Eur J Clin Pharmacol (1996) 50 : 457–461

PHARMACODYNAMICS

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Effect of *N*-acetylcysteine(NAC) treatment on HIV-1 infection: a double-blind placebo-controlled trial

Received: 25 October 1995/Accepted in revised form: 15 February 1996

Abstract *Objective*: In a double-blind placebo-controlled trial, human immunodeficiency virus (HIV)-seropositive patients with a CD4 lymphocyte cell count of more than $200 \times 10^6 \cdot l^{-1}$ were randomised to receive either 800 mg N-acetylcysteine (NAC) or placebo for 4 months. Before treatment low plasma cysteine levels, high free radical activity in neutrophils in the presence of autologous plasma – measured by the nitroblue tetrazolium (NBT) test – and increased tumor necrosis factor (TNF)-α levels were found in the HIV positive patients.
Results: After treatment the low plasma cysteine level in the NAC group increased to normal, and the decline of the CD4+ lymphocyte count before the study start, was less steep in the NAC group than in the placebo group after treatment. There was also a reduction in TNF-α level. However, NAC had no effect on the radical production by neutrophils, and although it did not increase the CD4+ cell count, it may have decreased the decline in CD4+ cells.
Conclusion: Further controlled trials with NAC are needed to devermine whether it has a beneficial effect in the treatment of asymptomatic HIV-infected individuals.

Key words N-acetylcysteine treatment, HIV-1 infection; oxidative stress, free radical generation

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Introduction

Human immunodeficiency (HIV)-infected patients are under continuous oxidative stress [1]. Early in the course of the infection, neutrophilic granulocytes [2, 3] and other phagocytes [3] release an increased amount of free oxygen radicals. As a consequence of the increased radical release, there is already in the early stage of the HIV infection increased lipid peroxidation, with an increased content of plasma malondialdehyde [4]. These radicals might also destroy or alter the function of different cells [5], including lymphocytes [6]. Furthermore, substances which protect the organism against radicals are apparently consumed during the HIV infection and are found in low amounts, such as selenium [7], vitamins A, E [8] and C [9], glutathione [10], and cysteine [11]. Oxidative stress has previously been shown to increase the amount of HIV in vitro [26]. In the long-lasting HIV infection, where oxidative stress seems to be present, an increase in cysteine and glutathione could be beneficial for the patients optimal defence against free radicals [12, 13]. As *N*-acetylcysteine (NAC), a thiol-containing compound, is known as a free radical scavenger, working either directly [14] or as a source of cysteine and glutathione [15], we decided to use NAC therapeutically with HIV-infected patients.

Patients and methods

Patients

A total of 45 HIV-seropositive individuals (40 men and 5 women), with CD4+ cell counts of over $200 \times 10^6 \cdot l^{-1}$, were included (mean age 36.9, range 22–65 y). Of the patients, 32 were homosexuals, eight had been heterosexually infected and five were former drug addicts. None of the patients had been treated with an antiretroviral drug. All patients were examined and entered the study within a 6-week period. Thirty-two of the patients were classified as belonging to Centers for Disease Control (CDC) group II and 13 patients to CDC group III. Early morning blood samples were taken from

each patient for all bio-chemical analysis. Sixteen non-HIV-infected controls, who were matched for age and sex, were tested in the same manner as were the patients. Both subjects and controls gave their informed consent for the study. The study protocol was approved of by the Ethics Committee, Karolinska Hospital.

Study design

The study was randomised and performed according to a double-blind placebo-controlled design. The patients were randomised to *N*-acetylcysteine (NAC) effervescent tablets 200 mg (KABI Pharmacia AB), two tablets twice daily, or placebo for 4 months.

Plasma analysis of thiols

Determination of total (reduced and oxidised) plasma concentration of cysteine and glutathione was performed according to the method by Johansson and Lenngren [16]. The method is specific and has a detection limit of 0.3 $\mu mol \cdot l^{-1}$ and a precision of 14%.

Nitroblue tetrazolium (NBT) test

The superoxide anion, an oxygen radical formed by the neutrophil, has the ability to reduce the almost colourless NBT to dark-blue formazan. The neutrophils are mixed with the NBT, with or without plasma. During 30 min incubation at 37 °C the formazan is formed in an amount that correlates with the amount of superoxide anions formed. The formazan can be quantitated spectrophotometrically [17, 18]. The effect of plasma from HIV-positive patients on neutrophils from six healthy non-HIV-infected donors was studied. The donors of the neutrophils had not been included in the control group of the main study . Plasma samples from three HIV-positive patients were frozen in −70 °C as was plasma from the donors. The three patients were selected firstly because their neutrophils exhibited a high NBT reduction and because a sufficient amount of plasma was available. All plasma samples were later used in NBT tests with neutrophils from the six donors. For each donor the result of the NBT test with autologous plasma as well as those with the plasma from each of the three HIV-positive patients was recorded (Table 1).

T-lymphocyte subset analyses

Blood samples were processed on the day of collection and tested as described previously [19].

Analysis of TNF-α

TNF-α was determined by an immunoradiometric assay (TNF-α-IRMA, Medigenic Diagnostics, Brussels, Belgium) as described previously [20].

Statistics

Statistical analysis was perfomed using Student's *t*-test and/or Wilcoxon´s rank sum test for continuous data. Multivariate regression analysis together with analysis of variance was used to study the linear regression with CD4+ cell count on time, prior to study start and after initiating the treatment. A P value < 0.05 was considered significant.

Results

Pretreatment conditions

Of the 45 patients, 37 completed the study. Poor compliance (six patients) and side effects (two patients), such as gastrointestinal disturbances, were the reasons for exclusion. Three of these eight patients took less than 75% of the drug amount and the other three were lost to follow-up for unknown reasons. An intention to treat analysis was done, including all patients randomised into the two groups . No differences as to symtoms, signs and CD4+ cell counts were observed between the groups. Before treatment, spontaneous NBT reduction of the neutrophils with autologous plasma was significantly higher in the HIV-infected patients than those of the controls ($P < 0.001$) (Fig. 1). Such an increase of the NBT reduction was not seen when the experiments were performed in the absence of plasma. Furthermore, plasma from each of three HIV-positive patients with high NBT values, induced a considerably higher NBT reduction in neutrophils from the six healthy donors than did autologous plasma (Table 1) ($P < 0.002$, 0.002, 0.001, respectively). The plasma cysteine levels of the HIV infected patients were lower than those of the controls ($P < 0.05$) (Table 2), while the TNF-α value was elevated in 50% of the HIV-infected patients compared to the upper normal limit (12.6 $pg \cdot ml^{-1}$, Medigenic Diagnostics); data not

Table 1 Influence of HIV plasma on the NBT reduction of neutrophils from six healthy donors

	Results of NBT test with autologous plasma	Results of NBT test with plasma from patient 1	Results of NBT test with plasma from patient 2	Results of NBT test with plasma from patient 3
Donor 1	0.17	0.45	0.51	0.35
Donor 2	0.27	0.58	0.62	0.57
Donor 3	0.56	1.06	1.30	0.88
Donor 4	0.21	0.51	0.70	0.38
Donor 5	0.39	0.96	1.02	0.76
Donor 6	0.73	1.42	1.66	0.90
Mean (SD)	0.39 (0.22)	0.83 (0.38)	0.97 (0.45)	0.64 (0.24)
P values vs controls		$P < 0.002$	$P < 0.002$	$P < 0.001$

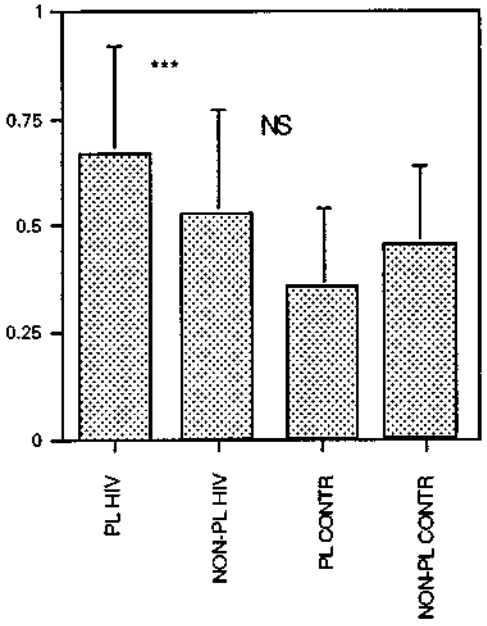

Fig. 1 Values of NBT reduction are means before treatment with NAC. *PL*, *NON-PL*, *HIV* and *CONTR* indicate with plasma, without plasma, HIV-infected patients and controls, respectively. *** indicates HIV+ vs controls, $P < 0.001$ and *NS* non-significant

shown. No significant difference was observed in the levels of glutathione and glutathione peroxidase between the HIV-positive patients and the controls.

Effect of treatment

A significant rise in the plasma cysteine level of the treated patients to the same level as that of the controls was seen after 4 months of treatment, while the corresponding level of the placebo group remained low (Table 2). As to the CD4+ lymphocyte cell counts (Table 2), no increase was observed in the NAC group. A multiple regression analysis of the CD4+ cells, as described previously [21], together with analysis of variance, did show a linear decline in both groups before study start with a median follow-up time of 36 months (range 1–50 months). However, a significant difference in the steepness of the slopes in the CD4+ cell losses was noted ($P < 0.05$), comparing the two groups during and immediately after the study. The NAC-treated patients showed a less steep slope than did the untreated patients. On treatment analysis of the 37 patients who completed the study further strenghtened this difference in steepness ($P < 0.005$). The TNF-α mean values were decreased in both groups. When on treatment analysis was performed on the 37 patients who completed the study, a significant decrease was found in the NAC group (Table 3). No significant change was seen in the NBT reduction of neutrophils in the presence of autologous plasma, or plasma levels of glutathione or glutathione peroxidase in thrombocytes.

After treatment the NBT reduction of neutrophils in the presence of autologous plasma was still

Table 2 NBT reduction of neutrophils and cysteine in HIV-infected patients (means and 95% confidence interval) before and after treatment with NAC or placebo

	Before treatment			After treatment		
	NAC	Placebo	Controls	NAC	Placebo	Controls
NBT reduction with plasma (OD)	0.69[a] (0.55–0.83)	0.62[a] (0.53–0.76)	0.36 (0.25–0.47)	0.69[b] (0.55–0.82)	0.60[b] (0.40–0.76)	0.34 (0.27–0.41)
Cysteine ($\mu mol \cdot 1^{-1}$)	7.96[c] (6.94–8.43)	7.38 (6.50–8.26)	9.36 (8.48–10.25)	10.60[d] (9.22–11.80)	7.74 (6.95–8.52)	9.81 (8.90–10.72

[a] $P < 0.001$ compared with controls
[b] $P < 0.001$ compared with controls
[c] $P < 0.05$ compared with controls
[d] $P < 0.005$ compared with before NAC treatment

Table 3 TNF-α values CD4+ and CD8+ counts (means and 95% confidence intervals) in HIV-infected patients before and after treatment with NAC or placebo.

	Before treatment		After treatment	
	NAC	Placebo	NAC	Placebo
TNF-α (pg/ml)	31.15 (17.18–45.12)	24.05 (12.88–35.81)	17.95[a] (9.27–26.63)	16.57 (7.46–25–67)
CD4+ ($10^9 l^{-1}$)	0.39 (0.34–0.45)	0.47 (0.40–0.55)	0.39 (0.29–0.49)	0.42 (0.34–0.50)
CD8+ ($10^9 l^{-1}$)	0.82 (0.58–1.08)	0.96 (0.67–1.25)	0.89 (0.66–1.13	0.92 (0.57–1.24)

[a] $P < 0.05$ compared with before treatment with NAC

significantly higher ($P < 0.001$) than that of the controls. No such difference was observed without plasma.

Discussion

In the present study, high oxygen radical production by neutrophils, in the presence but not in the absence of plasma, high TNF-α levels and low levels of cysteine in plasma were found in HIV-infected patients with slightly decreased CD4+ lymphocyte counts and no symtoms of HIV infection. The present data confirm earlier findings of increased TNF-α levels in serum [20] and activation of neutrophils to increased radical production [2, 3] in patients in the asymptomatic stage of HIV infection. Our results demonstrate a plasma-mediated stimulation of radical production of neutrophils in asymptomatic HIV-positive patients. This stimulation could be reproduced in vitro as plasma from HIV-positive patients with high NBT activity was allowed to act upon neutrophils from healthy donors. The plasma factor might consist of TNF-α, which is known to activate the neutrophils to an increased production of oxygen radicals [22, 23].

The low level of plasma cysteine is likely to be due to an increased stress on the defence mechanism against oxygen radicals, e.g. the glutathione, glutathione peroxidase system. Cysteine is a precursor of glutathione. The increased radical production in HIV-infected patients could lead to an increased lipid peroxidation [4] and an increased consumption of glutathione, cysteine [10] and other antioxidants [7–9]. These processes, which continue for several years in HIV- infected patients, are most likely to contribute to the tissue damage, cancer development and premature ageing seen in the AIDS stage. *N*-Acetylcysteine was primarily chosen to increase the levels of cysteine and glutathione, which both have an antioxidant effect.

The plasma levels of cysteine were normalised with NAC. Also the increased TNF-α levels in plasma were slightly decreased in the NAC group compared to the placebo group, while the decline in CD4+ lymphocytes was less evident. The slope of the CD4+ cells was less steep after treatment than before in the NAC group compared to the placebo group.

In spite of the treatment, however, the radical production of the neutrophils was unchanged. Probably the NAC treatment did not elevate the levels of glutathione and cysteine within the neutrophils. According to Dröge et al [24], a decrease in cysteine concentrations during HIV infection is responsible for the dysfunction of the lymphocytes. Also with flow cytometric techniques, an increase in intralymphocytic glutathione concentration has been observed in HIV-infected patients treated with oral NAC in a higher dosage than in this study [25].

Such high glutathione concentrations within lymphocytes together with the normalised concentrations of cysteine extracellularly seen in this study might protect the cell membranes of the lymphocytes against the radicals. The slightly decreased TNF-α level might be explained by a decreased HIV replication, as NAC is known to inhibit in vitro the lymphocyte transcription factor NF-kB [26].

NAC in a dose of 800 mg per day for 4 months was well tolerated and had no effect on the plasma-induced radical production by the neutrophils, but managed to keep the number of CD4+ lymphocytes unchanged and abated the slope of the CD4+ cell loss. NAC was also associated with a slight decrease in the TNF-α level.

This non-toxic drug, used in an even higher daily dosage than the one used in this study, and for a longer period of time, is a candidate for further controlled studies, to ascertain whether the drug has any beneficial effect on the course of the HIV infection.

Acknowledgements This study was supported by the Swedish Medical Council Grant No. 1086, Swedish Physicians Against AIDS Research Foundation and KABI Pharmacia AB, Sweden. Special thanks are due to Ms Margit Halvarson and Ms Elisabeth Eklund for their excellent help with patients and blood samples and to statistician Jan Kowalski, Karolinska Institute, and Dr Christer Lidman, Huddinge Hospital, for their assistance with the statistics.

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