



Comparison of Leukocyte Populations from Bronchoalveolar Lavage and Induced Sputum in the Evaluation of Cellular Composition and Nitric Oxide Production in Patients with Bronchial Asthma

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Abstract. Bronchoalveolar lavage (BAL) or induced sputum (IS) techniques may provide leukocytes for the evaluation of airway inflammatory response in bronchial asthma. The aim of the present study was to compare features of leukocyte populations obtained by the two different methods regarding the cell types and their activity in patients with bronchial asthma. The nitric oxide (NO) level released from the cells was measured as a marker of their activity. Pulmonary leukocytes were obtained from the BAL and IS of 11 asthmatic patients in stable condition at the time of the study. The BAL and IS leukocyte populations varied in cell count and NO production. Macrophages were the predominant leukocyte population in BAL (median (Me) = 83.0%, range 67.9–88.4%), whereas sputum sediments were found to consist mainly of neutrophils (Me = 55.7%, range 29.0–64.9%). The IS leukocytes released much more NO ($p = 0.0022$) than the BAL leukocytes. In spite of these quantitative differences, a similar pattern of NO production was observed in BAL and in IS cells. Both BAL and IS leukocyte populations produced almost the same amounts of NO before and after lipopolysaccharide stimulation ($p = 0.9063$, $p = 0.4801$, respectively). Furthermore, a slight positive correlation Spearman's rank ($R_s = 0.5578$, $p = 0.0594$) was noticed between the neutrophil percentages and NO levels produced by BAL cells, whereas in IS a statistically significant correlation between the percentage of neutrophils and the levels of NO ($R_s = 0.6643$, $p = 0.0184$) was observed. In conclusion, the BAL and IS leukocyte populations are different in cell type, their size and activity. Depending on the asthma severity and the type of cells needed in a study, either BAL or IS specimens may be chosen as a source of pulmonary leukocytes. The use of IS as a noninvasive technique is supposed to be potential value particularly in the study of the airway inflammatory response mediated mainly by neutrophils, i.e. during and/or after exacerbation of the disease. Based on our results, a possible contribution of neutrophils in the production of NO in the airways of asthmatic patients can be proposed apart from other cells such as macrophages.

Key words: bronchoalveolar lavage; induced sputum; pulmonary leukocytes; nitric oxide; neutrophils; macrophages; eosinophils; bronchial asthma.

Abbreviations used: BAL – bronchoalveolar lavage, IS – induced sputum, LPS – lipopolysaccharide, FEV₁ – forced expiratory volume in one second, Me – median.

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Introduction

Resident and infiltrating lung leukocytes, via production of nitric oxide (NO) and other mediators, have been postulated to participate in the ongoing inflammation in bronchial asthma^{4, 13}. The evaluation of the inflammatory processes has directly been assessed in material obtained mainly from bronchoalveolar lavage (BAL)^{30, 31} or, rarely, bronchial wash²⁰ and bronchial biopsy¹⁸. Our previous studies on the state of activity of inflammatory cells in bronchial asthma were performed using the BAL technique^{5–7}. However, widespread use of BAL is considered as an invasive method and limited rather to patients with mild asthma^{24, 31}. Over the past few years, an alternative, non-invasive method of sputum induction by inhalation of aerosolized hypertonic saline has been developed^{15, 24–27, 33}. Up till now, investigators' interests was mainly focused on comparing the cellular compositions of induced sputum (IS), BAL and bronchial biopsies^{12, 20, 29}. The results of their studies demonstrated the variability in differential cell count in the studied specimens. However, little attention was paid to the fact that in inflammatory processes the state of activation of inflammatory cells may be even more important than their number. Thus, besides the relationship between BAL and IS as to cellular content, the state of cell activation remains to be clarified.

The aim of the present study was to compare BAL and IS specimens taken from asthmatics with regard to the differential cell count on the one hand and the state of activity of the pulmonary leukocytes on the other. The release of NO by the leukocytes was used as a marker of degree of their activity.

Materials and Methods

Patients. Eleven bronchial asthma patients (6 women and 5 men) with a mean age of 40 ± 15 years (range 20–67) were enrolled in the studies (Table 1). Two of them were cigarette smokers. All of the patients met the diagnostic criteria of allergic asthma. Nine cases were assessed as severe asthma, while in two cases moderate stages of disease were diagnosed. Nine

of the asthmatics had systemic treatment with 10–20 mg prednisone (Encorton, Polfa, Pabianice, Poland) per day and inhaled about 1000 µg of budesonide (Budesonid, Glaxo Wellcome, Poznań, Poland) per day. In the past, all of the patients had undergone several courses of therapy with antibiotics (among which were mainly 1000 mg of amoxicillin/clavulanic acid (Augmentin, SmithKlineBeecham, Great Britain) twice a day, or 500 mg of ciprofloxacin hydrochloride (Cipronex, Polpharma, Poland) twice a day) due to respiratory infections. The intervals between the end of the therapy and the performance of BAL and IS ranged from 1 to 2 weeks. The BAL and IS procedures were performed at one- to two-day intervals at a time when the states of the patients were clinically stable. The study was approved by the Bioethics Committee of the Wrocław Medical University. Informed consent was obtained from all patients.

Bronchoalveolar lavage procedure. BALs were carried out according to a standardized protocol based on a recent recommendation³⁴ as described previously^{5–7}. Briefly, patients were premedicated intramuscularly with 50 mg of pethidine hydrochloride (Dolcontrol) and 0.8 mg of atropine sulphate (Atropinum sulfuricum), and local anesthesia was achieved with 2% lidocaine hydrochloride (Lignocainum hydrochloricum, Polfa Warszawa, Poland). Lavage was performed through a flexible fiberoptic bronchoscope (1 TP10, Olympus, Japan) wedged into a subsegmental bronchus of the middle lobe or lingula. Aliquots of a 0.9% sterile saline solution, pre-warmed to 37°C, were instilled 3 times with 50 ml and each of them was immediately withdrawn by gentle suction with a syringe. The lavage fluid was collected in a sterile container and immediately placed on ice.

Sputum induction. The procedure of sputum induction was carried out according to HOLZ et al.¹⁵. Sputum was induced by inhalation of hypertonic 4% saline aerosols. Sodium chloride solutions were nebulized at room temperature by an ultrasonic nebulizer (Aerodyne Omega, Kendal, Germany) at the maximal output setting of 3 ml/min. The aerosols were inhaled by the mouth. Every 5 min after the start of nebulization, patients were asked to rinse their mouths and throats with water and to expectorate sputum into clean plastic containers by coughing. Forced expiratory volume in one

Table 1. Characteristics of studied patients with bronchial asthma

Patients number	Age $\pm$ SD [yrs] (ranges)	Sex F/M	Smoking yes/no	Baseline FEV ₁ [% predicted] (ranges)
n = 11	40 $\pm$ 15 (20–67)	6/5	2/9	81.5 (72.0–107.3)

Results are given as medians and ranges in parentheses.

second (FEV₁) was monitored directly before and after the BAL procedure and IS, and whenever the subject felt uncomfortable.

Sputum processing. Sputum samples were processed according to the protocol recently validated by FAHY et al.¹⁰, with modification according to PANG et al.²⁶. Briefly, sputum was treated by adding 4 volumes of 10% Sputolysin (Calbiochem Corp., San Diego, USA) followed by 4 volumes of Dulbecco phosphate-buffered saline. To dissolve mucus plugs, the mixtures were vigorously shaken for 30 s and then incubated with gentle mixing in a water bath at 37°C for 15 min. Next, the samples were suspended in a PBS solution to a volume of 40 ml and were incubated with 100 U/ml of DNase (Sigma Co., St Louis, USA) at 37°C for 30 min to further dispersion. The sputum samples were filtered through a 48-µm nylon gauze to remove debris.

BAL and IS leukocyte isolation and analysis. The lavage fluid was filtered through wet double-layer sterile gauze and centrifuged at 400 g for 10 min at 4°C, whereas the filtrates of the homogenized sputum samples were centrifuged at 790 g for 10 min. The cell pellets were resuspended in physiological saline for total cell count and cell viability assessment by Trypan blue exclusion in a hemocytometer. For the determination of differential cell counts, the cell suspensions, at a concentration of 2×10^6 cells/ml, were spun in a cytocentrifuge (Cytospin, Shandon, Japan) and slides were stained with May-Grünwald-Giemsa stain. The slides were coded and counted blind by one investigator. Two hundred cells per slide were counted, with average counts of two slides for each patient. The results of differential leukocytes were expressed as a percentage of nucleated cells excluding squamous cells.

Cell culture system. As NO activity in the lung of asthmatics was found to depend not only on the type of producing cells, but also on the interaction between different cells³², we evaluated NO production in the whole populations of pulmonary leukocytes obtained from both BAL and IS. The pellets of the cells from BAL and IS were resuspended at a cell concentration of 5×10^6 /ml in RPMI 1640 medium without phenol red and supplemented with 5% heat-inactivated human AB serum and 2 mM of L-glutamine (Gibco, Edinburgh, Scotland), 100 U/ml penicillin, 100 mg/ml streptomycin and 5 µg/ml amphotericin B (Serva, Heidelberg, Germany). The cell suspensions were distributed and cultured in 24-well flat bottom culture plates (Costar, Cambridge, USA) for 48 h at 37°C with a 5% CO₂ atmosphere in the presence or absence of 5 µg/ml of lipopolysaccharide (LPS) from *E. coli*, serotype 055:B5 (Difco, Detroit, USA). All culture supplements

were freshly prepared from stock solutions by dilution with culture medium to the desired concentrations. At the end of the incubation period, the culture supernatants were collected, centrifuged and frozen at -20°C for further analysis.

Nitric oxide assay. NO synthesis was measured as the accumulation of nitrite (a stable oxidation product of NO) in the 48-hour leukocyte culture supernatants using a procedure based on the Griess reaction¹¹. Briefly, 100 µl samples were added in duplicate to 96-well microtiter plates. Then, 100 µl of Griess reagent (1% sulfanilamide in distilled water and 0.1% N-1-naphtylenediamine dihydrochloride in 2.5% H₃PO₄) (Sigma Co., St Louis, USA) was added to each well and the samples were incubated for 10 min at room temperature. Absorbances at λ=540 nm were read on an ELISA plate reader (Stat Fax x2100 Awareness, Technology Inc.). The values of nitrite concentrations in the culture fluid samples were calculated from a linear standard curve generated from 2 to 100 µM of sodium nitrite (NaNO₂) (Sigma Co., St Louis, USA).

Statistical analysis. Total differential cell counts, cell viability and NO levels are expressed as medians (ranges) and quartiles. To compare values Wilcoxon's signed rank test and the Mann-Whitney non-parametric U-test were used. Spearman's rank (R_s) correlation test was used for the assessment of interrelations. For statistical analysis, the computer program Statistica 5.1, StatSoft was used. Probability values <0.05 were accepted as statistically significant.

Results

Patients

Asthmatic patients demonstrated a wide range in lung function; their baseline FEV₁ were: median (Me) = 81.5%, range 72.0–107.0% of the predicted value (Table 1).

Analysis of the cell populations in BAL fluid and IS

Figure 1A and B present representative examples of slides with BAL and IS leukocyte populations of the asthmatic patient group. Macrophages obtained from BAL (Fig. 1A) are about 1.5- to 2-fold larger in size than those from IS (Fig. 1B). Table 2 summarizes the analysis of the two cell populations. There was a tendency to recover a higher ($p = 0.0567$) total number of the cells from sputa than from BAL specimens. Furthermore, a trend towards a lower viability of the cells

Table 2. Differential cell counts in bronchoalveolar lavages (BAL) and induced sputa (IS) obtained from patients with bronchial asthma

Cells	BAL	IS	BAL vs IS		
			differences ^a	correlations ^b	
				R _s	p ^c
	[%] of total nonsquamous cells		p		
Total cells × 10 ⁶	10.0 (4.6–44.0)	19.5 (2.5–63.9)	0.0567	0.2390	0.4543
Viability	89.3 (63.9–95.8)	78.0 (50.4–97.8)	0.1059	–0.1891	0.5560
Macrophages	83.0 (67.9–88.4)	31.3 (12.3–60.3)	0.0032	0.1155	0.7205
Lymphocytes	8.5 (5.6–12.0)	6.6 (2.0–20.0)	0.7125	–0.0371	0.9088
Eosinophils	5.0 (2.6–7.5)	7.5 (4.3–18.3)	0.0089	0.5298	0.0764
Neutrophils	3.7 (2.0–15.6)	55.7 (29.0–64.9)	0.0032	0.5964	0.0406

Data are presented as medians and ranges in parentheses.

^a Significance of differences were assessed using Mann-Whitney non-parametric U-test.

^b Correlations between percentages of cells in BAL and IS were assigned by Spearman's rank correlation test.

^c p less than 0.05 were accepted as statistically significant.

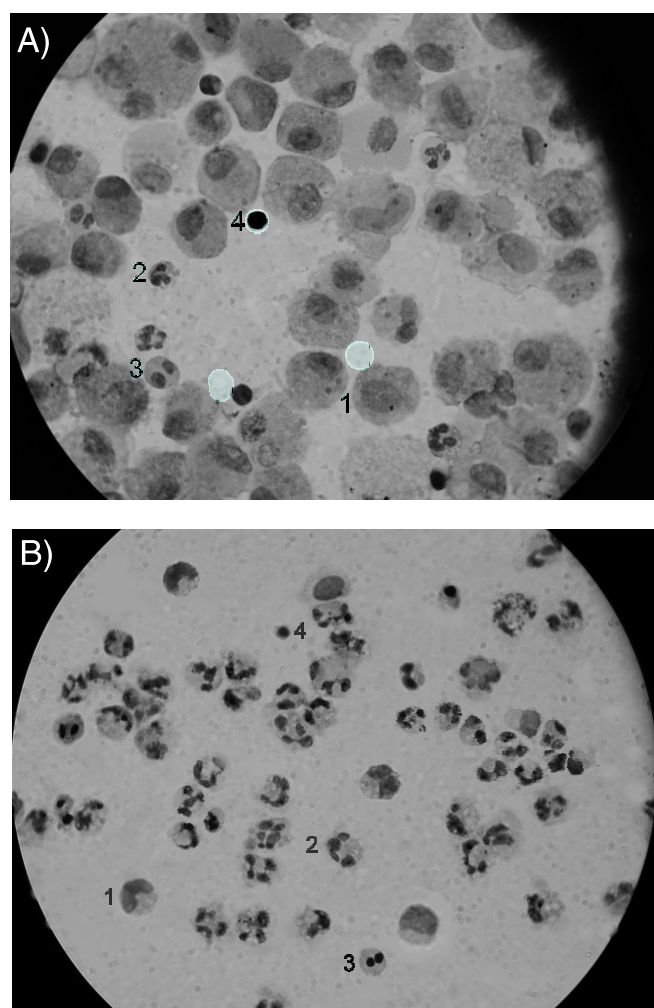


Fig. 1. Representative examples of leukocyte populations from BAL (A) and IS (B). 1 – macrophage, 2 – neutrophil, 3 – eosinophil, 4 – lymphocyte. May-Grünwald-Giemza stain. Magnification: ×500

in IS than in BAL (Me = 78.0% vs Me = 89.3%; $p = 0.1059$) was observed. The distributions of the cells in BAL and IS were significantly different. Macrophages were the predominant cells (Me = 83.0%) in bronchoalveolar lavages, whereas in sputa they were not (Me = 31.3%). IS cellular sediments were found to consist mainly of neutrophils (Me = 57.7%). Significant differences were noticed between BAL and IS in the percentages of macrophages ($p = 0.0032$), eosinophils ($p = 0.0089$) and neutrophils ($p = 0.0032$), but not in lymphocytes ($p = 0.7125$). Furthermore, there was a significant correlation in the percentage of neutrophils between BAL and IS ($R_s = 0.5964$, $p = 0.0406$) and a tendency to this in the case of eosinophils ($R_s = 0.5298$, $p = 0.0764$) (Table 2).

Spontaneous and LPS-induced NO release by BAL and IS leukocytes

Figure 2 shows spontaneous and LPS-induced NO release by the BAL and IS cell populations obtained from the patients studied. During the 48 h of incubation, non-stimulated and LPS-activated IS cells produced much higher ($p = 0.0022$) levels of NO than BAL cells. Despite quantitative differences, a similar pattern of NO response was observed, namely, stimulation of both BAL and IS cells with LPS did not change or even led to a reduction in the spontaneous release of NO (for BAL: Me = 13.5 μM , range 7.33–25.00 μM vs Me = 13.27 μM , range 8.44–20.75 μM ; $p = 0.9063$ and for IS: Me = 79.75 μM , range 17.7–99.5 μM vs Me = 66.5 μM , range 16.0–99.3 μM , $p = 0.4801$).

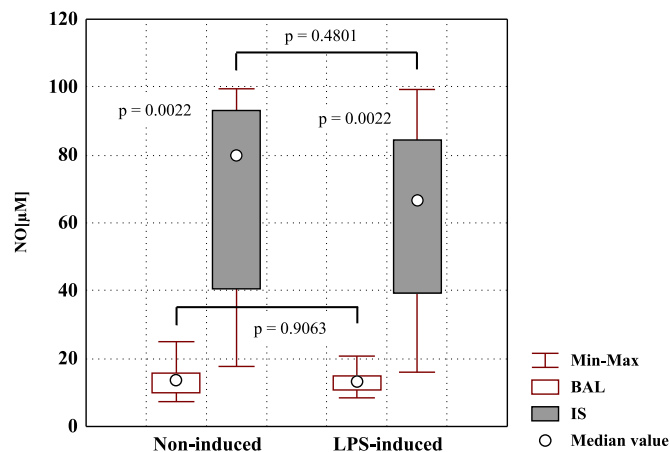


Fig. 2. Spontaneous and LPS-induced production of NO by bronchoalveolar leukocytes isolated from BAL and IS of patients with bronchial asthma. The BAL and IS cells at a concentration of 5×10^6 cells/ml were cultured for 48 h at 37°C in culture medium only, without exogenous inducers or with 5 μg/ml of LPS *E. coli*. The levels of NO were measured by determination of NO₂ (the end product of NO degradation) in both BAL and IS cell culture supernatants

Interrelationship between neutrophil and macrophage percentages and NO levels in BAL and IS

A trend towards a positive correlation was noted between nitric oxide levels and percentages of neutrophils in BAL ($R_s = 0.5578$, $p = 0.0594$) (Fig. 3), whereas in IS the correlation was statistically significant ($R_s = 0.6643$, $p = 0.0184$). In contrast, there was

no correlation between NO amounts and percentages of macrophages either in BAL ($R_s = 0.5017$, $p = 0.0964$) or in IS ($R_s = -0.4295$, $p = 0.1745$) (Fig. 4).

Discussion

Based on the assessment of the cellular composition of BAL and IS and the ability of the cells to NO release, the possible participation of neutrophils, apart from other cells, can be proposed in the production of NO in the airways of asthmatics. Despite significant differences in the differential cell counts of the BAL and IS specimens, a correlation between the percentages of neutrophils in BAL and IS and between NO levels and number of neutrophils in IS was noticed.

The different patterns of the cell populations from both of the specimens may be explained by the fact that the BAL and IS techniques collect cells from different locations within the airway tree. Accordingly, BAL recovers cells from peripheral airways and alveolar spaces²¹. In contrast to BAL, samples of IS may be taken only from a less extensive area, the central airways¹⁴. As asthma inflammation is considered to involve both the central as well as peripheral airway²¹, the cell population obtained from IS seems not to be fully representative of the entire ongoing local inflammatory process in bronchial asthma. Nevertheless, the predominance of neutrophils in IS and the noninvasiveness of the IS method may have a potential value, par-

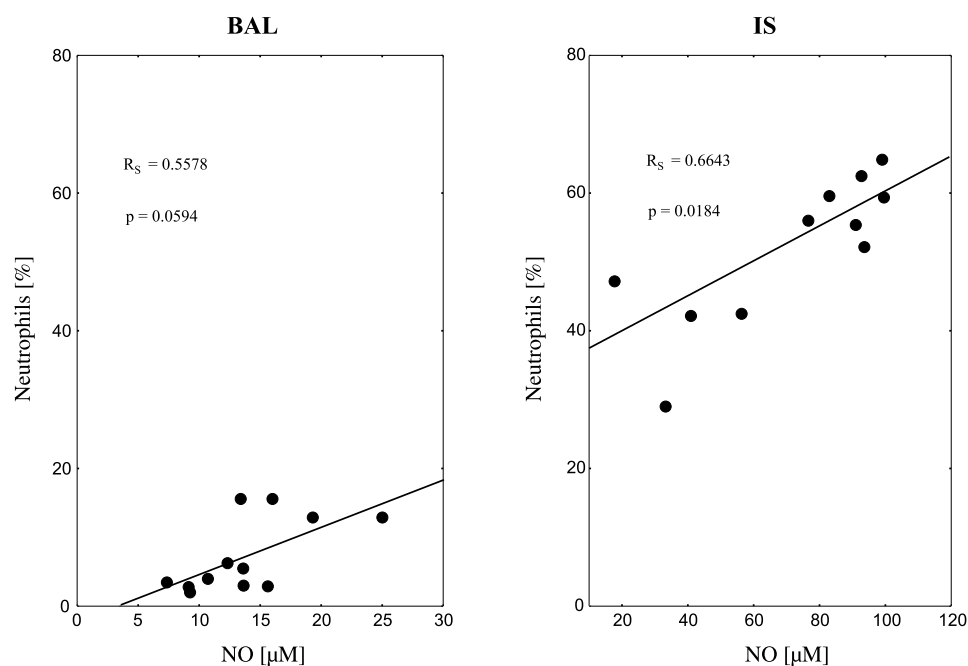


Fig. 3. Relationship between NO levels and neutrophil percentages in BAL and IS obtained from patients with bronchial asthma. The correlation was assigned by Spearman's rank (R_s) correlation test. Probability values less than 0.05 were accepted as statistically significant

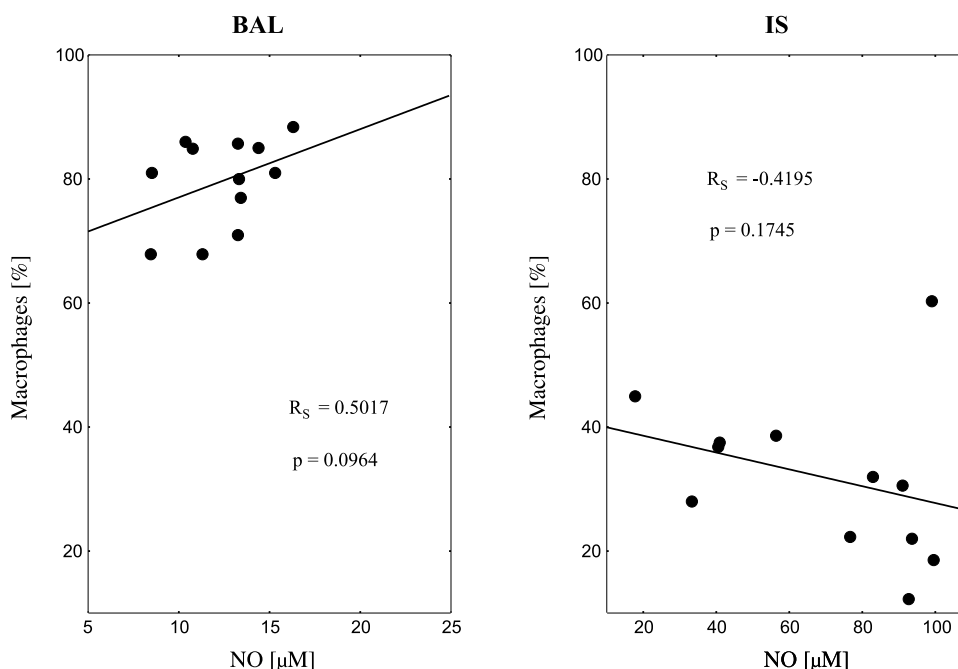


Fig. 4. Relationship between NO levels and macrophage percentages in BAL and IS obtained from patients with bronchial asthma. For explanation see Fig. 3

ticularly in the evaluation of local inflammation during exacerbation of asthma, in which neutrophils, rather than another cells, are supposed to play an important role^{10, 17}.

Our study was not designed to assess the clinical and inflammatory status of the patients. However, the group of asthmatics after corticosteroid and antibiotics therapy was the subject of our studies and the results need to be interpreted, even without comparison with a control group of patients. The high percentage of neutrophils and the elevated levels of NO in the IS of the patients in clinically stable condition at the time of the study seem to indicate that the presented parameters do not necessary relate to clinical indices. They may merely reflect the persistent lung inflammation in bronchial asthma, irrespective of disease severity. Furthermore, it cannot be excluded that the sputum neutrophilia and the high level of NO observed in our patients may reflect after-effects of past exacerbation of the disease and post-corticosteroid treatment. It seems to be in concordance with the fact that corticosteroids may prolong the survival of neutrophils in tissues and reduce their clearance by macrophages⁸. How and whether this refers to asthma severity remains to be elucidated by a study of well-defined subgroups of patients with varying disease severity and different treatment levels.

An explanation for the neutrophilic predominance and activity of the cells, expressed by higher NO secretion in IS than in BAL, is difficult. It is still not estab-

lished if the sputum induction procedure and processing may have an effect on the differential inflammatory cell percentage and high cell activity. It should be taken into consideration that the hypertonicity of the inhaled saline or reagents used in IS processing may have influence on the cells. However, some of the findings showed that, in comparison with isotonic saline inhalation, hypertonic saline inhalation did not significantly alter sputum cell percentages³. It was also shown that the mucolytic agent dithiothreitol (Sputolysin), which is usually used for IS processing, was not found to affect the release of eosinophil cationic protein by eosinophils or histamine by basophils and mast cells²³. However, it cannot be excluded that Sputolysin may have an effect on the other cells and the release of NO.

The findings of increased concentrations of NO in the exhaled air² and IS¹⁹ of asthmatic patients suggest its role, if any, in the pathogenesis of bronchial asthma. Up till now, the cellular source of NO in relation to the finding was not clearly defined. Generally, it has been known that NO is derived mainly by lung epithelial cells^{13, 28}. However, JANG and CHOI¹⁶ did not find a significant correlation between epithelial cell percentage and the level of NO in IS. On the other hand, LIM et al.²², in contrast to another investigators^{2, 9}, did not find a correlation between the amount of exhaled NO and eosinophilia in asthmatics. The interrelationship between cell composition and the ability of the cells to NO release in BAL and IS shown in our study may

suggest that NO may be produced rather by neutrophils but not by macrophages (Figs. 3 and 4). However, it should be taken into consideration that IS macrophages were found to be more functionally active than BAL cells¹. IS macrophages are believed to be persistently stimulated by inhaled environmental microorganisms and air pollutants which preferentially deposit in the bronchial airways than the alveolar spaces. Thus, even if there is a lower number of macrophages in IS than in BAL, they may be more active than alveolar macrophages and, subsequently, may be more extensively involved in the production of NO. On the other hand, in the present study we observed that macrophages obtained from IS were smaller in size than bronchoalveolar cells (Fig. 1A vs Fig. 1B). This may be in agreement with the findings that sputum leukocytes contain a distinct population of more active macrophages with shorter residence times in the lung and in less matured states than large alveolar macrophages with hypodense vacuolated cytoplasm¹.

In conclusion, the BAL and IS leukocyte populations are different in respect to cell composition, as well as cell size and activity. Depending on the asthma severity and the type of cells needed for study, BAL or IS specimens may be chosen as a source of pulmonary leukocytes. The use of the noninvasive technique of IS is supposed to have a potential value particularly in the study of airway inflammatory response mediated mainly by neutrophils, i.e. during and/or after exacerbation of the disease. Based on our results, a possible participation of neutrophils in the production of NO in the airways of asthmatic patients can be proposed apart from that of other cells, such as macrophages.

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