Lung Surfactant Suppresses Oxygen-Dependent Bactericidal Functions of Human Blood Monocytes by Inhibiting the Assembly of the NADPH Oxidase

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ABSTRACT. Surfactant is known to lower the surface tension in alveoli and affects the antibacterial functions of alveolar and peritoneal macrophages. We investigated the effects of surfactant on the bactericidal functions and oxidative metabolism of human blood monocytes and granulocytes. Monocytes incubated with surfactant ingest this material and subsequently exhibit an impaired ability to kill ingested bacteria. Granulocytes incubated with surfactant do not ingest this material, and their bactericidal functions are not affected. However, granulocytes that have ingested surfactant-coated Staphylococcus aureus display an impaired ability to kill these bacteria. Moreover, in monocytes and granulocytes that contain surfactant—the latter by ingestion of surfactant-coated S. aureus—the intracellular production of H$_2$O$_2$ is impaired due to inhibition of the assembly of the NADPH oxidase. Together these results demonstrate that surfactant inside monocytes and granulocytes inhibits the capacity of these cells to kill bacteria intracellularly by impairing oxygen-dependent killing mechanisms. Journal of Immunology, 1993, 150: 2391.

Under steady state conditions, alveolar macrophages are the predominant phagocytes of the lower airways and the alveoli. These cells function as scavengers and release proteases, reactive oxygen intermediates, arachidonic acid metabolites, chemotactic factors, and cytokines (1). Alveolar macrophages reside in a microenvironment rich in surfactant, the main function of which is to lower surface tension in the alveoli (2). In a previous study, we showed that the capacity of mouse alveolar macrophages to kill various bacteria intracellularly is very low compared to that of peritoneal macrophages and that the capacity of peritoneal macrophages to kill ingested bacteria is severely impaired after incubation with surfactant (3). Others have also reported that surfactant affects the antibacterial functions of alveolar macrophages (4–7).

Human alveolar macrophages exhibit a limited bactericidal activity compared to monocytes and granulocytes (8). Under normal conditions, monocytes migrate to the lungs and enter the alveoli where they differentiate into alveolar macrophages (9, 10). At that site, the monocytes come into contact with surfactant. Alveolar macrophages contain abundant amounts of this material (11). During an inflammatory reaction in the lungs due to an infection, increased numbers of monocytes as well as granulocytes can enter the alveoli (9, 12, 13). The present study focused on the question of whether surfactant affects the bactericidal functions of these phagocytes and if so, which mechanisms are involved.

Materials and Methods

Bacteria

Staphylococcus aureus (type 42D), S. epidermidis (serotype 4), Escherichia coli (type O54), Streptococcus pyogenes (group A), and Streptococcus pneumoniae (serotype
were cultured overnight in Nutrient broth no. 2. (Oxoid Ltd., Basingstoke, United Kingdom) at 37°C. Bacteria were harvested by centrifugation at 1500 \( \times \) g, washed twice with PBS, and resuspended in HBSS containing 0.1% gelatin (gelatin-HBSS) to a concentration of about 1 \( \times \) 10\(^7\) microorganisms/ml.

### Cells

Buffy coats from blood of healthy donors were diluted in PBS and subjected to differential centrifugation at 400 \( \times \) g for 20 min on a Ficoll-Hypaque gradient (\( \rho = 1.077 \) g/ml; Pharmacia, Uppsala, Sweden) (14). The monocyte-lymphocyte layer was washed four times with PBS containing 0.5 U heparin/ml and suspended to a concentration of 1 \( \times \) 10\(^7\) monocytes/ml gelatin-HBSS. This preparation consisted of about 30\% monocytes, 65\% lymphocytes, and less than 5\% granulocytes. After resuspension of the Ficoll-Hypaque pellet in PBS, the granulocytes were purified by plasma-sterile (Fresenius AG, Bad Hamberg, Germany) sedimentation for 10 min at 37°C followed by removal of the E by hypotonic lysis with distilled water; a cell suspension of 1 \( \times \) 10\(^7\) monocytes/ml gelatin-HBSS was then prepared. Viability of monocytes and granulocytes, as determined by trypan blue exclusion, was more than 95\%.

Sheep monocytes were isolated from fresh sheep blood as described above for human monocytes, except that differential centrifugation of blood on the Ficoll-Hypaque gradient was performed at 650 \( \times \) g for 45 min.

Human alveolar macrophages were isolated from BAL\(^3\) fluid from lung segments with no underlying pathologic condition that were collected after informed consent from patients who underwent BAL for diagnostic reasons. The final BAL cell suspension consisted of about 90 to 95\% alveolar macrophages, 4 to 9\% lymphocytes, and 1\% granulocytes. Viability of the cells exceeded 95\%, as measured by trypan blue exclusion.

### Isolation of surfactant

Surfactant was obtained from freshly excised sheep lungs by repeated lavage with 1.5 to 2 liters of saline. The airways were filled with saline until fully inflated, and then the fluid was removed. The pooled lavage fluid was centrifuged at 750 \( \times \) g for 20 min to remove cells and debris, the supernatant was collected and centrifuged at 21,000 \( \times \) g for 2 h, and the resulting pellet was resuspended in a small volume of saline and stored at \(-20^\circ\)C. The concentration of surfactant was expressed in mM organic phosphate, which was determined by Dr. J. Egberts (Department of Obstetrics, University Hospital, Leiden, The Netherlands) according to the method of Bartlett (15). The amount of organic phosphate is proportional to the total amount of phospholipid in surfactant; since surfactant consists of almost 90\% phospholipids (16, 17), the concentration of organic phosphate is representative of the concentration of surfactant. Human surfactant was prepared from BAL fluid as described for sheep surfactant.

### Labeling of surfactant with FITC

FITC-labeled surfactant (FITC-surfactant) was prepared by dialysis of surfactant at a concentration of 15 \( \mu \)mol phospholipid/ml 0.05 M carbonate buffer (pH 9.5) against 0.1 mg FITC (Isomer I; Sigma)/ml 0.05 M carbonate buffer for 18 h at 4°C. Free FITC was removed by centrifugation of the suspension at 21,000 \( \times \) g for 2 h; the material in the pellet was then dialyzed four times against 0.05 M carbonate buffer. The final suspension was centrifuged at 21,000 \( \times \) g for 2 h and the FITC-surfactant was suspended in saline at a concentration of about 30 mM.

### Incubation of monocytes and granulocytes with surfactant

Monocytes or granulocytes at a concentration of 1 \( \times \) 10\(^7\)/ml gelatin-HBSS were incubated with various concentrations of surfactant for 30 min at 37°C under slow rotation (4 rpm). After three washes with gelatin-HBSS and centrifugation at 250 \( \times \) g to remove excess surfactant, the cells were resuspended to a concentration of 1 \( \times \) 10\(^7\)/ml gelatin-HBSS. Viability of surfactant-incubated monocytes and granulocytes exceeded 90\%, as measured by trypan blue exclusion. Control monocytes and granulocytes were incubated with saline, the diluent of surfactant, under identical conditions.

### Measurement of the uptake of FITC-surfactant by monocytes and granulocytes

After incubation of monocytes or granulocytes with various concentrations of FITC-surfactant for various time periods at 37°C, the cells were washed four times with PBS, fixed with 1\% paraformaldehyde in PBS supplemented with 1\% BSA (PBS-BSA), and resuspended in PBS-BSA after one wash to remove paraformaldehyde. The amount of FITC-surfactant associated with monocytes and granulocytes was measured with a FACStar (Becton-Dickinson, Mountain View, CA) equipped with an argon-ion laser (excitation wavelength at 488 nm, laser power 300 mW) and a band pass filter of 530 nm (width, 20 nm). In each sample, 1 \( \times \) 10\(^4\) cells were analyzed; the results are expressed as the mean of their fluorescence intensity.

The localization of FITC-surfactant in phagocytes was studied by means of confocal laser scanning fluorescence microscopy. The plasma membranes of monocytes were stained immunocytochemically with the mAb 63D3 that recognizes the CD14 Ag (18), biotinylated horse-anti-mouse IgG, and streptavidin conjugated with phycoerythrin and Texas red (Southern Biotechnology Associates, Inc.,...
To acquire dual wavelength images, an excitation wavelength of 488 nm, a short-pass 560-nm filter, and a long-pass 630-nm filter were used in the confocal laser scanning fluorescence microscope (model MRC-500; Bio-Rad Laboratories, Richmond, CA). The results are presented as photographs of computer images.

Serum

Serum was prepared from the blood of healthy donors with blood group AB. The blood was clotted for 1 h at room temperature and centrifuged for 20 min at 1500 × g; serum was then collected and stored in 0.5 ml-aliquots at −70°C.

Phagocytosis of bacteria

The assay for phagocytosis was performed as described elsewhere (19). In short, 200 µl of the bacteria suspension containing 1 × 10⁷ bacteria/ml, 200 µl of the monocyte or granulocyte suspension containing 1 × 10⁶ cells/ml, and 40 µl fresh serum were incubated at 37°C under slow rotation (4 rpm). At the indicated times, a 100 µl-sample of the mixture was transferred to a vial containing 900 µl ice-cold gelatin-HBSS to stop phagocytosis. After centrifugation for 4 min at 110 × g to separate extracellular and cell-associated bacteria, the number of viable bacteria in the supernatant was determined by a microbiologic plate method. Phagocytosis is expressed as the percentage decrease in the number of viable extracellular bacteria.

To demonstrate that bacteria are truly ingested by and not merely adherent to the cells, monocytes preincubated with surfactant were allowed to phagocytose S. aureus for 3 min. The mixture was then washed three times with ice-cold gelatin-HBSS to remove noningested bacteria, and the cells with bacteria were exposed to 1 U lysostaphin/ml (Sigma) for 5 min at 4°C to lyse adherent S. aureus; cytocentrifuge preparations were then made. Next, the number of ingested bacteria per 100 cells before and after lysostaphin treatment was assessed by microscopy, and the number of cell-adherent S. aureus was calculated (20).

Pre-opsonization of bacteria

Bacteria were pre-opsonized with 10% serum for 30 min at 37°C under rotation (4 rpm), excess serum was removed by centrifugation, and the bacteria were resuspended in HBSS-gel to a concentration of 1 × 10⁷/ml. Where indicated, 1 × 10⁷ pre-opsonized S. aureus/ml were incubated with 4 mM surfactant for 30 min at 37°C, and then excess surfactant was removed by centrifugation; these bacteria were called pre-opsonized surfactant-coated bacteria. The coating of bacteria with surfactant was confirmed using FITC-surfactant and fluorescence microscopy.

Intracellular killing of bacteria

The assay for intracellular killing was performed, as described previously (19). In short, 200 µl pre-opsonized bacteria and 200 µl monocytes or granulocytes (1 × 10⁷/ml) were incubated for 3 min at 37°C under slow rotation; phagocytosis was stopped by shaking the tube in crushed ice. The noningested bacteria were removed by differential centrifugation and washing. Then 5 × 10⁶ phagocytes/ml containing ingested bacteria were reincubated in the presence of 10% AB serum at 37°C under rotation (4 rpm). At the indicated intervals, a 100 µl-sample was transferred to a vial containing 900 µl ice-cold water with 0.01% BSA, and the leukocytes were disrupted by vigorous shaking on a vortex mixer for 1 min. After serial dilution, the numbers of viable intracellular bacteria were determined microbiologically. At the end of the experiment, 1800 µl H₂O₂-BSA were added to the remaining cell suspension (200 µl), the cells were disrupted, and the number of intracellular viable bacteria determined as described above. Intracellular killing is expressed as the percentage decrease in the number of viable intracellular bacteria.

Incubation of monocytes with diphenyleneiodonium

To suppress oxygen-dependent killing mechanisms, 1 × 10⁷ monocytes/ml were incubated with 5 µM NADPH oxidase inhibitor DPI (21), a generous gift from Dr. A. R. Cross (Department of Biochemistry, University of Bristol, Bristol, United Kingdom), for 15 min at 37°C. As a control, monocytes were incubated with 0.5% DMSO, the diluent of DPI.

Measurement of extracellular and intracellular H₂O₂ production

H₂O₂ release by phagocytes without a stimulus and upon stimulation with 1, 5, or 25 ng PMA/ml was assayed by the horseradish peroxidase-mediated H₂O₂-dependent oxidation of homovanillic acid (22); the results are expressed as nmol H₂O₂/(10⁶ cells × 60 min). The H₂O₂-specific probe DCFH-DA (23) was used to measure the intracellular production of H₂O₂ upon stimulation with 100 ng PMA/ml. Briefly, after incubation of monocytes or granulocytes at a concentration of 1 × 10⁶/ml with 5 µM DCFH-DA (Eastman Kodak, Rochester, NY) for 15 min at 37°C, PMA was added to the cell suspension. The amount of the H₂O₂ reaction product, i.e., 2′,7′-dichlorofluorescein, was measured as described above. In each sample, 1 × 10⁶ cells were analyzed; the results are expressed as the mean of the fluorescence intensity.

Measurement of NADPH oxidase activity in a cell-free system

NADPH oxidase activity was measured by means of a Clark oxygen-electrode at 27°C as the rate of oxygen consumption by the cytosolic and membranous components of the NADPH oxidase that were isolated from neutrophils as described (24). In short, 400 µl of 10 mM HEPES containing 0.17 M sucrose, 75 mM NaCl, 0.5 mM EGTA, 1
mM MgCl₂, 2 mM NaN₃ (pH 7.0), and 10 μM GTPγS were mixed with 10 μl of membrane components and 10 μl of cytosol components, which are the equivalents of 1 × 10⁶ neutrophils. Assembly of the NADPH oxidase was initiated by the addition of SDS at a final concentration of 100 μM, and the reaction vessel was closed. After 3 min, NADPH was added at a final concentration of 250 μM, and oxygen consumption was measured next (24). The results are expressed as μM O₂/min.

Statistics

All results are the means and SD of at least three experiments. The significance of differences was determined with the Wilcoxon signed ranks test.

Results

Uptake of FITC-surfactant by monocytes and granulocytes

To determine whether monocytes or granulocytes ingest surfactant, this material was labeled with FITC. Monocytes incubated with FITC-surfactant showed an increase in fluorescence dependent on the concentration of FITC-surfactant and the incubation period (Fig. 1). Granulocytes did not bind or ingest detectable amounts of FITC-surfactant (Fig. 1). Since flow cytometry does not discern between FITC-surfactant inside the cells and on the cell surface, the localization of FITC-surfactant in monocytes was investigated by confocal laser scanning fluorescence microscopy. The results revealed that virtually all FITC-surfactant was localized inside the monocytes that were incubated with this material (Fig. 2). The uptake of surfactant was confirmed by transmission electron microscopy (results not shown).

Effect of surfactant on the phagocytosis of S. aureus by monocytes

After incubation of monocytes with various concentrations of surfactant, the phagocytosis of S. aureus at 60 min was decreased (p < 0.03) in a dose-dependent way, as determined microbiologically (Fig. 3a). Microscopic assessment of phagocytosis, using lysozyme to eliminate extracellular S. aureus, also revealed that significantly (p < 0.03) fewer bacteria were ingested by surfactant-incubated monocytes than by control monocytes, but that the percentage of bacteria adherent to the surface of surfactant-incubated monocytes and control monocytes did not differ (p > 0.2).

Effect of surfactant on the intracellular killing of S. aureus by monocytes

Intracellular killing of pre-opsonized S. aureus by monocytes incubated with surfactant was decreased compared to that by control monocytes (Table 1 and Fig. 3b). Concentrations of surfactant as low as 1 mM caused significantly (p < 0.05) reduced killing of S. aureus by monocytes, whereas 4 and 8 mM surfactant almost completely prevented the killing of ingested S. aureus (Fig. 3b). On the basis of these results, 4 mM surfactant was used in all additional experiments.

Incubation of monocytes with surfactant for periods shorter than 30 min also affected the intracellular killing of S. aureus: incubation for 1 and 5 min yielded killing values of 33 ± 17% and 22 ± 25% (n = 3), respectively, whereas the intracellular killing of S. aureus by control monocytes incubated with saline for these intervals was not affected. Since the effects of incubation with surfactant for 30 and 60 min were similar (intracellular killing values 19 ± 32% and 17 ± 25%, respectively, n = 3), a 30-min period was used in subsequent experiments.

To investigate whether impairment of the intracellular killing of S. aureus by monocytes incubated with surfactant is reversible, monocytes were incubated with 4 mM surfactant for 30 min at 37°C, washed four times in PBS, and cultured for various periods in medium without surfactant at 37°C. The intracellular killing of S. aureus by surfactant-incubated monocytes cultured for 0, 1, 2, or 3 days...
FIGURE 2. Ingestion of FITC-surfactant by monocytes assessed by confocal laser scanning fluorescence microscopy. Monocytes were incubated with 4 mM FITC-surfactant for 30 min at 37°C. Excess FITC-surfactant was removed by washing three times; the cells were then stained for the cell-surface Ag CD14 with biotinylated horse-anti-mouse IgG and streptavidin-labeled phycoerythrin and Texas red. Dual-wavelength images were obtained with a confocal laser scanning fluorescence microscope at an excitation wavelength of 488 nm, a short pass 530-nm filter, and a long pass 630-nm filter. Bar, 5 μm.

The amount of bacterial killing by monocytes amounted to 13 ± 17%, 45 ± 9%, 21 ± 17%, and 6 ± 10% (n = 3), respectively, all of which are significantly (p < 0.05) less than the respective values (66 ± 13%, 81 ± 6%, 69 ± 11%, and 50 ± 15%; n = 3) found for control monocytes incubated with saline. These results demonstrate that the surfactant-induced impairment of the monocyte antibacterial functions is irreversible.

Since the intracellular killing of bacteria by monocytes requires continuous membrane stimulation by serum components (25), we investigated whether surfactant inhibits the interaction between serum components and the corresponding receptors on monocytes. When surfactant was added at various times after the initiation of intracellular killing by serum, the intracellular killing of S. aureus was not affected during the first 10 to 15 min; subsequently, however, the number of intracellular bacteria did not decrease but increased (results not shown).

Since sheep surfactant and human monocytes were used in most of the experiments, the effect of human surfactant on the intracellular killing of S. aureus by human monocytes and also the effect of sheep surfactant on the killing of S. aureus by sheep monocytes were investigated. The intracellular killing of S. aureus by human monocytes incubated with human surfactant was significantly decreased (p < 0.02) compared to that found for control monocytes (respectively, values 10 ± 14% and 59 ± 10%, n = 4). The moderate killing of S. aureus by sheep monocytes (31%) was completely inhibited by incubation of sheep monocytes with sheep surfactant. The inhibitory effect of surfactant on the intracellular killing of S. aureus by monocytes was not due to LPS contamination of surfactant, since incubation of monocytes with 3 ng LPS/ml (equivalent to the concentration of LPS found in 4 mM surfactant) did not affect the killing of S. aureus by these cells (data not shown).

Since bacteria invading the alveoli also come into contact with surfactant, the phagocytosis and intracellular killing of surfactant-coated S. aureus by monocytes were investigated. The phagocytosis of pre-opsonized surfactant-coated and pre-opsonized control S. aureus did not differ, as determined by microscopy using lysostaphin to eliminate adherent S. aureus (data not shown). The intracellular killing of surfactant-coated bacteria by normal monocytes was significantly impaired compared to that of control bacteria (18 ± 21% and 66 ± 18%, respectively, n = 5).

Effect of surfactant on the phagocytosis and intracellular killing of other bacteria by monocytes

Monocytes incubated with 4 mM surfactant exhibited significantly decreased (p < 0.05) phagocytosis of S. pneumoniae 69 ± 9% (control cells 86 ± 5%) and E. coli 72 ± 10% (control cells 82 ± 10%) (n = 4). Surfactant did not affect the in vitro growth of these bacteria (data not shown). The phagocytosis of S. epidermidis and S. pyogenes by monocytes was not affected by surfactant (data not shown). The intracellular killing of S. pneumoniae, S. pyo-
Surfactant suppresses monocyte bactericidal functions

**Figure 3.** Effect of surfactant on the phagocytosis (a) and intracellular killing (b) of opsonized *S. aureus* by human monocytes. Monocytes were incubated with various concentrations of surfactant for 30 min at 37°C. Control monocytes were incubated with saline. To determine phagocytosis, monocytes and *S. aureus* were incubated at a ratio of 1:1 in the presence of AB serum (*n* = 6 experiments). To determine intracellular killing, monocytes and pre-opsonized *S. aureus* were incubated at a ratio of 1:1; after 3 min of phagocytosis, extracellular bacteria were removed by washing, and killing of the ingested bacteria was initiated by the addition of AB serum (*n* = 4 experiments). Results are expressed as the percentage phagocytosis or intracellular killing at 60 min.

**Table 1**

<table>
<thead>
<tr>
<th>Surfactant (mM)</th>
<th><em>S. aureus</em> (%)</th>
<th><em>S. epidermidis</em> (%)</th>
<th><em>E. coli</em> (%)</th>
<th><em>S. pyogenes</em> (%)</th>
<th><em>S. pneumoniae</em> (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>62 ± 14</td>
<td>90 ± 2</td>
<td>94 ± 3</td>
<td>71 ± 17</td>
<td>86 ± 9</td>
</tr>
<tr>
<td>4</td>
<td>18 ± 19</td>
<td>72 ± 11</td>
<td>78 ± 10</td>
<td>57 ± 15</td>
<td>69 ± 9</td>
</tr>
</tbody>
</table>

*a* Determined as the percentage decrease in the number of viable intracellular bacteria at 60 min (*n* = 4).

*b* Final concentration of surfactant expressed as mM organic phosphate.

*p* < 0.05 for the difference between monocytes incubated with surfactant and control monocytes.

Effect of DPI on the intracellular killing of bacteria by monocytes

The NADPH oxidase inhibitor DPI significantly (*p* < 0.05) inhibited the intracellular killing of *S. aureus*, *S. epidermidis*, and *E. coli*, but not *S. pyogenes* by monocytes (Table II).

Phagocytosis and intracellular killing of *S. aureus* by granulocytes incubated with surfactant

Incubation of human granulocytes with 4 or 8 mM surfactant for 30 min did not affect either the phagocytosis or the intracellular killing of *S. aureus* (Table III). The intracellular killing of *S. aureus* also was not affected when granulocytes were incubated with surfactant for 1 or 10 min (intracellular killing 93 ± 5% and 91 ± 6%, respectively, *n* = 6).

Because granulocytes do not ingest surfactant, it was asked whether the capacity of granulocytes to kill bacteria intracellularly is affected when surfactant is present inside these cells. Therefore, pre-opsonized *S. aureus* were coated with surfactant, and these bacteria were used to introduce surfactant into granulocytes. The phagocytosis of pre-opsonized surfactant-coated and pre-opsonized control *S. aureus* by granulocytes did not differ, when assessed microscopically using lysostaphin (data not shown). The intracellular killing of pre-opsonized surfactant-coated *S. aureus* by granulocytes in the absence of extracellular serum was significantly impaired compared to that of pre-opsonized control bacteria (28 ± 14% and 78 ± 6%, respectively, *n* = 4), indicating that the presence of surfactant within granulocytes leads to impaired killing of bacteria.

H$_2$O$_2$ release by monocytes and granulocytes incubated with surfactant

To investigate whether the decreased killing of bacteria by monocytes after incubation with surfactant was due to im-

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*genes, S. epidermidis, and E. coli* by monocytes incubated with 4 mM surfactant was decreased compared to that by control monocytes (Table I).

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H$_2$O$_2$ release by monocytes and granulocytes incubated with surfactant

To investigate whether the decreased killing of bacteria by monocytes after incubation with surfactant was due to im-
paired oxygen-dependent bactericidal mechanisms, we measured the release of \( \text{H}_2\text{O}_2 \) by surfactant-incubated monocytes and granulocytes upon stimulation with PMA. Monocytes incubated for 30 min with 4 mM surfactant showed significantly \((p < 0.01)\) decreased \( \text{H}_2\text{O}_2 \) release upon stimulation with 5 ng PMA/ml but not after stimulation with 25 ng PMA/ml (Table IV). After incubation with 0.4 mM surfactant for 20 h, monocytes exhibited a significant \((p < 0.05)\) inhibition of their release of \( \text{H}_2\text{O}_2 \) upon stimulation with 5 and 25 ng PMA/ml (Table IV). Granulocytes incubated for 30 min with 4 mM surfactant displayed a reduced \( \text{H}_2\text{O}_2 \) release upon stimulation with 1 and 5 ng PMA/ml but not after stimulation with 25 ng PMA/ml; incubation of these cells for 20 h with 0.4 mM surfactant did not result in a decreased \( \text{H}_2\text{O}_2 \) release upon stimulation with 1 and 25 ng PMA/ml (Table IV).

Intracellular production of \( \text{H}_2\text{O}_2 \) by monocytes and granulocytes incubated with surfactant

Since surfactant inhibits the intracellular killing of \( S. \text{aureus} \) inside phagocytes, it was essential to investigate the effect of surfactant on the intracellular production of \( \text{H}_2\text{O}_2 \) by monocytes and granulocytes. The results revealed that surfactant abolished the production of intracellular \( \text{H}_2\text{O}_2 \) in monocytes stimulated with 100 ng PMA/ml (Fig. 4). The loading of control monocytes and monocytes incubated with surfactant with DCFH-DA was comparable, as measured spectrophotometrically, in the lysates of these cells after the addition of \( \text{H}_2\text{O}_2 \) (data not shown).

Granulocytes incubated with surfactant were not impaired in their PMA-induced intracellular production of \( \text{H}_2\text{O}_2 \) compared to control cells (Fig. 4). However, granulocytes that had ingested pre-opsonized surfactant-coated \( S. \text{aureus} \) did not exhibit \((p > 0.05)\) enhanced intracellular production of \( \text{H}_2\text{O}_2 \) upon stimulation with PMA, whereas the intracellular production of \( \text{H}_2\text{O}_2 \) by granulocytes that had ingested pre-opsonized control \( S. \text{aureus} \) was significantly \((p < 0.02)\) enhanced upon stimulation with PMA (Fig. 5).

Effect of surfactant on the assembly of the NADPH oxidase

The NADPH oxidase consists of cytoplasmic and membranous components; after addition of a stimulus, these components assemble to form a functional enzyme by which electrons are transferred from NADPH to molecular oxygen (26). A cell-free system was used to investigate whether surfactant impairs the assembly of the components of the NADPH oxidase or the transfer of electrons. Oxygen consumption by the NADPH oxidase complex was inhibited by sheep as well as human surfactant in a concentration-dependent fashion when this material was added to a mixture of the components of the NADPH oxidase before addition of SDS, i.e., before assembly of the components into a functional enzyme complex (Fig. 6). The possibility that inhibition of oxygen consumption by surfactant was due to interference with the electron transfer was excluded by adding surfactant to the system after assembly of the various components into an active NADPH oxidase had occurred. The respective values before and after addition of 0.4 mM sheep surfactant were 13 ± 2.1 \( \mu \text{M/min} \) and 11 ± 1.6 \( \mu \text{M/min} \).

Antibacterial functions of alveolar macrophages

Human alveolar macrophages displayed efficient phagocytosis (83 ± 18%, \( n = 4 \)) but limited killing of ingested \( S. \text{aureus} \) (14 ± 21%, \( n = 8 \)). \( \text{H}_2\text{O}_2 \) release upon stimulation of these cells with 25 ng PMA/ml was low \((3 ± 1.8 \text{nmol/}(10^6 \text{cells} \times 60 \text{min}), n = 5 \)) compared to that by monocytes (Table IV).

Discussion

The main conclusion to be drawn from the present results is that surfactant when located within monocytes and granulocytes inhibits the intracellular killing of bacteria by impairing oxygen-dependent killing mechanisms through inhibition of the assembly of components of the NADPH oxidase. This conclusion is based on the following arguments. 1) Monocytes incubated with surfactant ingest this material. 2) These cells display an impaired intracellular production of \( \text{H}_2\text{O}_2 \) upon stimulation with PMA. 3) Granulocytes do not ingest surfactant and their capacity to kill bacteria or to produce \( \text{H}_2\text{O}_2 \) intracellularly is not affected.

<table>
<thead>
<tr>
<th>DPI (( \mu \text{M} ))</th>
<th>( \text{S. aureus} ) %</th>
<th>( \text{S. epidermidis} ) %</th>
<th>( \text{E. coli} ) %</th>
<th>( \text{S. pyogenes} ) %</th>
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<tr>
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<td>5</td>
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<td>52 ± 10 b</td>
<td>48 ± 34 ab</td>
<td>63 ± 10</td>
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</table>

a Determined as the percentage decrease in the number of viable intracellular and cell-associated bacteria at 30 min \((n = 3)\).

b \( p < 0.05 \) for the difference between monocytes incubated with DPI and control monocytes.

<table>
<thead>
<tr>
<th>Surfactant (mM)</th>
<th>Phagocytosis a (%)</th>
<th>Intracellular Killing a (%)</th>
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<td>77 ± 16</td>
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</tr>
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<td>8</td>
<td>76 ± 7</td>
<td>86 ± 11</td>
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a Determined as the percentage decrease in the number of viable extracellular bacteria at 30 min \((n = 3)\).

b Determined as the percentage decrease in the number of viable intracellular bacteria at 60 min \((n = 3)\).
Table IV
Effect of surfactant on the hydrogen peroxide release by monocytes and granulocytes

<table>
<thead>
<tr>
<th>Incubation of Cells with</th>
<th>Time of Incubation</th>
<th>H$_2$O$_2$ Release by Monocytes upon Stimulation with*</th>
<th>H$_2$O$_2$ Release by Granulocytes upon Stimulation with*</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>5 ng PMA/ml</td>
<td>25 ng PMA/ml</td>
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<tr>
<td>Saline</td>
<td>30 min</td>
<td>13 ± 2.7</td>
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<tr>
<td>4 mM surfactant</td>
<td>30 min</td>
<td>8 ± 3.6b</td>
<td>14 ± 2.1</td>
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<td>Saline</td>
<td>20 h</td>
<td>7 ± 1.6</td>
<td>11 ± 1.7</td>
</tr>
<tr>
<td>0.4 mM surfactant</td>
<td>20 h</td>
<td>4 ± 0.3b</td>
<td>8 ± 2.3b</td>
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</tbody>
</table>

*p < 0.05 for the difference between cells incubated with surfactant and the respective control.

RESULTS

FIGURE 4. Effect of surfactant on the intracellular H$_2$O$_2$ production in monocytes (a) and granulocytes (b) upon stimulation with PMA. Monocytes and granulocytes were incubated with 4 mM surfactant (●) or saline (○) for 30 min at 37°C. After loading the cells with DCFH-DA, PMA (100 ng/ml) was added as a stimulus. Fluorescence intensity, as a measure of intracellular H$_2$O$_2$ production, was determined with a FACStar. Results are expressed as the mean of the fluorescence intensity; a representative of three experiments is shown.

after incubation with surfactant. 4) The introduction of surfactant into granulocytes, by allowing these cells to ingest pre-opsonized surfactant-coated S. aureus, leads to both impaired killing of these bacteria and intracellular production of H$_2$O$_2$. 5) Surfactant reduces the oxygen consumption by the NADPH oxidase by inhibiting the assembly of cytoplasmic and membranous components into a functional enzyme. The mechanism for the inhibition of the assembly of the NADPH oxidase by surfactant is unknown.

To justify the conclusion that intracellular surfactant inhibits the bactericidal functions of phagocytes, it was crucial to demonstrate the exact localization of FITC-surfactant in monocytes and granulocytes incubated with this material. By means of confocal laser scanning fluorescence microscopy, it could be demonstrated that FITC-surfactant is ingested by human monocytes but not by granulocytes. Studies with transmission electron microscopy confirmed the intracellular localization of surfactant in monocytes.

For practical reasons, sheep surfactant instead of human surfactant was used in this study. There are no major differences in the composition of sheep and human surfactant (16, 17). Comparable results were found when human monocytes were incubated with surfactant of sheep and human origin, and incubation of sheep monocytes with sheep surfactant also resulted in complete impairment of their capacity to kill ingested S. aureus. Sheep and human surfactant were also equally effective in inhibiting the assembly of the NADPH oxidase in a cell-free system. Therefore, we concluded that it was justifiable to use sheep surfactant and human phagocytes. It is likely that the concentration of surfactant used in the present study is similar to that encountered by monocytes when they enter the alveoli, because the ratio between the amount of surfactant and the number of phagocytes used in the present study was comparable to such ratios calculated for the lungs of sheep (27), rabbits (2, 28), and rats (2, 29). The effect of surfactant on the antibacterial functions of monocytes could not be due to LPS contamination of surfactant, since incubation of monocytes with concentrations of LPS comparable to those found in surfactant did not affect the intracellular killing of S. aureus by monocytes.

The results described in the present study are in agreement with our earlier finding that the bactericidal functions of mouse peritoneal macrophages are inhibited after incubation with sheep surfactant (3). It has also been reported
that surfactant inhibits the killing of Candida albicans (5) and Streptococcus agalactiae (6) by newborn rabbit alveolar macrophages and impairs the PMA- or opsonized zymosan-induced chemiluminescence by rabbit alveolar macrophages (4). Surfactant also affects the functional activities of T lymphocytes (30, 31). It is important to realize that in vivo the functions of various types of cell localized in the alveoli are influenced by the presence of surfactant. Monocytes incubated with surfactant resemble alveolar macrophages as far as the intracellular presence of surfactant (11), their limited antibacterial functions (Ref. 8 and the present study), and their capacity to release H₂O₂ upon stimulation with PMA (Refs. 8, 32, and the present study) are concerned. Granulocytes that are not affected by incubation with surfactant display impaired bactericidal functioning after ingestion of surfactant-coated bacteria. The question of the mechanisms responsible for the removal of bacteria—which may be coated with surfactant—from the alveoli remains unanswered. Bacteria ingested by alveolar macrophages, monocytes, and granulocytes leave the alveoli as a result of migration of these phagocytes to the upper parts of the respiratory tract and expulsion from the body via mucociliary movement and forced respiration.

In addition to the inhibition of both intracellular killing of bacteria and the intracellular production of H₂O₂ by surfactant, another important finding was the reduced release of H₂O₂ by both monocytes and granulocytes incubated with surfactant. Since this material is not ingested by granulocytes, extracellular surfactant must account for the impaired H₂O₂ release resulting from inhibition of the assembly of the NADPH oxidase in the plasma membrane. These results could mean that in vivo surfactant plays an important role in the protection of alveolar epithelium from the injury caused by reactive oxygen intermediates released by phagocytes.

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