**Effects of Exogenous Surfactant on Acute Lung Injury Induced by Intratracheal Instillation of Infant Formula or Human Breast Milk in Rabbits**

Kahoru Nishina, M.D.,* Katsuya Takao, M.D.,† Nobuhiro Maekawa, M.D.,‡ Hidefumi Obara, M.D.§

**Background:** An animal experimental model of acute lung injury after intratracheal instillation of acidified milk products has been recently demonstrated. Exogenous administration of surfactant has proved to be successful treatment for acute lung injury induced by many causes including acid aspiration. The authors conducted this study to investigate whether exogenous surfactant can reduce the magnitude of lung damage induced in rabbits by acidified milk products.

**Methods:** The lung injury was induced by intratracheal instillation of acidified human breast milk or acidified infant formula (0.8 ml/kg, pH 1.8). Thirty minutes after the insult, some animals were treated with intratracheal surfactant 100 or 200 mg/kg. Lung compliance and alveolar-to-arterial oxygen gradient were recorded during ventilation. After 4 or 12 h, the lungs were excised to determine physiologic and histologic lung damage. Albumin, interleukin-8, and eicosanoids in bronchoalveolar lavage fluid and superoxide production by neutrophils were measured.

**Results:** The acidified milk products increased A-aDo₂ (550 ± 52 and 156 ± 28 mmHg; mean ± SD in saline solution and infant formula groups, respectively), lung wet-to-dry weight ratio (6.6 ± 0.5 and 5.6 ± 0.2), %neutrophils in bronchoalveolar lavage fluid (84 ± 4% and 8 ± 2%), and decreased compliance (0.76 ± 0.09 and 1.90 ± 0.11 ml/cm H₂O). Surfactant improved these variables in a dose-dependent manner (A-aDo₂ = 363 ± 50 and 237 ± 55 mmHg in 100-mg/kg and 200-mg/kg surfactant groups). Surfactant attenuated extensive histologic changes caused by the milk products. Superoxide production was less in rabbits receiving surfactant than in those not receiving it.

**Conclusion:** Exogenous surfactant improved physiologic, histologic, and biochemical lung injury induced by acidified milk products in a dose-dependent manner. The effectiveness of surfactant may be caused, in part, by inhibition of neutrophils' sequestration and activation. These data indicate that intratracheal instillation of surfactant may be a promising therapeutic modality in acute lung injury resulting from aspiration of acidified milk products. (Key words: Acute respiratory distress syndrome; aspiration pneumonia; cytokines; lung edema.)

**INTRATRACHEAL** instillation of acidified breast milk or infant formula has been recently shown to cause acute lung injury in rabbits, as assessed by oxygenation, lung mechanics, and histopathologic changes, including neutrophil activation. From the perspective of pediatric clinical practice, the animal model of acid aspiration-induced lung injury using these acidified milk products may be more clinically relevant than using HCl alone.

Many experiments have demonstrated that the pulmonary surfactant system, which prevents collapse of alveoli by decreasing its surface tension, is harmed in acute lung injury induced by a variety of conditions. Surfactant replacement therapy prevents or attenuates deterioration of gas exchange in the experimental model of acute lung injury induced by HCl instillation, endotoxin, viral pneumonia, oleic acid, hyperoxia, repetitive lung lavage, or acidified breast milk through restoration of surfactant function. Those milk products with a low or native pH cause damage to the alveolar-capillary membrane, alveolar hemorrhage, and influx of protein-rich edema fluid into alveolar spaces. The blood components and plasma exudates inhibit surfactant function, leading to hypoxemia caused by atelectasis and mismatch of the ventilation-perfusion ratio. Thus, exogenous administration of surfactant may also provide

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* Instructor in Anaesthesiology.
† Assistant Professor in Anaesthesiology.
‡ Associate Professor of Anaesthesiology.
§ Professor of Anaesthesiology.

Received from the Departments of Anaesthesiology, Kobe University School of Medicine, Osaka, Japan. Submitted for publication September 14, 1998. Accepted for publication March 10, 1999. Support was provided solely from institutional and/or departmental sources. Presented in part at the annual meeting of the American Society of Anaesthesiologists, San Diego, California, October 18-22, 1997.

Address reprint requests to Dr. Nishina: Departments of Anaesthesiology, Kobe University School of Medicine, Kusunoki-cho 7, Chuo-ku, Kobe 650-0017, Japan.
beneficial effects on oxygenation in this setting. Furthermore, immunological mechanisms are thought to be involved in the development and progress of acute lung injury induced by aspiration of gastric contents \(^{11-15}\) as well as other causes. In particular, neutrophils recruited to the lung in response to chemotaxins (e.g., interleukin-8 [IL-8]) are speculated to play an important role in the pathogenesis of acute lung injury by releasing superoxide anion (O\(_2^-\)), elastase, and lipid metabolites, regardless of underlying disease or conditions.\(^ {5,14}\) In addition to biophysical properties, surfactant has been documented to possess immunomodulatory activities.\(^ {15-22}\) They include inhibitory effects on production of reactive oxygen species, elastase, and cytokines.\(^ {20-22}\) Therefore, exogenous surfactant, in addition to treatment of symptoms, may prevent or attenuate progression of acidified milk-induced acute lung injury by suppressing these inflammatory mediators.

To test these hypotheses, we determined the effects of treatment with exogenous surfactant on acute lung injury induced by these milk products. To make this assessment, we compared physiologic, histologic, and biochemical findings of the acute lung injury between rabbits receiving surfactant and those not receiving surfactant using two time-course (short [4 h] and long [12 h]-term) experiments.

Materials and Methods

Animal Preparation and Experimental Protocol

This study was conducted according to the guidelines of the animal care review board of Kobe University School of Medicine. Our study consisted of four parts: studies 1, 2, 3, and 4 (four groups each study). We used 121 male Japanese White rabbits (body weight 2.1-2.6 kg), which were anesthetized with 15 mg/kg of ketamine injected intravenously and intubated with a 3.5-mm endotracheal tube through a tracheotomy. A catheter was inserted into an ear vein for infusion of fluids. Anesthesia was maintained with infusion of ketamine at a rate of 10 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) h\(^{-1}\). An arterial catheter was placed through a cut-down in the right femoral artery to monitor blood pressure and take samples for blood gas analysis and peripheral leukocyte count. Muscle relaxation was attained with pancuronium bromide, and the lungs were mechanically ventilated using an infant ventilator (IV100B, Sechrist, Anaheim, CA) with an inspired oxygen concentration of 100%. The initial tidal volume was set to 10 ml/kg measured by pneumotachograph and 5 cm H\(_2\)O of positive end-expiratory pressure was added. Respiratory rate was adjusted to produce normocapnia (\(P_{\text{a},C_{O_2}} = 32-38\) mmHg). The ventilator settings were adjusted (with increased inspiratory pressure, flow, and respiratory rate) to maintain the tidal volume and \(P_{\text{a},C_{O_2}}\) at the initial values, as the lung compliance decreased after the lung injury. Central venous pressure was also monitored via a catheter inserted through the femoral vein. All rabbits were placed supine on a heating pad under a radiant heat lamp so that the esophageal temperature could be kept at 37.6-40.1°C. Lactated Ringer’s solution was administered intravenously at a rate of 8 ml \(\cdot\) kg\(^{-1}\) \(\cdot\) h\(^{-1}\). Fifteen to 20 min after the start of ventilation, baseline values of lung compliance and hemodynamics were measured, and the first arterial blood sample was taken for determination of \(P_{\text{a},O_2}\), \(P_{\text{a},C_{O_2}}\), and peripheral leukocyte counts.

Preparation of Exogenous Surfactant

The surfactant used in the current study (Surfacten, Tokyo Tanabe, Tokyo, Japan) is a freeze-dried modified natural surfactant isolated from bovine lungs. It consists of approximately 85% phospholipids and 1% hydrophobic surfactant-associated proteins (SP-B, SP-C), the remainder being other lipids, such as glyceride, and free fatty acids. This surfactant preparation does not contain SPA. These components of Surfacten are basically the same as Survanta (Abbott, North Chicago, IL), which is commercially available in the United States. Surfactant was suspended in saline at a concentration of 50 mg dry weight/ml for use.

Grouping of Animals

Study 1. Study 1 used 28 rabbits randomly divided into four groups. Three groups received acidified infant formula (0.8 ml/kg, pH 1.8; Lai, Yukijirushi, Sapporo, Japan). This infant formula contains a protein composition similar to breast milk. Each study solution was titrated to a pH level of 1.8 by the addition of 6 N HCl. The pH level was determined using a pH meter (Horiba F-8L, Kyoto, Japan), which has a precision of 0.01 pH units over the entire pH range. The electrode was calibrated using standard buffers at pH values of 1 and 4. Thirty minutes after instillation of the acidified infant formula, two groups received intratracheal surfactant 100 mg/kg and 200 mg/kg, whereas the rabbits in the third group received no pharmacologic therapeutic treatment. The fourth group received acidified saline instillation (0.8 ml/kg, pH 1.8) and no treatment.

Study 2. Study 2 also consisted of four groups. Three groups received acidified breast milk (0.8 ml/kg). As in
Another group received intratracheal acidified saline. The acidified saline, milk products, and surfactants were prepared and given in the same manner as in studies 1 and 2. However, we observed the animals for 12 h in studies 3 and 4.

Studies 3 and 4. Group assignments in studies 3 and 4 was similar to those in studies 1 and 2, respectively. The acidified saline, milk products, and surfactants were prepared and given in the same manner as in studies 1 and 2. However, we observed the animals for 12 h in studies 3 and 4.

Assessment of Acute Lung Injury

Arterial Blood Gas Analysis and Lung Mechanics. Data of hemodynamics and lung compliance were recorded at specified points. Arterial blood samples for gas analysis and peripheral leukocyte counts were also obtained during the study. Arterial blood gases were analyzed for PaO₂, PaCO₂, and pH (ABL2, Radiometer, Copenhagen, Denmark), and the number of leukocytes was measured with a counter (Sysmex K-1000, Toa Iyou, Kobe, Japan). The alveolar-arterial oxygen tension difference (A-aDO₂) was calculated as follows:

\[
\text{A-aDO₂} = F_{\text{IF}} \times \left[ \text{PB} - \text{PH₂O} \right] - \frac{[\text{PaCO₂/RQ}]}{[\text{PaO₂}]} - 713 - \frac{\text{PaCO₂}}{0.8} - \frac{\text{PaO₂}}{1.14}
\]

Lung mechanics were measured by the passive expiratory flow-volume technique.²⁵ The airflow was measured with a Fumech 00 pneumotachograph and a differential pressure transducer (model MP045, Validyne Engineering, Northbridge, CA). Airway pressure was measured at the proximal end of the pneumotachometer with a semiconductor pressure transducer (model P-300 501G, Copal Electronics, Tokyo, Japan). The volume was determined for each breath by digital integration of airflow using a respiration monitor (Alvision, Tokyo, Japan). The static compliance and resistance of the total respiratory system were then calculated by personal computer (PC9801 VM11, NEC, Tokyo, Japan).

At the end of observation (4 h after induction of the lung injury in studies 1 and 2, and 12 h in studies 3 and 4), the thorax was opened and blood (15 ml) was drawn into a heparinized syringe (20 U/ml) from the pulmonary artery for the chemiluminescence assay. The rabbits were killed by an injection of thiomyal (overdose). The heart and lungs were removed en bloc. The lungs were divided into three segments: the right lobe was used for bronchoalveolar lavage, the left upper lobe for wet-to-dry weight ratio, and the left lower lobe for histologic examination.

Lung Wet-to-Dry Weight Ratio. The left upper lobe of each rabbit was weighed and then dried to constant weight at 60°C for 24 h in an oven. The ratio of wet weight to dry weight was calculated to assess tissue edema.

Analysis of Bronchoalveolar Lavage Fluid. Through the right mainstem bronchus, 35 ml saline solution with EDTA-2Na at 4°C was slowly infused and withdrawn to obtain bronchoalveolar lavage (BAL) fluid. This procedure was repeated three times. Indomethacin was added to the BAL fluid to inhibit the further metabolism of arachidonic acid to prostaglandins during analysis. The BAL fluid was analyzed for cell count and cell differentiation. A cytocentrifuged preparation (Cytopsin 2, Shandon, Pittsburgh, PA) of the BAL fluid was stained with Wright-Giemsa stain for cell differentiation. The cells present in the fluid were counted by a counter and the Bürker-Türk method. The fluid was centrifuged at 250g at 4°C for 10 min to remove the cells. The cell-free supernatant was divided into several aliquots and stored at −70°C until assay. Several substances, metabolites, and mediators in the BAL fluid were measured: Albumin concentrations were determined by nephelometry with immunoglobulin G fraction of goat antirabbit albumin (Cappel, Durham, NC); concentrations of IL-8 were measured by enzyme immunoassay (Amersham, Buckinghamshire, UK); and concentrations of thromboxane A₂ (TXA₂) and prostacyclin (PGI₂) were quantified by radioimmunoassay (Amersham, Buckinghamshire, UK) as thromboxane B₂ and 6-keto prostaglandin F₁α, the stable metabolites, respectively.

Chemiluminescence Assay. Reagents. Cypridina luciferin analog, (CLA; 2-methyl-6-phenyl-3,7-dihydroimidazo [1,2-a] pyrazin-3-one), dimethyl sulfoxide (DMSO), Hank’s balanced salt solution (HBSS), Histopaque-1119, Histopaque-1077, and N-formyl-methionyl-leucyl-phenylalanine (FMLP) were obtained from Sigma Chemical (St. Louis, MO). The CLA was dissolved to 56 μg/ml in distilled water. The solution was stored in 1-ml aliquots at −70°C. The CLA concentrations were based on €410 nm = 8,900 · M⁻¹ · cm⁻¹. FMLP, 5 mg, was dissolved in 1.14 ml DMSO. The solution was stored at −70°C. Just before use, the stored solution was diluted with 50% DMSO/50% HBSS to 100 μM.

Isolation of Neutrophils. Histopaque-1119, Histopaque-1077, and whole blood were layered and cen-
trifuged at 700g for 30 min at room temperature. The layer containing granulocytes (at the interface between HISTOQUE-1077 and -1119) was transferred to another tube. The cells were washed in HBSS and centrifuged twice at 200g for 10 min. The resultant leukocytes were suspended to 1 × 10^7 cells/ml in HBSS and were kept at 0°C for no longer than 3 h before use. The cell analysis showed that more than 98% of the cells were neutrophils, and the trypan blue dye exclusion test confirmed that more than 96% of the cells were viable. For the reference value, neutrophils were also separated from the rabbit not given intratracheal installation of fluids.

**Measurement of Chemiluminescence.** Measurement of chemiluminescence was based on the method of Sugioka et al. The incubation mixture contained 4 × 10^7 neutrophils, 1 μM FMLP, and 1 μM CLA, and the total volume was brought to 2 ml with HBSS. Cells and HBSS were preincubated for 3 min and the reaction initiated by the simultaneous addition of the other two components. CLA-dependent chemiluminescence, which is thought to represent mostly O_2^- production, was monitored with a luminescence reader (Lumicounter-1000, Nichion, Chiba, Japan). During luminescence measurement, the incubation mixture was agitated at 37°C in the luminescence reader. Peak chemiluminescence by neutrophils from normal rabbits (n = 4) was 1.5 ± 0.1 × 10^4 counts/min. Ketamine used as an anesthetic in the current study is thought to have no effect on O_2^- production by neutrophils at doses used in the clinical setting.

**Histopathologic Examination.** Immediately after the rabbits were killed (4 h and 12 h after the acidified milk products in studies 1 and 2 and studies 3 and 4, respectively), the left lower lobe was fixed by instillation of 10% formaldehyde solution through the left lower bronchus at 20 cm H_2O. The specimens were embedded in paraffin wax, stained with hematoxylin and eosin, and examined under a light microscope. The acute lung injury was scored by a blinded observer according to the following four items: alveolar congestion, hemorrhage, infiltration or aggregation of neutrophils in air space or vessel wall, and thickness of alveolar wall/hyaline membrane formation. Each item was graded according to a five-point scale: 0 = minimal (little) damage, 1 = mild damage, 2 = moderate damage, 3 = severe damage, and 4 = maximal damage.

**Statistics**

The lung injury score data are given as median (range), whereas the other data are expressed as mean ± SD. Parametric data were analyzed using a two-way analysis of variance with Tukey-Kramer test for between-group comparisons at each treatment interval, and repeated-measurement analysis of variance for comparisons within groups. The lung injury score was analyzed using the Kruskall-Wallis rank test. P < 0.05 was deemed significant.

**Results**

Of 121 rabbits enrolled in the study, 9 rabbits were excluded from analysis because of death from hypoxemia during the experiments: 5 and 4 rabbits receiving infant formula and breast milk, respectively. However, all of the animals that had received exogenous surfactant survived throughout the study. Thus, we accomplished our protocol in 112 rabbits (n = 7 in each group).

**Changes in Oxygenation**

As shown in figure 1, intratracheal instillation of acidified saline caused a transient increase of A-aDO_2 (P < 0.05 vs. basal values), but this returned to a normal level by the end of the study. Intratracheal instillation of acidified milk products increased A-aDO_2 dramatically, and this variable remained high at 4 h after induction of the acute lung injury. Treatment with surfactant (100 mg/kg or 200 mg/kg) successfully improved oxygenation deteriorated by acidified milk products. The degree of improvement was greater in rabbits receiving surfactant 200 mg/kg than in those receiving surfactant 100 mg/kg (fig. 1).

Figure 2 indicates that deterioration of oxygenation caused by the acidified milk products still persisted at 12 h. Surfactant at a dose of 200 mg/kg restored oxygenation to basal levels in the two models of lung injuries. Although surfactant 100 mg/kg attenuated the increase in A-aDO_2, oxygenation was not completely back to normal levels by 12 h after treatment.

**Change in Hemodynamics and Peripheral Leukocyte Counts**

There was no difference in arterial pressure, central venous pressure, or heart rate among the groups at any point in studies 1-4 (data not shown). Peripheral circulating leukocyte counts gradually reduced with instillation of the infant formula or breast milk, reached the lowest level 3 h after administration, and remained low until 4 h after induction of the acute lung injury (table 1). The peripheral leukopenia was less severe in rabbits receiving surfactant at 3 h and thereafter. The number of
leukocytes after instillation of the acidified milk products tended to increase to the basal values in rabbits receiving surfactant.

**Lung Mechanics**

As shown in figure 3, lung static compliance immediately after the start of mechanical ventilation was not different between the groups. There was no difference in resistance between the groups at basal point (data not shown). Intratracheal instillation of the acidified milk products dramatically decreased compliance (fig. 3) and increased resistance (data not shown). Both doses of surfactant improved compliance and resistance. In rabbits receiving surfactant 200 mg/kg, compliance was restored to values similar to the saline groups (fig. 3) at 4 h postinduction and thereafter remained at these levels in the two models (fig. 4). However, surfactant 100 mg/kg could not completely improve compliance to levels of the saline groups by 12 h after the acute lung injury induction.

**Lung Edema**

As shown in table 2, the lung wet-to-dry weight ratio was increased in the rabbits receiving milk products compared with those receiving saline in short-term ex-
### Table 1. Changes in Peripheral Leukocyte Counts ($\times 10^5$ cells/$\mu L$)

<table>
<thead>
<tr>
<th></th>
<th>Time after Induction of Acute Lung Injury</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>1 h</td>
<td>2 h</td>
<td>3 h</td>
<td>4 h</td>
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<tr>
<td>Study 1</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>S</td>
<td>56 ± 8</td>
<td>47 ± 11§</td>
<td>43 ± 7§</td>
<td>42 ± 6§</td>
<td>42 ± 5§</td>
</tr>
<tr>
<td>IF</td>
<td>54 ± 9</td>
<td>31 ± 9§</td>
<td>24 ± 4§</td>
<td>16 ± 2§</td>
<td>19 ± 1§</td>
</tr>
<tr>
<td>IF-PSF100</td>
<td>58 ± 9</td>
<td>39 ± 8§</td>
<td>30 ± 4§</td>
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<td>26 ± 4§</td>
</tr>
<tr>
<td>IF-PSF200</td>
<td>52 ± 7</td>
<td>38 ± 6§</td>
<td>31 ± 4§</td>
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<tr>
<td>Study 2</td>
<td></td>
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<td></td>
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<tr>
<td>S</td>
<td>51 ± 8</td>
<td>45 ± 7</td>
<td>44 ± 9§</td>
<td>46 ± 12§</td>
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<td>BM</td>
<td>57 ± 8</td>
<td>29 ± 9§</td>
<td>19 ± 5§</td>
<td>14 ± 1§</td>
<td>18 ± 2§</td>
</tr>
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<td>BM-PSF100</td>
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<td>35 ± 7§</td>
<td>27 ± 8§</td>
<td>22 ± 4§</td>
<td>25 ± 6§</td>
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<tr>
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<td>40 ± 8§</td>
<td>34 ± 8§</td>
<td>31 ± 6§</td>
<td>34 ± 7§</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>S</td>
<td>60 ± 12</td>
<td>52 ± 10§</td>
<td>56 ± 11</td>
<td>51 ± 9§</td>
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</tr>
<tr>
<td>IF</td>
<td>59 ± 10</td>
<td>17 ± 4§</td>
<td>25 ± 4§</td>
<td>32 ± 6§</td>
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<tr>
<td>IF-PSF100</td>
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<td>36 ± 7§</td>
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</tr>
<tr>
<td>IF-PSF200</td>
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<td>32 ± 8§</td>
<td>42 ± 9§</td>
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<td>Study 4</td>
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<td></td>
</tr>
<tr>
<td>S</td>
<td>54 ± 9</td>
<td>48 ± 11§</td>
<td>52 ± 9</td>
<td>47 ± 10§</td>
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</tr>
<tr>
<td>BM</td>
<td>61 ± 12</td>
<td>19 ± 3§</td>
<td>26 ± 4§</td>
<td>35 ± 8§</td>
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<td>28 ± 7§</td>
<td>37 ± 8§</td>
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<td>BM-PSF200</td>
<td>52 ± 8</td>
<td>33 ± 9§</td>
<td>42 ± 10§</td>
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</tbody>
</table>

S = intratracheal saline; IF = intratracheal infant formula; BM = intratracheal breast milk; IF-PSF100, BM-PSF100 = intratracheal infant formula or breast milk followed by intratracheal surfactant 100 mg/kg; IF-PSF200, BM-PSF200 = intratracheal infant formula or breast milk followed by intratracheal surfactant 200 mg/kg.

Values are mean ± SD.

* $P < 0.05$ versus group IF or BM.

† $P < 0.05$ for groups PSF200 versus groups PSF100.

‡ $P < 0.05$ versus group S.

§ $P < 0.05$ versus basal values within groups.
experiments (4 h). Surfactant treatment decreased wet-to-dry weight ratio. The attenuating effect was greater in surfactant at dose of 200 mg/kg than 100 mg/kg. The elevation of wet-to-dry weight ratio induced by milk products remained for 12 h after administration (table 3). The two doses of surfactant successfully attenuated the increase in this variable to levels in the saline groups 12 h after treatment (table 3).

**Analysis of BAL Fluid**

Recovery percentages of BAL fluid in the groups in the four studies ranged between 77% and 85%, indicating no significant differences among the groups. As shown in figure 5, the total number of leukocytes recovered in BAL fluid was higher in the groups receiving the acidified milk products than in the intratracheal saline groups. After 12-h experiments, the counts were less with higher doses of surfactant than with lower doses (fig. 6). Differential counts revealed that macrophages occupied as much as 90% of total leukocytes in BAL fluid (fig. 5). In the groups in which acidified milk products were instilled, the number of neutrophils was elevated in BAL fluid. The neutrophils-to-total leukocytes ratio (%neutrophils) was signifi-

### Table 2. Analysis of Bronchoalveolar Lavage Fluid and Acute Lung Injury (ALI) Score in Study 1 and Study 2

<table>
<thead>
<tr>
<th></th>
<th>Study 1</th>
<th>Study 2</th>
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<tbody>
<tr>
<td></td>
<td>S</td>
<td>IF-PSF100</td>
</tr>
<tr>
<td>W/D ratio</td>
<td>5.6 ± 0.2</td>
<td>6.0 ± 0.2†</td>
</tr>
<tr>
<td>Albumin (mg/ml)</td>
<td>2 ± 2</td>
<td>9 ± 5†</td>
</tr>
<tr>
<td>IL-8 (fmol/ml)</td>
<td>2 ± 3</td>
<td>10 ± 4†</td>
</tr>
<tr>
<td>TxB2 (pg/ml)</td>
<td>113 ± 49</td>
<td>127 ± 52</td>
</tr>
<tr>
<td>6-keto-PGF1,2</td>
<td>174 ± 97</td>
<td>171 ± 104</td>
</tr>
<tr>
<td>ALIS</td>
<td>1 (0-3)</td>
<td>9 (8-12)†</td>
</tr>
</tbody>
</table>

S = intratracheal saline; IF = intratracheal infant formula; BM = intratracheal breast milk; IF-PSF100, BM-PSF100 = intratracheal infant formula or breast milk followed by intratracheal surfactant 100 mg/kg; IF-PSF200, BM-PSF200 = intratracheal infant formula or breast milk followed by intratracheal surfactant 200 mg/kg; IL-8 = interleukin-8; TxB2 = thromboxane B2; PGE1 = prostaglandin E1; W/D ratio = wet weight/dry weight ratio.

Values are mean ± SD or median (range). The acute lung injury score (ALIS) consists of four items as follows: [a] alveolar congestion, [b] hemorrhage, [c] infiltration or aggregation of neutrophils in air space or vessel wall, and [d] thickness of alveolar wall/hyaline membrane formation. Scores of each item range from 0 to 4+ as follows: 0 = minimal (little) damage; 1 = mild damage; 2 = moderate damage; 3 = severe damage; 4 = maximal damage. Maximum and minimum possible scores are 16 and 0, respectively.

* P < 0.05 versus group IF or BM.
† P < 0.05 for group PSF200 versus group-PSF100.
‡ P < 0.05 versus group S.
Table 3. Analysis of Bronchoalveolar Lavage Fluid and Acute Lung Injury (ALI) Score in Study 3 and Study 4

<table>
<thead>
<tr>
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<th>Study 4</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>IF</td>
</tr>
<tr>
<td>W/D ratio</td>
<td>5.8±0.3</td>
<td>7.3±0.6†</td>
</tr>
<tr>
<td>Albumin (mg/ml)</td>
<td>1±1</td>
<td>27±12†</td>
</tr>
<tr>
<td>IL-8 (fmol/ml)</td>
<td>1±1</td>
<td>2±1</td>
</tr>
<tr>
<td>TxB2 (pg/ml)</td>
<td>124±46</td>
<td>168±67</td>
</tr>
<tr>
<td>6-keto-PGF1α (pg/ml)</td>
<td>173±86</td>
<td>212±101</td>
</tr>
<tr>
<td>ALIS</td>
<td>0(0-3)</td>
<td>11(6-15)‡</td>
</tr>
</tbody>
</table>

Values are mean ± SD or median (range). See Table 2 for abbreviations. The acute lung injury score (ALIS) consists of four items as follows: [a] alveolar congestion, [b] hemorrhage, [c] infiltration or aggregation of neutrophils in air space or vessel wall, and [d] thickness of alveolar wall/hyaline membrane formation. Scores of each item range from 0 to 4+ as follows: 0 = minimal (little) damage; 1+ = mild damage; 2+ = moderate damage; 3+ = severe damage; 4+ = maximal damage. Maximum and minimum possible scores are 16 and 0, respectively.

- *P < 0.05 versus group IF or BM.
- †P < 0.05 for group PSF200 versus group -PSF100.
- ‡P < 0.05 versus group S.

The BAL fluid concentrations of albumin were higher in the rabbits into which milk products were instilled than in the saline-treated rabbits in short- and long-term experiments (tables 2 and 3). Surfactant at both doses attenuated the increase in albumin concentrations.

The BAL fluid concentrations of IL-8 and thromboxane B2 were elevated in the rabbits receiving the acidified milk products but did not increase in surfactant-treated groups compared with those receiving saline solution after 4 h (table 2). These mediators returned to control levels by 12 h after the acute lung injury induction point (table 3). We found no differences in 6-keto-PGF1α levels in BAL fluid between the groups.

**Chemiluminescence**

The FMLP-stimulated, CLA-dependent chemiluminescence (representing O2 production) by neutrophils isolated from pulmonary artery 4 h after induction of lung injury was significantly higher in the group in which milk products were instilled than in the saline-instilled group (fig. 7). Treatment with exogenous surfactant attenuated the increase in chemiluminescence. On the other hand, chemiluminescence by neutrophils measured 12 h after instillation of milk products was not increased.

**Histopathology**

No remarkable damage was found in the saline-instillation groups (fig. 8A). Light microscopic findings 4 h after instillation of infant formula included extensive morphologic lung damage: edema, hemorrhage, ruptured and thickened alveolar walls, infiltration of inflammatory cells in alveolar spaces, and diffuse proteinaceous exudate.
Fig. 6. Effects of surfactant on the number of white blood cells (WBCs) and percentage of neutrophils (%neutrophils) in bronchoalveolar lavage fluid (BALF) in study 3 and study 4. Data are expressed as mean ± SD. Definitions of study groups are shown in the legend of figure 1. #P < 0.05 versus group S; *P < 0.05 versus group IF or BM. †P < 0.05 for group PSF200 versus group PSF100. (A and C) WBC counts. (B and D) Percentage of neutrophils.

exudate (fig. 8B). These changes were less severe in the rabbits treated with surfactant (figs. 8C and 8D). Morphologic lung injury 12 h after instillation of infant formula was less pronounced compared with that in the short-term (4 h) experiment (fig. 9). We observed that histologic findings in the lung after instillation of breast milk were similar to those after infant formula (data not shown).

The acute lung injury scores at 4 h after receiving infant formula or breast milk were greater than those in the saline groups (table 2). The scores were reduced by surfactant. This lowering effect was greater with surfactant 200 mg/kg than with 100 mg/kg (table 2). The acute lung injury scores 12 h after instillation of the acidified milk products were improved spontaneously compared with those at 4 h postinstillation but still remained high (table 3). Surfactant 200 mg/kg lowered the scores at 12 h postinstillation to near the levels of the saline groups.

Discussion

In the current study, we have confirmed the previous observation by Lerman et al. that intratracheal instillation of the acidified milk products caused physiologic and histologic lung damage. Furthermore, we have found that this lung injury persisted at least 12 h, and the severity of lung injury was similar between infant formula and breast milk. We have also shown that exogenous surfactant successfully attenuated the early (4 h) and late (12 h) phases of acute lung damages in a dose-dependent manner. We have confirmed neutrophil activation as assessed by chemiluminescence 4 h after the administration of acidified milk products in accordance with the past experiment. This activation was attenuated by surfactant. However, the neutrophil activation spontaneously subsided by 12 h after aspiration. Although mortality rate was not examined in the current study, surfactant replacement may have improved survival of the animals with the aspiration-induced lung injury. In the current study, surfactant failed to exhibit perfect improvement of the lung injury during observation period. Surfactant treatment after BAL by which inhibitors of surfactant are removed from alveolar spaces has been shown to provide better improvement of gas-exchange.

Fig. 7. Effects of surfactant on cypridina luciferin analog-dependent chemiluminescence (peak) by neutrophils isolated from pulmonary artery. Data are expressed as mean ± SD. Neutrophils are stimulated by formyl-methionyl-leucil-phenylalanine. Definitions of study groups and symbols of significant difference are shown in the legends of figure 1 and figure 6, respectively.
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in HCl aspiration-induced lung injury compared with surfactant instillation alone. This combined therapeutic strategy may have elicited a complete improvement of the lung injury in the current study.

The rationale for the dose and timing of surfactant treatment in the current study was based on the following past reports. Previous animal experiments demonstrated that surfactant at doses of 100 mg/kg or 200 mg/kg attenuated acute lung injury induced by various conditions, including HCl instillation, endotoxin, viral pneumonia, hyperoxia, N-nitroso-N-methylurethane, or repetitive lung lavage. Lachmann et al. have demonstrated the superior efficacy of surfactant at larger doses (280–350 mg/kg) in restoring oxygenation in severe lung injury caused by intravenous anti-lung serum. It may have been worthwhile trying treatment with these high doses of surfactant in our study. In our preliminary study, however, several rabbits receiving 300 mg/kg of surfactant died of severe transient hypoxemia resulting from loading large volume of saline in which surfactant was suspended. It has been recommended that surfactant should be given as early as possible after acid aspiration in experiments using rats. Our clinical experiences of surfactant replacement therapy reveal that we are able to administer the drug within 20–30 min after our determination of its use under diagnosis of ARDS. Thus, this timing of surfactant administration after witnessing gastric aspiration is clinically feasible in a hospital setting.

The precise mechanism underlying acute lung injury associated with instillation of infant formula or breast milk is not fully understood. Similar to other models of acid aspiration-induced lung injury, the acidified milk products probably lead to increase in permeability and
consequently to influx of protein-rich edema fluid into the alveolar spaces by initial direct physiochemical damage to the alveolar-capillary membrane. These damages are likely to occur within 10 min after aspiration of the milk products. The intraalveolar plasma exudates are potent surfactant inhibitors. The impairment of surfactant is thought to be caused by competition for space at the air-liquid interface. Surfactant dysfunction increases surface tension on the alveolar wall and subsequently causes atelectasis and mismatch of the ventilation-perfusion ratio. The functional surfactant deficiency further accumulates edema fluid into the alveolar space because of increased retractive forces across the alveolar-capillary membrane. Thus, a vicious cycle of surfactant dysfunction is constituted. To overcome this critical situation, it is pertinent to replenish surfactant using high doses of exogenous surfactant with the aim of recovery of favorable surfactant-to-inhibitor ratio. Because we administered surfactant 30 min after instillation of the milk products in the current study, the drug was theoretically unable to prevent initiation of the direct injuries to the alveolar-capillary membrane. In the short-term (4-h) experiment, surfactant seems to have readily improved gas exchange by interrupting the vicious cycle of surfactant inhibition.

The acute lung injury progresses as lung damage mediated by many cellular (e.g., macrophages) and humoral (e.g., chemical mediators) immunological components, which constitute a complex network to amplify inflammatory responses, leading to acute lung injury. In particular, activated neutrophils are thought to play an important role in formation of the network by releasing reactive oxygen species, proteases, and cytokines. These potent mediators primarily attack the endothelial

Fig. 9. Light micrograph (hematoxylin and eosin, × 100) of the left lower lobe of lung in study 3 (long-term experiment). A = group S, B = group IF, C = group IF-PSF100, D = group IF-PSF200.
and epithelial cells. In this pathway, chemotaxins including IL-8 and thromboxane B2 promote accumulation of neutrophils in the lung. In the current study, intratracheal instillation of infant formula or breast milk increased infiltration of neutrophils into the alveolar spaces as well as decreasing the peripheral leukocyte counts. These changes were less prominent in the surfactant-treated rabbits. Our observations suggest that surfactant treatment mitigated recruitment of neutrophils to the lung. Surfactant is known to suppress production of chemotaxins in alveolar macrophages. Phospholipids of surfactant are also reported to inhibit thromboxane B2 release. In the current study, surfactant treatment decreased the chemotaxin levels in BAL fluid. This may contribute to successful attenuation of neutrophils' sequestration and resultant lung injury. Surfactant has been demonstrated to inhibit production of reactive oxygen species by the phagocytes. In agreement with these previous studies, we have shown that surfactant treatment reduced O2•− production by neutrophils obtained from the pulmonary artery. In the current study, 100% oxygen was used to ventilate the animal lungs because of severe hypoxemia induced by acidified milk. High levels of inspired oxygen have been reported to deteriorate acid-induced lung injury. Thus, surfactant instillation may be effective to reduce the damage from reactive oxygen species during ventilation with high inspired oxygen tension. Lung edema formation was a key finding in that acute lung injury induced by acidified milk products caused severe pulmonary edema, as evidenced by increases in the lung wet-to-dry weight ratio and albumin concentrations in BAL fluid. These variables were lower in rabbits receiving surfactant, suggesting that the drug attenuated lung edema formation by dynamic (retractive forces) and immunologic (secondary epithelial-capillary membrane damage) mechanisms.

In conclusion, we have shown that early (30 min after insult) treatment with exogenous surfactant attenuated the magnitude of physiologic and histologic lung damage induced by acidified milk products in a dose-dependent fashion. We have observed the effectiveness of the drug in both short (4-h) and long (12-h)-term experiments. Improvement of the lung injury may be attributable not only to restoration of surfactant function in alveoli by supplementation of the large dose but also to inhibition of neutrophils' recruitment and activation in the lung, as evidenced by reduction of neutrophil counts and chemiluminescence. This study is a basis for a clinical trial to elucidate whether exogenous surfactant is a promising therapeutic approach in pediatric patients with acute lung injury or acute respiratory distress syndrome induced by aspiration of milk products.

The authors thank Tokyo-Tanabe Pharmaceutical for a generous supply of Surfacten.

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Anesthesiology, V 91, No 1, Jul 1999