

The Contribution of Endotoxins Present in the Respiratory Tract to Overproduction of Nitric Oxide Associated with Impaired Interleukin 6 Release in Bronchoalveolar Leukocytes from Lung Cancer Patients

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Abstract. The purpose of the study was to assess the relation between the levels of endotoxins circulating in airways of patients with lung cancer and the ability of bronchoalveolar lavage (BAL) leukocytes for *ex vivo* release of nitric oxide (NO) and interleukin 6 (IL-6) and for *in vitro* lipopolysaccharide (LPS)-induced production of the mediators. Leukocytes isolated from the BAL of 11 patients and from 5 healthy individuals were cultured in the absence or presence of LPS_{E. coli}. The levels of endotoxins in the BAL fluids (BALF) and the amounts of NO released *ex vivo* from unstimulated cells from the patients were highly ($p = 0.0025$) elevated in comparison with those from healthy individuals. The release of NO was significantly correlated ($R_s = 0.638$, $p = 0.047$) with the levels of endotoxins in BALF. In contrast, production of IL-6 remained very low and a negative correlation ($R_s = -0.623$, $p = 0.0542$) was observed between the amounts of NO and IL-6. It was also found that, in response to LPS, bronchoalveolar leukocytes from patients with lung cancer express a reduced capacity for *in vitro* production of NO and IL-6. Our data suggest that, in patients with lung cancer, the activation of BAL cells by endotoxins circulating in the airways may contribute, at least in part, to overproduction of spontaneous NO and, subsequently, the NO may reduce IL-6 production. Moreover, the exposure *in vivo* of the BAL cells to LPS renders them unable to respond to the second signals.

Key words: endotoxins; respiratory tract; nitric oxide; interleukin 6; bronchoalveolar leukocytes; lung cancer.

Introduction

The suppressed cytokine-response of bronchoalveolar lavage (BAL) cells from patients with lung cancer

has been already documented^{3, 4}, while the mechanism of this phenomenon is poorly understood. It has been generally assumed that the event may reflect the existence of the immunosuppressive factors related to tumor

Abbreviations used: BAL – bronchoalveolar lavage, BALF – bronchoalveolar lavage fluids, PBS – phosphate buffered saline, LPS – lipopolysaccharide, NDV – Newcastle disease virus, NO – nitric oxide, IL-6 – interleukin 6, LAL – *Limulus amoebocyte* lysate, EU – endotoxin unit, SCLC – small cell lung cancer, NSCLC – non-small cell lung cancer, R_s – rank correlation coefficient.

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growth²³. Tumor cells are known to secrete a number of immunosuppressive molecules, such as transforming growth factor- β (TGF- β), interleukin 10 (IL-10) and prostaglandin E2 (PGE2)^{1, 8, 11} as well as nitric oxide (NO)⁹. On the other hand, the contribution to the immunosuppressive activity in lung cancers of mediators released by pulmonary immunocompetent cells and activators of these cells has remained unclear.

Some studies indicate that respiratory infections caused mainly by Gram-negative bacteria are very frequent in the course of lung cancer¹⁶. These findings prompted us to look for bacterial products such as endotoxins (LPS) in the lower respiratory tract by estimation of the levels of LPS in bronchoalveolar lavage fluids (BALF). With respect to the possibility that LPS is considered as one of the activators of pulmonary immunocompetent cells¹⁰, we assessed in patients with lung cancer the relation between endotoxins present in the airways and the secreted markers of the BAL cells' activation, such as NO and IL-6.

Materials and Methods

Patients and control subjects. The study population consisted of 11 randomly selected patients (2 women, 9 men) with previously undiagnosed and untreated disease. They had initially undergone bronchoscopy because of a chest radiography that had revealed a tumor mass. The diagnosis was confirmed on the basis of histological and cytological examination of the selected

materials by bronchofibroscopy or transthoracic thin-needle aspiration of the tumor. The patients had not been receiving any therapy at least one month before the bronchofibroscopy procedure. All of them were current smokers (Table 1). The tumor types were: non small cell lung cancer (NSCLC) – 3 patients, and small cell lung cancer (SCLC) – 8 patients. Eight patients had stage 2 disease and the other three had stage 3 disease, according to the TNM classification²⁴. The control group comprised 5 non-smoking patients (1 woman and 4 men), who had undergone diagnostic examination because of non-organic, neurologic symptoms or cancer phobia. They were found to be without clinical or radiographic evidence of lung cancer. The study was approved by the Ethic Committee of the University Medical School of Wrocław, Poland. Informed consent was obtained from all of the patients.

Bronchoalveolar lavage leukocytes' isolation and culture. The BAL procedure was performed by a routine method as described previously^{3, 4}. Briefly, lavage was filtered through wet double-layer sterile gauze and centrifuged at 400 g for 10 min at 4°C. After 2 washings, the pellet was resuspended at a cell concentration of 5×10^6 per 1 ml in RPMI medium supplemented with 5% heat-inactivated human AB serum and 2 mM of L-glutamine (Gibco, Edinburgh, GB), 100 U/ml penicillin, 100 μ g/ml streptomycin. The cell suspensions were distributed and cultured in 24-well flat bottom culture plates (Costar, Cambridge, MA, USA). The cells were enumerated in a Thoma chamber and their viability was ascertained by the trypan blue exclusion

Table 1. Characterization of the studied patients and BAL data

Parameters	Healthy (n = 5)	Patients with lung cancer (n = 11)	p (Mann-Whitney test)
Age (years)	31 (21–45)	50 (40–67)	0.0043*
Sex F/M	1/4	2/9	
Smoking Y/N	0/5	11/11	
FEV (% of predicted)	98 (94–108)	96 (65–103)	0.117
Total cells $\times 10^6$	8.1 (4.2–15.8)	133.0 (14.6–186.0)	0.0050*
Viability (%)	95.0 (86.0–98.0)	88.0 (48.0–94.0)	0.1489
Macrophages (% total)	94.0 (91.0–96.0)	81.0 (34.0–93.0)	0.0032*
Lymphocytes (% total)	6.0 (4.0–6.0)	12.0 (5.0–35.0)	0.0032*
PMN, others (% total)	0.0 (0.0–4.0)	4.0 (1.0–54.0)	0.0380*

* Differences were considered significant at a p level less than 0.05.

Data are expressed as medians. Ranges are shown in parentheses.

test. Differential cell counts were performed by analyzing May-Grünwald-Giemsa-stained smears, identifying at least 200 cells.

Determination of NO release. NO production by bronchoalveolar cells was quantified by measuring the accumulation of its stable degradation product nitrite (NO_2^-) in the culture supernatants by using the Griess reaction according to Ding et al.⁵. Briefly, cell-free supernatants (100 μl) were removed from 48-hours-BAL leukocyte cultures and placed onto 96-well plate. Then 100 μl Griess reagent (0.1% naphthylenediamine dihydrochloride (Sigma Chemicals Co., USA) in water and 1% sulphanilamide (Sigma Chemicals Co., USA) in 5% H_3PO_4 (v/v) was added to each well. The incubation was carried out for 10 min at room temperature and adsorbance was measured at $\lambda = 540$ nm using an ELISA plate reader (Stat Fax 2100, Awareness Technology Inc., USA). NO_2 concentrations were calculated from a linear standard curve generated from 2 to 100 μM of sodium nitrite (NaNO_2 ; Sigma Chemicals Co., USA).

Assay for IL-6 activity. Production of IL-6 was determined by using the IL-6-dependent hybridoma cell line 7TD1 38 as was described previously^{3, 4}. Briefly, the cells were cultured in 96-well plates (Costar, MA, USA) at a concentration of 2×10^3 cells/well in the presence of serial dilutions of the non-stimulated or LPS-stimulated BAL cell culture supernatants. After 72 h, the surviving cells were estimated colorimetrically by staining of the cells with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemicals Co., USA), and lysis of the cells with SDS/DMF solution (13.5%, w/v) sodium dodecyl sulfate (SDS) in 45% water solution (v/v) N, N-dimethylformamide (DMF; Sigma Chemicals Co., USA). One unit of IL-6 activity was calculated as the inverse dilution of a supernatant sample where a half-maximal proliferation of 7TD1 cells was registered. In our assay, one unit corresponded to 0.1 ng of recombinant (*E. coli*), human IL-6 (R&D Systems, USA). The sensitivity of the bioassay was 5 U/ml.

Measurement of endotoxins. Cell-free fluids from bronchoalveolar lavage were 10-fold concentrated by lyophilization and dissolved in phosphate buffered saline (PBS). The amounts of endotoxins were determined in the BAL fluids, culture media and PBS by the end-point method⁷ using the chromogenic *Limulus ameobocyte* lysate (LAL) test (Manufacturer of LAL, Charleston, SC, USA). The tests samples were diluted 1:10 with sterile endotoxin-free water and heated for 5 min at 75°C. Next, 100 μl samples of BALF of patients or healthy individuals and diluted control standard endotoxins were incubated at 37°C for 5 min and then mixed with 100 μl of LAL and

further incubated for 16 min at 37°C. Two hundred μl of chromogenic substrate was then added, vigorously mixed and the incubation was continued for an additional 8 min. The reaction was stopped by the addition of 20% acetic acid. From the contents of the test tubes, 200 μl samples were transferred to a 96-well microplate and the adsorbance was read in an Elisa Reader Dynatech 5000 (USA) at the wavelength of 405 nm.

Statistical analysis. The data were presented as medians, quartiles (25–75%) and ranges (Min.–Max.). Significance of differences between groups was determined using the Mann-Whitney *U*-test. Differences between pairs of measurements in the same patients were tested using Wilcoxon's matched-pairs signed-ranks test. Spearman's rank correlation test was used for determination of the relationship between the levels of endotoxins, NO and IL-6. Analysis was done using the computer program Statistica Version 5.1, Stat-Soft (USA), taking $p < 0.05$ as statistically significant.

Results

Patients and BAL data

Table 1 summarizes the clinical data and BAL parameters. BAL was well tolerated by all subjects. The total number of BAL cells obtained from lung cancer patients was higher ($p = 0.005$) than that from healthy subjects. However, viability of the cell populations was similar ($p = 0.1489$) in the two groups. Differential cell counts for lung cancer patients are representative of the inflammatory state within the lower respiratory tract because of the increase ($p = 0.0032$) in the number of lymphocytes and granulocytes in comparison with healthy individuals.

Assessment of endotoxin levels in BALF

Endotoxin contamination in culture media and in PBS was undetectable or below 0.2 EU/ml (Table 2). The BALF obtained from healthy donors and studied patients with lung cancer were positive with regard to the presence of endotoxins. However, the levels of LPS in BALF obtained from NSCLC and SCLC patients were significantly elevated ($p = 0.00045$) in comparison with that from healthy subjects (Table 2).

Spontaneous and LPS-induced secretion of NO

The levels of NO released from unstimulated BAL cells of patients with lung cancer were estimated. More-

Table 2. Concentration of endotoxins in media and bronchoalveolar lavage fluids

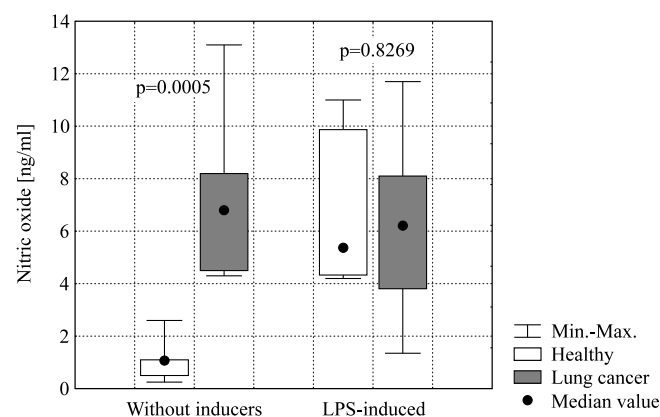
Fluids	Endotoxin concentration [EU/ml]
Phosphate buffered saline (PBS)*	0.000 (0.000–0.110)
RPMI + 5% human serum*	0.167 (0.153–0.185)
BALF **	
healthy (n = 5)	0.353 (0.183–0.460)
lung cancer (n = 11)	0.631 (0.562–0.875)

* The estimations were done on 4 of the different batches of media used to the study.

** Bronchoalveolar lavage fluids (BALF) were 10-fold concentrated.

Results are presented as medians and ranges are given in parentheses.

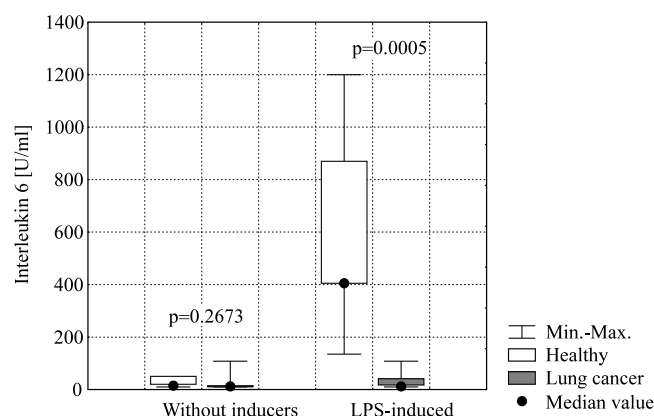
over, *in vitro* the ability of the cells to production of NO in response to LPS was also studied and compared with that of healthy controls. As shown in Fig. 1, the unstimulated BAL cells from healthy subjects produced a low level of NO, whereas stimulation with LPS resulted in an increase of the amounts of NO (median 1.07, range 0.25–2.6 pg/ml vs median 5.3, range 4.26–11.0 pg/ml). In contrast, a significantly ($p = 0.0004$) higher production of NO was observed in unstimulated cells of lung cancer patients than in healthy controls, whereas stimulation of the cells with LPS was accompanied by a decrease in the levels of NO (median 7.3,

**Fig. 1.** Production of nitric oxide by bronchoalveolar lavage leukocytes obtained from patients with lung cancer and healthy individuals. The BAL cells at a concentration of 5×10^6 cells per 1 ml were cultured for 48 h in the absence or presence of LPS (5 μ g/ml). The amounts of NO were estimated in cell-free culture media. Results are presented as median values and quartiles (25–75%). Ranges are shown as whiskers plots

range 4.5–13.1 pg/ml vs median 6.2, range 3.7–11.7 pg/ml).

Production of IL-6 by unstimulated and LPS-stimulated BAL cells

The *in vitro* unstimulated cells from lung cancer patients released a level of IL-6 similar to that found in healthy subjects (median 12.0, range 10.0–108.0 U/ml vs median 15.0, range 10.0–45.0 U/ml; $p = 0.2673$). It must be noted that in healthy individuals the stimulation of BAL cells with LPS resulted in elevated ($p = 0.0005$) amounts of IL-6 while in both NSCLC and SCLC patients the level of IL-6 remained as low as in intact cells ($p = 0.8269$; Fig. 2).

**Fig. 2.** Production of interleukin 6 by bronchoalveolar lavage leukocytes obtained from patients with lung cancer and healthy individuals. For further explanation see Fig. 1

Relationship between the levels of endotoxins present in the airways and the ability of BAL cells to produce NO and IL-6

The levels of NO and IL-6 released from BAL cells were assessed in relation to the concentration of endotoxins in BALF. A high production of NO was significantly correlated with the levels of endotoxins in BALF ($R_s = 0.717$, $p = 0.0017$; Fig. 3). In contrast, secretion of IL-6 remained very low and a negative correlation was observed between the amounts of NO and IL-6 ($R_s = -0.616$, $p = 0.012$; Fig. 4).

Discussion

Our study for the first-time provides evidence for endotoxin contamination in the lower respiratory tract of patients with lung cancer. We suggest that arterial

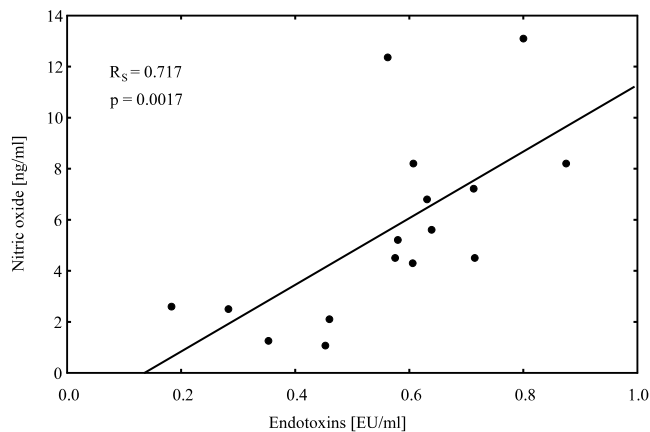


Fig. 3. Relationship between endotoxin levels in bronchoalveolar lavage (BAL) fluids and production of nitric oxide by BAL leukocytes obtained from patients with lung cancer

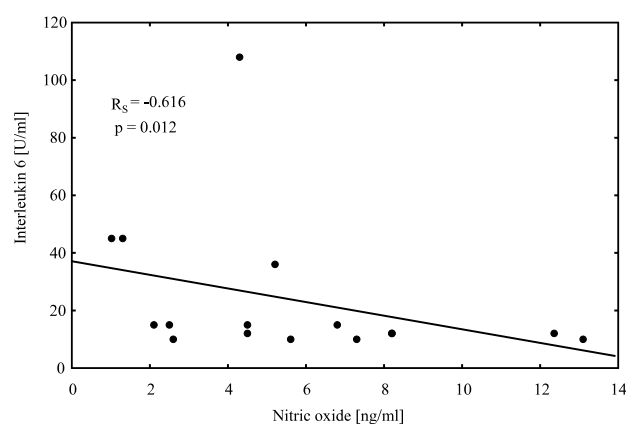


Fig. 4. Relationship between production of nitric oxide and interleukin 6 by bronchoalveolar lavage leukocytes obtained from patients with lung cancer

products, such as LPS, which occupied the airways of the studied patients could contribute, at least in part, to the suppressed cytokine-response of bronchoalveolar leukocytes by triggering NO release.

The presence of LPS in the lower respiratory tract may reflect the process of pulmonary Gram-negative bacteria infections. Indeed, the high frequency of the pattern of respiratory infections was firmly established by retrospective examination in which mainly Gram-negative bacteria were found in sputum, bronchial aspirate and lung specimens isolated from deceased patients with lung cancer¹⁶. Infections associated with lung cancer are usually undiagnosed because of the tumor related abnormalities². Moreover, the routine microbiological studies to determine the respiratory infectious etiology are of limited value because of controversy concerning the list of pathogens and the frequency with which they cause the infections²⁰.

We have tried to exclude the possibility suggested

by NELSON et al.¹⁸ that LPS contents in the salines instilled through the bronchoscope can stimulate BAL cells to NO production. We have given special attention to clean bronchoscopes and to use LPS-free physiological salines (Table 1) for lavage procedures. We have also used culture media containing endotoxins in a quantity which is insufficient to induce NO and cytokine release (our data not shown).

Our observations provide indirect evidence for *ex vivo* stimulation by endotoxins of BAL leukocytes from lung cancer patients. We have found a significant positive correlation between the amounts of NO produced by unstimulated *in vitro* BAL cells and endotoxin levels in BALF. Additionally, suppressed NO response to LPS *in vitro* may confirm the suggestion that the exposure *in vivo* of the BAL cells to LPS renders them unable to respond to the second signals. Our results are in agreement with the finding of DUGAS et al.⁶ that NO synthase in human alveolar macrophages is not expressed constitutively, but needs stimuli to be induced. In other words, endotoxins, which are known as the most potent immune stimuli and which were found by us in the lower respiratory tracts of the studied patients with lung cancer, may be considered as inducers of NO overproduction in BAL cells.

However, an other kind of stimuli can not be excluded. Tumor cells may also be involved in the production of NO by immunocompetent cells. Some studies on mice models have indicated that direct stimulation of alveolar as well as peritoneal macrophages by a membranous fragment of tumor cells can lead to the secretion of NO¹³. Likewise, NO synthase mRNA and iNOS protein were found in human monocytes after stimulation by colon carcinoma cells²⁰. It should be noted that, in addition to immunocompetent cells, NO may also be produced by lung tumor cells themselves, depending on the type of the cancer⁹.

Anyway, on the basis of our results, we propose that the increased NO production may be attributed not only to the tumor itself, but also to the tumor-associated non-specific immunological processes. We suggest that such processes may reflect, at least in part, the effect on BAL leukocytes of environmental stimuli, including LPS. Moreover, the response of the cells seem to be irrespective of the localization of the cells. They can be located even in the distant surroundings of the tumor, such as in the contralateral lobe.

Our previous study^{3, 4} demonstrated that bronchoalveolar leukocytes isolated from lung cancer patients appear to be immunodeficient because they did not spontaneously secrete TNF- α , IFNs and IL-6 and could not responded to LPS and NDV. This is in concordance

with the present observation concerning attenuated IL-6 production in such patients. The immunodeficient state of BAL cells seems to be discrepant with the fact that differential cell counts of the cell population obtained from the studied patients reflect the inflammatory status of the entire lower respiratory tract, as was established by HUNNINGHAKE et al.¹². This may be explained by the proposal that the state of activation of the cells may be more important than their number.

Nitric oxide was found to be an attractive potential mediator of inflammatory reactions by downregulation of LPS-induced cytokines in lung macrophages^{17, 19}. Overproduction of NO associated with an impaired release of IL-6, which was observed by us in lung cancer patients, suggests that NO may be involved in the diminished response of the cytokine. Indeed, it was established by THOMASSEN et al.²² that NO can inhibit TNF- α and IL-1 β production by human alveolar macrophages which were primarily activated with LPS. This seems to be the case in our study concerning IL-6.

Based on our results and the findings that NO can decrease production of inflammatory cytokines by lung macrophages¹⁷ and reduce Con A-induced lymphocyte proliferation^{14,15} in lung cancer patients, one can speculate that NO may be responsible, at least in part, for the immunosuppressive state of the patients. However, the production of NO may be consequent on tumor-related or tumor-unrelated processes. Further investigation of the possible role of NO in the metabolism and behavior of lung cancer depending on the origin of NO are needed.

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