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BRIEF COMMUNICATION

Effect of N-acetyl-L-cysteine on Cytokine Production by Human Peripheral Blood Mononuclear Cells

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تأثير N - أسيتايل L - سيستين في إنتاج السايتوكين من قبل الخلايا وحيدة النواة في دم الإنسان المحيطي

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الملخص: الهدف: هذه الدراسة المختبرية تبحث في تأثير الأدوية المضادة للأكسدة (N - أسيتايل L - سيستين) بإنتاج السايتوكين من قبل الخلايا وحيدة النواة في دم الإنسان المحيطي . **الطريقة:** تم الحصول على الخلايا وحيدة النواة بواسطة فيكول هايبيك وحفزت بمضادات الأجسام من نوع (CD3). والراصَّةُ الدَمَوِيَّةُ النَباتِيَّة . وعَديدُ السَّكاريدِ الشَّحمِيّ لمدة 24 ساعة مع وبدون 5 مل من N - أسيتايل - L سيستين . تم قياس السايتوكين المنتج بواسطة المُقايَسَةُ الامْتِصاصِيَّةُ المَناعِيَّةُ للإنْزِيمِ المُرْتَبِط. **النتائج:** إضافة N - أسيتايل - L سيستين زاد من إنتاج ((IL-1β, IL-5, IFN-γ بينما قلل من إنتاج (IL-10) . بعد إضافة مضادات الأجسام (CD3) أو الراصَّةُ الدَمَوِيَّةُ النَباتِيَّة بشكل معتد إحصائيا . لكن ليس بعد تحفيز عَديدُ السَّكاريدِ الشَّحمِيّ . كذلك يزيدN - أسيتايل - L سيستين بشكل معتد إحصائيا إفراز مجموع (IL-12) بعد إضافة مضادات الأجسام (CD3) ولكن ليس بتحفيز الراصَّةُ الدَمَوِيَّةُ النَباتِيَّة أو عَديدُ السَّكاريدِ الشَّحمِيّ . و (IL-12p40) بعد تحفيز مضادات (CD3) . والراصَّةُ الدَمَوِيَّةُ النَباتِيَّة وعَديدُ السَّكاريدِ الشَّحمِيّ . **الخلاصة:** هذه النتائج تشير إلى أن N - أسيتايل L - سيستين يزيد إنتاج السايتوكين ما قبل الالتهابي . ويقلل إنتاج السايتوكين المضاد للالتهاب من قبل الخلايا وحيدة النواة في دم الإنسان المحيطي . بوسيلة ربما تكون مرتبطة بزيادة مستويات الجلوتاثايون . المطلوب مزيدا من البحث لتبيان فيما إذا كانت زيادة أو قلة انتاج السايتوكين كانت بسبب التأثير المباشر ل N - أسيتايل L - سيستين .

مفتاح الكلمات: مضاد الأكسدة. N - أسيتايل L - سيستين. سايتوكين. خلايا وحيدة النواة في دم الإنسان المحيطي.

Objectives: This study investigates the in vitro effect of the antioxidant drug, N-acetyl-L-cysteine (NAC), on cytokine production by peripheral blood mononuclear cells (PBMC). ***Methods:*** PBMC were isolated by Ficoll-Hypaque, and stimulated with anti-CD3 antibodies, phytohaemagglutinin (PHA), lipopolysaccharide (LPS) for 24 hours in the presence or absence of 5 mM NAC. The cytokines produced were measured by enzyme-linked immunosorbent assay (ELISA). ***Results:*** Treatment with NAC significantly up-regulates the secretion of IL-1β, IL-5 (interleukin) and IFN-γ (interferon) and down regulates IL-10 production, after anti-CD3 or PHA (p<0.05), but not after LPS stimulation. NAC also significantly increased total IL-12 secretion after anti-CD3 (but not PHA or LPS) stimulation and IL-12p40 after anti-CD3, PHA, and LPS stimulation (p <0.05). ***Conclusion:*** These results indicate that NAC up-regulated the production of pro-inflammatory cytokines, and down regulated anti-inflammatory cytokine production by PBMC, in a process which may be associated with increased levels of glutathione (GSH). Further work is required to determine whether this increase or decrease in cytokine production is due to direct effect of NAC.

Key words: Antioxidant; N-acetylcysteine; Cytokines; Peripheral blood mononuclear cells.

N-acetyl-L-cysteine (NAC) is a well-known medication which is commonly used as a mucolytic agent in a variety of respiratory illnesses, in the treatment of paracetemol overdose, and in conditions characterised by decreased antioxidants or oxidative stress such as HIV infection, cancer, heart disease and cigarette smoking. This drug works as a free radical scavenger, as a glutathione (GSH) precursor, or as structural analogue of GSH, enriching the GSH pool in the cellular environment. The beneficial effects of NAC are attributed either to its ability to reduce extracellular cysteine to cysteine, or as a source of sulfhydryl (SH) metabolites, which are important for GSH synthesis.[1,2]

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There are limited studies investigating the effects of NAC on cytokine production by peripheral blood mononuclear cells (PBMC). A study by Viora et al., using PBMC, demonstrated that NAC up-regulated the secretion of interleukin, IL-1β, IL-2, IL-12, and IL-15 after phytohaemagglutinin (PHA) stimulation.[3] Several reports have shown that NAC treatment induced a significant up-regulation of IL-12 production by isolated monocytes.[4,5] In addition, pre-treatment with NAC reduced lipopolysaccharide (LPS)-mediated secretion of TNF-α (tumour necrosis factor) and IL-6 by human alveolar macrophages[6] and by rat epithelial cells.[7]

Based on the hypothesis that antioxidant drugs such as NAC can act as modulators of the immune response and can be used in antioxidant therapy in many human diseases with oxidative imbalance,[1,2] we studied the *in vitro* effect of NAC (as an active antioxidant agent) on the production of some of pro/anti-inflammatory cytokines by peripheral blood mononuclear cells isolated from normal healthy individuals. Also, we examined whether NAC can influence several cell-activation pathways using different stimuli.

METHODS

Peripheral blood (5ml) was collected in sodium heparin-containing tubes from 14 normal volunteers (10 males and 4 females, aged between 23-37 years) at the College of Medicine and Health Sciences, Sultan Qaboos University (SQU), Muscat, Oman. All the volunteers provided informed consent, and completed a questionnaire to assess their health status. The study was approved by the Research Committee of the College of science Medicine at SQU.

PBMC were isolated by Ficoll density gradient centrifugation (Sigma Diagnostics, USA) following the manufacturer's instructions. Buffy coats were harvested and cells were washed twice (centrifuged at 300g for 5 minutes) in Hanks solution (GIBCO, Paisley, UK) and resuspended in an RPMI-1640 medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum (FCS), (Sigma Diagnostics, USA), L-glutamine and penicillin/streptomycin (GIBCO, USA, UK) (complete medium).

The antioxidant compound NAC (Sigma Diagnostics, USA) was dissolved in RPMI1640, the pH was adjusted to 7.4 by addition of sodium hydroxide (NaOH), and used at final concentration of 5 mM, after addition to cell suspension. The trypan blue dye exclusion test was performed in order to exclude any drug toxicity.

All the materials for this assay were purchased from Sigma Diagnostics (USA). Total glutathione (reduced and oxidized GSH) was measured in PBMC lysates after 24 hour incubation with 5 mM NAC. Cells were lysed in 2ml of 0.05% v/v Triton- X-100 in buffer (125 mM sodium phosphate, 6.3 mM sodium ethylenediamine tetra acetic acid (Na-EDTA), adjusted to pH 7.5). The samples were assayed by enzymatic recycling procedure as described previously.[8]

PBMC were resuspended at concentration of 1 x 10^6 cell/ml (for IL-1β, IL-1ra, IL-10, IL-12p40, and IFN-γ) or 5 x 10^6 cell/ml (for IL-5 and IL-12) in complete medium, and cultured in 24-well plates (1ml/well). Cells were stimulated with mouse anti-human CD3 (Serotec, UK, MCA 463XZ; 10μg/ml), PHA, Sigma Diagnostics; 10μg/ml), or lipopolysaccharide (LPS, *Escherichia coli* serotype 055:B5, Sigma Diagnostics; 200ng/ml), in the presence or absence of 5 mM NAC. Cells were incubated for 24 hr at 37°C / 5% CO_2. Supernatants were harvested and the cytokine content (except for IL-1β) was measured using commercially available sandwich enzyme-linked immunosorbent assays (ELISA) (R&D Systems, USA), performed according to the manufacturer's instructions.

Monocytes were isolated by an adherence method as previously described.[9] The plastic-adherent cells were cultured for 24 hours in a complete medium containing 100 ng/ml LPS in the presence or absence of 5 mM NAC.

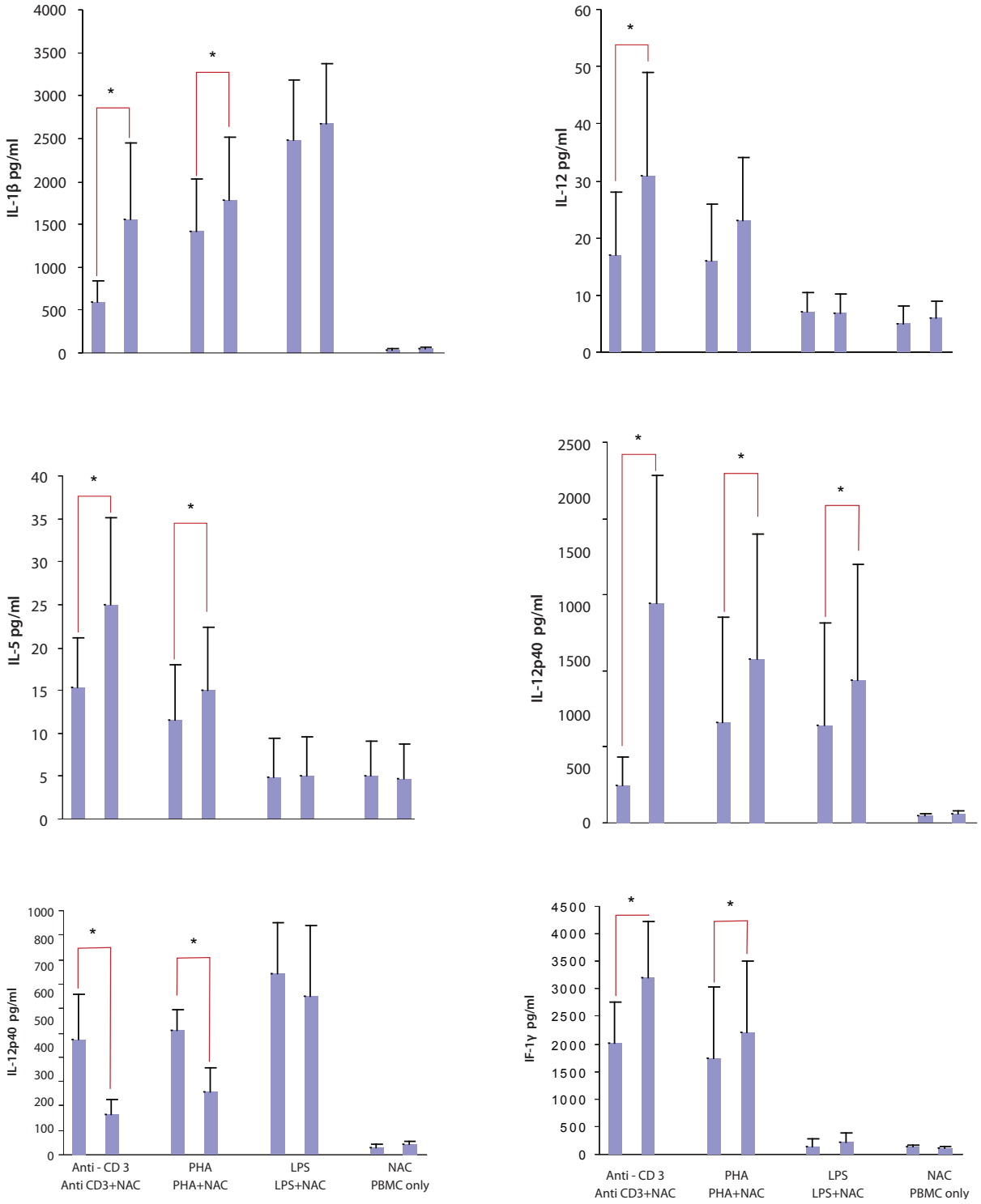
A sandwich ELISA was developed in our laboratory using a method previously described.[10] ELISA was performed to determine the production of IL-1β in the supernatant of the cell cultures (PBMC and isolated monocytes).

The SPSS (Statistical Package for the Social Sciences) software programme was used for data analysis. As all the data were normally distributed, pair-wise group (NAC-treated cells and un-treated cells) comparison was carried out using Student's t-test. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Incubation of PBMC with 5mM NAC for 24 hours resulted in a two-fold increase in total GSH levels in all treated samples. The range of GSH concentration was 0.56 - 0.85 and 1.2 - 1.5nmol/10^6 cells before and after treatment with 5mM NAC respectively.

As shown in Figure 1, NAC induced significant secretion of IL-1β after anti-CD3 and PHA stimula-



IL = interleukin; NAC = N-acetyl-L-cysteine ; PHA = phytohaemagglutinin; LPS = lipopolysaccharide ; PBMC = peripheral blood mononuclear cells

Figure 1: N-acetyl-L-cysteine (NAC) modulates cytokine production. Anti-CD3/PHA/LPS-stimulated peripheral blood mononuclear cells (PBMC) were treated with 5 mM NAC for 24 hr. Results are expressed aspg/ml. Data presents the mean value ± S.D. of at least six experiments for six different blood volunteers. * = Significant *p* value <0.05.

tion (p <0.05), and statistically insignificant secretion after LPS stimulation. Incubation of plastic-adhered monocytes with NAC in the presence of LPS resulted in increased IL-1β production. Although this increase is not statistically significant, it was a consistent in all five experiments (p value = 0.061).

Low concentrations of IL-5 were detected after stimulation of PBMC with anti-CD3 (mean value of 15pg/ml), PHA, (12pg/ml) and LPS (5pg/ml) when compared to other cytokines. In the presence of NAC, significant IL-5 secretion was further increased after anti-CD3 or PHA (but not with LPS) stimulation, this achieved statistical significant (p <0.05) [Figure 1].

NAC had an inhibitory effect on IL-10 secretion by PBMC. In the presence of NAC, a significant decrease (p <0.05) in IL-10 production was observed after anti-CD3, and PHA stimulation, but not after LPS stimulation. IL-10 production, following LPS stimulation in the absence or presence of NAC, was twice the concentration achieved after anti-CD3 or PHA stimulation [Figure 1].

The levels of IL-12 obtained after all three stimulants were low, similar to those of IL-5. However, stimulation with anti-CD3 or PHA induced better IL-12 secretion than with LPS. The addition of NAC caused a significant increase in IL-12 production after anti-CD3 (p <0.05); a slight increase was observed after PHA stimulation, but this was not significant. There was no significant change in Il-12 levels after LPS stimulation [Figure 1].

Unlike total IL-12, stimulation of PBMC with anti-CD3, PHA, or LPS induced substantial amounts of IL-12p40. The presence of NAC with all three stimulants elicited higher levels (p <0.05) of IL-12p40. This was highest after anti-CD3 stimulation [Figure 1].

Stimulation of PBMC with anti-CD3 or PHA (but not LPS), induced high levels of IFN-γ, compared to un-stimulated cells. LPS (100 ng/ml) failed to induce a significant amount of IFN-γ. As shown in Figure 1, exposure of PBMC to NAC elicited a significant up-regulation (p <0.05) of IFN-γ after anti-CD3 or PHA stimulation and a slight increase after LPS stimulation.

DISCUSSION

NAC significantly up-regulated IFN-γ, IL-1β, IL-5, IL-12, and IL-12p40, and significantly down-regulated IL-10 production by PBMC after cellular stimulation. A number of studies investigated the effect of both NAC on cytokine production by several cell types including alveolar epithelial cells,[4] alveolar macrophages,[11] and PBMC.[3] Some of our results (increase in IL-1β and decrease in IL-10 production) are in partial agreement with a study carried out Viora et al. which demonstrated a significant up-regulation of IL-1β, IL-2, IL-12 and IL-15 production and an insignificant increase in IL-10 and IFN-γ by PHA-stimulated PBMC in presence of NAC.[3] This discrepancy in the production of IL-10, may be related to the genetic variation among individuals, hence, immense differences in response to various stimulations and cytokine production was observed.

T cells and monocytes are major sources of inflammatory mediators and can communicate with each other as well as with other cells[12] This communication is mediated by soluble factors and by direct cell-cell contact.[12] As traditionally known, potent cytokine polypeptides have pleiotropic activities and functional redundancy, acting in a complex intermingled network where one cytokine can influence the production of, and response to, many other cytokines.[13] For example, Th1 cells release IFN-γ, and to a lesser extent IL-2 and IL-12, promoting a proinflammatory response. Th2 cells secrete IL-4, and sometimes IL-5, IL-6, IL-10 and IL-13, and enhance anti-inflammatory responses.[14] Therefore, we were not able to prove that the decrease in IL-10 or the increase of other inflammatory cytokines in our study is due to the direct effect of NAC.

Moreover, our data showed that an increase in IL-1β production after anti-CD3 activation, is most likely due to the effect of T lymphocytes activation in this system.

The effects of NAC on cytokine production are still controversial. On the one hand, several studies (including this study), showed that, NAC up-regulated proinflammatory cytokines.[3] On the other hand, some studies reported that NAC down regulated pro-inflammatory cytokines such IL-6, TNF-α IL-1β, and IL-8.[7,15,16] In addition, Stanislaus et al. reported that NAC treatment blocked induction of TNF-α, IL-1β, and IFN-γ.[17] From the above discussion, it appears that, the effect of NAC on cytokine production depends on cell types.

CONCLUSION

NAC up-regulates the production of pro-inflammatory cytokines and down regulates anti-inflammatory

cytokines by PBMC, in a process associated with increased levels of GSH. Also this study indicated a side effect of NAC when used as an anti-inflammatory drug. Further studies are required to confirm whether this increase or decrease in cytokine production is due to a direct effect of NAC.

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CONFLICT OF INTEREST: None declared

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