Superoxide anion production by bronchoalveolar lavage cells in relation to cellular composition and lung function in sarcoidosis and chronic bronchitis

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KEY WORDS
bronchoalveolar lavage, chronic bronchitis, oxidative stress, sarcoidosis, superoxide anion

ABSTRACT

INTRODUCTION Increased generation of superoxide anion (O$_2^-$) by bronchoalveolar lavage (BAL) cells has been reported in various inflammatory disorders. However, the clinical relevance of this phenomenon is unclear.

OBJECTIVES The aim of the study was to investigate whether production of O$_2^-$ is enhanced in smoking-related chronic bronchitis and sarcoidosis, and to assess a relationship between O$_2^-$ generation and lung function impairment and changes in BAL cellular pattern.

PATIENTS AND METHODS Forty-two patients with sarcoidosis, 24 smokers with chronic bronchitis, and 17 controls were examined. A number/percentage of BAL cells was calculated. Spontaneous and phorbol myristate acetate (PMA)-stimulated O$_2^-$ production was measured in BAL cells. Spirometry was performed.

RESULTS Patients with smoking-related chronic bronchitis produced more O$_2^-$ spontaneously (6.42 ± 1.24 vs. 15.39 ± 2.47 nmol/10$^6$ cells, $P = 0.003$) and after stimulation (3.73 ± 1.32 vs. 14.76 ± 2.79 nmol/10$^6$ cells; $P = 0.001$). PMA-stimulated excess production correlated with the percentage of neutrophils ($r = 0.66$, $P = 0.0005$). In sarcoidosis, only spontaneous production of O$_2^-$ was higher (vs. 18.07 ± 2.49 nmol/10$^6$ cells, $P = 0.004$) and correlated with the percentage of BAL lymphocytes. There was no correlation between O$_2^-$ production and lung function parameters.

CONCLUSIONS Patients with smoking-related chronic bronchitis produce more O$_2^-$, and this phenomenon is related to BAL neutrophils. In sarcoidosis, spontaneous release of O$_2^-$ from BAL cells is related to the extent of lymphocytic alveolitis. Higher O$_2^-$ generation did not impair lung function.
peroxidation, \textsuperscript{6} oxidized proteins, \textsuperscript{3} and nitrotyrosine. \textsuperscript{7} A new marker of oxidative stress, 8-iso-prostanate, has been recently proposed. \textsuperscript{8}

Bronchoalveolar lavage (BAL) is a procedure widely used in the diagnosis and differentiation of various lung diseases. The BAL fluid is a biological material often used to assess oxidative stress in the lung. The measurement of $O_2^-$ generation by BAL fluid cells is a common method. However, the real source of ROS and clinical significance of these measurements are not known. Taking the above into consideration, we undertook this study to answer the following questions:

1. Does $O_2^-$ generation by BAL fluid cells in patients with sarcoidosis and smoking-related chronic bronchitis differ from healthy controls?
2. Does the amount of $O_2^-$ produced by BAL fluid cells correlate with impaired lung function?
3. Is $O_2^-$ generation related to changes in BAL fluid cellular pattern that favor any particular cell type?

**PATIENTS AND METHODS**

**Study population** Bronchoscopy with BAL was performed in 2 separate groups of patients: with sarcoidosis ($n = 42$, 14 women, age 40 ±11 years), and smoking-related chronic bronchitis ($n = 24$, 6 women, age 52 ±10 years). All patients were treatment-naive. Patients with sarcoidosis were diagnosed according to the guidelines. \textsuperscript{9} All were nonsmokers, there were 5 former smokers with maximum cumulative consumption below 10 pack years (mean ± standard error of the mean [SEM] = 1.6 ±0.7).

Stage 1 disease was diagnosed in 14 patients, stage 2 in 22, and stage 3 in 6. All had an active disease, based on the presence of symptoms, radiological progression, or progressive lung function impairment. Smoking-related chronic bronchitis was diagnosed in active smokers with chronic productive cough, macroscopic and microscopic signs of chronic inflammation. Other causes of cough were excluded. \textsuperscript{10} These patients had normal spirometry except 3 subjects who fulfilled the criteria for stage 1 COPD. \textsuperscript{11}

A control group consisted of 17 healthy never-smokers (7 women, aged 44 ±4 years), who were compared with sarcoidosis and chronic bronchitis patients.

All patients gave written informed consent. The study was approved by the Ethics Committee at the Medical University of Lodz (No. RNN/81/2001/KE).

**Lung function tests** Spirometry was performed according to the European Respiratory Society/American Thoracic Society (ERS/ATS) standards, \textsuperscript{12} on a computer-based spirometer (Jaeger, Germany). Forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV\textsubscript{1}) were measured, and the Tiffenau index (FEV\textsubscript{1}/FVC) was calculated. Lung diffusing capacity for carbon monoxide was measured only in sarcoidosis patients with Lungtest 1000 SB (MES, Poland) using a single breath method, according to the ERS/ATS standards. \textsuperscript{13} Values of diffusing capacity of the lung for carbon monoxide were corrected for hemoglobin concentration (DLCO) and recalculated for alveolar volume. All data (except the Tiffenau index) were presented as a percentage of predicted value.

Bronchoscopy was performed with a flexible bronchoscope (Pentax, Japan) according to the British Thoracic Society guidelines. \textsuperscript{14} Patients optionally received midazolam and atropine before the examination, 2% lidocaine was used as a topical anesthetic.

The BAL fluid was collected from medial lobe or lingula by instillation and subsequent withdrawal of $4 \times 50$ ml of 0.9% NaCl. The fluid recovery was 52.1 ±12.0%. Crude BAL fluid was filtered through a gauze, centrifuged and the pellet was suspended in a phosphate buffer. The total cell count (TCC) was presented as $n \times 10^6$. Cytospin slides were prepared and stained by the May-Grünwald-Giemsa stain. Macrophages, lymphocytes, neutrophils, and eosinophils were calculated using light microscopy and expressed as a percentage of the TCC. Moreover, the total number of cells and particular cell types were presented as the number of cells ($n \times 10^4$) per ml of recovered fluid. BAL was classified as unsatisfactory in the case of low recovery (below 30%), presence of blood, or cell viability below 90%. The lowest number of total BAL cells was 4.8 × 10\textsuperscript{4}/ml.

$O_2^-$ production by BAL fluid cells was measured colorimetrically, as previously described, \textsuperscript{15} with Pharmacia Biotech, Ultraspec 2000, Sweden, at 550 nm. Cells after centrifugation were suspended in phosphate buffer ($10^6$ cells/ml).

Reduction of cytochrome C by released O\textsubscript{2} led to decreased absorbance (the decrease of 0.100 equals the reduction of 9.8 nmol of cytochrome and release of the same amount of O\textsubscript{2}). Superoxide dismutase was used to inhibit the reaction (reference sample), and the difference between uninhibited and inhibited samples were used for calculations. We measured spontaneous and excess release after stimulation with phorbol myristate acetate (PMA) (1 ng/ml, for 20 min). Values were expressed as nmol O\textsubscript{2}^-/10\textsuperscript{6} cells. Because lymphocytes could be excluded as a source of oxidants, we presented these values as nmol O\textsubscript{2}^-/10\textsuperscript{6} cells potentially producing O\textsubscript{2}^- (macrophages, neutrophils, eosinophils). This value $[C]$ was calculated according to the following formula: $C = n \times 100/100-y$, where $n$ is a production of $O_2^-$ by all BAL fluid cells, and $y$ is a percentage of BAL fluid lymphocytes.

**Statistical analysis** Data were expressed as mean ± SEM, except age (mean ± standard deviation). The Kolmogorow-Smirnoff test was used to assess normality. Median with 25 and 75 percentile was also provided, for normally distributed data. The unpaired t-test (for normally distributed data) or the Mann-Whitney test (for data without normal distribution) was used to compare sarcoidosis and chronic bronchitis patients.
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with controls. To compare more than 2 groups, we used the one-way ANOVA and Bonferroni post-hoc test (for data with Gaussian distribution) or Kruskall-Wallis followed by Dunn’s multiple comparison test (for data without normal distribution). The Spearman test was used to assess correlation. $P \leq 0.05$ was considered statistically significant.

**RESULTS** Differences between sarcoidosis, smoking-related chronic bronchitis, and control groups The sarcoidosis group differed from controls in the number and percentage of BAL fluid lymphocytes and macrophages (Table 1).

There were no significant differences in BAL fluid cell number and percentage between sarcoidosis stages. There were no differences between these subgroups in terms of lung function test (LFT). DLCO for stage 1 was 84.82 ±5.37, for stage 2 – 73.82 ±4.23, and for stage 3 – 66.05 ±11.69% (nonsignificant).

In chronic bronchitis, both spontaneous and PMA-stimulated $O_2^-$ production were significantly higher (Figure 1Ab).

In sarcoidosis, only spontaneous $O_2^-$ release was higher compared with controls (Figure 2A). Differences were more significant in a subgroup of patients with lymphocyte percentage >25% (Figure 2A). PMA-stimulated $O_2^-$ production, although not different between controls and all sarcoidosis patients, achieved the level of significance in the subgroup with high lymphocyte percentage (Figure 2B). Spontaneous release was significantly higher in patients with stage 2 sarcoidosis (Figure 3A), and PMA-stimulated production was significantly elevated in stage 1 (Figure 3B).

There were no significant differences in LFT parameters between the study groups (Table 2).

**Correlation between BAL fluid superoxide anion concentration and lung function test parameters** There was no correlation between spontaneous or PMA-stimulated $O_2^-$ production by BAL fluid cells and LFT parameters, either in sarcoidosis or chronic bronchitis group.

**Correlation between BAL fluid superoxide anion production and BAL fluid cells** In sarcoidosis, spontaneous $O_2^-$ production was correlated with BAL fluid lymphocyte percentage (Figure 4) and lymphocyte number/ml of recovered BAL fluid ($r = 0.35, P = 0.03$).

In chronic bronchitis patients, $O_2^-$ production after stimulation with PMA was correlated with BAL fluid neutrophil percentage (Figure 5) and neutrophils/ml of recovered BAL fluid ($r = 0.52, P = 0.009$).

**BAL fluid recovery** BAL fluid recovery was significantly lower in patients with chronic bronchitis (Table 1). Only in this group it correlated inversely with the number of neutrophils/ml, but not with...
stimulated $O_2^-$ production was statistically higher in the group of smokers with chronic bronchitis. Although this finding is not surprising, it should be emphasized that even in smokers who do not fulfill the criteria for COPD, oxidative stress is already present. Similar results were obtained by Montuschi et al., who found elevated concentrations of 8-isoprostane in exhaled breath condensate of healthy smokers compared with healthy nonsmokers.

In sarcoidosis patients, we found an increased spontaneous release of $O_2^-$ PMA-stimulated production of $O_2^-$ was significantly higher only in patients with stage 1 disease, and in patients with high lymphocyte percentage in the BAL fluid. Spontaneous production was also higher in patients with stage 2 disease and with high lymphocyte percentage. These findings could be explained by overall higher level of inflammation in stages 1 and 2, and frequent acute and symptomatic sarcoidosis (Löfgren syndrome). Of note, these patients have a better prognosis.

**Discussion**

Our study was conducted on 2 different groups of patients: with sarcoidosis and smoking-related chronic bronchitis. Both diseases differ in terms of the type of cells involved in pathogenesis and predominant location of pathological changes (conductive airways in chronic bronchitis, lung parenchyma in sarcoidosis). Sarcoidosis is a lymphocytic inflammation, in which mutual interactions between CD4+ lymphocytes and macrophages lead to granuloma formation. In smoking-related chronic bronchitis, neutrophils and their products, including ROS, are the key components of inflammation.

Does superoxide anion generation by BAL fluid cells in patients suffering from sarcoidosis and smoking-related chronic bronchitis differ from healthy controls? Both spontaneous and stimulated $O_2^-$ production was statistically higher in the group of smokers with chronic bronchitis. Although this finding is not surprising, it should be emphasized that even in smokers who do not fulfill the criteria for COPD, oxidative stress is already present. Similar results were obtained by Montuschi et al., who found elevated concentrations of 8-isoprostane in exhaled breath condensate of healthy smokers compared with healthy nonsmokers.

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However, they ignored significance of spontaneous $O_2^-$ production. All cited authors reported significantly elevated levels of oxidant production in response to PMA, but spontaneous release in most of these studies was not different compared with healthy controls, or was not studied. Only in a study by Fels et al., spontaneous hydrogen peroxide release by BAL fluid alveolar macrophages was higher (but not significantly) when compared with controls.19 Our study, conducted on a larger group of patients, showed higher spontaneous production related to lymphocytic alveolitis. It might be suggested that alveolar macrophages isolated from patients with sarcoidosis could be naturally prestimulated, and an additional trigger does not result in a further significant increase of $O_2^-$ production in the majority of patients.

Correlation between lung function and the amount of superoxide anion produced by BAL fluid cells

Other authors have already confirmed higher $O_2^-$ production by alveolar macrophages in patients with active when compared with inactive sarcoidosis.4,17,18 Cassatella et al. found higher PMA-stimulated $O_2^-$ production by BAL fluid macrophages of patients with high percentage of lymphocytes in the BAL fluid and intensity of $^{67}$Ga uptake.4 However, they ignored significance of spontaneous $O_2^-$ production. All cited authors reported significantly elevated levels of oxidant production in response to PMA, but spontaneous release in most of these studies was not different compared with healthy controls, or was not studied. Only in a study by Fels et al., spontaneous hydrogen peroxide release by BAL fluid alveolar macrophages was higher (but not significantly) when compared with controls.19 Our study, conducted on a larger group of patients, showed higher spontaneous production related to lymphocytic alveolitis. It might be suggested that alveolar macrophages isolated from patients with sarcoidosis could be naturally prestimulated, and an additional trigger does not result in a further significant increase of $O_2^-$ production in the majority of patients.
Correlation between BAL fluid neutrophils (percentage of all cells) and spontaneous \( \cdot \cdot \) production (nmol/10^6 cells)

\[ r = 0.66 \]
\[ P = 0.0005 \]

**FIGURE 5** Correlation between BAL fluid neutrophils (percentage of all cells) and superoxide anion production after stimulation with PMA in smokers with chronic bronchitis

Abbreviations: see FIGURE 1

generated from inflammatory cells, i.e., hydrogen peroxide in expired breath condensate (EBC), and secondary markers, such as lipid peroxidation products in EBC. But several other authors did not confirm such a relationship, e.g., when EBC 8-isoprostane was analyzed.

In contrast to our results, Groen et al. observed that higher stimulated \( \cdot \cdot \cdot \) production was associated with a significantly lower total lung capacity and pulmonary diffusing capacity in sarcoidosis patients. Other authors, however, did not confirm these observations. Maier et al. reported that oxidized methionine residues in the BAL fluid were significantly increased in patients with IPF (and the effect was mediated by neutrophils) but not in patients with sarcoidosis. In another study, protein-carbonyls in the BAL fluid were normal in sarcoidosis but increased several times in IPF, a disease characterized by severe nonreversible fibrosis. This marker was strongly correlated with mRNA expression for interleukin (IL)-8, which may indicate neutrophil involvement.

**Generation of superoxide anion in relation to BAL fluid cellular pattern** In this study we observed that the higher the percentage of lymphocytes in the BAL fluid of patients with sarcoidosis, the higher the spontaneous release of \( \cdot \cdot \) from BAL fluid cells. We have not found such a correlation with PMA-stimulated \( \cdot \cdot \) production. This is inconsistent with the results of other investigators. In a few studies on \( \cdot \cdot \) production in sarcoidosis, the PMA-stimulated (but not spontaneous) generation was closely related to a degree of lymphocytic alveolitis.

The spontaneous release in the cited studies was either negligible or not evaluated at all. Because the main source of ROS in sarcoidosis are alveolar macrophages, our observation regarding the importance of spontaneous release of \( \cdot \cdot \) is consistent with the data indicating that macrophages in this disease are constantly stimulated by spontaneously released interferon \( \gamma \) from T lymphocytes, a key cytokine in granuloma formation. Another important cytokine for stimulation of alveolar macrophages and granuloma formation is IL-2. Tumor necrosis factor-\( \alpha \), a cytokine released mainly from macrophages but also from T lymphocytes, may be responsible for priming macrophages, neutrophils, and eosinophils to increased ROS production. It plays an important role in granulomatous inflammation, but is also one of the key cytokines in the pathogenesis of COPD. On the other hand, oxidants released from BAL cells may work as chemoattractants for lymphocytes, stimulating their proliferation and differentiation to T helper type 1 cells.

Although it is supposed that ROS originate mainly from numerous activated alveolar macrophages, in vitro studies have shown that on the per cell basis neutrophils and eosinophils are much stronger producers of oxidants. There are data showing that higher percentage of neutrophils and eosinophils in the BAL fluid are associated with more severe sarcoidosis.

Stimulated \( \cdot \cdot \) production is related to BAL fluid neutrophils. Phorbol esters (e.g., PMA) activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a membrane-bound enzyme which is strongly represented in these cells. This relation was found in smokers with chronic bronchitis. The spontaneous generation, however, was not correlated with BAL fluid neutrophils. Our results regarding \( \cdot \cdot \) production and the involvement of neutrophils in smoking-related chronic bronchitis are consistent with current knowledge.

The final question to consider is a possible relation of our findings to BAL fluid recovery. Other studies have shown that the worse recovery of the BAL fluid, the higher the percentage of recovered neutrophils and the lower the recovery of lymphocytes. We have observed lower recovery of the BAL fluid in smokers with chronic bronchitis, and a negative correlation between BAL fluid recovery and the number (but not percentage) of neutrophils. There was no correlation with BAL fluid lymphocytes in any of the groups. Because we have not found any correlation between \( \cdot \cdot \) production and BAL fluid recovery, we believe that the influence of BAL quality on our results is minimal.

In summary:

1. BAL fluid cells produce more \( \cdot \cdot \) in patients suffering from sarcoidosis (spontaneously) and smoking-related chronic bronchitis (also after stimulation). Patients with sarcoidosis of potentially better prognosis (stages 1 and 2) and with more extensive lymphocytic alveolitis tend to generate more \( \cdot \cdot \).
2. Spontaneous \( \cdot \cdot \) release correlates with lymphocytes detected in the BAL fluid of sarcoidosis patients. Neutrophils, although in low numbers in the BAL fluid, may be an important source of oxidants, which are generated by stimulation of NADPH oxidase in smokers with chronic bronchitis.
3 Generation of $O_2^-$ was not correlated with impaired lung function, either in sarcoidosis or chronic bronchitis.

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REFERENCES


ARTYKUŁ ORYGINALNY

Produkcja anionorodnika ponadtlenkowego przez komórki popłuczyn oskrzelowo-pęcherzykowych w odniesieniu do składu komórkowego i czynności płuc w sarkoidozie i przewlekłym zapaleniu oskrzeli

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SŁOWA KLUCZOWE
popłuczyny oskrzelowo-pęcherzykowe, przewlekłe zapalenie oskrzeli, anionorodnik ponadtlenkowy, sarkoidoza, stres oksydacyjny

STRESZCZENIE

WProwadzenie W chorobach zapalnych układu oddechowego stwierdza się zwiększenie produkcji anionorodnika ponadtlenkowego (O_2^-) przez komórki popłuczyn oskrzelowo-pęcherzykowych (bronchoalveolar lavage – BAL). Znaczenie kliniczne tego zjawiska nie jest jasne.

CELE Celem badania była ocena, czy w przewlekłym zapaleniu oskrzeli, a także u chorych na sarkoidozę produkcja O_2^- jest zwiększona i czy istnieje związek pomiędzy produkcją O_2^- a upośledzeniem czynności układu oddechowego i zmianami w składzie komórkowym BAL.

PACJENTI I METODY U 42 chorych na sarkoidozę, 24 chorych na przewlekłe zapalenie oskrzeli i 17 osób z grupy kontrolnej obliczono liczbę/odsetek komórek BAL. Zbadano spoczynkową i stymulowaną octanem mirystynianu forbolu (phorbol myristate acetate – PMA) produkcję O_2^- przez komórki BAL. Wykonano spirometrię.

WYNIKI U chorych na przewlekłe zapalenie oskrzeli stwierdzono większe spoczynkowe i stymulowane uwalnianie O_2^- do BAL. Dodatkowa produkcja PMA korelowała z odsetkiem neutrofilów (r = 0,66; P = 0,0005). W sarkoidozie tylko spoczynkowa produkcja O_2^- była większa (vs 18,07 ± 2,49 nmol/10^4 komórek; P = 0,004) i korelowała z odsetkiem limfocytów w BAL. Nie stwierdzono korelacji pomiędzy produkcją O_2^- a czynnością układu oddechowego.

WNIOSKI Komórki BAL u osób z przewlekłym zapaleniem oskrzeli palących tytoń wytwarzają więcej O_2^- i jest to zjawisko zależne od neutrofilów w BAL. W sarkoidozie spoczynkowe uwalnianie O_2^- z komórki BAL zależne jest od stopnia zapalenia limfocytarnego pęcherzyków płucnych. Wysoki poziom produkcji O_2^- nie jest związany z pogorszeniem parametrów czynnościowych płuc.