as human and other surfactants. Generally, when antibodies inactivate surfactant from more than one animal species, they inactivate heterologous surfactants comparably to the extent to which they inactivate the surfactant to which they are directed. Immune complexes between anti-surfactant antibodies and surfactant have been described in the course of neonatal respiratory distress syndrome. The potential pathophysiological importance of anti-surfactant antibodies may therefore lie in their ability to inactivate administered surfactant, other similar surfactants and endogenous surfactant. In so doing, these antibodies may potentiate surfactant deficiency or pulmonary injury initiated by other stimuli.

Keywords anti-surfactant antibodies neonatal respiratory distress syndrome surfactants

INTRODUCTION

Neonatal respiratory distress syndrome (RDS) reflects pulmonary immaturity and insufficient surfactant production and secretion (Avery & Mead, 1959). Neonatal RDS is characterized by end expiratory airway collapse, poor arterial oxygenation and pulmonary compliance, patent ductus arteriosus, intracranial hemorrhage and bronchopulmonary dysplasia (Kendig & Sinkin, 1988). The severity of these complications and their implications for life-long morbidity in this age group make development of effective therapy for neonatal RDS a pressing concern.

Surfactant therapy, in conjunction with mechanical ventilation should theoretically improve the prognosis of neonatal RDS. Several investigators have recently reported the therapeutic utility of several types forms of surfactant to treat RDS. After surfactant instillation, arterial/alveolar oxygen ratio improves rapidly, and it becomes possible to reduce mechanically assisted ventilation. Patients receiving mammalian surfactants intratracheally usually survive better than do control patients, and develop complications of RDS less severely and less frequently (Merritt *et al.*, 1986; Kendig & Sinkin, 1988; Merritt & Hallman, 1988; Collaborative European Multicenter Study Group, 1988; Ware *et al.*, 1988).

Although some totally synthetic surfactants have been used to treat neonatal RDS (Morely, 1987; U.S. Exosurf Pediatric Study Group, 1989), most surfactants used therapeutically come from animal sources and contain protein. In these preparations, the surfactant protein is essential for surface activity (Yu & Possmayer, 1986; Revak *et al.*, 1986).

Surfactant proteins of varying sizes and hydrophobicity have been identified (Possmayer, 1988). Surfactant protein A (SP-A), a glycoprotein of approximately 28–35 kD, is the most hydrophilic of the three known surfactant proteins. It appears to stabilize tubular myelin structure characteristic of surfactant

Correspondence: David S. Strayer, MD, PhD, Department of Pathology, University of Texas Health Science Center, 6431 Fannin Street, Houston, TX 77030, USA.

morphology (Hawgood, Benson & Hamilton, 1985), its role in lowering surface tension is not clear. Surfactant protein B (SP-B) is about 9 kD. It forms homodimers *in vivo* and appears to be important for surfactant surface activity *in vitro* and in animal models (Whitsett *et al.*, 1986a; Revak *et al.*, 1988). Surfactant protein C (SP-C) is very hydrophobic and weighs approximately 4 kD, depending upon the animal species. Its function is not yet clear.

The presence of proteins and phospholipids in surfactant preparations used to treat RDS raises the issue of surfactant immunogenicity. It is important to consider the potential role of such antibodies on the course of neonatal and subsequent lung injury, and in the development of adult respiratory distress syndrome. We have described the immunogenicity of human surfactant and the potential importance of antibodies to HSRF to RDS (Strayer, Hallman & Merritt, 1988; Strayer *et al.*, 1989, 1990). Here we describe the immunogenicity of bovine and porcine surfactants and their immunologic crossreactivity with each other and with human surfactants.

MATERIALS AND METHODS

Animals

Female F-344 rats were obtained from Harlen Company, Indianapolis, IN.

Surfactants

Porcine surfactant (Curosurf) was obtained from Drs Bengt Robertson and Tore Curstedt, St Goran's Hospital, Karolinska Institute, Stockholm, Sweden, and bovine surfactant (CLSE) from the late Dr Donald Shapiro, University of Rochester, NY. Human amniotic fluid surfactant was prepared according to Hallman, Merritt & Schneider (1983).

Antibodies

The procedures for producing antisera and rat monoclonal antibodies to surfactant preparations, and for assaying their reactivity against surfactant by ELISA have been reported (Strayer *et al.*, 1989, 1991).

SDS-PAGE and Western blotting

Proteins were electrophoresed in gels that were 0·1% SDS–15% acrylamide and 6 M urea and transferred to Immobilon (Millipore). Binding of surfactant proteins by individual monoclonal antibodies was assayed separately for monoclonal antibodies as culture supernatants, followed by incubation of the filters with ^{125}I-*Staphylococcus aureus* protein A and then by autoradiography (Strayer *et al.*, 1989).

Surface activity

We measured surface activity of mixtures of surfactants, combined with monoclonal antibodies as culture supernatants or with control preparations, using a pulsating bubble surfactometer (Enhorning, 1977; Strayer *et al.*, 1989). The final concentrations of surfactant phospholipid and protein were 5 mg/ml and 60–130 μg/ml, respectively. Surface tension is calculated by the law of Young & La Place (Enhorning, 1977), and expressed as mN/m 5 min after bubble formation.

RESULTS

Antibodies to Curosurf

Fifteen monoclonal antibodies, P1–P15, were identified by their ability to bind Curosurf in ELISA. They were analysed for specific protein reactivity by immunoblotting (Fig. 1, summarized in Table 1). Antibodies P1, P6 and P9 bind a protein of about 10 kD, while P3, P4, P5, P10 and P15 recognize proteins of 7–8 kD. P3, P10 and P15 also recognize higher molecular weight proteins: 17, 30 and 29 kD, respectively. Several

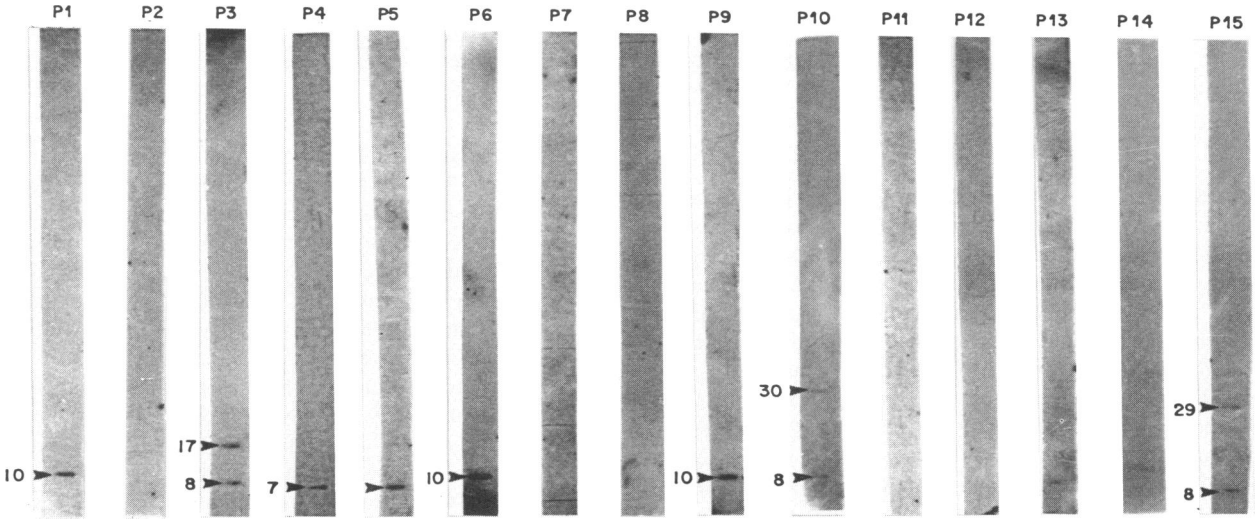

Fig. 1. Western blot analysis of monoclonal antibodies directed to the porcine surfactant preparation, Curosurf. Rat monoclonal antibodies to Curosurf were prepared as described in Materials and Methods. Fifteen clones were identified after subcloning twice (P1–P15). These were analysed for their ability to recognize specific protein constituents of Curosurf by Western blotting. Curosurf was boiled in reducing sample buffer, electrophoresed and transferred to Immobilon. Individual lanes were exposed to antibodies as neat culture supernatants, followed by rabbit anti-rat IgG. Finally, blots were exposed to ^{125}I-*Staphylococcus aureus* protein A and protein binding was visualized by autoradiography.

Table 1. Binding of proteins in Curosurf, CLSE, and human surfactants by monoclonal antibodies directed to Curosurf

	Surfactant		
Antibody	Curosurf	Human	CLSE
P1	9 kDa	10 kD	—
P2	—	—	—
P3	8, 17 kD	7, 18 kD	—
P4	8 kD	—	—
P5	7 kD	—	—
P6	10 kD	—	—
P7	(9 kD)	—	—
P8	—	—	—
P9	9 kD	—	—
P10	—	—	—
P11	—	—	—
P12	—	—	—
P13	—	—	—
P14	—	—	—
P15	8, 29 kD	—	—

Surfactants from human, porcine and bovine sources were obtained as described in Materials and Methods. Monoclonal antibodies to porcine surfactant (Curosurf) were raised. The various surfactant preparations were electrophoresed on SDS-PAGE and transferred to filters, then exposed to the monoclonal antibodies. Subsequently, filters were treated with rabbit anti-rat IgG and then with ^{125}I-*Staphylococcus aureus* protein A. Binding patterns were visualized by autoradiography and compared with protein standards.

CSLE, cow lung surfactant extract.

antibodies that bind Curosurf by ELISA do not recognize porcine surfactant proteins sufficiently to visualize their binding activity using Western blotting.

Antibodies to CLSE

Four monoclonal antibodies to CLSE were identified, C1–C4. We examined these antibodies by Western blot analysis for reactivity to CLSE. Results (Fig. 2, summarized in Table 2) show that antibody C1 recognizes an 11-kD protein, C2 a 9-kD protein and C4 a 36-kD protein. C3 does not bind a specific protein, assayed using Western blotting.

Cross-reactivity of anti-Curosurf and anti-CLSE antibodies with other species of surfactants

We examined these antibodies to determine the degree to which they cross-react with surfactant preparations from other animals. By Western blot, we found that P1 and P3 monoclonal antibodies to Curosurf recognize proteins of 9 kD and 9 and 17 kD, respectively in preparations of human surfactant. They do not, however, bind proteins in other surfactant preparations detectably (CLSE, murine, rabbit; data not shown). Protein binding activities for anti-Curosurf antibodies thus resemble patterns of reactivity against Curosurf. Anti-CLSE monoclonal antibodies were tested against human and bovine surfactants (Table 2). C3 alone recognizes proteins of 8 and 30 kD in a heterologous surfactant (Curosurf).

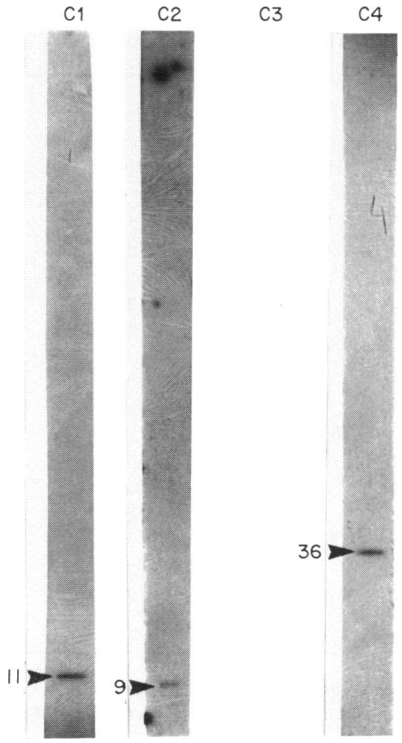

Fig. 2. Western blot analysis of monoclonal antibodies directed to cow lung surfactant extract (CLSE). Rat monoclonal antibodies to CLSE were prepared as described in Materials and Methods. Four reactive clones were identified after subcloning twice (C1–4). These were analysed for their ability to recognize specific protein constituents of CLSE by Western blotting. CLSE proteins were separated by electrophoresis in 15% acrylamide and then transferred to Immobilon. Individual lanes were separated, incubated with antibodies and then with rabbit anti-rat IgG. Finally, blots were exposed to ^{125}I-*Staphylococcus aureus* protein A and protein binding was visualized by autoradiography.

Table 2. Binding of human, bovine and porcine surfactants by monoclonal antibodies directed to CLSE, as determined by Western blot

	Surfactant		
Antibody	Curosurf	Human	CSLE
C1	—	—	8 kD
C2	—	—	8 kD
C3	8, 30 kD	—	—
C4	—	—	10, 32 kD

Surfactants from human, porcine and bovine sources were obtained as described in Materials and Methods. Monoclonal antibodies to bovine surfactant were raised. The various surfactant preparations were electrophoresed on SDS-PAGE and transferred to filters, then exposed to the monoclonal antibodies. Subsequently, filters were treated with rabbit anti-rat IgG and then with ^{125}I-*Staphylococcus aureus* protein A. Binding patterns were visualized by autoradiography and compared with protein standards.

Curosurf, porcine surfactant preparation; CSLE, cow lung surfactant extract.

Table 3. Effect of monoclonal antibodies to Curosurf on the surface activity of Curosurf, CLSE and human surfactant (γ_{min} in mN/m)

	Surfactant		
Antibody	Human	Curosurf	CLSE
—	0±0	2·3±2·5	0±0
Medium	8±1·7	5·7±1·2	7±1·2
P1	—	14±1	18±4
P2	—	18·7±1·5	10·7±2·9
P3	—	8·7±0·6	—
P4	—	11±1	31·3±0·6
P5	—	0±0	—
P6	—	12·3±1·5	—
P7	28·7±2·1	13·7±0·6	19·3±1
P8	—	13·3±1·5	22·3±3·2
P9	—	10·7±0·7	—
P11	—	5±2·6	—
P12	25·3±1·3	20·7±2·5	5·3±2·5
P13	—	17·7±0·5	16±1
P14	—	9±1	16±1
P15	—	23·7±6	16·7±4·1

The monoclonal antibodies derived from animals immunized with Curosurf were mixed in equal volumes with Curosurf, CLSE or human surfactant, at final phospholipid concentrations of 5 mg/ml phospholipid for 30 min at 37°C. The resulting mixture was examined in the pulsating bubble surfactometer. Antibodies were used as culture supernatants at rat IgG concentrations of 300–600 ng/ml, in RPMI 1640 with 5% horse serum. Control medium is this RPMI + 5% horse serum. Data shown are the γ_{min} at 5 min, representing the means of at least three separate assays ± s.e.m.

Curosurf, porcine surfactant preparation; CLSE, cow lung surfactant extract.

Table 4. Effect of monoclonal antibodies to CLSE on the surface activity of CLSE, Curosurf and human surfactant (γ_{min} at 5 min, in mN/m)

	Surfactant		
Antibody	Human	CLSE	Curosurf
—	0±0	4·3±1·6	2·3±2·5
Medium	0±0	3·1±1·4	5·7±1·2
C1	10±1·7	18·1±0·8	15±1·7
C2	14±5·2	18·4±0·7	8±0
C3	18·3±0·6	20·4±1·5	13±0·6

The monoclonal antibodies derived from animals immunized with CLSE were incubated with CLSE, Curosurf and human surfactant at final phospholipid concentrations of 5 mg/ml phospholipid for 30 min at 37°C. The resulting mixtures were examined in the pulsating bubble surfactometer. Antibodies were used as culture supernatants at rat IgG concentrations of 300–600 ng/ml, in RPMI 1640 with 5% horse serum. Control medium is this RPMI + 5% horse serum. Data shown are the means of at least three separate determinations ± S.E.M. Antibody C4 was not available in sufficient quantities to perform these studies.

CLSE, cow lung surfactant extract; Curosurf, porcine surfactant preparation.

Functional studies on Curosurf using anti-Curosurf antibodies

We then measured the ability of monoclonal antibodies to Curosurf to affect its function *in vitro*. The various monoclonal antibodies as culture supernatants were added to Curosurf, and the surface activity of the resultant mixtures measured in the pulsating bubble surfactometer. Rat IgG (anti-Curosurf antibody) concentrations in these preparations ranged from 300 to 600 ng/ml. Control preparations consisted of culture medium alone (RPMI 1640 + 5% horse serum) + Curosurf. Most of the monoclonal anti-Curosurf antibodies altered the surface activity of Curosurf (Table 3). Some inhibit Curosurf activity profoundly, most do so moderately, and several not at all. Of the seven monoclonal antibodies recognizing species of 8–10 kD, P15 severely impair Curosurf activity. Four (P1, P4, P7 and P9) inhibit Curosurf moderately. One antibody (P5) augments Curosurf activity.

Functional studies on CLSE using anti-CLSE antibodies

In the same manner, monoclonal antibodies C1–C3 against CLSE were examined for their ability to inactivate CLSE (Table 4). All three monoclonal antibodies inhibit CLSE surface activity.

Cross-inhibition of anti-CLSE and anti-Curosurf antibodies for Curosurf, human surfactant and CLSE

A major question in understanding the implications of surfactant immunogenicity is the degree to which heterologous surfactants cross-react immunologically with each other and with human surfactant. Antibodies directed to CLSE were tested for their ability to inhibit Curosurf activity as described above. We found (Table 4) that two anti-CLSE antibodies inhibit Curosurf activity moderately (C1, C3). All three anti-CLSE antibodies inhibit human surfactant function moderately. We also examined the ability of monoclonal antibodies to Curosurf to inactivate CLSE and human surfactant. Several monoclonal antibodies to Curosurf inhibit CLSE (Table 3) moderately. One (P4) did so greatly. The two anti-Curosurf antibodies tested, P7 and P12, inhibit human surfactant function almost completely. Generally, antibodies that inhibit Curosurf seem to inhibit CLSE and human surfactant comparably. Exceptions to this rule include P12 that inhibits Curosurf strongly but CLSE not at all and P4 that inhibits Curosurf moderately but CLSE strongly.

DISCUSSION

These studies examine the antigenicity of bovine and porcine surfactants and the effects of antibodies raised to these surfactants on their ability to lower surface tension *in vitro*. We raised and characterized monoclonal antibodies to Curosurf and CLSE and determined the degree to which these antibodies alter surface activity of these surfactants. In addition, we tested these antibodies for their crossreactivity, both in binding and in functional assays with the opposite surfactant preparation and with human surfactant.

We identified 15 different monoclonal antibodies to Curosurf and four to CLSE. Of these, seven of those against Curosurf and three of those against CLSE bind protein constituents detectably by Western blot analysis. The failure of the remaining monoclonal antibodies to do so is not unusual. In our experience, about half of the monoclonal antibodies to surfac-

tant that bind ligands by ELISA do not bind identifiable protein species by Western blot (Strayer *et al.*, 1989, 1990). Antigens may be altered sufficiently by denaturation during electrophoresis and then binding to filters so as to be unrecognizable by antibodies specific for only one epitope. Also, immunization and ELISA were done using phospholipid-containing surfactant preparations. Thus, we cannot rule out the possibility that some of the monoclonal antibodies react with phospholipid components or with epitopes formed by interactions of phospholipids and proteins.

Of 15 anti-Curosurf antibodies identified, seven bind species of approximately 8–10 kD. One (P5) binds a 7-kD protein in this preparation. One (P3) of the antibodies that recognizes a species of 8–10 kD also binds a 17-kD protein. We are currently testing purified Curosurf proteins to confirm the identities of the proteins in question. The nature of the lower molecular weight species recognized by P5, similarly, is not clear. It may represent small aggregates of SP-C molecules, incomplete breakdown products of larger SP-C precursors, or a breakdown product of SP-B. As investigators using purified SP-C have been unable to elicit anti-SP-C antibodies (J. A. Whitsett & B. J. Benson, personal communications), it is most likely that the 7-kD protein recognized by P5 is an SP-B breakdown product.

Two antibodies to Curosurf recognize a larger protein as well as lower molecular weight proteins. The nature of this larger protein is as yet uncertain. Curosurf is reported to contain no SP-A (Suzuki *et al.*, 1986; Curstedt *et al.*, 1987). Thus, this larger species could represent a precursor of one of porcine lower molecular weight surfactants or aggregated SP-B molecules. Both of these possibilities are under study.

Several antibodies (P1, P3) recognize proteins of similar size (9 and 17 kD) in Curosurf and human surfactant. These data are consistent with recognition of SP-B monomers and homodimers by these monoclonal antibodies and suggest that human and porcine SP-B are antigenically similar.

Most of the monoclonal antibodies against Curosurf species Curosurf function as measured using the pulsating bubble surfactometer. Some of these antibodies (P2, P7, P8, P12 and P13) do not react with protein antigen on Western blot, while others appear to recognize a protein of approximately 8–10 kD. Some of the antibodies (P3, P5) that recognize proteins of this size in Curosurf do not inactivate Curosurf functionally.

Antibodies to CLSE recognize molecular species in CLSE of molecular weights 8–10 kD, as do anti-Curosurf antibodies. Two of these antibodies bind a larger protein: C4 binds a 32-kD protein in CLSE and C3 binds proteins of 8 and 30 kD in Curosurf but does not bind proteins in CLSE by Western blot. We believe it is most likely that the 8–10-kD species involved are the respective SP-Bs. The fact that C4 recognizes a protein of 32 kD recalls similar reactivity by anti-Curosurf antibodies. SP-A is felt not to be present in CLSE (Whitsett *et al.*, 1986b; Warr *et al.*, 1987). Thus, reactivity of C4 with the larger proteins may reflect binding of SP-B aggregates, or of precursor protein(s).

On the whole, inactivation of surfactants different from that to which individual antibodies were raised parallels the patterns of inactivation of the original surfactant. However, some antibodies that inactivate Curosurf do not alter activity of other surfactants (e.g. P12). Some of these antibodies alter surface activity of other surfactants more than they inhibit Curosurf. Thus, P4 only slightly inhibits Curosurf activity but inhibits CLSE activity profoundly. P7 inhibits Curosurf moderately, but human surfactant strongly. Regarding anti-CLSE antibodies, C1 and C3 inhibit human surfactant and Curosurf comparably to CLSE. C2 inhibits Curosurf function only slightly but inhibits human surfactant moderately. Thus, both in terms of binding and functional inactivation, there is considerable immunologic crossreactivity among human, porcine and bovine surfactants.

Antibodies elicited by porcine and bovine surfactants may recognize each other as well as human surfactant. Anti-human surfactant antibodies may recognize and inactivate surfactants from both bovine and porcine sources (Strayer *et al.*, 1991). It is thus reasonable to suggest that antibodies elicited by exposure to heterologous surfactant could inactivate endogenous surfactant if a subsequent lung injury occurs that involves leakage of plasma proteins into the lung.

We have sought here to determine the immunogenicity and degree of immunologic cross-reactivity of animal pulmonary surfactants currently used as experimental therapy of RDS. We felt that because of expanded use of surfactants to treat neonatal RDS and also adult RDS, it is important to examine the potential for immunologically mediated side effects such as antibody- or immune complex-mediated tissue injury (Strayer *et al.*, 1986, 1989; Taeusch, 1989; Bartmann *et al.*, 1989; Kobayashi *et al.*, 1989). We and others have documented the presence of such antibodies, the efficacy of intratracheal instillation as a route of immunization against surfactant and the potential pathogenicity of anti-surfactant antibodies *in vivo*. One should recognize the spectrum of injury that attends neonatal and adult RDS and its treatment. Therapy of RDS should take into account not only the possibility that cross-reactive immunogenic substances may be introduced into patients with RDS, but also the possibility that effective treatment of RDS may decrease the immune system's exposure to immunogenic endogenous surfactants and so mitigate potential future damage due to surfactant immunogenicity. Our findings of surfactant inactivation *in vitro* need not necessarily limit the therapeutic usefulness of heterologous surfactants. Clinical trials of Curosurf and CLSE have shown rapid and significant improvement in infants receiving these surfactants. However, as with many agents administered to neonates, their life-long implications may initially be incompletely understood.

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