ORIGINAL ARTICLE

Arch. Immunol. Ther. Exp., 2006, **54**, 201–207
PL ISSN 0004-069X

DOI 10.1007/s00005-006-0025-z

Nitric oxide production by pulmonary leukocytes from induced sputum in patients with asthma and its effect on epithelial cell viability

Małgorzata Bieńkowska-Haba[1], Jerzy Liebhart[2] and Monika Cembrzyńska-Nowak[1]

[1] Laboratory of Virology, Institute of Immunology and Experimental Therapy, Polish Academy of Science, Wrocław, Poland
[2] Department of Internal Medicine and Allergology, Wroclaw Medical University, Wrocław, Poland

Received: 2005.06.01, **Accepted:** 2006.02.06, **Published online first:** 2006.05.31

Abstract

Introduction: Nitric oxide (NO) is one of many factors potentially involved in lung remodeling in asthma. The aim of the study was to assess the effect of pulmonary leukocytes from patients with bronchial asthma on alveolar epithelial cell damage in relation to NO production.
Materials and Methods: Induced sputum samples were obtained from 25 patients with bronchial asthma and 10 healthy volunteers. Twelve asthmatics were on inhaled corticosteroid treatment and 13 were corticosteroid free. Type II-like alveolar epithelial (A549) cells were cultured for 48 h in the presence of cell-free media from a 24-h culture of leukocytes obtained from the induced sputa (IS-Su). The level of NO was measured in supernatants from the cell cultures and the viability of the A549 cells was established.
Results: The levels of NO in IS-Su from corticosteroid-free asthmatics were significantly higher ($p=0.001$) than those in IS-Su from healthy controls. Furthermore, NO production by A549 cells exposed to IS-Su from steroid-free asthmatics (group A) was significantly higher than that from asthmatics on corticosteroid therapy (group cA) as well as from healthy controls ($p=0.01$ and $p=0.001$, respectively). Lower viability of the epithelial cells exposed to IS-Su was observed in group A compared with controls (median: 72% vs. 97.5%; $p<0.001$). In addition, a negative correlation ($R_S=–0.706$, $p<0.001$) was found between the levels of NO produced by pulmonary leukocytes and the viability of epithelial cells.
Conclusions: The results suggest that in the course of asthma, pulmonary leukocytes may interact with alveolar epithelial cells by inducing an excessive production of NO which, in turn, may contribute to epithelium impairment.

Key words: nitric oxide, asthma, lung remodeling, pulmonary leukocytes, alveolar epithelial cells.

Abbreviations: IS – induced sputum, IS-Su – cell-free media from 24-h culture of leukocytes obtained from induced sputa, NO – nitric oxide.

Corresponding author: Jerzy Liebhart, Ph.D., Department of Internal Medicine and Allergology, Wrocław Medical University, Traugutta 57/59, 50-454 Wrocław, Poland, tel.: +48 71 370-00-221, e-mail: Jerzy.Liebhart@dilnet.wroc.pl

INTRODUCTION

Asthma is a chronic inflammatory disease of the airways. Consecutive structural changes in the airway wall are referred to as airway remodeling. These changes may be the result of the interaction of inflammatory mediators with stromal cells or of tissue injury (as a result of altered repair). In the course of asthma in the airway lumen, the mucus is mixed with activated pulmonary leukocytes and shed epithelial cells. Structural cells of the airway respond to inflammation in a characteristic, coordinated fashion in an attempt to repair the damage. Recent studies have shown that remodeling in asthma not only encompasses the bronchial tree, but also alveolar tissue [10]. Not much is known about the processes of repair in this part of lung. The recognition of local interactions between pulmonary leukocytes and epithelial cells may be an important step towards improving our knowledge of lung impairment in asthma.

Nitric oxide (NO) is one of many factors potentially involved in lung remodeling in asthma. NO is a multifunctional particle and it may play diverse roles in physiological and pathological processes. Elevated concentrations of NO were observed in the exhaled air of asthmatic patients. Recently, the level of this mediator in exhaled air was considered a useful marker in monitor-

ing inflammation, disease severity, and exacerbations in asthmatics [16]. NO exerts its effects through interactions with numerous signal transduction pathways and transcription factors. Large amounts of NO may affect numerous proteins and enzymes critical for cell survival and signaling. These include signaling molecules involved in cytokine signaling such as the JAK or STAT proteins, the NFκB/IκB pathway as well as MAPK, and some G proteins and transcription factors. The nitration of cysteines in these proteins may lead to their activation or inactivation [1, 3, 18]. NO acts as a double-edged sword in allergic inflammation and asthma. On the one hand, it inhibits mast cell activation and vascular adhesion molecule expression, but on the other it may induce Th2-dependent IgE synthesis [4, 25]. Its role in asthma is still unclear. It may be produced by various types of cells and it exerts diverse effects on the airways. NO synthases have been localized in a variety of cells in the respiratory tract, including epithelial cells, smooth muscle cells, as well as pulmonary leukocytes such as macrophages, eosinophils, and neutrophils. Depending on concentration, NO can be involved in neurotransmission, regulation of the smooth muscle and vascular tone, vascular permeability, and in immune response. Studies with NO donors have proved that NO can have cytotoxic effects on epithelial cells. In general, NO at low concentrations exerts predominantly advantageous impacts on asthma (smooth muscle relaxation, reduction of bronchial hyperreactivity), while its main detrimental effect of cytotoxicity is supposedly observed at higher concentrations. However, threshold values are, in this case, difficult to establish in an *in vivo* experiment.

The aim of this study was to assess the effect of pulmonary leukocytes from patients with bronchial asthma on alveolar epithelial cell damage in relation to NO production by pulmonary leukocytes.

MATERIALS AND METHODS

Patients

Twenty-five patients (14 males) aged 35–76 years with bronchial asthma and 10 healthy volunteers (5 males) aged 22–77 years consented to induction and to providing induced sputa (IS). All the patients met the diagnostic criteria of allergic asthma established by GINA [8]. Asthma severity according to these criteria ranged from sporadic to persistently severe and the symptom intensity score ranged from 1–3. Twelve asthmatics were on systemic and/or inhaled corticosteroid therapy (group cA), whereas 13 were currently corticosteroid free – corticosteroid naive or at least 1 month after discontinuation of glucocorticoid treatment (group A). The control group consisted of persons without lung diseases and allergies. Detailed characteristics of the patients are summarized in Table 1. There were no statistically significant differences in the relevant parameters of age, disease duration, spirometric predicted values, and sex distribution between controls and asthmatics. The study was approved by the Bioethics Committee of the Wroclaw Medical University. Informed consent was obtained from all the subjects examined.

Table 1. Characteristics of patients and healthy controls

Parameters	C n=10	A n=13	cA n=12
Age (years)* $\bar{x}$ (±SD)	46.1 (±21.7)	54 (±11.7)	50.7 (±6.7)
Sex* F/M	5/5	7/6	5/7
Smoking* yes/no	3/7	4/9	4/8
Symptom intensity score median (min–max)	–	2 (1–3)	2 (1–3)
FVC (%)** $\bar{x}$ (±SD)	91.3 (±20.1)	72.4 (±28.9)	74.0 (±19.1)
FEV (%)** $\bar{x}$ (±SD)	92.5 (±22.7)	66.2 (±28.7)	67.1 (±24.3)
Disease duration (years)** $\bar{x}$ (±SD)	–	15.9 (±9.7)	16.2 (±12.1)
Dose of inhaled corticosteroids (μg/day) n=12 $\bar{x}$ (±SD)	–	–	1542 (±967.1)
Dose of systemic corticosteroids (mg of prednisone/day)** n=22 $\bar{x}$ (±SD)	–	39.7 (±25.6)	25.8 (±14.2)

A – asthmatics corticosteroid naive or 1 month after corticosteroid therapy, cA – asthmatics on systemic and/or inhaled corticosteroids, C– healthy controls, FEV – forced expiratory volume in one second, FVC – forced vital capacity.
* There were no statistically significant differences between controls and asthmatics.
** There were no statistically significant differences between the asthmatic groups (A vs. cA).

Symptom intensity score

The symptom intensity score (ranging from 1–4) was assessed according to GINA 2002 guidelines [8] using the asthma severity scheme without taking into account corrections related to treatment.

Sputum induction

The procedure of sputum induction was carried out according to Holz et al. [14]. 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 mouth. Every 5 min after the start of nebulization, the patients were asked to rinse their mouths and throats with water and to expectorate sputum into clean plastic containers by coughing.

IS leukocyte isolation and culture system

Sputum samples were processed according to the protocol recently validated by Fahy et al. [5], with the modification according to Pang et al. [21]. Briefly, sputum was treated by adding four volumes of 10% Sputolysin (Calbiochem Corp., San Diego, USA) followed by four 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 incubated with 100 U/ml of DNAse (Sigma Co., St. Louis, MO, USA) at 37°C for 30 min to further dispersion. The sputum samples were filtered through a 48-µm nylon gauze to remove debris and 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. The pellets of the cells from IS were resuspended at a cell concentration of 5×10^5 cells/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 mg/ml amphotericin B (Serva, Heidelberg, Germany). The cell suspensions were distributed and cultured in 24-well flat-bottom culture plates (Costar, Cambridge, USA) for 24 h at 37°C with a 5% CO_2 atmosphere. At the end of the incubation period the culture supernatants were collected, centrifuged, and filtered through 0.22-µm filters (Amicon) and frozen at –80°C for further analysis.

Scheme of experiments

Type II-like alveolar epithelial cells (A549) were cultured in tissue flasks incubated in a 5% CO_2 atmosphere at 37°C in Dulbecco's medium without phenol red containing 10% heat-inactivated fetal calf serum (FCS) supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin. Twenty-four-hour monolayers of A549 cells (10^5 cells/ml) were cultured in duplicate in 24-well flat-bottom culture plates (Costar, Cambridge, MA, USA) for 48 h at 37°C in a 5% CO_2 atmosphere in the presence of cell-free media from the 24-h culture of leukocytes obtained from the IS (IS-Su) of asthmatics and healthy controls (diluted 1:1 v/v in culture medium supplemented with FCS and antibiotics). At the end of the incubation period, cell-free culture supernatants were centrifuged and frozen at –20° C until further analysis and the viability of the A549 cells was determined by the MTT test.

Determination of viability of A549 cell

The viability of A549 cells was established after 48 h of culture by the MTT test. Following staining of the target cells with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5--diphenyltetrazolium bromide; Sigma Chemical Co. St. Louis, MO, USA) and lysis of the cells with SDS/DMF solution (13.5% (w/v) sodium dodecyl sulfate (SDS) in 45% (v/v) N,N-dimethylformamide (DMF; Sigma Chemical)), spectrophotometric measurement at 570 nm was done.

Measurement of NO

The level of NO was measured in the supernatants from the cell cultures. In aqueous solutions, NO is metabolized rapidly into nitrite. NO production was measured by the Griess reaction as nitrite (NO_2) accumulated in the culture medium [24]. The samples were incubated with 100 µl of Griess reagent (1% sulfanilamide and 0.1% N-1-naphtylenodiamine dihydrochloride (Sigma Corp., USA) in 2.5% H_3PO_4) at room temperature. Absorbance was measured at 540 nm using an ELISA plate reader (Stat Fax 2100 Awareness, Technology Inc.). Sodium nitrite was used as the standard. The detection limit for the assay was 1 µM. Production of NO in A549 cells exposed to supernatants of IS leukocyte (IS-Su) cultures was estimated according to the formula:

$$NO_{A549+IS\text{-}Su} - NO_{IS\text{-}Su}$$

were $NO_{IS\text{-}Su}$ is the level of NO in the supernatants of cultures of leukocytes from IS (added on A549 cultures) and $NO_{A549+IS\text{-}Su}$ the level of NO in the supernatants of cultures of A549 cells exposed to IS-Su.

Statistical analysis

Statistical analysis of the data was performed using Statistica 6.0 (StatSoft, BXXP 308C083428FA) software. Comparison of nitric oxide levels and A549 cell viability between all groups examined was done using the Kruskal-Wallis test. Significance of differences between groups was assessed by the Mann-Whitney U test and interrelationships between the parameters were

examined by the Spearman's rank (R_S) correlation test. A value of $p<0.05$ was accepted as the level of significance.

RESULTS

Differential cell count

The analysis of the differential cell counts in the induced sputum showed a significant increase (at the expense of macrophages) in the percentage of neutrophils and eosinophils in asthmatics compared with controls (Table 2), while there were no significant differences between subgroups A and cA.

Viability of A549 cells treated with supernatants from IS leukocytes (IS-Su)

Figure 1 (A–F) presents examples of slides with A549 cell cultures after 48-h incubation with and without cell-free media from 24-h cultures of IS leukocytes (IS-Su) from healthy volunteers and asthmatic patients. A cytotoxic effect on epithelial cells was observed only in the case of cell cultures exposed to IS-Su from asthmatics.

Figure 2 presents the viability of A549 cells exposed to IS-Su. The Kruskal-Wallis test showed statistically significant differences ($p=0.0001$) between the viability of epithelial cells treated with the supernatants from the cultures of pulmonary leukocytes from the studied

Table 2. Differential inflammatory cell counts in induced sputa obtained from patients with bronchial asthma and from healthy subjects

Parameters	C n=10	A n=13	cA n=12	p^1	p^2
Total cell ($\times 10^5$)	8.6 (2.0–50.9)	11.45 (3.3–83.9)	8.3 (2.3–40.7)	0.576	0.373
Viability (%)	81.9 (50–91)	88.5 (50–97)	85.8 (52–99)	0.576	0.468
Macrophages (%)*	64.2 (54.0–69.0)	40.0 (16.0–60.3)	55.0 (29.0–58.0)	0.002	0.171
Lymphocytes (%)*	4.6 (1.1–7.7)	3.0 (0.0–9.0)	3.0 (1.0–4.0)	0.059	0.939
Neutrophils (%)*	28.9 (23.1–39.0)	51.0 (29.0–70.5)	39.0 (30.0–65.0)	0.008	0.323
Eosinophils (%)*	0.6 (0.0–5.9)	7.1 (0.0–15.0)	5.0 (1.0–10.0)	0.009	0.196

Data are presented as medians; ranges (min–max) are given in parentheses.
*Percent of total nonsquamous cells.
A – corticosteroid-free asthmatics, cA – asthmatics on systemic and/or inhaled corticosteroids, C– controls.
p^1 – Mann-Whitney test for healthy controls vs. total asthmatic group (A+cA).
p^2 – Mann-Whitney test for A vs. cA.

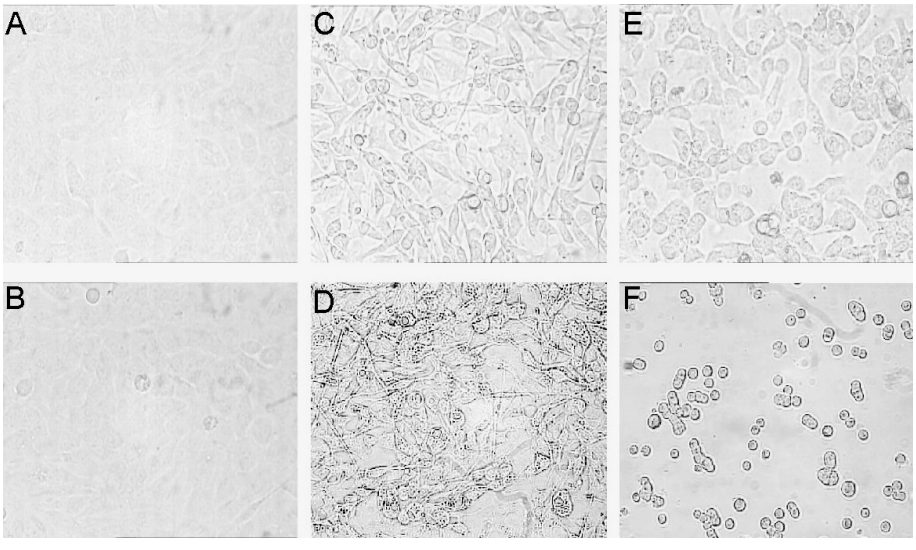

Fig. 1. Representative examples of A549 cultures treated with: culture medium only (**A**); cell-free media from 24-h cultures of pulmonary leukocytes obtained from the induced sputa (IS-Su) of healthy controls (**B**) and asthmatic patients (**C**, **E** – from asthmatics on corticosteroid therapy, **D**, **F** – from asthmatics who were corticosteroid free).

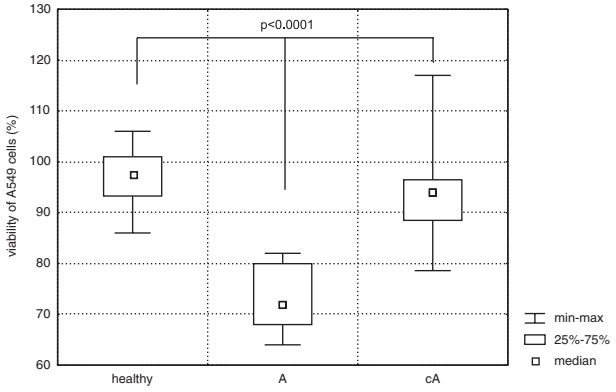

Fig. 2. Viability of A549 cells exposed to cell-free media from 24-h cultures of leukocytes isolated from induced sputa (IS-Su) obtained from healthy controls and asthmatic patients. Results are presented as median values and quartiles (25–75%). Ranges are shown as whisker plots.

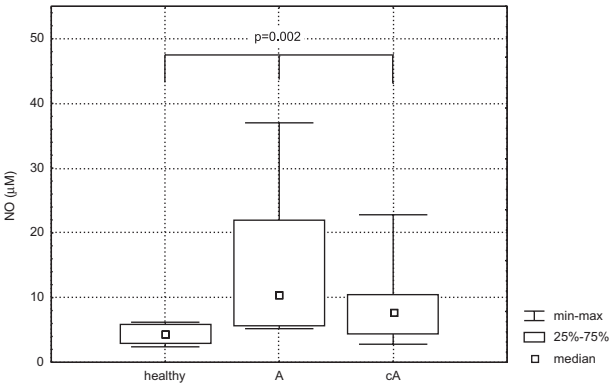

Fig. 3. Production of NO by pulmonary leukocytes isolated from IS obtained from asthmatics and healthy controls. Results are presented as median values and quartiles (25–75%). Ranges are shown as whisker plots.

groups. The viability of A549 cells treated with IS-Su from corticosteroid-free asthmatics (group A) was significantly lower than that of cells treated with IS-Su from healthy controls and from group cA, i.e. asthmatics on corticosteroids (median: 72% vs. 98% and 94%; p=0.00006 and p=0.0001, respectively). The values for group cA were insignificantly lower than those of controls (median: 94% vs. 98%; p=0.12).

Production of NO by pulmonary leukocytes

Leukocytes from asthmatics (Fig. 3) produced significantly different (p=0.002) amounts of NO from those from control subjects. The production of NO by pulmonary leukocytes from corticosteroid-free asthmatics (group A) was significantly higher than that from asthmatics on corticosteroid treatment (group cA) as well as from healthy controls (median: 10.4 μM vs. 7.8 μM and 4.4 μM; p=0.001 and p=0.021, respectively). There was no statistically significant difference in NO production by leukocytes isolated from the two groups of asthmatic patients (median: 10.4 μM vs. 7.8 μM; p=0.1).

Production of NO by A549 cells treated with cell-free media from pulmonary leukocyte cultures (IS-Su)

There were statistically significant differences (p=0.03) in NO production by A540 cells treated with supernatants from cultures of pulmonary leukocytes (IS-Su) from all the studied groups (see Fig. 4). Nitric oxide production by epithelial cells exposed to IS-Su from steroid-free asthmatics (group A) was significantly higher than that from asthmatics on corticosteroid therapy (group cA) as well as from healthy controls (median: 3.2 μM vs. 1.05 μM and 0.95 μM; p=0.01 and p=0.001, respectively). The levels of NO produced by A549 cells exposed to IS-Su from healthy controls and

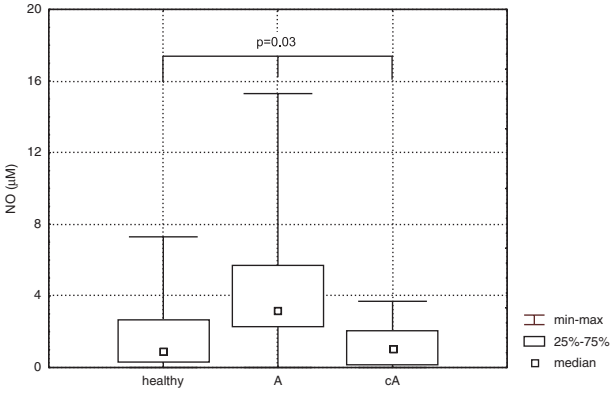

Fig. 4. Production of NO by A549 cells treated with cell-free media from 24-h cultures of pulmonary leukocytes (IS-Su) obtained from the induced sputa of asthmatics and healthy controls. Results are presented as median values and quartiles (25–75%). Ranges are shown as whisker plots.

asthmatics on corticosteroids did not differ significantly (median: 0.95 μM vs. 1.05 μM; p=0.56).

Relationship between NO levels and the viability of epithelial cells treated with cell-free media from 24-h cultures of IS leukocytes

Negative correlation was noted between the levels of NO produced by pulmonary leukocytes from IS and the viability of A549 cells treated with cell-free media from 24-h cultures of IS leukocytes (IS-Su) (R_S=–0.731; p<0.001). Furthermore, negative correlation was also observed between the production of NO by A549 cells treated with IS-Su and the viability of these epithelial cells (R_S=–0.342; p=0.044).

Relationship between patient symptom intensity score and the production of NO and the viability of epithelial cells exposed to cell-free media from 24-h cultures of IS leukocytes

We analyzed the relationship between the obtained results and patient symptom intensity scores. Statistical analysis showed positive correlation between the symptom intensity scores of the studied patients and the levels of NO produced by pulmonary leukocytes obtained from IS (R_S=0.336; p=0.048). There was also negative correlation between the viability of epithelial cells treated with cell-free media from cultures of leukocytes from IS and patient symptom score (R_S=–0.428; p=0.01).

DISCUSSION

Chronic airway inflammation with damage to the epithelium in asthma patients prompts repair mechanisms, which lead to structural and functional changes in the respiratory tract. Airway remodeling is currently considered as characteristic of this disease as allergic inflammation. Recently, Holgate et al. [12, 13] proposed a new paradigm for asthma pathogenesis termed the "epithelial-mesenchymal trophic unit". According to this concept, signaling between injured epithelium and fibroblasts activates growth factors that stimulate the growth and survival of mesenchymal cells. Fundamental features of asthma are increased bronchial epithelium susceptibility to injury through the activation of caspase-3/apoptosis as well as impaired epithelial repair. However, in this theory the mechanisms of lung tissue impairment are not a matter of consideration.

There are some reports which suggest that NO may contribute to airway remodeling in asthma. Gabazza et al. [6] showed that the ratio of airway wall thickness to lumen diameter in asthmatics significantly correlated with sputum nitrite/nitrate levels. Other authors found a correlation in patients with asthma between NO output and reticular basement membrane thickness or concentrations of bronchoalveoalar lavage cytokines associated with remodeling, such as TGF-β_1 and TIMP-1/MMP-9 [16, 22]. However, it is not clear whether NO is a cause or a bystander of these processes. The mechanisms of lung tissue impairment in asthma are definitely less recognized. A predominant role is ascribed to IL-1β as well as to neutrophil and macrophage infiltrates [15].

In this study we evaluated the relationship between NO production by leukocytes obtained from IS of asthmatic patients and the viability of lung epithelial-like cells. NO was produced in significantly higher amounts by the leukocytes of asthma patients than of controls. The analysis of the differential cell counts of IS suggested that this increase in NO production was probably generated mainly by neutrophils. NO production significantly inversely correlated with the viability of lung epithelial-like cells and positively correlated with symptom intensity score. Cytotoxic NO effects on A549 cells were observed only in cultures treated with cell-free media from cultures of leukocytes obtained from asthmatic patients. These harmful effects were attenuated in samples obtained from patients on chronic corticosteroid therapy. In our experiment we did not determine the type of alveolar cell death. Data from the literature are conflicting. Gavino et al. [7] reported that apoptosis is involved, while Narula et al. [19] suggested non-apopototic mechanisms. Though our results provided only indirect evidence, we can speculate that the NO produced by pulmonary leukocytes not only reflects the intensity of inflammation, but may also contribute to alveolar epithelium damage. This thesis is in line with the results of Gavino et al. [7], who found increased apoptosis in fetal rat type II pneumocytes (the stem cells of the alveolar epithelium) under the influence of exposure to hyperoxia and NO. A cytotoxic effect of NO on airway epithelial cells has also been described on the basis of an *in vitro* study with NO-donors and in an animal model where NO was given by inhalation [11]. However, in this model the epithelial cells did not have direct contact with either pulmonary leukocytes or the reactive forms of NO produced by these leukocytes.

We also observed negative correlation between the levels of NO produced by epithelial cells treated with supernatants from pulmonary leukocyte cultures and the viability of these epithelial cells. This finding suggests that pulmonary leukocytes may also activate the production of NO by epithelium in asthma, which is another self-perpetuating process.

We consider this study as a first step in our attempt to better elucidate the role of NO in lung remodeling in asthma. In order to demonstrate clearly the causation of NO products on cellular viability, further experiments with inhibitors of NO synthesis, potential inducible NO synthase (iNOS) upregulation, as well as epithelial cell apoptosis assays have been planned.

At present there is no satisfactorily effective therapy available to prevent lung remodeling in asthma. Corticosteroids have shown limited effects on structural changes in the lung tissue [2, 20, 23]. Further elucidation of the role of NO in this process may potentially offer a novel therapeutic target. There are some expectations from the introduction of selective inhibitors of iNOS that may allow limiting the production of NO to close to constitutive levels [9]. Such compounds might be considered as complementary to corticosteroids in the treatment of asthmatic patients.

We are aware of all the difficulties and potential pitfalls in transposing the results of *ex vivo* experiments to *in vivo*, real situations. Among other things, one must remember that NO is produced not only by neutrophils, macrophages, eosinophils, and epithelial cells, but also by various other cells. It also combines with hemoglobin to form small quantities of methemoglobin, preventing spread of its effect [17]. Despite all these objections, we believe on the basis of our results that in the course of asthma, pulmonary leukocytes may interact with alveolar epithelial cells by inducing excessive NO production,

which in turn may contribute to epithelium impairment. This effect is attenuated in patients on chronic corticosteroid therapy.

Acknowledgment: The study was supported by a grant awarded by the State Committee for Scientific Research (grant KBN 6 PO5B 102 21). The authors wish to thank Ewa Liebhart, Rafał Dobek, Anna Dor, Wojciech Barg, and Aleksandra Kulczak for their help in collecting the clinical material.

REFERENCES

1. Bogdan C. (2001): Nitric oxide and the immune response. Nat. Immunol., **2,** 907–916.
2. Chakir J., Shannon J., Molet S., Fukakusa M., Elias J., Laviolette M., Boulet L. P. and Hamid Q. (2003): Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF-β1, IL-11, IL-17, and type I and type III collagen expression. J. Allergy Clin. Immunol., **111**, 1293–1298.
3. Coleman J. W. (2001): Nitric oxide in immunity and inflammation. Int. Immunopharmacol., **1**, 1397–1406.
4. Eastmond N. C., Banks E. M. and Coleman J. W. (1997): Nitric oxide inhibits IgE-mediated degranulation of mast cells and is the principal intermediate in IFN-gamma--induced suppression of exocytosis. J. Immunol., **159**, 1444–1450.
5. Fahy J. V., Kim K. W., Liu J. and Boushey H. A. (1995): Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. J. Allergy Clin. Immunol., **95**, 843–852.
6. Gabazza E. C., Taguchi O., Tamaki S., Murashima S., Kobayashi H., Yasui H., Kobayashi T., Hataji O. and Adachi Y. (2000): Role of nitric oxide in airway remodelling. Clin. Sci., **98**, 291–294.
7. Gavino R., Johnson L. and Bhandari V. (2002): Release of cytokines and apoptosis in fetal rat type II pneumocytes exposed to hyperoxia and nitric oxide: modulatory effects of dexamethasone and pentoxifylline. Cytokine, **20**, 247–255.
8. Global Strategy for Asthma Management and Prevention NIH Pub. No 02-3659, January 1995 (updated 2002) www.ginasthma.com.
9. Hansel T. T., Kharitonov S. A., Donnelly L. E., Erin E. M., Currie M. G., Moore W. M., Manning P. T., Recker D. P. and Barnes P. J. (2003): A selective inhibitor of inducible nitric oxide synthase inhibits exhaled breath nitric oxide in healthy volunteers and asthmatics. FASEB J., **17**, 1290–1300.
10. Harmanci E., Kebapci M., Metintas M. and Ozkan R. (2000): High-resolution computed tomography findings are correlated with disease severity in asthma. Respiration, **69,** 420–426.
11. Ho Y. S., Liou H. B., Lin J. K., Jeng J. H., Pan M. H., Lin Y. P., Guo H. R., Ho S. Y., Lee C. C. and Wang Y. J. (2002): Lipid peroxidation and cell death mechanisms in pulmonary epithelial cells induced by peroxynitrite and nitric oxide. Arch. Toxicol., **76**, 484–493.
12. Holgate S. T., Davies D. E., Puddicombe S., Richter A., Lackie P., Lordan J. and Howarth P. (2003): Mechanisms of airway epithelial damage: epithelial-mesenchymal interactions in the pathogenesis of asthma. Eur. Respir. J. Suppl., **44**, S24–29.
13. Holgate S. T., Peters-Golden M., Panettieri R. A. and Henderson W. R. Jr. (2003): Roles of cysteinyl leukotrienes in airway inflammation, smooth muscle function, and remodeling. J. Allergy Clin. Immunol., **111** (suppl. 1), S18–34.
14. Holz O., Kips J. and Magnussen H. (2000): Update on sputum methodology. Eur. Respir. J., **16**, 355–359.
15. Lappalainen U., Whitsett J. A., Wert S. E., Tichelaar J. W. and Bry K. (2005): Interleukin-1beta causes pulmonary inflammation, emphysema, and airway remodeling in the adult murine lung. Am. J. Respir. Cell Mol. Biol., **32**, 311–318.
16. Mahut B., Delclaux C., Tillie-Leblond I., Gosset P., Delacourt C., Zerah-Lancner F., Harf A. and de Blic J. (2004): Both inflammation and remodeling influence nitric oxide output in children with refractory asthma. J. Allergy Clin. Immunol., **113,** 252–256.
17. Milner A. D. (1994): Nitric oxide. Eur. J. Pediatr., **153** (suppl. 2), s7–s11.
18. Moilanen E. and Vapaatalo H. (1995): Nitric oxide in inflammation and immune response. Ann. Med., **27**, 359–367.
19. Narula P., Xu J., Kazzaz J. A., Robbins C. G., Davis J. M. and Horowitz S. (1998): Synergistic cytotoxicity from nitric oxide and hyperoxia in cultured lung cells. Am. J. Physiol., **274** (3 Pt 1), L411–416.
20. Olivieri D., Chetta A., del Donno M., Bertorelli G., Casalini A., Pesci A., Testi R. and Foresi A. (1997): Effect of short-term treatment with low-dose inhaled fluticasone propionate on airway inflammation and remodeling in mild asthma: a placebo-controlled study. Am. J. Respir. Crit. Care Med., **155**, 1864–1871.
21. Pang G. T., Clancy R. L. and Reeves G. E. (1995): Isolation and functional characterization of T cells from human sputum. Clin. Exp. Immunol., **102**, 642–648.
22. Payne D. N., Rogers A. V., Adelroth E., Bandi V., Guntupalli K. K., Bush A. and Jeffery P. K. (2003): Early thickening of the reticular basement membrane in children with difficult asthma. Am. J. Respir. Crit. Care Med., **167**, 78–82.
23. Sont J. K., Willems L. N., Bel E. H., van Krieken J. H., Vandenbroucke J. P. and Sterk P. J. (1999): Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long--term treatment. The AMPUL Study Group. Am. J. Respir. Crit. Care Med., **159** (Pt 1), 1043–1051.
24. Stuehr D. J. and Marletta M. A. (1985): Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. Proc. Natl. Acad. Sci. USA, **82**, 7738–7742.
25. Xiong Y., Karupiah G., Hogan S. P., Foster P. S. and Ramsay A. J. (1999): Inhibition of allergic airway inflammation in mice lacking nitric oxide synthase 2. J. Immunol., **162**, 445–452.