Oral N-acetylcysteine reduces selected humoral markers of inflammatory cell activity in BAL fluid from healthy smokers: correlation to effects on cellular variables.

A. Eklund*, Ö. Eriksson**, L. Håkansson***, K. Larsson****, K. Ohlsson†, P. Venge**, H. Bergstrand†, A. Björnson‡, R. Brattstrand†, C. Glennow†, M. Linden‡‡, E. Wieslander††

Oral N-acetylcysteine reduces selected humoral markers of inflammatory cell activity in BAL fluid from healthy smokers: correlation to effects on cellular variables. A. Eklund, Ö. Eriksson, L. Håkansson, K. Ohlsson, P. Venge, H. Bergstrand, A. Björnson, R. Brattstrand, C. Glennow, M. Linden, E. Wieslander.

ABSTRACT: Bronchoalveolar lavage (BAL) was performed on eleven healthy smokers before and after eight weeks of oral treatment with N-acetylcysteine (NAC) 200 mg t.i.d. The concentrations of selected eosinophil and neutrophil granule constituents and of selected proteases and protease inhibitors, albumin and endotoxin were determined in the recovered BAL fluid and in plasma or serum samples. In addition, in vitro chemotactic activities for neutrophils and eosinophils were assessed in the BAL fluid. Significant reductions in BAL fluid content of lactoferrin (LF), eosinophil cationic protein (ECP), anti-chymotrypsin (ACT) and chemotactic activity for neutrophils were recorded after NAC treatment. The levels of other examined markers tended to be reduced or were not affected. In serum/plasma, the concentrations of myeloperoxidase (MPO) and elastase were reduced after NAC treatment whereas concentrations of other constituents examined were unaltered. These data, together with previously reported findings, suggest that oral NAC may influence the activity of inflammatory cells in the bronchoalveolar space of smokers.

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Oral treatment of patients with chronic bronchitis with N-acetylcysteine (NAC) may lead to a reduction in the rate of exacerbations [1–3]. Although NAC is often referred to as a mucolytic agent [1, 2] or a radical scavenger, the reason for the possible beneficial effect of NAC in chronic bronchitis is unclear. Chronic bronchitis is often associated with smoking and smoking in turn is associated with an increased incidence of infectious diseases in the lung [4, 5]. Smoking also influences the abundance and activity of inflammatory cells in the airways [6]. Thus, the number of cells that can be recovered by lavage from the bronchoalveolar space is markedly higher in smokers than in nonsmokers [7–11]. Moreover, the capacity of alveolar macrophages and peripheral leukocytes to generate oxygen radicals and leukotriene B4 (LTB4) when stimulated may be altered for cells from smokers [7, 9, 11–14]. Smoking is also reported to alter proportions of immunoregulatory T-cell subsets in peripheral blood and in bronchoalveolar lavage fluid [11, 15–17]. Therefore in addition to the postulated mucolytic effect of NAC or its supposed interference with mucus hypersecretion [1, 18], the NAC-induced reduction in exacerbation rates in patients with chronic obstructive lung disease could possibly also result from an improvement in the host response to infectious agents. To examine this possibility, we decided to assess whether NAC can directly or indirectly influence inflammatory cells of smokers, particularly cells in the airways. We therefore analysed bronchoalveolar lavage (BAL) fluid and plasma or serum from healthy smokers for selected markers of inflammatory cell activity before and after oral treatment with NAC.

The present report summarizes the study and gives the results recorded for soluble constituents of the BAL fluid. The results recorded with respect to cellular content/composition and functions are reported separately [10, 13]. The results as a whole suggest that the oral NAC treatment may to some extent normalize the alterations in inflammatory cell activity induced by smoking.
Patients and methods

Subjects

Eleven healthy smokers (four men), with a mean age of 29±2 yrs, participated in the study. All subjects gave their informed consent and the study had the approval of the local Ethics Committee. All had consumed twenty or more cigarettes per day for at least five years and the mean cigarette consumption was 14.5±2.8 pack-years. No subject changed smoking habits markedly during the study. Seven had no respiratory symptoms, the remaining four having productive cough in the mornings. Lung function tests, chest X-ray and ECG were examined [9, 12] before each BAL and did not deviate from normal.

Table 1. - Bronchoalveolar lavage (BAL) fluid and serum/plasma levels of eosinophil cationic protein (ECP), lysozyme, lactoferrin (LF), myeloperoxidase (MPO), anti-leukoprotease (ALP), elastase, albumin, α₁-protease inhibitor (α₁-PI), α₂-macroglobulin (α₂-M), anti-chymotrypsin (ACT), endotoxin and eosinophil and neutrophil chemotactic activity in smoking individuals before and after oral treatment with NAC for eight weeks.

<table>
<thead>
<tr>
<th></th>
<th>BAL fluid</th>
<th>Serum/plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td></td>
<td>n=11</td>
<td>n=10</td>
</tr>
<tr>
<td>ECP (µg·l⁻¹)</td>
<td>22.6</td>
<td>13.7*</td>
</tr>
<tr>
<td>Lysozyme (µg·l⁻¹)</td>
<td>3.2</td>
<td>1.0</td>
</tr>
<tr>
<td>LF (µg·l⁻¹)</td>
<td>413.0</td>
<td>287.0</td>
</tr>
<tr>
<td>MPO (µg·l⁻¹)</td>
<td>50.9</td>
<td>4.4</td>
</tr>
<tr>
<td>ALP (µg·l⁻¹)</td>
<td>104.5</td>
<td>45.4*</td>
</tr>
<tr>
<td>Elastase (µg·l⁻¹)</td>
<td>14.5</td>
<td>4.3</td>
</tr>
<tr>
<td>Albumin (µg·l⁻¹)</td>
<td>36.3</td>
<td>32.6</td>
</tr>
<tr>
<td>α₁-PI (µg·l⁻¹)</td>
<td>6.8</td>
<td>7.4</td>
</tr>
<tr>
<td>α₂-M (µg·l⁻¹)</td>
<td>1.34</td>
<td>0.82</td>
</tr>
<tr>
<td>Albumin (µg·l⁻¹)</td>
<td>0.41</td>
<td>0.12</td>
</tr>
<tr>
<td>ACT (µg·l⁻¹)</td>
<td>0.085</td>
<td>0.044*</td>
</tr>
<tr>
<td>Endotoxin (pg·ml⁻¹)</td>
<td>0.032</td>
<td>0.008</td>
</tr>
<tr>
<td>Eosinophil chemotactic activity (µm·h⁻¹)</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>Neutrophil chemotactic activity (µm·h⁻¹)</td>
<td>25</td>
<td>18*</td>
</tr>
</tbody>
</table>

ECP, lysozyme, LF and MPO were examined in serum whereas ALP, elastase, albumin, α₁-PI, α₂-M and ACT were assessed in plasma. Figures given are mean±SEM and are shown for n=11 and n=10 (one individual excluded; see text). The figures given for albumin, α₁-PI and ACT are in µg·l⁻¹ (BAL fluid) and in g·l⁻¹ (plasma). ND: not determined. *: p<0.05; **: p<0.01; †: p=0.059.
After the first BAL, the subjects received oral medication with NAC (Draco, Lund, Sweden) 200 mg i.d. for eight weeks. One and a half hours after the last dose the second BAL was performed. The study was performed during October, November, and December.

**Bronchoalveolar lavage**

Bronchoscopy was performed through the mouth with a flexible fiberoptic bronchoscope (Olympus Type 4B2), under local anaesthesia with 2% lignocaine (Xylocaine®, Astra, Södertälje, Sweden), after premedication with morphine-scopolamine and phenemal. The bronchoscope was wedged in a middle lobe bronchus and the volume was measured. The cells were pelleted at 400 g for 5 min at +4°C and then resuspended in RPMI 1640 supplemented with gentamycin (50 µg·mL⁻¹) and 5% foetal calf serum. After transport (at approximately 4°C), differential and total cell counts, viability tests (Trypan blue) and examination of superoxide and LTB₄ generation and phagocytic capacity were all performed, as has been reported in detail previously [10, 13]. The cell-free BAL fluid supernatant (and plasma/serum samples harvested at the same time for each BAL) were analysed, according to procedures previously described in detail, for eosinophil cationic protein (ECP) [19], lysozyme [20], lactoferrin (LF) [20, 21], myeloperoxidase (MPO) [20, 21], anti-leukoprotease (ALP) [22] and elastase [23]. Albumin, alpha-1-protease inhibitor (α₁-PI), α₂-macroglobulin (α₂-M), and anti-chymotrypsin (ACT) were quantified using electroimmunoassay [24]. Endotoxin was determined in BAL fluid supernatants with the aid of the Limulus assay [25]. Eosinophil and neutrophil chemotactic activities in crude BAL fluid supernatants were determined as described previously [26].

**Statistical analyses**

Statistical analyses were performed using the RS/1 programme (BBN Software Products Corporation, Cambridge, MA, USA) which utilizes either Student’s paired t-test or, if the sample population shows indication of not being normally distributed, Wilcoxon’s test on paired observations. Pearson’s correlation coefficients were tested against zero with Student’s t-test.

**Results**

BAL fluid and plasma/serum levels of selected granule constituents of inflammatory cells, as well as levels of selected proteases/antiproteases, were examined before and after eight weeks of treatment with oral NAC. Chemotactic activity for neutrophils and eosinophils in vitro were assessed in BAL fluid only. The results are shown in table 1. Significant reductions in BAL fluid levels of eosinophil cationic protein (ECP),
effects of NAC. For that purpose log (preNAC/postNAC) was calculated for each variable and patient (n=11); for chemotactic activities preNAC-postNAC values were used. Out of 276 examined correlations only those showing significance at the p<0.01 level are shown. The corresponding figures obtained when one individual is excluded are given on the right. For variable abbreviations see table 1.

Table 3. – Correlation coefficients (r) recorded at examination of correlation between degrees of NAC treatment-induced changes in various examined variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>Variable</th>
<th>n=11</th>
<th>t</th>
<th>n=10</th>
<th>t</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECP, BAL</td>
<td>MPO, BAL</td>
<td>0.835</td>
<td>4.57**</td>
<td>0.723</td>
<td>2.95*</td>
</tr>
<tr>
<td>Lysozyme, BAL</td>
<td>LF, BAL</td>
<td>0.798</td>
<td>3.97**</td>
<td>0.757</td>
<td>3.27**</td>
</tr>
<tr>
<td>Lysozyme, BAL</td>
<td>ALP, BAL</td>
<td>0.897</td>
<td>6.10***</td>
<td>0.846</td>
<td>4.49***</td>
</tr>
<tr>
<td>Lysozyme, BAL</td>
<td>Endotoxin, BAL</td>
<td>0.741</td>
<td>3.31***</td>
<td>0.649</td>
<td>2.41*</td>
</tr>
<tr>
<td>LF, BAL</td>
<td>MPO, BAL</td>
<td>0.849</td>
<td>4.81**</td>
<td>0.496</td>
<td>1.61</td>
</tr>
<tr>
<td>LF, BAL</td>
<td>ALP, BAL</td>
<td>0.799</td>
<td>3.98**</td>
<td>0.867</td>
<td>4.92**</td>
</tr>
<tr>
<td>LF, BAL</td>
<td>Elastase, BAL</td>
<td>0.766</td>
<td>3.57**</td>
<td>0.040</td>
<td>0.11</td>
</tr>
<tr>
<td>LF, serum</td>
<td>MPO, serum</td>
<td>0.862</td>
<td>5.10***</td>
<td>0.900</td>
<td>5.84**</td>
</tr>
<tr>
<td>MPO, BAL</td>
<td>Elastase, BAL</td>
<td>0.823</td>
<td>4.34**</td>
<td>0.489</td>
<td>1.59</td>
</tr>
<tr>
<td>MPO, serum</td>
<td>Cellular, LTBl</td>
<td>0.769</td>
<td>3.36**</td>
<td>0.742</td>
<td>2.92*</td>
</tr>
<tr>
<td>ACT, BAL</td>
<td>Chemotactic activity, EOS</td>
<td>0.767</td>
<td>3.28**</td>
<td>0.684</td>
<td>2.48*</td>
</tr>
<tr>
<td>α1-PL, plasma</td>
<td>Albumin, BAL</td>
<td>0.778</td>
<td>3.71**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α1-PL, BAL</td>
<td>ACT, BAL</td>
<td>0.836</td>
<td>4.30**</td>
<td>0.768</td>
<td>3.17*</td>
</tr>
<tr>
<td>α1-PL, BAL</td>
<td>α1-PL, plasma</td>
<td>0.913</td>
<td>6.34***</td>
<td>0.865</td>
<td>4.56**</td>
</tr>
</tbody>
</table>

Log (preNAC/postNAC) was calculated for each variable and patient (n=11); for chemotactic activities preNAC-postNAC values were used. Out of 276 examined correlations only those showing significance at the p<0.01 level are shown. The corresponding figures obtained when one individual is excluded are given on the right. For variable abbreviations see table 1.

** * 0.01<p<0.05; ** * 0.001<p<0.01; *** * p<0.001

lactoferrin (LF), anti-chymotrypsin (ACT) and neutrophil chemotactic activity were recorded, whereas the levels of other variables examined were not significantly affected by the NAC treatment. In plasma/serum, only the levels of elastase, myeloperoxidase and albumin were significantly affected by the treatment. The concentrations in BAL fluid or serum/plasma of ECP, LF, ACT, MPO, and elastase recorded after NAC treatment approached the values normally recorded for these constituents in samples from apparently healthy nonsmokers (data not shown).

Two of the individuals showed signs of upper airway infection at the first bronchoscopy examination. For one of these individuals, high levels were recorded for all BAL fluid humoral markers (but not for cellular activity markers [10, 13]). If the patient with these high levels is excluded from the results and calculations are performed based on the remaining ten individuals, significant reductions in BAL fluid levels of elastase are still recorded, whereas the significant reductions in BAL fluid levels of neutrophil chemotactic activity and serum level of MPO induced by NAC treatment are not observed (table 1).

The results recorded in the present study with respect to cell recovery, differential cell count and viability, with respect to oxygen radical generation, LTBl generation and phagocytosis have all been detailed elsewhere [10, 13]. In table 2 we show a schematic summary of the findings of the present study.

The conspicuous finding of this study seems to be that oral treatment of smokers with NAC tends to reduce the levels of supposed markers of inflammatory cell activities in BAL fluid and to normalize smoking-induced activation/deactivation of selected BAL cell function(s). We therefore examined (with the recorded data as the basis) whether a correlation existed between the various effects of NAC. For that purpose log (preNAC/postNAC) was calculated for each variable and patient as a measure of the change induced by NAC treatment; for the chemotactic activities preNAC-postNAC values were used since for these variables negative recordings could occur. We then examined the linear correlation between (preNAC/postNAC) values for the various variables. In these calculations, figures for cellular yield and for α1-macroglobulin were not included; as mentioned previously the latter is not detectable in BAL fluid. Furthermore, superoxide radical generation was represented by figures recorded at triggering with PMA. Since 24 different humoral and cellular variables were thus followed, a total of 276 different correlations were examined. Fourteen of these (collected in table 3) were found to be significant at the levels of p<0.01. Two to three of the fourteen correlations would thus be expected to have occurred by chance. However, it is of interest that the changes, apparently induced by NAC treatment, in BAL fluid levels of humoral markers of granulocyte activity often correlated significantly, as did changes induced by NAC treatment in the serum levels of lactoferrin and myeloperoxidase. Conversely, no convincing correlation was recorded between effects of NAC treatment on any pair of examined variables of cellular activation (LTBl generation or phagocytic activity) or between such an activity and endotoxin levels in BAL [10, 13].

**Discussion**

A beneficial effect of oral treatment with N-acetylcysteine on the exacerbation rate in patients with chronic bronchitis has been documented in at least two multicentre studies [1, 2]. A third study [3], which examined patients with more advanced disease reported similar results but the difference did not reach statistical significance.
The reason for such an effect of NAC on the exacerbation rate in patients with chronic bronchitis is not known. However, it is now realized that following oral treatment, NAC can hardly be considered to exert mucolytic activity in the airways [27]. Since the possibility that smoking may induce changes in the immune and inflammatory system(s) and, therefore, may contribute to the disease is now supported by numerous studies (for reviews see [6, 11]) we speculated that NAC could in some way reverse a smoke-induced defect in an “antimicrobial” host response. To examine this possibility we assessed the influence of oral NAC treatment on selected variables of the airway immune response (in a broad sense) in healthy smokers. The present report gives the results with respect to the effects of NAC treatments on examined humoral components. Previous reports [10, 13] have detailed effects of the treatment recorded for cellular constituents of BAL fluid. We found that NAC treatment significantly reduced the BAL fluid levels of the eosinophil and the neutrophil granule constituents ECP and lactoferrin and also seemed to reduce those of some (but not all) of the other granulocyte constituents examined. Serum/plasma levels of examined variables were not significantly affected, with the exception of myeloperoxidase and, possibly, elastase (both neutrophil constituents) and albumin.

Another conspicuous finding in this study was that NAC treatment of smokers did not affect BAL or plasma/serum concentrations of proteases/protease inhibitors examined, with two possible exceptions. These were elastase (previously reported to be elevated in BAL fluid as well as in plasma/serum of smokers [28, 29]) and anti-chymotrypsin. The BAL fluid concentration of both of these components tended to be reduced following NAC treatment (table 1). On the other hand, immunoreactive \( \alpha_1 \)-protease inhibitor level in BAL was not changed by NAC treatment; in fact, the level of this component (and possibly also that of ALP) was, if anything, higher in BAL fluid of smokers than in that of healthy nonsmokers (unpublished data). This is in apparent contrast to previous reports of reduced activity of \( \alpha_1 \)-protease inhibitor in BAL from smokers compared to normals [30–32].

Together with the results recorded on cellular variables [10–13] (table 2) and those independently recorded for nonsmoking individuals (unpublished results), the general finding of this study is that NAC treatment tends to normalize a dysfunction induced by cigarette smoke. Such an interpretation is underlined by the recordings of significant correlations between effects of NAC treatment on different but apparently related variables, e.g. LF serum and MPO serum, whereas apparently “false” correlations (those which are difficult to explain) are few (table 3). In this context we are aware of the fact that lactoferrin in BAL is not a neutrophil-specific constituent but is also produced by serous cells.

The correlation analysis also provides other important information. Firstly, since no correlation exists between any examined cellular activity and endotoxin levels in BAL, the altered cellular activity previously recorded for cells from smokers [10, 13] is less likely to be due to endotoxin “contamination” of the BAL fluid. Secondly, several “expected” or easily explained correlations between humoral, supposedly granule-derived variables are noted but few, if any, between any humoral and cellular variables or between different variables reflecting cellular activity. Therefore, the examined cellular activities may be similarly triggered and/or regulated by independent mechanisms. It has previously been observed in studies in vitro that the system for neutrophil superoxide generation is physically separate and induced independently from that of degranulation [33, 34]; in the human basophils histamine release system, various secretagogues induce mediator release independently [35, 36]. The present data therefore seem to indicate that various functions of a granulocyte/mononuclear cell may be regulated independently in vivo also.

The basis for the suggested effect of NAC remains unclear. However, the finding that distinct examined variables, like BAL content of the eosinophil marker ECP and the supposed neutrophil constituent lactoferrin are affected by the NAC treatment suggests that either NAC exerts an indirect effect on granulocyte function, e.g. by influencing production of principles governing the activity of granulocytes, or that NAC may influence a biochemical pathway which is important in the function of eosinophils as well as neutrophils. The former alternative is substantiated by findings that components of the smoke may directly induce activation of cells or may stimulate lymphocytes to produce factors which in turn activate phagocytes [11]. With respect to, e.g. oxygen radical production, different lymphocyte-derived principles have been attributed roles as inducers of such a primed state. \( \gamma \)-interferon is a necessary and sufficient inducer of that activity in human macrophages [37]. Conversely, \( \beta \)-interferon is reported to reduce macrophage oxygen radical generating capacity [38]. Therefore, one speculative explanation for the effect of NAC treatment would be that NAC affects/normalizes the production by mononuclear cells of factors with chemotactic and/or priming activity for effector cells in the airways. It is also possible that NAC treatment influences the activity of granulocytes more directly leading to reduced levels of their products. Such a possibility is strengthened by, e.g., the recorded reduction by NAC treatment of elastase and myeloperoxidase serum/plasma levels (table 1). This effect, in turn, is significantly correlated to that on serum lactoferrin (table 3). Since neither NAC nor an increased cysteine level can clearly be demonstrated in BAL fluid after oral treatment with the drug [26], any action of NAC or a NAC derivative on peripheral granulocytes would seem more probable than an action locally in the lung.

At the biochemical level also, the explanations for the effect(s) seen with NAC are not known. NAC has previously been reported to modulate various biochemical parameters of rat alveolar macrophages [39]. Recent experience also indicates that NAC markedly enhances the effectiveness of nitro compounds with respect to vascular smooth muscle relaxation [40, 41] and inhibition of platelet aggregation [42]. These effects of nitro compounds are thought to be partly due to their capacity
to increase generation of cyclic guanosine monophosphate (cGMP) [43]. Since NAC treatment is claimed to lead to increased plasma levels of cysteine [44] one may speculate that NAC in some way fulfills the requirement for SH-compounds which seems to exist at activation of soluble cellular guanylate cyclase [45].

Only further detailed studies on the influence of NAC on the activity of immune cells in smokers and patients with chronic bronchitis, together with biochemical examinations of its supposed influence on guanylate cyclase can clarify whether this may be the basis for the observed effect of the drug.

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RÉSUMÉ: Le lavage broncho-alvéolaire (BAL) a été pratiqué chez 11 fumeurs bien portants, avant et après un traitement oral de huit semaines au moyen de N-acétylcystéine (NAC; 200 mg trois fois par jour). Les concentrations des constituants sélectifs des granules éosinophiles et neutrophiles, ainsi que ceux de protéases et d’inhibiteurs de protéases d’albumine et d’enzyme detoxine, ont été déterminées dans le liquide de lavage ainsi que dans le plasma ou le sérum des individus concernés. En outre, les activités chemotactiques pour les neutrophiles et les éosinophiles ont été mesurées dans le liquide de lavage in vitro. Des diminutions significatives du contenu du liquide de lavage broncho-alvéolaire en lactoferrine (LF), en protéines cationiques éosinophiles, en antioxydant et en activité chemotactique pour les neutrophiles, ont été décelées après traitement à la NAC. Quant aux autres marqueurs examinés, leurs niveaux sont tendance à diminuer, soit n’étaient pas modifiés. Dans le sérum/plasma, les concentrations de myéloperoxidase (MPO) et d’elastase, ont été respectivement diminuées après traitement à la NAC, alors que celles des autres constituants examinés restaient inchangées. Ces données, associées à des résultats rapportés antérieurement concernant l’influence du traitement à la NAC sur le nombre et la fonction des diverses cellules du lavage broncho-alvéolaire, suggèrent que la NAC orale peut influencer l’activité des cellules “inflammatoires” dans l’espace broncho-alvéolaire des individus fumeurs.