Pharmacological Reports
2010, 62, 131–138
ISSN 1734-1140

N-acetylcysteine inhibits IL-8 and MMP-9 release and ICAM-1 expression by bronchoalveolar cells from interstitial lung disease patients

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Abstract:
N-acetylcysteine (NAC), owing to its antioxidant, mucolytic and anti-inflammatory properties, is used in the treatment of various pulmonary disorders. However, the direct effects of NAC on bronchoalveolar lavage (BAL) cells from patients suffering from interstitial lung diseases have not yet been studied. Therefore, the aim of the present work was to evaluate the effect of NAC on interleukin-8 (IL-8) and matrix metalloproteinase-9 (MMP-9) production as well as intercellular cell adhesion molecule-1 (ICAM-1) expression by BAL cells from interstitial lung diseases. The study was performed on BAL cells from nine patients with interstitial lung disease: four patients with idiopathic pulmonary fibrosis (IPF) and five patients with sarcoidosis. Cultured unstimulated BAL cells were treated with increasing doses of NAC (1–30 mM). Production of IL-8 and MMP-9 was evaluated by specific enzyme-linked immuno-sorbent assays and ICAM-1 expression was studied by immunohistochemistry. NAC exerted a dose-dependent inhibitory effect on IL-8 and MMP-9 release and ICAM- expression by BAL macrophages and lymphocytes from patients with IPF and sarcoidosis. In conclusion, NAC inhibits production of factors playing a key role in the etiopathogenesis of interstitial lung diseases, thus suggesting its possible therapeutic potency in the treatment of these disorders.

Key words:
N-acetylcysteine, bronchoalveolar lavage, intercellular adhesion molecule-1, interleukin-8, interstitial lung diseases, matrix metalloproteinase

Abbreviations: BAL – bronchoalveolar lavage, ELISA – enzyme-linked immunosorbent assay, ICAM-1 – intercellular cell adhesion molecule-1, IL – interleukin, IPF – idiopathic pulmonary fibrosis, Lym – lymphocytes, Mac – macrophages, MMP-9 – matrix metalloproteinase-9, NAC – *N*-acetylcysteine, NF-κB – nuclear factor kappa B, TNF – tumor necrosis factor, VCAM-1 – vascular cell adhesion molecule

Introduction

N-acetylcysteine (NAC) is an L-cysteine derivative displaying direct and indirect antioxidant properties [7, 23]. Thanks to a sulfhydryl group, it has a direct

ability to neutralize free oxygen radicals. It may also serve as a precursor of glutathione, a key factor responsible for cellular oxidant/antioxidant balance. In addition to its antioxidant properties, NAC is well known as a mucolytic agent and has been found to exert anti-inflammatory activity [7, 23]. The anti-inflammatory activity of NAC may manifest through its ability to inhibit the expression and release of a variety of pro-inflammatory cytokines [17] and to downregulate cytokine-stimulated expression of leukocyte adhesion molecules [19, 21]. It may also affect fibroblast proliferation and collagen synthesis [3, 14], and has been reported to inhibit the release of matrix metalloproteinase 9 (MMP-9) [16, 17].

Owing to its antioxidant, mucolytic, and anti-inflammatory properties, NAC is used in the treatment of various pulmonary disorders such as chronic bronchitis, respiratory distress syndrome, chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis (IPF) [23, 27]. IPF and sarcoidosis belong to interstitial lung diseases of unknown etiology [27]. Pathogenesis of IPF is manifested by an abnormal lung tissue repair process due to repeated lung micro-injury, leading to irreversible loss of pulmonary function. Briefly, it appears that injuries induce epithelial cell apoptosis followed by fibroblast proliferation and enhanced synthesis as well as a decreased degradation of extracellular matrix, leading to progressive lung fibrosis [3, 27]. On the other hand, sarcoidosis, another example of interstitial lung disease, is a chronic systemic granulomatous disorder characterized by the accumulation of activated T-lymphocytes and macrophages in the lung [22]. Sarcoidosis-associated alveolitis results in pulmonary tissue destruction that is often followed by fibrosis. The etiopathogenesis of interstitial lung diseases may involve the participation of numerous cytokines, growth factors and tissue proteases. It has been demonstrated that bronchoalveolar lavage fluid from sarcoidosis and IPF patients may contain increased levels of IL-1, IL-2, IL-6, IL-8, TNF [6, 13] and metalloproteinases (MMP) [5, 16]. Analysis of bronchoalveolar lavage (BAL) cells has also revealed an increased expression of intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) [12].

It is possible that beneficial NAC effects in interstitial lung diseases are related to its anti-inflammatory activity. However, the direct effects of NAC on immune cells from patients suffering from interstitial lung diseases have not yet been studied. Therefore, the aim of the present work was to elucidate the effects of NAC on IL-8 and MMP-9 production as well as ICAM-1 expression by BAL cells from interstitial lung diseases patients.

Materials and Methods

Patients

The study involved 9 patients with interstitial lung diseases: 4 patients with idiopathic pulmonary fibrosis and 5 patients with pulmonary sarcoidosis. Bronchoscopic BAL was performed in each patient for clinical reasons, either for the diagnostic purposes or to assess the disease activity. IPF groups were diagnosed according to ATS/ERS criteria [1]. Clinical (compatible history, digital clubbing, inspiratory crackles on auscultation, pulmonary function test results) and radiological signs (high-resolution thoracic computed tomography scan with bibasilar reticular abnormalities with minimal ground opacities and honeycombing present) were typical for IPF. A confirmatory open lung biopsy was performed in 2 IPF patients who showed high lymphocytosis (53.1% and 32%) in their BAL fluid. The histological diagnosis (interstitial fibrosis and alveolar inflammatory cells) was usual interstitial pneumonia.

Diagnosis of sarcoidosis in patients was based on compatible clinical (cough, dyspnea, fever, erythema nodosum, polyarthralgia) and radiographic findings, increased CD4/CD8 ratio in BAL fluid and the exclusion of other granulomatous lung diseases. Three out of 5 patients with sarcoidosis had biopsy confirmation of pulmonary BBS. According to the chest X-ray findings, BBS was classified as stage I in four patients and stage II in one patient.

Exclusion criteria were smoking, any pharmacological therapy during the 3 months prior to the onset of the study or any coexisting systemic disease. The study has been accepted by the local ethical committee and all patients signed an informed consent to the study. Basic demographical data of the patients are shown in Table 1.

Tab. 1. Basic demographic data and BAL characteristics of patients with idiopathic pulmonary fibrosis and sarcoidosis

	IPF[a]	Sarcoidosis
Patients data		
Age (years)	62.7 ± 5.8 (range: 56 – 70)	35.5 ± 13.3 (range: 22–50)
Gender (female/male)	3/1	4/1
BAL characteristics		
Volume (ml)	137.5 ± 15.0	154.0 ± 18.2
Cell count ($\times 10^{-4}$/ml)	33.6 ± 5.0	55.1 ± 22.0
Macrophages (%)	58.9 (range: 41.3–72.9)	74.5 (range: 66.8–84.0)
Lymphocytes (%)	31.4 (range: 18.4–53.1)	24.7 (range: 15.5–33.3)
Neutrophils (%)	3.9 (range: 2.4–5.7)	0.4 (range: 0–0.6)
Eosinophils (%)	5.8 (range: 0.5–15.5)	0.2 (range: 0–0.5)
CD4/CD8 ratio	3.3 (range: 2.6–4.4)	6.8 (range: 2.3–13.6)

Data are presented as the mean ± SD. Range is shown in parentheses where applicable. [a] idiopathic pulmonary fibrosis

Isolation and culture of BAL cells

BAL cells were obtained during diagnostic bronchoscopy by the standard procedure [9]. In brief, a total volume of 200 ml (4 × 50 ml) of sterile 0.9% saline solution at room temperature was instilled through a flexible fiberoptic bronchoscope with harvesting of the fluid under immediate gentle vacuum.

BAL fluids were filtered through sterile gauze and centrifuged at 400 × g for 10 min. The cell viability was > 90% as judged by trypan blue exclusion. For differential cell counts, cytosmears were routinely stained with May-Grunwald-Giemsa. CD4 and CD8 positive cells were identified by specific monoclonal antibody staining procedure ($LSAB^+$ Kit, DAKO, Denmark) according to detailed description provided by the manufacturer. The cells were counted under a light microscope. Characteristics of BAL cells are shown in Table 1.

BAL cells were cultured in 2 ml of RPMI medium (GIBCO, Paisley, UK) supplemented with 10% heat inactivated bovine serum, glutamine, and a mixture of penicillin and streptomycin (final concentration 100 U $\times$ ml^{-1} and 10 mg $\times$ ml^{-1}, respectively) at a density of $2 \times 10^6 \times ml^{-1}$ in tissue culture tubes (BD, Bioscience, Belgium), at 37°C and in 5% CO_2 in air atmosphere. *N*-acetylcysteine (Sigma-Aldrich, St. Louis, MO, USA) was added to obtain final concentrations ranging from 1 mM to 30 mM. Cultures without NAC served as controls. After 24 hours, cell-free culture supernatants were collected and stored frozen at –70°C until used for IL-8 and MMP-9 evaluation. The remaining cells were vigorously washed out with ice-cold Ca^{2+}-free PBS. The recovery was always > 85% of the input cell number. The recovered cells were used for cytosmear preparations and ICAM-1 immunohistochemical staining. NAC did not affect cell viability at all tested concentrations as judged by trypan blue exclusion test.

Evaluation of ICAM-1 positive BAL cells

Numbers of ICAM-1 (CD54) positive BAL cells, macrophages and lymphocytes were estimated following 24-h culture by indirect immunoperoxidase staining using a specific monoclonal antibody and the N-visione kit (DAKO). The specimens were routinely counterstained with hematoxylin. ICAM-1 positive lymphocytes and macrophages were identified using a light microscope at a final magnification 1000×. The results are expressed as the percentage of ICAM positive cells.

Quantification of IL-8 and MMP-9 in culture supernatants

The levels of IL-8 and total MMP-9 in culture supernatants from NAC-treated and control BAL cells were quantified by commercial specific enzyme-linked-immuno-sorbent-assays (ELISA) (R&D Systems, Abingdon, UK), according to the detailed description of the manufacturer. All standards and samples were tested in duplicate and the mean values were taken for calculations.

Statistical analysis

All data are shown as a the mean ± SD. Differences between groups were evaluated by one-way ANOVA for paired samples and *post-hoc* Tukey HSD test. The differences were considered significant at a p value < 0.05.

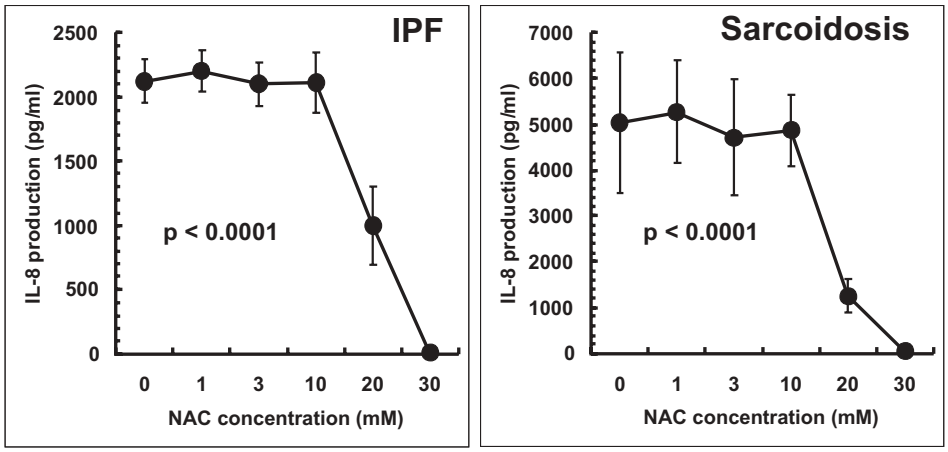

Fig. 1. The effect of *N*-acetylcysteine (NAC) on spontaneous IL-8 production by BAL cells from patients with idiopathic pulmonary fibrosis (IPF) and sarcoidosis. Data are presented as the mean ± SD, p values computed by one-way ANOVA are also shown

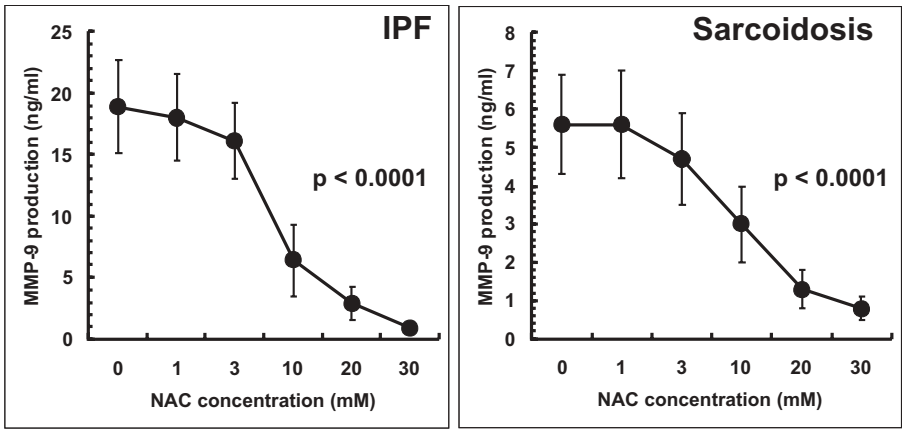

Fig. 2. The effect of *N*-acetylcysteine (NAC) on spontaneous MMP-9 production by BAL cells from patients with idiopathic pulmonary fibrosis (IPF) and sarcoidosis. Data are presented as the mean ± SD, p values computed by one-way ANOVA are also shown

Results

Effect of NAC on IL-8 production by BAL cells

The results of the effect of NAC on spontaneous IL-8 production by BAL cells are shown in Figure 1. Unstimulated BAL cells from both IPF and sarcoidosis patients spontaneously released significant amounts of IL-8. Interestingly, patients with sarcoidosis produced two times more IL-8 than IPF patients. NAC exerted a significant, dose-dependent inhibitory effect on IL-8 production by BAL cells from both groups of patients (both $p < 0.0001$ by one-way ANOVA). This effect was significant at 20 and 30 mM NAC ($p < 0.01$ by Tukey HSD test). IL-8 production was completely inhibited by 30 mM NAC.

Effect of NAC on MMP-9 production by BAL cells

Unstimulated BAL cells from both IPF and sarcoidosis patients spontaneously released significant amounts of MMP-9 (Fig. 2). Production of this metalloproteinase by BAL cells from IPF patients was three times higher than from sarcoidosis patients. In both groups of patients, NAC exerted a significant, dose-dependent inhibitory effect on MMP-9 production (both $p < 0.0001$ by one-way ANOVA). A significant decrease of MMP-9

release in both groups was seen at 10 mM and higher NAC concentrations. A NAC concentration of 30 mM almost completely inhibited MMP-9 release.

Effect of NAC on ICAM-1 (CD54) expression by BAL cells

The effect of NAC on ICAM-1 expression (CD54) was assessed on both BAL macrophages and lymphocytes and the results are shown in Figure 3. In both groups of patients, NAC treatment resulted in a significant, dose-dependent decrease of BAL $CD54^+$ macrophage numbers (both $p < 0.0001$ by one-way ANOVA). A similar effect was also seen in the case of BAL $CD54^+$ lymphocytes. A significant inhibitory effect of NAC on CD54 expression by macrophages was seen already at a 10 mM concentration ($p < 0.05$ by Tukey HSD test), whereas downregulation of CD54 in lymphocytes was observed at a 20 mM NAC concentration ($p < 0.05$ by Tukey HSD test). At 30 mM NAC, CD54 expression was completely inhibited in both macrophages and lymphocytes. NAC treatment did not affect cell number or viability, which was > 90% as judged by trypan blue exclusion.

Discussion

In the present study, we show for the first time that spontaneous *in vitro* production of IL-8, MMP-9 and ICAM-1 expression by BAL cells from IPF and sarcoidosis patients may be inhibited by NAC. This effect was dose-dependent and complete inhibition was seen at the highest (30 mM) concentration of this agent. These results provide evidence for a direct and

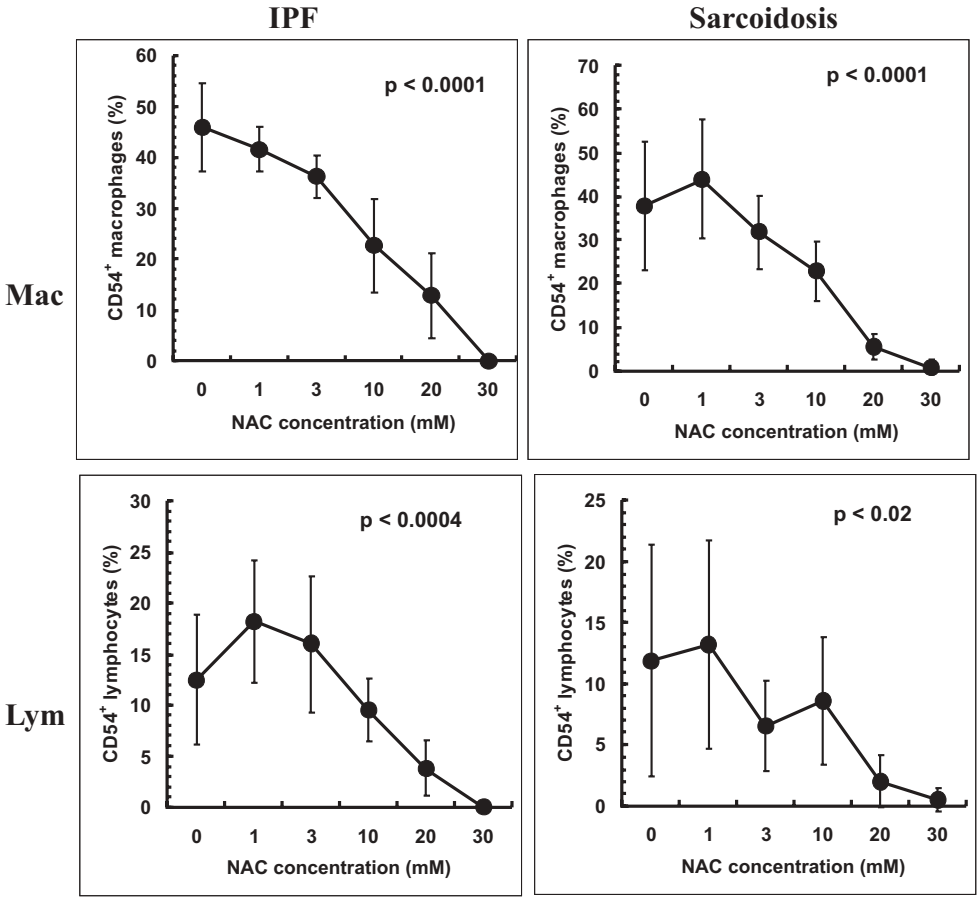

Fig. 3. The effect of *N*-acetylcysteine (NAC) on spontaneous ICAM-1 (CD54) expression by BAL macrophages (Mac) and lymphocytes (Lym) from patients with idiopathic pulmonary fibrosis (IPF) and sarcoidosis. Data are presented as the mean ± SD, p values computed by one-way ANOVA are also shown

broad anti-inflammatory effect of NAC on alveolar leukocytes from interstitial lung diseases.

We found that BAL cells from both IPF and sarcoidosis patients spontaneously release IL-8 and MMP-9, which are markers of inflammatory and connective tissue remodeling. This finding is consistent with previous observations that the levels of IL-8 and MMP-9 in BAL fluid are significantly increased in interstitial lung diseases when compared with healthy subjects and that they may participate in the etiopathogenesis of these disorders [5, 10, 16, 20]. Spontaneous release of IL-8 and MMP-9 by BAL leukocytes possibly reflects their disease-specific activation, e.g. by classical inflammatory cytokines such as TNF, IL-1β, IL-18 and others, which appear to be key factors in the etiopathogenesis of interstitial lung diseases [18, 22, 27]. Interestingly, our study shows that spontaneous release of IL-8 by BAL cells from sarcoidosis patients is much higher than from IPF patients, and this result is consistent with previously reported data [30]. In accordance with studies on BAL fluid [10, 20], BAL cells from IPF patients released much more MMP-9 than cells from sarcoidosis patients, and this finding may suggest that higher levels of this enzyme reflect different etiopathogenetic features of both disorders. High levels of MMP-9 production by BAL cells may play a crucial role in extracellular matrix turnover [4, 10, 16] and new vessel formation in the course of fibrosis [2, 4, 16]. This observation supports the view that connective tissue remodeling and angiogenesis play a key role in the etiopathogenesis of IPF.

The mechanism by which NAC may inhibit spontaneous IL-8 and MMP-9 release as well as ICAM-1 expression by BAL cells remains to be clarified. We have previously shown that NAC inhibits TNF- or IL-1β-induced IL-8 release and ICAM-1 expression by endothelial and epithelial cell lines [20]. It is therefore plausible that the inhibitory NAC effect may be related to inhibition of production and attenuation of the stimulatory effect of inflammatory cytokines. Inflammatory cytokines induce the expression of other cytokines and adhesion molecules *via* activation of nuclear factor-kappa B (NF-κB) transcription factor [7, 25, 29]. Indeed, NAC has been reported to inhibit NF-κB activation and consecutive IL-8 and IL-6 production in patients with sepsis [19]. It was also reported to inhibit NF-κB activation, the production of TNF, IL-6, IL-8, the metabolism of arachidonic acid and MMP-9 and plasminogen activator activity in fetal human membranes and synovial fibroblasts *in vitro* [17, 25]. Inhibition of NF-κB activation by NAC may be due to its anti-oxidant function and modulation of the cellular oxidant/antioxidant balance [7, 11, 15, 23].

Inhibition or reduction of the spontaneous production of inflammatory cytokines by BAL cells or alveolar macrophages has previously been reported in the cases of other therapeutic agents routinely used in the treatment of interstitial lung diseases such as steroids, pentoxifylline and thalidomide [24, 26, 28, 30]. Our present study shows that NAC exerts similar anti-inflammatory effects. However, it is difficult to compare the effectiveness of NAC with other therapeutic agents.

It should be stressed that in our study, significant NAC inhibitory effects on IL-8 and MMP-9 production and ICAM-1 expression by BAL cells were seen at 10–30 mM concentrations. Similarly, most of the other *in vitro* studies on NAC have also been performed using high doses of this agent, even up to 100 mM [17, 23]. These concentrations are much higher than concentrations obtained in blood plasma or BAL fluid following conventional oral administration of NAC during lung disease treatment [8, 23]. Nevertheless, it has been postulated that a significant *in vivo* anti-inflammatory effect of NAC may be achieved not only by administration of the very high doses but also by a chronic administration of low doses of this agent. Accordingly, anti-inflammatory effects following the chronic administration of relatively low doses of NAC (< 3 mM), used in interstitial lung diseases for example, may be explained by generation of a cumulative effect of this drug *via* free radical scavenging and modulation of redox status in cells [23]. This, however, requires further *in vivo* investigations.

We also provide evidence that NAC inhibits the release of MMP-9. The effect of anti-inflammatory agents on the production of MMP-9 by alveolar cells has not been studied so far. MMP-9 appears to play a crucial role in the etiopathogenesis of interstitial lung diseases as a regulator of tissue remodeling and fibrosis [4, 16]. Therefore, properties of NAC as a potent inhibitor of fibrosis in the lung may be of clinical significance.

In conclusion, the results shown in the present study confirm the beneficial effects of NAC in the treatment of IPF [8, 11, 15, 24, 28] and provide evidence that the therapeutic potency of this agent may be related to its direct and broad anti-inflammatory effect on alveolar leukocytes. Our present study may also suggest that NAC might be of some therapeutic

significance and may improve therapeutic regimens in the treatment of sarcoidosis. However, there are no available data on a possible therapeutic usefulness of NAC in sarcoidosis thus far, and the precise clinical value of this agent in the treatment of this disorder requires further experimentation in clinical trials.

Acknowledgments:
The study was supported by grants from the Medical University of Warsaw (1M15/N/07) and the National Tuberculosis and Lung Disease Research Institute.

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Received:
April 14, 2009; in revised form: February 2, 2010.