Effects of oral N-acetylcysteine on cell content and macrophage function in bronchoalveolar lavage from healthy smokers

M. Linden*, E. Wieslander*, A. Eklund**, K. Larsson***, R. Brattsand*

ABSTRACT: Bronchoalveolar lavage (BAL) was performed in fourteen healthy non-smokers and eleven healthy smokers. In smokers BAL was performed before and after eight weeks' treatment with N-acetylcysteine (NAC; 200 mg t.i.d.). Cell number, composition and viability were determined in the BAL fluid. Alveolar macrophages (AMs) were cultured before examination of their phagocytic capacity and their ability to produce leukotriene B\(_4\) (LTB\(_4\)). BAL fluid from smokers contained more cells than that from non-smokers (p < 0.001). This was mainly attributable to increases in both proportion and absolute number of AMs (p < 0.001) and to an increase in absolute number of neutrophils (p < 0.05). However, there was a decrease in proportion of lymphocytes in BAL fluid from smokers (p < 0.001). Phagocytic capacity of adherent cells and capacity of AMs to generate LTB\(_4\) after stimulation with opsonized zymosan (OZy) were decreased in smokers (p < 0.05 and p < 0.01 respectively). NAC treatment of smokers did not affect cell number but resulted in an increased proportion of lymphocytes in BAL fluid (p < 0.05). The phagocytic capacity of AMs was not significantly altered but was improved in five of eleven smokers after NAC treatment. NAC also enhanced the decreased LTB\(_4\) secretion by smokers' AMs (p < 0.05). We conclude that smoking leads to reduced phagocytic capacity and LTB\(_4\) secretion of AMs and that oral NAC treatment may improve the function of AMs.

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Many forms of chronic lung disease are associated with smoking [1]. The reasons for this have not been fully established, but much attention has been focused on smoke-induced changes in the character and content of bronchoalveolar lavage (BAL) cells such as the alveolar macrophage (AM). The AM plays a major role as a scavenger of particles and microbes in the lung. The antimicrobial function of AMs requires a combination of phagocytosis and generation of reactive oxygen species. Furthermore, AMs recruit other phagocytes, such as neutrophils, to the lung by secretion of chemotactic factors, e.g. LTB\(_4\). Studies of AMs from smokers have demonstrated a decreased [2] or normal [3, 4] phagocytic capacity. Moreover, decreased metabolism of arachidonic acid, by means of lowered capacity of prostaglandin E\(_2\) (PGE\(_2\)) and LTB\(_4\) secretion by AMs from smokers, has been recently reported by LAVIOLETTE and co-workers [5, 6] and by WIESLANDER and co-workers [7, 8]. Considering these effects of cigarette smoking and its association with an increased incidence of infectious diseases in the lung [9, 10], we compared the LTB\(_4\) secretion and the phagocytic activity of AMs from smokers and non-smokers in this study.

N-acetylcysteine (NAC) has previously been reported to reduce the number of exacerbations in patients with chronic bronchitis [11-13], a condition associated with cigarette smoking. Although NAC is often referred to as a mucolytic agent [11, 12] or a radical scavenger, the reason for the beneficial effect of NAC is not clear. We therefore examined whether oral NAC treatment of smokers influences the content and the composition of BAL cells and the function of AMs by means of their phagocytic capacity and LTB\(_4\) secretion capacity.

Patients, materials and methods

Subjects

Fourteen healthy non-smoking volunteers (three females) with a mean age of 27 ± 1 yrs and eleven healthy smokers (seven females) with a mean age of 29 ± 2 yrs participated in the study. The mean cigarette consumption was 14.5 ± 2.8 pack-years. All subjects had a normal ECG and chest X-rays. Five of the smokers had productive cough in the morning. All subjects gave their informed consent and the study had the approval of the local Ethical Committee.
Lung function

Lung volumes were measured in a body plethysmograph and dynamic spirometry was performed with a rolling seal spirometer (Ohio Model 840) as previously described [14]. The lung function was within normal limits in all participating subjects.

NAC treatment

After BAL the smokers received oral medication with NAC (Draco, Sweden) 200 mg t.i.d. for eight weeks. One and a half hours after the final dose, a second BAL was performed.

Bronchoalveolar lavage

Bronchoscopy was performed through the mouth with a flexible fibreoptic bronchoscope (Olympus Type 4B2) under local anaesthesia with 2% lignocaine (Xylocain®, Astra, Sweden) after premedication with morpine-scopolamine and phenemal [15]. The bronchoscope was wedged in a middle lobe bronchus and 250 ml of sterile saline solution (pH 7.4) at 37°C was instilled in aliquots of 50 ml. After each instillation the fluid was gently aspirated and collected in a siliconized plastic bottle kept on ice. Total and differential cell counts and cell viability were also determined directly after the transport. There was no viability caused by the transport.

Cytocentrifuge preparations were made using the Shandon (GB). Approximately 5 x 10^4 cells suspended in 100 μl of RPMI 1640 were centrifuged at 500 rpm for 3 min. Cell differentials were performed on cytocentrifuge preparations stained with May-Grünewald Giemsa reagents. Eight hundred cells per preparation were counted. The remaining BAL cells were repelleted by centrifugation for 5 min at 4°C and then resuspended in 9 ml of saline. One hundred μl of this cell suspension was used for a total cell count in a Bürker chamber. The viability of the cells was determined by means of Trypan blue exclusion.

Cell collection and characterization

The BAL fluid was filtered through a double layer of Dacron nets and the volume was measured. Cells were pelleted at 400 g for 5 min at +4°C and then resuspended in 9 ml of saline. One hundred μl of this cell suspension was used for a total cell count in a Bürker chamber. The viability of the cells was determined by means of Trypan blue exclusion. Cytocentrifuge preparations were made using the Cytopsin 2 (Shandon, GB). Approximately 5 x 10^4 cells suspended in 100 μl of RPMI 1640 were centrifuged at 500 rpm for 3 min. Cell differentials were performed on cytocentrifuge preparations stained with May-Grünewald Giemsa reagents. Eight hundred cells per preparation were counted. The remaining BAL cells were repelleted by centrifugation for 5 min at 400 g. This cell pellet was resuspended in 4 ml of RPMI 1640 (Flow Lab, Sweden) supplemented with gentamycin (50 μg·ml^-1) and 5% foetal calf serum. The cell suspension was then kept on ice during transportation (5-7 h). Total and differential cell counts and cell viability were also determined directly after the transport. There was no change in cell number, cell composition or cell viability caused by the transport.

Phagocytosis assay

The phagocytic activity of adherent BAL cells was determined according to the method described by Hed [16]. Thus, Saccharomyces cervisiae (baker’s yeast) were killed by boiling in a water bath for 30 min. The heat-killed yeast particles were labelled with fluorescein isothiocyanate (FITC, Sigma Chemical, St Louis, USA) in a 0.5 M carbonate buffer at pH 9.5 containing 10^8 yeast particles and 0.1 mg FITC per ml. The suspension was incubated for 60 min at 37°C and then washed four times with Hank’s buffer. FITC-labelled yeast particles were resuspended in Hank’s buffer (10^8 cells·ml^-1) and stored at -20°C. These particles were opsonized with human serum (1:1, 30 min at 37°C) just before use. The BAL cells were seeded on multispot microscope slides (Novakemi, Enskede, Sweden), 10^5 cells per slide, and incubated for 1 h at 37°C. Non-adherent cells were removed by washing three times with prewarmed Hank’s buffer. After the washing, the adherent cells were fed with FITC-labelled yeast particles (2.5 x 10^6 of opsonized FITC-labelled yeast particles per slide) and incubated at 37°C for 5, 15, 30 or 60 min. At the end of incubation the supernatant was decanted and phagocytosis was stopped by placing the slides in ice-cold Hank’s buffer. Immediately before microscopic examination the excess of Hank’s buffer was removed from the slides and a drop of Trypan blue (5 mg·ml^-1 in saline) was added. This dye quenched the fluorescence of the extracellularly located FITC-labelled yeast particles, whereas the ingested ones remained fluorescent [17]. Two hundred cells were examined in duplicate slides using fluorescence and light microscopy, and the percentage of ingesting cells was calculated.

Alveolar macrophage culture

The BAL cells were seeded in fresh growth medium (1 x 10^6 cells in 2 ml per well) in tissue culture 24-well multidishes (Nunc, Denmark). They were incubated at 37°C in an atmosphere of 5% CO₂ and 95% air for 1 h. The non-adherent cells were then removed by washing three times with Ca²⁺-, and Mg²⁺-free phosphate buffered saline. The adherent cells were further incubated in fresh medium (2 ml per well) overnight.

LTB₄ analysis

After overnight culture, AMs were incubated with human serum opsonized zymosan (OZY) suspended in Hank’s buffer (1 mg·ml⁻¹ and well), or in Hank’s buffer only, for 90 min at 37°C. The multidish was then transferred to an ice bath and the supernatants were collected, centrifuged at 200 g for 10 min at 4°C, decanted and stored at -70°C. A 100 μl aliquot of the supernatant was analysed for LTB₄ using a radioimmunoassay kit (Amersham Int, UK) [18].

DNA analysis

The number of AMs in cultures was determined by measurement of DNA content. Cells were disrupted by sonication in ice-cold phosphate buffered saline (PBS) (1 ml per well) and 200 μl aliquots of the homogenates were used for determination of DNA by the bisbenzimidazole (Hoechst 33258, Sigma Chemicals, St Louis, USA) method as described by Labarca and Paigen [19].
Results are presented as mean ± SEM and compared by Student's t-test for paired and unpaired observations. P values < 0.05 are considered significant.

Results

BAL fluid recovery, cell viability and cell counts

The recovered volume of BAL fluid was similar in non-smokers and smokers, before and after NAC treatment (68 ± 2, 64 ± 3, and 67 ± 2% respectively). There was a significant increase in total cell number, both before and after NAC treatment, in BAL from smokers, compared with non-smokers (p < 0.001; table 1). This was due to an increase in the proportion and absolute number of macrophages recovered from the lungs of smokers (table 1). There was no difference in proportion of neutrophils or eosinophils between smokers and non-smokers. However, the absolute number of neutrophils was significantly increased in smokers (p < 0.05). The proportion of lymphocytes in BAL was significantly lower in smokers than in non-smokers (p < 0.001).

NAC treatment resulted in a significant increase in

![Graph](https://via.placeholder.com/150)

Fig. 1 Phagocytic activity of adherent AMs from non-smokers (n = 7; ○—○) and from smokers before (n = 11; △——△) and after NAC treatment (n = 11; △——△). AMs (10⁵ cells per slide) were incubated with FITC-Labelled yeast particles (2.5 × 10⁶ particles per slide) for 5, 15, 30, and 60 min at 37°C. *: p < 0.05 compared with non-smokers (Student's t-test).
the proportion of lymphocytes (p<0.05). However, the lymphocyte proportion was still significantly lower than in non-smokers (p<0.05). When the absolute number of lymphocytes was calculated from the total cell yield, smokers had as many lymphocytes as non-smokers (table 1). In smokers the proportions and absolute numbers of AMs and neutrophils remained unchanged after NAC treatment.

The viability of the BAL cells was similar in all three groups; 74±3% for cells from non-smokers, 80±2% for cells from smokers before as well as after NAC treatment. There was no relationship noted between cell viability and the functional parameters that will be described.

Phagocytic activity of alveolar macrophages

After 30 and 60 min of incubation, AMs from smokers had a significantly decreased phagocytic activity, i.e. lower percentage of ingesting cells, compared with AMs from non-smokers (fig. 1). The mean of phagocytic activity of smokers’ AMs was not significantly influenced by the NAC treatment (fig. 1). However, the phagocytic activity of AMs was enhanced in five of eleven treated smokers (fig. 2).

$LTB_4$ secretion by alveolar macrophages

$LTB_4$ secretion in unstimulated AM cultures from smokers was significantly lower than in unstimulated AM cultures from non-smokers. AMs from both smokers and non-smokers significantly increased the secretion of $LTB_4$ when they were stimulated with OZy (results not shown). However, the increase of $LTB_4$ secretion in AM cultures from smokers was significantly lower than the increase in AM cultures from non-smokers (p<0.01; fig.3). NAC treatment of smokers improved the secretion of $LTB_4$ by AMs (p<0.05). However, this improvement did not return secretion of $LTB_4$ to the value found in OZy stimulated cultures of AMs from non-smokers (p<0.05; fig. 3).

Discussion

The present report provides data on effects of oral NAC treatment on cell composition and AM function in BAL from healthy smokers. It confirms previous reports concerning cell composition and numbers [20], however, the absolute number of lymphocytes was similar in smokers and non-smokers. In the
present study oral NAC treatment resulted in a significant increase in lymphocyte proportion in BAL from smokers. The basis of this effect is not known.

Phagocytic ability of AMs is an important component of pulmonary clearance of inhaled particles. A possible alteration of phagocytic capacity of AMs caused by cigarette smoke has been investigated by many researchers [21] and contradictory results have been reported. Although most investigators did not find an altered phagocytic activity of AMs from smokers, MARTIN and WARR [2] reported that the activity of smokers' AMs was less than that of non-smokers. It seems that choice of important assay parameters, such as test particles, time of cultivation of the AMs, time of incubation of the AMs with test particles, greatly influences the results of phagocytosis assay. Furthermore, the phagocytic assays used in previous studies did not always distinguish between particles that had been ingested and particles that had remained on the cell membrane without being engulfed. The present work provides data which indicate that smokers' AMs have reduced phagocytic activity, assayed as their capacity to ingest heat-killed yeast particles. The mean phagocytic capacity of the smokers' AMs was not significantly affected by NAC treatment; it was, however, improved in five of eleven smokers. Although only further experiments can clarify the action of NAC in this respect, there may be a parallel between our findings and those of NIELSON and BONDE [22]. They found that Biostim PVRPV, an immunostimulating agent, enhanced the phagocytic activity of blood monocytes from patients with chronic bronchitis.

The recent reports from LAVIOLETTE and co-workers [6] and from our laboratory [7, 8] on the depressed synthesis of LTB₄ by smokers' AMs were the reason for examining whether oral NAC treatment could affect this impaired function. In fact, the LTB₄ production by cultured AMs from smokers was significantly improved as a result of oral NAC treatment. The reason why NAC enhances this synthesis is not known at present. According to DNA determinations, we found no differences in the adherence of AMs from non-smokers or smokers before and after NAC treatment. Furthermore, there was no significant correlation between the phagocytic ability of AMs to ingest yeast particles and the stimulation of LTB₄ secretion by OZY (r = 0.28). Thus, these two cellular events seem to be regulated by separate mechanisms. This is in agreement with the data reported by Rouzer et al. [23], which indicate that phagocytosis is not a prerequisite for formation of prostaglandin E₂, another metabolite in the arachidonic acid pathways, whereas an interaction between a ligand and its receptor is a critical step in this process.

In conclusion, since AMs are involved in the early stages of immunological and inflammatory processes, alteration of their function may change these processes in the lungs of smokers. The data provided by the present work as well as the report of BERGSTRAND et al. [24] on the partial reduction of the enhanced superoxide radical generation by smokers' AMs as a result of oral NAC treatment, suggest an influence of NAC on AMs as a possible explanation for the action of this drug. This influence may help to explain the reported capacity of NAC to reduce the frequency of exacerbations in chronic bronchitis [11–13].

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References
Prostaglandin Smoking

A simple, rapid and sensitive DNA assay for leukotriene assay procedure. 

20. Ingested glutaraldehyde-fixed red blood cells in phagocytosing murine macrophages.


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