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Presence of Bleomycin-Detectable Free Iron in the Alveolar System of Preterm Infants[1]

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Chronic lung disease (CLD) is a major cause of long term morbidity in preterm infants. Reactive oxygen species (ROS) play an important role in the pathogenesis of CLD. We show that a high percentage (63 to 83%) of the investigated bronchoalveolar secretions (BAS) of neonates contain bleomycin-detectable free iron concentrations (0.04–0.124 nmol/μg SC, median range). Beside the presence of redox-active iron several iron-binding proteins like transferrin, ferritin and lactoferrin were determined in BAS. Comparison of protein distribution within the first three days of life showed slight differences between the group of preterm infants who developed CLD and the neonates who recovered from RDS. Because of the existence of free iron we suggest a higher risk of hydroxyl radical formation in the alveolar space. In an artificial system with addition of iron and hydrogen peroxide we were able to demonstrate OH-radical production in BAS by electron paramagnetic resonance (EPR). OH-radical formation by H_2O_2 and iron in buffer solution was slightly enhanced in the presence of BAS, indicating the absence of OH-radical-scavengers in BAS. © 1999 Academic Press

Key Words: **free iron; iron-binding proteins; hydroxyl radical; respiratory distress syndrome; chronic lung disease; bronchoalveolar secretions.**

There is growing evidence that reactive oxygen species (ROS) play an improtant role in the pathogenesis of pulmonary diseases in adults (1) and especially in preterm infants (2). ROS have been shown to be generated by endothelial cells via the xanthine-xanthine-oxidase reaction (2) and activation of macrophages or granulocytes in the alveolar system and the pulmonary tissue (3).

After birth the immature lung of the neonate is often confronted with high inspiratory oxygen concentrations leading to oxidative damage of the epithelial lining fluid and associated cells (4). As a consequence macrophages and granulocytes accumulate in the area of lung injury and release superoxide anion, hydrogen peroxide and hypochlorous acid (5). Protective antioxidant enzymes or molecules like catalase, glutathione peroxidase and glutathione are diminished in the alveolar surrounding of the neonate (6,7).

Under these conditions the impaired scavenger system and the oxygen radical generating system can promote and induce radical development, e.g. hydroxyl radicals via the Fenton reaction (8). The production of superoxide anion and the following dismutation to H_2O_2 can play an important role in the oxidative tissue damage. H_2O_2 itself is not very toxic (9) but can react together with free ferrous iron (Fe^{2+}) via the Fenton reaction to form the toxic and highly reactive hydroxyl radical.

$$Fe^{2+} + H_2O_2 + H^+ \xrightarrow{\text{Fenton-reaction}} Fe^{3+} + {}^{\bullet}OH + H_2O$$

The required iron can be present as loosely bound iron to proteins or chelated with molecules like citrate (10).

In this study we have evaluated the role of free iron under conditions of enhanced oxidative stress in the lung of neonates. In addition the relationship between iron-binding and -scavenging proteins has been analyzed.

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Abbreviations: ARDS, adult respiratory distress syndrome; BAS, bronchoalveolar secretions; BSA, bovine serum albumin; CLD, chronic lung disease; EPR, electron paramagnetic resonance; MDA, malondialdehyde; PBS, phosphate buffered saline; RDS, respiratory distress syndrome; ROS, reactive oxygen species; SC, secretory component of IgA.

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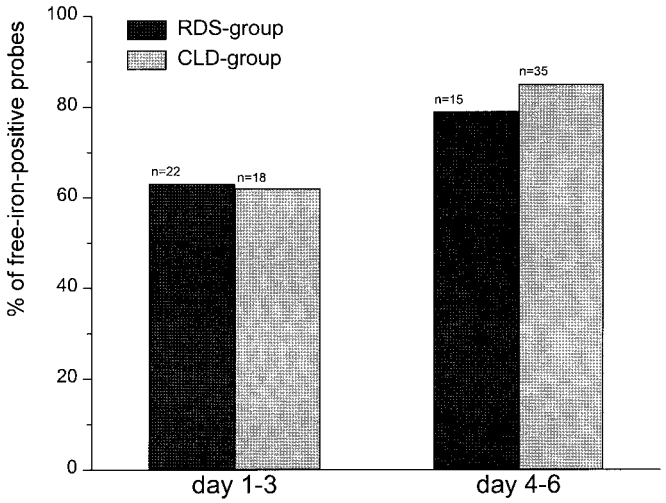

FIG. 1. Percentage of free iron positive BAS: Comparison of the RDS and CLD-group on day 1–3 and 4–6 of life.

MATERIALS AND METHODS

Patients and samples. BAS of 35 mechanically ventilated preterm infants (birth weight 885 grams, gestational age 27 ± 3 weeks (median range)) was sequentially obtained within the first six days of life. Preterm infants who recovered from RDS (n = 18) were compared to infants who developed CLD (n = 17). All preterm infants had been treated at the Department of Neonatology, University Children's Hospital, Tübingen from January 1997 to December 1997. CLD was defined as signs of persisting respiratory distress and oxygen dependency on day 28 of life.

BAS was aspirated in a standardized manner at least three times daily by instillation of 0.5 ml 0.9% (w/v) NaCl-solution into the endotracheal tube. Secretions were suctioned and collected in sterile specimen traps. BAS was diluted with 0.9% (w/v) NaCl-solution to a total volume of 0.5 ml, centrifuged at 1000 × g for 5 minutes and cell-free supernatants were immediately frozen and stored at −20°C prior to analysis.

Correction of determined protein and iron concentrations by secretory component of IgA (SC). To avoid errors due to the sampling procedure, values were related to concentrations of the SC as the reference protein (11). SC was measured by direct enzyme-linked immunosorbent assay as described in (5). Highly purified and biochemically characterized SC from human colostrum was used as standard.

Albumin assay. Albumin was measured by single radial immunodiffusion (LC and VLC-Partigen, Behring, Marburg, Germany).

Lactoferrin assay. Lactoferrin was measured by an enzyme immuno assay according to the manufacturers instructions (Oxis International, Portland, USA).

Transferrin assay. Transferrin was measured by a specially developed enzyme immuno assay in our laboratory using antibodies from ICN (Eschwege, Germany). Briefly, 96-well plates (Nunc, Roskilde, Denmark) were coated with a monoclonal mouse anti-human transferrin antibody (1:4000) overnight. Wells were washed and blocked with PBS/Tween/2.0% (w/v) BSA at 37°C over 90 minutes. Wells were washed and standards (human transferrin, Sigma, München, Germany) or diluted samples were added to the wells and incubated at 37°C for 2 h. Wells were washed and the second polyclonal horseradish peroxidase-conjugated chicken anti-human transferrin antibody (1:1000) was added and incubated at 37°C for 2 h. Wells were washed and 100 μl of OPD as substrate and H_2O_2 (20 mM) was added. The plate was incubated in the dark at room temperature for at least 20 minutes. The reaction was stopped after adding 50 μl H_2SO_4 (2M) and the absorbance was read at 490 nm against 650 nm with an ELISA-reader (Milenia, Los Angeles, CA).

Ferritin assay. Ferritin was measured using a commercially available DELFIA-time resolved immunofluorescence test-kit (Wallac Oy, Turku, Finland).

Free iron determination by the bleomycin assay. Free iron was measured using the bleomycin assay (10). Briefly, DNA (1 mg/ml), $MgCl_2$ (50 mM), Tris buffer (10 mM, pH 7.4), bleomycin sulphate (0.75 U/ml) and BAS was mixed and the reaction was started by adding ascorbate (0.8 mM). The mixture was incubated at 37°C for 1 hour under slow shaking. In the presence of oxygen ferrous iron generated ROS are able to degrade DNA with the release of malondialdehyde (MDA) from its deoxyribose moiety. After reaction with thiobarbituric acid MDA was detected spectrophotometrically at 532 nm. Contaminating iron was removed previously from all solutions by Chelex-treatment (Chelex-100, BioRad, Hercules, CA). All values were quantitated according to a standard curve using iron atomic absorption standard (Sigma, München, Germany), the pH was carefully observed during the whole assay.

Measurement of total iron. Total iron was determined by electrothermal atomic absorption spectrometry (ETAAS) using a Philips PU9200X AAS as recommended by the manufacturer. Iron standards were prepared in reagent water type II acidified with HNO_3 (0.5 M). BAS was diluted similar to the standards and $Mg(NO_3)_2$ (0.5%, w/v) was added as a matrix modifier. Samples and standards were automatically injected in a pyro-coated graphite furnace. The furnace operation conditions were: drying-temperature: 140°C, ashing-temperature: 1000°C, atomization-temperature of 2500°C, wavelength was set to 248.3 nm.

Measurement of hydroxyl radicals. In order to investigate whether production of OH-radicals in BAS is principally possible, the generation of hydroxyl radicals was determined by electron paramagnetic resonance (EPR) using a Bruker ESP 300 E spectrometer with a TM_{110} cavity. Briefly, OH-radicals were trapped by 5,5 dimethyl-1-pyrroline-N-oxide (DMPO) (50 mM). 100 μl BAS was mixed with H_2O_2 (10 μM) and $FeSO_4$ (5 μM) in 0.9% (w/v) NaCl-solution (total sample volume 1000 μl) and the spectra of trapped OH-radicals were measured immediately using a flat quartz cell. For control, H_2O_2 and $FeSO_4$ were mixed in 0.9% (w/v) NaCl-solution and measured without addition of BAS.

Statistics. All values are given as median ranges because of a considered not-normal distribution of data.

RESULTS

Free Iron in BAS

The measurement of free iron in BAS indicated that a high percentage of investigated samples were iron

TABLE 1

Free Iron in BAS of Preterm Infants

	RDS-Group		CLD-Group	
Days	1–3	4–6	1–3	4–6
Median	0.043	0.124	0.04	0.05
25% quartile	0.016	0.035	0.012	0.018
75% quartile	0.149	0.16	0.103	0.113
	n = 22	n = 15	n = 18	n = 35

Note. Free iron [nmol/μg SC] was determined by the bleomycin assay.

TABLE 2

Calculation of Free Iron Concentration Relative to the Total Iron Concentration

	RDS-Group		CLD-Group	
Day	2	5	2	5
Mean	0.24	0.31	0.31	0.46
±S.D.	0.25	0.33	0.29	0.29
n	7	4	5	10

Note. Shown is the quotient free iron/total iron.

positive. 63% of all samples had detectable free iron in BAS measured within the first three days of life. The percentage of free iron containing BAS increased to 83% in the following time-period day 4–6, with no great differences between the group of children who developed CLD and the group who recovered from RDS (Fig. 1).

In Table 1 the measured iron concentrations of each group is demonstrated (median, 75% and 25% quartile). The median value of the free iron concentration on day 1–3 of life was nearly identical in the RDS and the CLD-group. However in the second time period (day 4–6 of life) the RDS-group showed higher iron BAS-levels than the CLD-group.

Calculations of positive free iron concentration relative to the measured total iron amount in BAS demonstrated a higher ratio in the CLD-group compared to the RDS-group (Table 2).

Iron-Binding Proteins and Albumin

Table 3 summarizes iron-binding proteins (transferrin, lactoferrin and ferritin) and albumin according to their distribution in both groups and days of life. In the first three days of life the concentrations of all iron-binding proteins are higher in the group of CLD-neonates compared to infants with RDS. At day 4–6 the RDS-group had higher lactoferrin and albumin levels compared to the CLD-group.

Hydroxyl-Radical Formation

The presence of free or loosely bound iron in BAS allows the production of OH-radicals in the presence of H_2O_2, generated e.g. by activated granulocytes or the xanthine/xanthine-oxidase reaction. We therefore asked whether other possible scavanger compounds of BAS are able to prevent OH-radical fomation. The experiment in Fig. 2 demonstrates that BAS did not reduce, but even enhanced OH-radical formation in an artificial system containing H_2O_2 and iron. That indicates a lack of scavenging capacity of BAS against OH-radical formation.

DISCUSSION

In recent years the potential role of reactive oxygen species in the development of chronic lung disease of preterm infants has been carefully evaluated (12,13). Products of lipid peroxidation or protein carbonylation were detected during the first week of life in neonates who developed CLD (14,15). The presence of free iron in extracellular surroundings has been implicated in the pathogenesis of many diseases like rheumatoid arthritis and adult respiratory distress syndrome (16,17). Reduced ferrous iron is able to react with hydrogen peroxide to form the highly reactive and toxic hydroxyl radical (8). In animal experiments an involvement of superoxide anion, iron and hydroxyl radicals was suggested because superoxide dismutase and desferrioxamine mesylate scavenged and reduced oxidative damage of fetal lung tissue (18).

In this study we investigated the content of free iron and furthermore the concentration of iron-binding proteins like transferrin, ferritin and lactoferrin in bronchoalveolar secretions of preterm infants with RDS and CLD.

TABLE 3

Iron-Binding Proteins and Albumin in BAS of Preterm Infants

	RDS-Group		CLD-Group	
Days	1–3 (*n = 35, **n = 36)	4–6 (*n = 19, **n = 18)	1–3 (*n = 28, **n = 34)	4–6 (*n = 41, **n = 40)
Transferrin (μg/μg SC)				
Median	0.46 (0.26/1.06)*	0.49 (0.33/1.05)*	0.62 (0.32/1.28)*	0.45 (0.25/0.82)*
Ferritin (ng/μg SC)				
Median	1.85 (1.08/6.17)*	5.66 (1.11/30.75)*	4.98 (2.64/15.7)*	5.36 (2.19/2.45)*
Lactoferrin (μg/μg SC)				
Median	0.41 (0.16/0.77)*	0.91 (0.68/1.26)**	0.67 (0.34/0.99)**	0.70 (0.61/1.23)**
Albumin (μg/μg SC)				
Median	14.79 (8.3/38.2)**	20.47 (11.4/39.6)**	30.78 (13.1/49.9)*	15.53 (7.7/24)**

Note. Shown are the values related to SC as standard protein, values are given as median (25% and 75% quartiles).

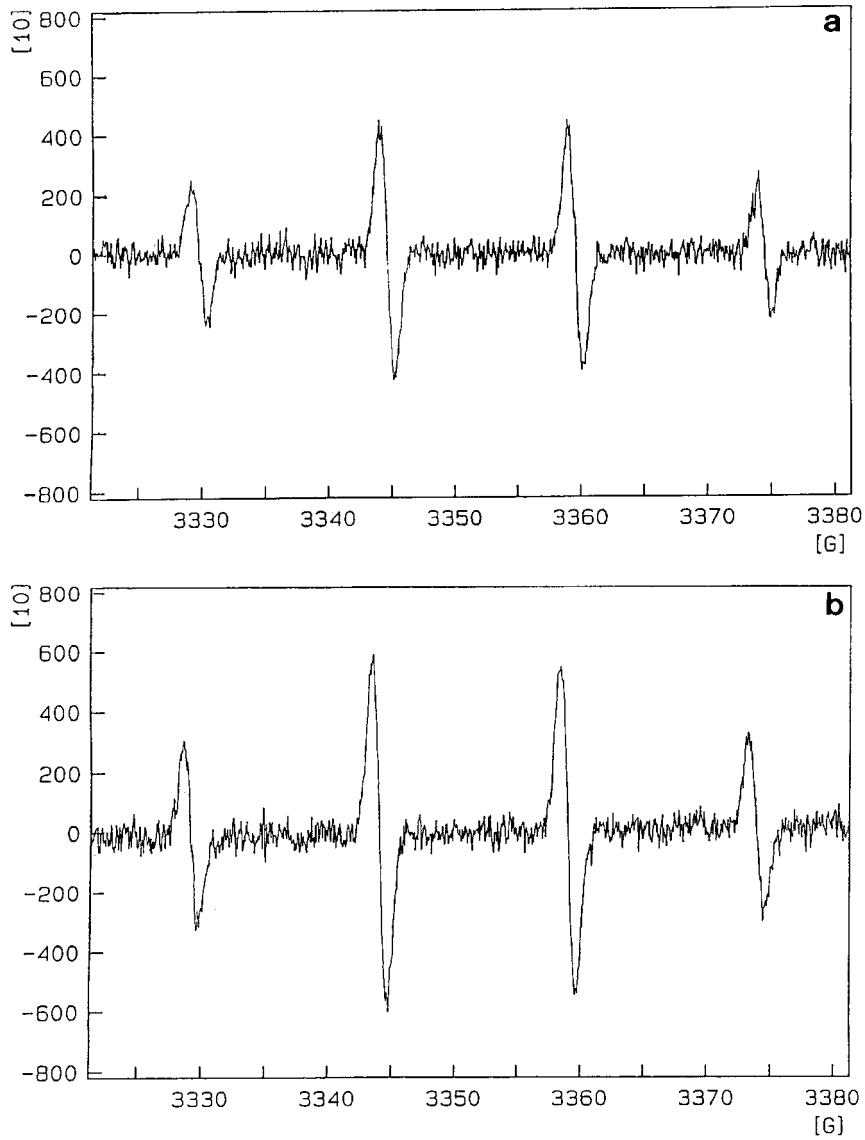

FIG. 2. Influence of BAS on the generation of OH-radicals (EPR spectra) by H_2O_2 [10 μM] and $FeSO_4$ [5 μM]. (a) Without BAS (control). (b) With BAS.

Gutteridge and coworkers (19) analysed bronchoalveolar lavage fluid from healthy volunteers and patients with adult respiratory distress syndrome (ARDS). They demonstrated that free iron was present in all airway secretions of healthy adults and ARDS patients who survived, but not in non-survivors. This surprising result was explained by the leakage of antioxidants into the damaged lung. Kime et al. (20) documented by HPLC-analysis the presence of free iron in 12% of the investigated BAS samples of ventilated preterm infants.

Surprisingly we found no great differences in the free iron concentrations of BAS between the two investigated groups, infants who recovered from RDS and patients who developed chronic lung disease. The amount of free iron relative to the total iron concentration of day 2 and 5 of life demonstrates clearly the higher ferrous iron concentration to the total measurable iron, indicating a reduced iron-binding capacity of proteins.

According to the data of Evans et al. (21) a reduced iron-binding capacity of the neonatal proteins is feasible. Infants with developing CLD had elvated levels of iron-binding proteins within the first three days of life, normally indicating a sufficient iron-binding capacity. Since every protein identified in BAS can be an indicator of cellular or tissue damage of the lung of preterm infants, the increased transferrin and albumin levels most likely reflect an increased alveolar damage. In addition the high ferritin levels can result from cellular damage or from an increased ferritin production as a result of increased iron concentrations. Lactoferrin,

normally present in high concentrations in neutrophils, can be a direct marker for degranulation of activated granulocytes. Concerning the activation of neutrophils and macrophages it is noteworthy that these cells contain large amounts of proteases, which can be involved in degradation not only of the surrounding tissue but as well of antioxidant and iron-binding proteins. Moreover, macrophages exposed to neutrophil elastase and cathepsin G have been shown to be primed for an increased release of toxic oxygen radicals (22).

The proteins found in BAS indicate an enhanced leakage of the epithelial lining in the lung of preterm infants. Suggesting a decrease of iron-binding capacitiy, the lung of the neonate is exposed to enhanced oxidative stress in the presence of free iron.

The enhanced generation of OH-radicals in BAS compared to buffer solution demonstrates a lacking OH-radical-scavenger system in BAS. Therefore we hypothesize a possible involvement of reactive oxygen species, especially of hydroxyl radicals in combination with the presence of free iron in BAS of preterm infants.

At the moment investigations are in progress to enhance the sensitivity of the OH-radical-assay in order to identify directly the OH-radical formation in BAS.

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