LYSOPHOSPHATIDYLCHOLINE ABROGATES THE CR1 PRESERVING EFFECT OF SURFACTANT ON QUARTZ-EXPOSED HUMAN GRANULOCYTES

G. ZETTERBERG, T. CURSTEDT, J. LUNDAHL, and A. EKLUND

1Department of Medicine, Division of Respiratory Medicine
Kaulinska Hospital

2Department of Clinical Chemistry
Danderyd Hospital and Kaulinska Hospital

3Department of Clinical Immunology, Kaulinska Hospital, Stockholm, Sweden

Abstract—The effects of pulmonary surfactant on granulocytes were studied by flow cytometry. Cells from hemolyzed blood were first activated by N-formylmethionyl-leucyl-phenylalanine (fMLP) which mobilizes complement receptor 1 (CR1) from the intracellular pool to the cell surface. The reduced CR1 expression observed after quartz incubation was abolished by a porcine surfactant preparation containing phospholipids and the hydrophobic surfactant proteins. Phospholipids alone had no preservative capacity. However, addition of the surfactant proteins to the phospholipids did not restore the CR1 values to that of intact surfactant, probably due to changed protein structure during the purification procedure. Heating of surfactant at 37°C up to 72 h reduced the preservative effect of surfactant on CR1 expression. Congruent results of CR1 expression were achieved when 1–10% lysophosphatidylcholine was added to the surfactant preparations. Our results imply that lysophosphatidylcholine formed during storage of surfactant at elevated temperatures influences CR1 expression on granulocytes.

INTRODUCTION

Surfactant, which is a mixture of lipids and proteins, is secreted from alveolar type II cells. It covers the alveolar surface and plays an important physiological role by decreasing air-liquid surface tension at end-expiration, thereby keeping the airways open and reducing airway resistance (1, 2). Presently surfactant replacement therapy is routinely used in premature neonates suffering from respiratory distress syndrome (RDS) (3, 4). Surfactant also counteracts the accumulation of luminal fluid and mucosal edema (1). Furthermore, it exerts effects on the immune system, by influencing cytokine production (5) and on the host
defense system through its anti-bacterial (6, 7) and anti-viral (8) properties. The
alveolar lining fluid may modify the cellular response to inhaled particles and
substances, including nonspecific binding of noxious agents (9, 10), but anti-
inflammatory or immunosuppressive effects have also been reported (11–13).

An inflammatory reaction occurs in lungs exposed to quartz, with recruit-
ment of neutrophils and mononuclear phagocytes, leading to development of
pulmonary fibrosis and functional impairment (14). The expression of functional
cell surface receptors, such as complement receptor 1 (CR1) is altered on accumu-
lated leukocytes (15). CR1 is an intracellularly stored opsonin receptor for
complement factor C3b and is mobilized to the cell surface upon activation. CR1
is expressed on leukocytes and erythrocytes (16), and according to earlier reports
(17) soluble CR1 has anti-inflammatory effects.

Activated granulocytes from peripheral blood express less CR1 on the cell
surface after incubation with quartz, whereas complement receptor 3 (CR3)
expression remains unaffected (15). The shedding of CR1 is protease-dependent.

We have earlier demonstrated that the quartz-induced shedding of CR1 on
activated granulocytes in vitro was abolished by a simultaneous addition of a
porcine surfactant preparation (18). The preparation, which is used in treatment
of RDS contains phospholipids and the two hydrophobic surfactant proteins B
and C (SP-B and SP-C) (3, 19).

Our hypothesis was that an intact surfactant preparation is required to exert
an optimal CR1-preserving effect. Storage of surfactant, especially at elevated
temperatures, will spontaneously decompose phosphatidylcholine to lysophos-
phatidylcholine, which may inhibit some properties of surfactant. The objective
of the present study was to investigate the relative contribution of the lipid and
protein fractions, respectively, with special attention given to storage conditions,
and to the overall CR1-preserving effect of surfactant on quartz-exposed human
granulocytes.

MATERIAL AND METHODS

Preparation of Porcine Surfactant, Surfactant Fractions and Lipid Mixtures. Surfactant was
prepared from minced pig lungs by a combination of washing, chloroform-methanol extraction, and
liquid-gel chromatography (19). The isolated polar lipid fraction was dissolved in chloroform (20
ml/g of surfactant) and sterilized using a high-pressure filter system. Subsequent steps, including
evaporation of the solvent and suspension of surfactant in normal saline by gentle sonication at
a phospholipid concentration of 80 mg/ml were performed under sterile conditions. The prepara-
tion consists of 98–99% phospholipids (30–35% dipalmitoylphosphatidylcholine) and 1–2% of the
hydrophobic surfactant proteins B and C. A similar preparation is also commercially available (Curo-
surf®, Chiesi Farmaceutici, Parma, Italy).

Modified preparations were obtained by separation of the hydrophobic proteins SP-B and
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SP-C from the phospholipids by chromatography on Sephadex LH-60 in chloroform/methanol 1:1 (v/v) containing 5% 0.1 M HCl (20). To the phospholipid fraction, eluted between 60-120% of the column volume, methanol and water were added (final proportions of chloroform/methanol/water, 8:4:3 by volume) (21). After separation the nonpolar phase was evaporated by dryness and analyzed for phospholipids and proteins. The purified phospholipids were suspended in normal saline by sonication and repeated freezing and thawing. The percentage of remaining proteins was 50.1%.

The proteins SP-B and SP-C, eluted between 0-60% of the column volume, were evaporated to dryness (20, 21) and added to the phospholipid fraction or to a synthetic lipid mixture containing dipalmitoylphosphatidylcholine/phosphatidylglycerol/palmitic acid in the proportions 68:22:9 (w/w/w).

Lysophosphatidylcholine (1-palmitoyl-sn-glycero-3-phosphocholine) was added at different concentrations (0-10%) to the porcine surfactant preparation. For some experiments, the porcine surfactant preparation was kept in a waterbath for different lengths of time (0-72 h) at 37°C and 55°C, respectively. All preparations were suspended in normal saline at a concentration of 50 mg/ml.

Synthetic lipids were purchased from Sigma Chemical Co., St. Louis, Missouri.

Preparation of Quartz. Quartz (kindly provided by Prof. Å. Svensson, Karolinska Hospital, Stockholm, Sweden) was prepared from natural sand by sedimentation in water. It was not freshly crushed, and consisted of 98.3% crystalline and 1.7% amorphous silica. With the use of a projection light microscope (VisioPan projection microscope, Reichert, Austria) the geometric mean of the particles was measured to be 1.2 μm, with a geometric standard deviation of 1.6 μm. There were no endotoxins in the preparation, as assessed by a Limulus assay. The quartz was suspended in RPMI medium (Northumbria Biologicals Ltd., Cramlington, UK) without fetal calf serum to a concentration of 1600 μg/ml. The suspension was ultrasonicated for 10–30 sec, centrifuged and resuspended with RPMI to a concentration of 400 μg/ml.

Preparation of Leukocytes. Blood from healthy blood donors (age 18-65 ys) was collected in tubes containing EDTA. The blood was hemolyzed in 100 μl portions by dilution in 2 ml 4°C isotonic lysing buffer (1.54 mM NH₄Cl, 1.5 mM KHCO₃, 0.1 mM EDTA, pH 7.2). After incubation (5 min, 4°C) the cells were centrifuged (300g, 5 min, 4°C), washed with 2 ml cold 0.15 M phosphate-buffered saline (pH 7.3) supplemented with 0.1 mM EDTA and 0.02% Na-azide (PBS-EDTA), and therefore centrifuged again (300g, 5 min, 4°C). The Ethics Committee at Karolinska Hospital approved the study.

Receptor Mobilization. Leukocytes (except tubes for background controls that were kept on ice) were incubated (15 min, 37°C) in Hepes-buffered RPMI-medium (RPMI-1640, Northumbria Biologicals Ltd.) (pH 7.3) supplemented with 5 x 10⁻⁷ M N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma Chemical Co.) for mobilization of intracellularly stored CR1 to the cell surface (22). After centrifugation and washing in PBS-EDTA, only the fMLP-activated samples were kept on ice during the following quartz incubation.

Incubation Procedures. The fMLP-activated leukocytes were incubated (15 min, 37°C) with either quartz (100 μl) and RPMI (25 μl) or quartz (100 μl), RPMI (20 μl) and different surfactant preparations (5 μl) (final concentration 2 mg/ml). The cells were then washed twice in PBS-EDTA.

Immunostaining and Flow Cytofluorometry. Expression of CR1 on granulocytes was analyzed by incubating the leukocyte pellets (30 min, 4°C) on ice in the dark with 10 μl monoclonal anti-CR1 of mouse IgG₁ (DAKO AS, Glostrup, Denmark) and 100 μl PBS-EDTA. After two washes with 4°C PBS-EDTA, the leukocyte pellets were resuspended in 100 μl cold fluorescein isothiocyanate-conjugated rabbit F(ab')₂ fragments anti-mouse immunoglobulins (RAM-FITC) (DAKO) diluted 1:20 in PBS-EDTA. After incubation (30 min, 4°C) on ice in the dark, the cells were washed twice with 4°C PBS-EDTA and resuspended in 0.5 ml cold PBS-EDTA before examination. Isotype-matched control antibody (FITC-conjugated mouse IgG₁, Coulter Inc., Hialeah, Florida) was used to define the nonspecific binding which was subtracted from the specific binding.

The leukocytes were examined in a flow cytofluorometer (Epics XL Cytometer, Coulter Inc.)
using light scattering properties for differentiation. Calibration was performed daily with Standard-Brite (Coulter Inc.) to ensure the same fluorescence level in each experiment. Well-separated clusters in two-parameter scatter plot histograms represent granulocytes, lymphocytes, and monocytes. A discrimination frame was placed around the granulocyte cluster and at least 5,000 cells were counted for analysis. The instrument gives mean fluorescence intensity (MFI) of the cell population within the discrimination frame.

Statistical Analysis. Results are presented as medians and ranges of the groups, together with individual values in figures. A two-sided non-parametric Mann-Whitney U-test was used and results were considered statistically significant at $P < 0.05$.

**RESULTS**

*Effects of Different Surfactant Components on CR1 Expression.* Incubation of fMLP-activated granulocytes with quartz reduced the CR1 expression, measured as mean fluorescence intensity (MFI), from median (range) MFI 47.5 (37.6–55.3) to 21.6 (17.8–30.1) ($N = 8$) ($P < 0.001$) (Figure 1). The quartz-

![Fig. 1. The expression of CR1 on fMLP-activated granulocytes was significantly reduced by addition of quartz. Native porcine surfactant added to the quartz-exposed cells abolished this reduction. After removal of the proteins (i.e. SP-B and SP-C) from surfactant the remaining phospholipid fraction (PL) lost the preserving effect. Addition of the proteins did not restore CR1 expression. Synthetic lipids (i.e. lipids) with or without proteins did not differ from only quartz-exposed cells. MFI is presented as median with individual data; $N = 8$. ***$P < 0.001$ vs. fMLP.](image-url)
induced downregulation of CR1 was inhibited by the porcine surfactant preparation, giving an MFI-value of 39.4 (30.0–47.5), and did not significantly differ from fMLP-stimulated granulocytes. In contrast, the protein-depleted surfactant (phospholipid-fraction) or the recombined phospholipid and protein fractions did not restore CR1 expression to the levels registered when intact surfactant was used. The MFI-values were 19.8 (13.0–26.4) and 19.3 (14.6–25.7), respectively, and similar MFI-values, 25.3 (17.8–32.2) and 26.8 (19.4–34.3), were registered when the synthetic lipid mixture, alone or together with the protein fraction, were incubated with quartz and fMLP-activated granulocytes.

Effects of Heated Surfactant on CR1 Expression. The CR1 expression of fMLP-activated granulocytes was significantly lowered from MFI 47.1 (35.7–63.2) to 23.1 (14.0–31.2) after incubation with quartz \( (P < 0.01) \) \( (N = 7) \) (Figure 2). Addition of porcine surfactant inhibited this downregulation of CR1, giving an MFI of 34.6 (30.8–51.9). The preserving effect on CR1 expression was significantly reduced if surfactant was kept at 37°C for 1–72 h before incubation with quartz and fMLP-activated granulocytes. For surfactant stored at 37°C for 72 h before use the CR1 expression registered was MFI 27.1 (23.0–42.6). This impaired effect was even more pronounced when surfactant was heated at 55°C for 1–72 h (data not included).

Fig. 2. Addition of quartz to fMLP-activated granulocytes reduced CR1 expression, and addition of surfactant (not heated, “S-0 h”) inhibited this effect. Exposure to surfactant stored at 37°C for 1–72 h (“S-1 h” to “S-72 h”) significantly reduced CR1 expression. MFI is presented as median with individual data; \( N = 7 \). *\( P < 0.05 \), and **\( P < 0.01 \) vs. fMLP.
Effects of Lysophosphatidylcholine Added to Surfactant on CR1 Expression.

Lysophosphatidylcholine was added at increasing concentrations to the surfactant preparation to simulate changes occurring when heating surfactant (N = 9) (Figure 3). The fMLP-stimulated cells had an MFI of 42.8 (31.5–52.9), and after incubation with quartz a reduction of CR1 was recorded, to 16.6 (12.9–26.4) (P < 0.001). Addition of surfactant to the quartz-exposed cells reduced downregulation of CR1 to MFI 32.9 (19.6–39.1). When lysophosphatidylcholine was added the preserving effect by surfactant was already reduced at a concentration of 1%, giving a CR1 expression of 21.2 (16.7–31.2). By increasing the lysophosphatidylcholine concentration to 10% a more marked reduction was observed, MFI 15.2 (12.1–22.5), which was similar to that observed after only quartz exposure.

Inhibition of Quartz-Induced Shedding of CR1 by Surfactant.

We summarized the observations of CR1 expression (N = 24) in Table 1, based on experiments presented in Figures 1–3. Non-activated granulocytes, kept at 4°C, had a low CR1 expression of MFI 6.3 (2.8–13.0) (baseline value). Activation by fMLP (15 min, 37°C) increased the CR1 expression to 47.2 (31.5–63.2) (P < 0.001). Incubation with quartz (15 min, 37°C) reduced the CR1 expression to 21.3 (12.9–31.2) (P < 0.001). When porcine surfactant (2 mg/ml) was added together with quartz, the CR1 expression was MFI 34.5 (19.6–51.9) (P < 0.001 vs. both fMLP activation only and vs. fMLP and quartz).
DISCUSSION

The objective for this study was to identify the roles of different surfactant component(s) responsible for the mechanisms behind the observation that a porcine surfactant preparation, containing phospholipids and the hydrophobic proteins SP-B and SP-C, can neutralize quartz-provoked downregulation of CR1 from activated granulocytes (18). Neither the phospholipid fraction from porcine surfactant, after separation of the proteins, nor the protein fraction recombined with the phospholipid fraction or a synthetic lipid mixture retained the same CR1-preserving effect as porcine surfactant. Furthermore, the protective effect of surfactant was reduced if it was warmed up before use. A similar effect on CR1 expression was observed if an increasing percentage of lysophosphatidylcholine was added to the surfactant preparation.

Native surfactant includes phospholipids and at least four surfactant proteins, SP-A, -B, -C and -D (2, 23, 24). The hydrophilic proteins SP-A and SP-D have structural similarities and are collagenous-like glycoproteins (2, 24), but they are not included in our preparation from minced and purified pig lungs, whereas the hydrophobic SP-B and SP-C were. SP-B and SP-C, which are not commercially available, are of importance for the reduction of surface tension and the fast spreading of surfactant on epithelial surfaces.

In this study we separated phospholipids from proteins in order to elucidate the influence they may have on some surfactant properties. However, fractionation of surfactant into phospholipid and protein fractions included the use of acidified organic solvents, and thus proteins may have been methylated during the purification procedure (25). Furthermore, the procedure may also change the

| Background | 4°C | fMLP | fMLP + Quartz | fMLP + Quartz + Surfactant |
|------------|-----|------|---------------|----------------------------|
| 6.3        | 47.2a | 21.3b | 34.5bc         |
| 2.8-13.0   | 31.5-63.2 | 12.9-31.2 | 19.6-51.9 |

Results are represented as mean fluorescence intensity (MFI) and were summarized from information on Figure 1–3, expressed as medians and ranges (n = 24).

P < 0.001 vs. background
P < 0.001 vs. fMLP
P < 0.001 vs. fMLP and quartz
three-dimensional structure of the proteins (25, 26). Such changes may be the reason why addition of the protein fraction to the phospholipids or to the synthetic lipid mixture did not restore CR1 expression. Our results indicate that the composition of the phospholipids is of significant importance, since addition of lysophosphatidylcholine or decomposition of phospholipids by heating had significant influences on CR1 expression. However, any effects of SP-B and SP-C cannot be ruled out since the purification procedure may alter the protein structures.

Surfactant and polymorphonuclear leukocytes (PMN) may interact in different ways. Liau and coworkers (27) have studied the effects of activated PMN on surfactant. They used leukocyte elastase that degraded the surfactant proteins. This modified surfactant had a slow adsorption and a high surface tension. In another in vitro study PMN activated by phorbol myristate acetate impaired function of surfactant, measured as e.g. surface tension, this effect mainly being mediated by oxidant radicals, and these altered properties may be important in the pathogenesis of adult respiratory distress syndrome (ARDS) (28). In contrast to this latter study, we used fMLP for activation of PMN and measured how surfactant influenced the phenotype.

Jarstrand et al. (29) have reported that surfactant inhibits lipopolysaccharide-induced release of oxygen radicals from human peripheral blood neutrophils. From another group we also know that a surfactant preparation containing the hydrophobic proteins stimulated basal eicosanoid production by rat alveolar macrophages, but inhibited silica-induced thromboxane A2 and leukotriene B4 production (30). Based on these findings the authors suggested that surfactant inhibits the reactivity of alveolar macrophages to various stimuli. A parallel finding on PMN was demonstrated in our study, in which the CR1 expression was restored by surfactant after exposure to quartz. Others have reported that when fMLP-activated PMN were exposed to the same surfactant preparation as we used, elastase release was decreased, but the release was uninfluenced if only phospholipids and lipids (Exosurf®) were used (31).

Storage of surfactant at elevated temperatures decomposes phospholipids, thus increasing the formation of lysophosphatidylcholine. Already at a lysophosphatidylcholine concentration of 1% the effect of intact surfactant was reduced, and this was even more pronounced at 10%. The proportion of lysophosphatidylcholine in native porcine surfactant is very low (21, 32). A slight increase of lysophosphatidylcholine influences CR1 expression. This is also supported by other preliminary results from our group, where surfactant stored at 4–8°C for more than a year caused a reduced CR1 expression (data not included). Moreover, this surfactant had retained function on the surface tension. However, Fornasier et al. (33) reported that heated porcine surfactant with a lysophosphatidylcholine content of 18% did not improve lung mechanics in premature animals with surfactant deficiency. From our study we can not conclude if the observed
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effect of lysophosphatidylcholine on the immune system has any clinical implications.

In conclusion, surfactant prevents the protease-dependent shedding of CR1 from the cell surface of quartz-exposed granulocytes. Neither by fractionating surfactant into phospholipid and protein fractions, nor by recombining the two fractions could an equivalent protective effect be observed. Thus, the roles of SP-B and SP-C in this respect are difficult to discern. Separation of phospholipids from proteins may have changed the structure of the proteins, thereby altering their function. Addition of lysophosphatidylcholine to surfactant or storage of surfactant at elevated temperatures reduced the protective effect on the CR1 expression. Therefore, it seems crucial that surfactant is stored at low temperatures.

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