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Improvement of immune functions in HIV infection by sulfur supplementation: Two randomized trials

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Abstract To determine the therapeutic effect of sulfur amino acid supplementation in HIV infection we randomized 40 patients with antiretroviral therapy (ART; study 1) and 29 patients without ART (study 2) to treatment for 7 months with *N*-acetyl-cysteine or placebo at an individually adjusted dose according to a defined scheme. The main outcome measures were the change in immunological parameters including natural killer (NK) cell and T cell functions and the viral load. Both studies showed consistently that *N*-acetyl-cysteine causes a marked increase in immunological functions and plasma albumin concentrations. The effect of *N*-acetyl-cysteine on the viral load, in contrast, was not consistent and may warrant further studies. Our findings suggest that the impairment of immunological functions in HIV⁺ patients results at least partly from cysteine deficiency. Because immune reconstitution is a widely accepted aim of HIV treatment, *N*-acetyl-cysteine treatment may be recommended for patients with and without ART. Our previous

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report on the massive loss of sulfur in HIV-infected subjects and the present demonstration of the immunoreconstituting effect of cysteine supplementation indicate that the HIV-induced cysteine depletion is a novel mechanism by which a virus destroys the immune defense of the host and escapes immune elimination.

Key words HIV infection · Viral load · Immune reconstitution in HIV infection · NK cell activity · *N*-Acetyl-cysteine

Abbreviations ART: Antiretroviral therapy · GSH: Glutathione · HAART: Highly active antiretroviral therapy including a protease inhibitor · HIV: Human immunodeficiency virus · IL-6: Interleukin-6 · NAC: N-Acetyl-cysteine · NK: Natural killer · PE: Point estimate of shift

Introduction

Previous studies on macaques infected with simian immunodeficiency virus have revealed markedly increased intracellular sulfate and decreased glutathione (GSH) levels in the skeletal muscle tissue, suggesting that this retroviral infection causes an increase in the muscular cysteine catabolism at the expense of the cysteine and GSH pools [1]. In line with this notion, we and others [2, 3, 4, 5, 6, 7] have shown that patients infected with human immunodeficiency virus (HIV) have abnormally low plasma cysteine concentrations and low GSH levels, although some authors have not observed decreased GSH levels in peripheral blood cells [8, 9]. A more recent study revealed that the peripheral tissue of HIV⁺ patients with or without highly active antiretroviral therapy (HAART) releases large amounts of sulfate that would account for a loss of approximately 5 g cysteine per day if extrapolated to a person of approximately 70 kg body weight [10]. A complementary investigation on 64 asymptomatic HIV⁺ patients and 65 HIV⁻ subjects revealed increased plasma sulfate levels in the HIV⁺ patients.

The analysis of the daily urinary excretion of sulfate and urea of HIV⁺ patients and healthy HIV⁻ subjects confirmed (a) that HIV⁺ patients experience a massive loss of sulfur, and (b) that this loss is not ameliorated by (HA)ART. The net loss of sulfur in asymptomatic patients was equivalent to a net loss of about 7 g cysteine per day [10]. If extrapolated, this would correspond to an alarming negative balance of approximately 2 kg cysteine per year under the assumption that the normal sulfate excretion equivalent to approximately 3 g per day is balanced by a standard Western diet. The abnormally high sulfate/urea ratio suggested that this process drains largely the GSH pool [10]. To ameliorate the immunological consequences of the virus-induced cysteine and GSH deficiency in HIV infection we have previously proposed treatment with N-acetyl-cysteine (NAC) [11, 12, 13]. In the meantime, several clinical studies on the effects of NAC have been performed [14, 15, 16, 17, 18, 19]. These results of these studies have in general been promising, but they are controversial because the doses of NAC were relatively low, or the observation period was relatively short [15, 16, 17, 18, 19]. One investigation [14] with relatively high doses suggested that NAC improves the 2-year survival rate, but this investigation was not randomized, and the doses were chosen arbitrarily and may have been too high for some patients, for reasons discussed below. A placebo-controlled randomized study on the effects of NAC on immunological functions and virus load has not been described previously.

Our two randomized studies on HIV⁺ patients with and without ART involved 7 months of treatment with individually adjusted doses of NAC. Outcome measures included NK cell activity and several proliferative T cell responses. These immunological functions are decreased relatively early in HIV infection [20, 21, 22, 23, 24]. Several reports suggest that NK cells play a protective role in HIV infection [25, 26, 27, 28]. Additional outcome measures were viral load, CD4⁺, CD8⁺ and CD16⁺/CD56⁺ cell numbers, body cell mass, plasma albumin, interleukin-6 (IL-6), thioredoxin and glutamine levels. Plasma glutamine is decreased relatively early in HIV infection [29], and a decrease in the extracellular glutamine level impairs lymphocyte functions [30, 31, 32, 33].

Patients and methods

Double-blind randomized clinical trial on the effect of NAC in combination with antiviral therapy (study 1)

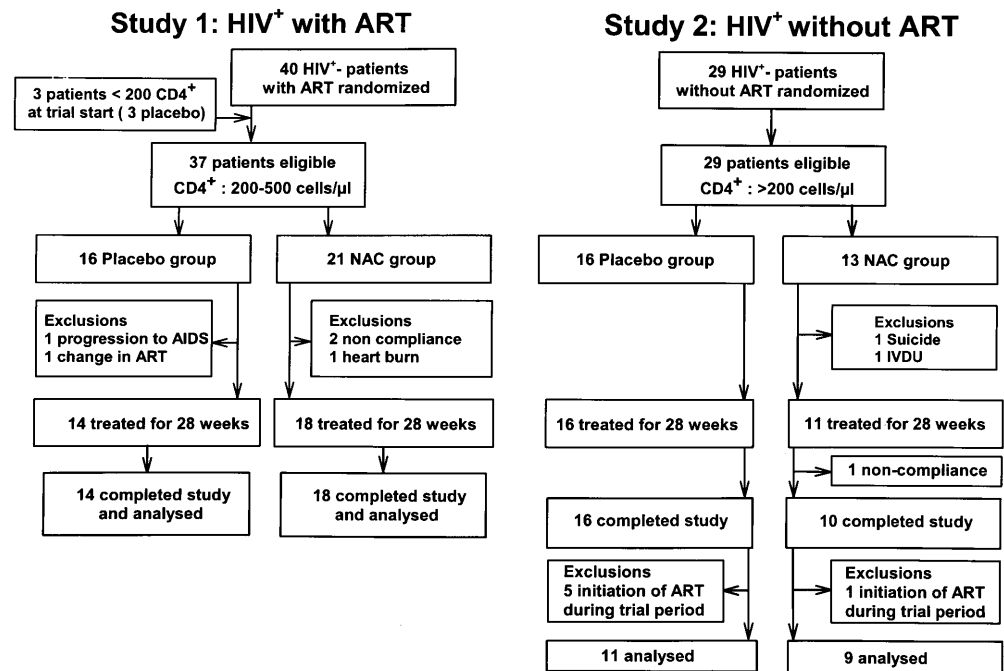
Clinically asymptomatic HIV⁺ patients (CDC I, 1993, age over 18 years) with 200–500 CD4⁺ cells/mm³ were eligible as determined less than 2 months before recruitment. Exclusion criteria were endocrinological diseases, liver cirrhosis, serum creatinine higher than 1.5 mg/dl, cardiorespiratory insufficiency, alcohol or drug abuse. Female patients had to use contraception throughout the study. At study entry the clinical laboratory data were in the normal range. All patients had been under constant ART for at least 6 months before and throughout the study (Table 1). The trial profile is shown in Fig. 1. Block randomization (individual) was performed by the Biostatistics Unit of the German Cancer Research Center (L.E.). The code was known only to L.E. After generation of the assignment by L.E., the patients were recruited, and the blinded assignment was executed by R.B. at the clinical centers in Mannheim. The code for the assignment to one or another group was broken after completion of the last measurement. The code for the assignment to verum vs. placebo was broken after completion of the statistical analysis. The study was approved by the ethics committee of the Medical Faculty of the University of Heidelberg/Mannheim and was conducted according to the guidelines of good clinical and laboratory practice and the principles of the Declaration of Helsinki.

The patients were examined before the start of therapy within 3 days after informed consent and 4 weeks, 4 months, and 7 months later. NAC (600 mg tablets or placebo with same taste) was administered every second day during this period. Empty blisters were returned by the patients to the physicians to confirm compliance. The initial daily dose was 3.6, 2.4, 1.2, and 0.6 g for patients with plasma glutamine levels lower than 450, 450–500, 500–600, and higher than 600 µM, respectively. After the subsequent examinations the dose was either increased by 2.4, 1.8, 1.2, not changed, decreased to half of the previous dose, or decreased to 0.6 g/day if the patients had plasma glutamine levels lower than 450, 450–500, 500–600, 600–700, 700–800, or 800–900 µM, respectively.

Double-blind randomized clinical trial on the effect of NAC without antiviral therapy (study 2)

Eligible were clinically asymptomatic ART-naive HIV⁺ patients (CDC I, 1993) with more than 200 CD4⁺ cells/mm³ as determined less than 2 months before recruitment. For other details see study 1.

Fig. 1 Trial profiles of studies 1 and 2



Sample size

The two studies were originally planned as a single study with two strata defined by different inclusion criteria. A sample size of $n=30$ per treatment group was calculated with the aim to detect a significant difference in the treatment group of the combined strata at the significance level of 5% by a one-tailed paired t test with a power of 80%. In the absence of information about the range and variability of the viral load and immunological parameters in patients with and without NAC treatment, we determined that sample size on the basis of plasma cystine levels such that an increase in the cystine level by $10.0 \mu\text{M}$ after two cycles with a standard deviation of $15 \mu\text{M}$ was detectable. A drop-out rate of 10–20% was assumed. In order to adjust for multiple endpoints of the plasma cystine level and other variables a maximum of 14 comparisons was considered, and the error per experiment was set to $0.05/15=0.0035$ to achieve a multiple significance level of 0.05. The same sample size of $n=30$ was planned for the placebo control.

Physical, biochemical, and functional tests

Plasma amino acids were determined as described [3, 4, 5, 29] using postabsorptive blood samples from the cubital vein. The intra-assay and inter-assay variation ($100 \times \text{SD}/\text{mean}$, $n=20$) of the glutamine assay was 0.83% and 2.80%, respectively. The HIV RNA levels were determined by the HIV-1 QT Nuclisens-test kit (Organon Teknika) [34, 35] and cell types by three-color staining with CD3, CD4, CD8, CD14, CD16, CD19, CD45, and CD56 antibodies (Becton Dickinson/Simulstest) on a FACScan. The proliferative responses against phytohemagglutinin and immobilized CD3 plus CD28 antibodies were determined as described [24] with minor modifications. Specifically, peripheral blood lymphocytes were incubated for 3 days without and another 18 h with [^3H]thymidine prior to harvesting. The response against tetanus toxin (Behring, Marburg, Germany) was determined as described [22] with an incubation period of 7 days plus 18 h. The NK cell activity was determined as described [21]. For comparison and validation of the assays, all patient samples were run in parallel to at least one healthy donor sample on the same day (see following section). Plasma thioredoxin and albumin levels were determined as described [36, 37], and IL-6 was assayed by the IL-6 EASIA test kit (Biosource, Ratingen, Germany) [38].

HIV⁻ control group

NK cell activities, cell types, proliferative activities, plasma amino acids, and albumin levels were determined during the trials also in 87, 56, 74, 107, and 42 HIV⁻ control subjects, respectively (see Fig. 2).

Statistical analysis

With the exception of Table 2, our analysis was carried out “per protocol” (i.e., by “as treated analysis”) to most closely reflect the scientific model underlying the protocol. The baseline data of the drop-outs (Table 1) suggest no bias of this procedure compared with an “intent-to-treat analysis.” The statistical analysis was performed under blinded conditions. The relative changes in outcome parameters of the two different treatment groups were compared by the Wilcoxon rank-sum test (Figs. 4, 5), and individual changes between baseline and terminal examinations in each treatment group were analyzed by the Wilcoxon sign-rank test (Fig. 3). P values <0.05 were regarded as statistically significant. Hodges-Lehmann point estimate shifts (PE) with exact 95% confidence intervals, arithmetic or geometric means, and standard errors of the mean together with graphic box plots were used as descriptive statistics. The “intent-to-treat analysis” of the main parameters is shown in Table 2.

Results

Baseline data from studies 1 and 2 and control data from HIV⁻ subjects

The baseline data of the two studies were comparable in the respective treatment groups before the start of the trial (Table 1). The NK cell activity, stimulation indices, plasma glutamine, and albumin levels from all groups of HIV⁺ patients were abnormally low in comparison with HIV⁻ subjects regardless of whether the patients had been treated with or without ART or with highly active

Table 1 Baseline characteristics of studies 1 and 2 (SI stimulation index)

	Study 1		Study 2	
	NAC group (n=21)	Placebo group (n=16)	NAC group (n=13)	Placebo group (n=16)
Males/females	11/10	10/6	8/5	8/8
Monotherapy with Nucleoside analogs (n)	2 (ddI/AZT)	1 (ddI)	0	0
Two nucleoside analogs (n)	12	10	0	0
HAART (n)	7	5	0	0
Mean age (years)	39.7±2.4	40.1±2.6	32.6±2.3	39.2±2.9
CD4 ⁺ cells (mm ⁻³)	367±32	366±37	503±70	491±56
Virus load (log copies/ml)	4.1±3.62	3.6±3.2	4.7±4.51	4.4±3.9
NK (lytic units per 10 ⁷ cells)	31.6±9.7	58.8±15.6	43.9±11.5	46.6±9.9
NK (lytic units per CD3 ⁻ /CD16 ⁺ /CD56 ⁺ cell)	0.22±0.06	0.43±0.13	0.33±0.13	0.54±0.20
SI αCD3/CD28	364±73*	1045±296*	785±164	568±107
SI phytohemagglutinin	746±137	909±264	779±163	996±138
SI tetanus toxin	25±14	41±18	40±30	35±13
Plasma albumin (μM)	637.7±13.7	650±13.1	573.4±33.9	584.4±25.9
Plasma IL-6 (ng/ml)	44.4±5.9	31.0±5.3	54.1±4.2	64.6±7.0
Plasma glutamine (μM)	568.9±18.6	577.1±16.9	585.5±32.2	570.6±24.0
Plasma arginine (μM)	47.3±3.9	56.1±6.8	59.7±5.8	60.3±4.5
Plasma cystine (μM)	42.0±2.1	41.2±2.9	38.6±2.3	45.7±2.5

**P*=0.03 for the difference between the corresponding NAC and placebo groups

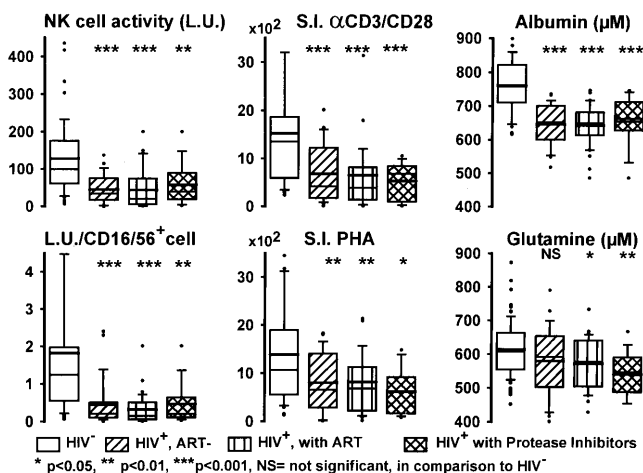


Fig. 2 Baseline characteristics of HIV⁺ persons with and without ART and data from HIV⁻ subjects. The four columns represent the HIV⁻ control group (see text), ART-naïve HIV⁺ persons (baseline data of study 2), patients with ART (baseline data of study 1) and patients from study 1 treated with HAART (i.e., a protease inhibitor plus one or two nucleoside analogs; *n*=12). *Box plot* describe the first (25%) and third (75%) quartile of the distribution, the arithmetic mean (*strong line*) and the median (*thin line*). The NK cell activity is expressed as lytic units per 10⁷ peripheral blood mononuclear cells (*upper left panel*) or as lytic units per CD16⁺/CD56⁺ cell

antiretroviral therapy (HAART) involving at least one protease inhibitor (Fig. 2).

NAC treatment with individually adjusted doses

The plasma glutamine level was used as a guideline against NAC overdosing because an excessive cysteine catabolism in the liver is associated with the production of

protons and may inhibit urea production in favor of glutamine production eventually to the point that the glutamine forming capacity is exceeded, and toxic ammonia accumulates [39]. In pilot investigations on several NAC-treated HIV⁺ patients we occasionally observed abnormally high plasma glutamine levels (>900 μM) and cystine levels (>150 μM: W.D., unpublished observations). As a rule, the plasma glutamine level was found to increase or decrease whenever the dose of NAC was increased or decreased, respectively, as shown by the example in Fig. 3A. We therefore did not give a constant dose of NAC to all patients, but decreased the dose of NAC whenever the plasma glutamine level exceeded 700 μM (see protocol). The mean dose of NAC that was administered according to the defined protocol every second day during the three intervals of the observation periods is shown in Fig. 3B and C and was about 3 g NAC at the end of the trials.

The effects of NAC in combination with or without ART: Reconstitution of immunological and biochemical parameters and effect on viral load

NAC caused a strong consistently increase in all immunological test parameters in both studies (Fig. 4, NK cell activity in study 1: PE 732.8, 95% CI 212–2128; study 2: PE 363.1, 95% CI 64–2185; for units see legend to Fig. 4). The analysis on the basis of “intent-to-treat” revealed in the case of study 1 identical results and in the case of study 2 similar results. Table 2 shows the “intent-to-treat” analysis of the most relevant parameters. The NK cell activity of NAC treated patients increased to almost normal levels (Fig. 3). The changes in CD4⁺ (Table 2) and CD8⁺ cell numbers and plasma thioredoxin levels (not shown), in contrast, did not differ significantly between the treatment groups in the two studies.

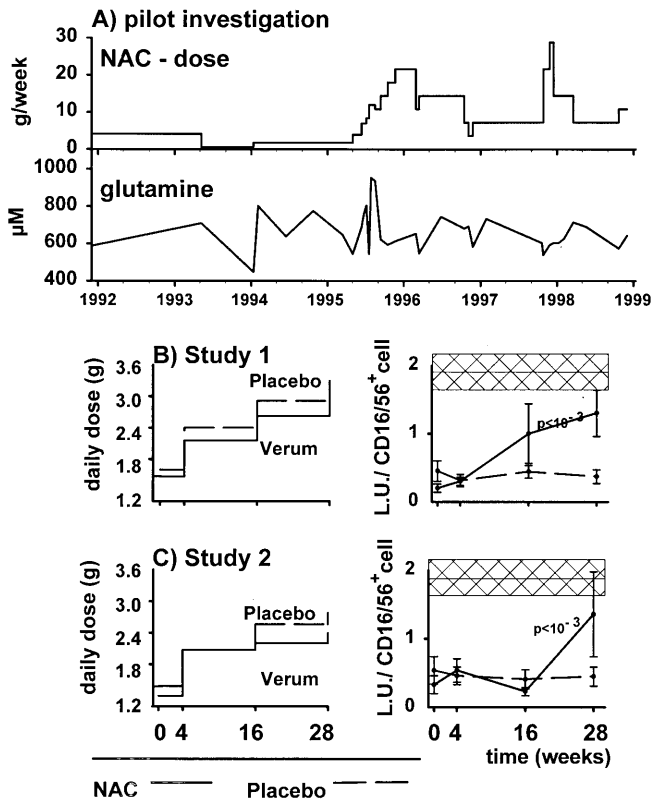


Fig. 3A–C Longitudinal changes in HIV-infected individuals during a NAC treatment. **A** Pilot investigation. A 50-year-old male HIV-infected healthcare worker with a CD4⁺ cell count of 175/mm³ and without clinical symptoms started to take NAC (0.6 g/day) in 1991. As a rule, the patient received the minimum dose of NAC to maintain plasma glutamine at a level higher than 600 µM (i.e., the mean of healthy subjects). From 15 December 1995 the patient also received ART. At the time of submission of this report, the patient is still symptom-free. **B** Study 1. *Left* the means of the doses of NAC or placebo that were administered every second day according to the treatment scheme. The NK cell activity is given as lytic units per CD3⁺/CD16⁺/CD56⁺ cell. *Error bars* SEM; *shaded area* mean \pm SEM of HIV⁻ persons (see description of HIV⁻ control group in text). *P* values refer to differences between terminal and baseline examination. **C** Study 2. For details see **B**

The placebo group of study 1, i.e., patients who received stable ART for more than 6 months before plus 7 months throughout the study showed on average a significant increase in viral load during the observation period ($P=0.01$ for the longitudinal change within the group) indicative of ART failure (Fig. 5, Table 2). This increase was not seen in the NAC-treated group (PE 152.0, 95% CI -22.7 to -432.1 , $P=0.014$ for the comparison between treatment groups). However, in view of the differences between the mean virus loads of the NAC group and the placebo group at baseline examination, the differential increase in virus load may be explained by the higher baseline level in the NAC-treated group rather than by the effect of NAC (Table 2). Also, there was no significant effect of NAC on the virus load in study 2.

Finally, NAC increased the mean plasma glutamine level (PE 16.0, 95% CI 6.0–26.0, $P<0.01$, data not

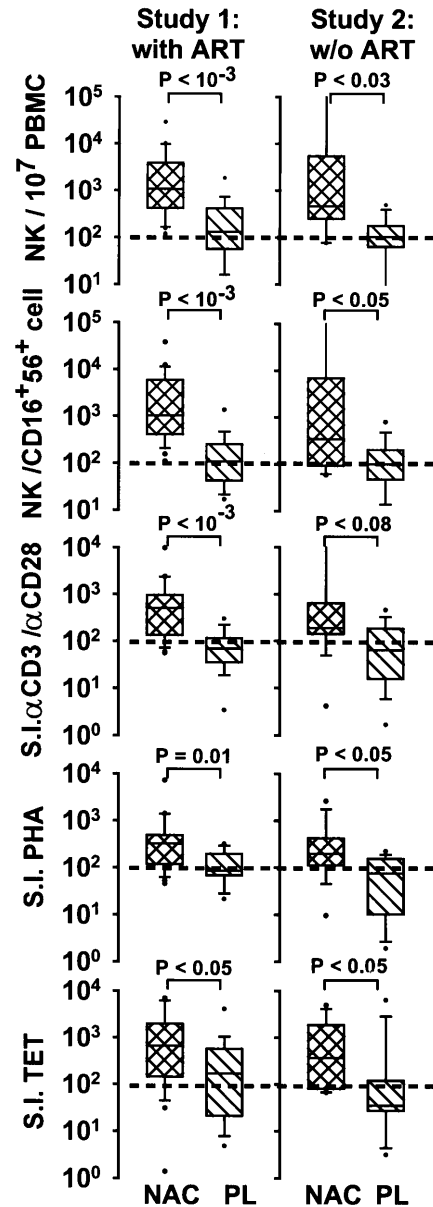
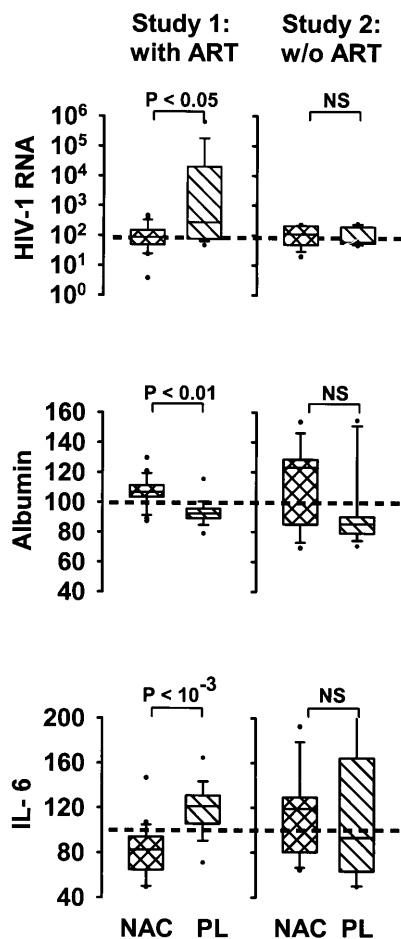


Fig. 4 Effect of NAC on the relative changes in NK cell activity and T cell stimulation indices. Median and box plots of the relative changes in NK cell activity per 10^7 peripheral blood mononuclear cells, NK cell activity per CD3⁺/CD16⁺/CD56⁺ cell, and the relative changes in the stimulation indices in response to immobilized CD3 plus CD28 antibodies, phytohemagglutinin (PHA) and tetanus toxin (TET) from single individuals at the last examination (i.e., after 7 months of therapy) expressed as percentage of the corresponding individual baseline values. *P* values are given for the difference between the NAC and placebo group. *Dashed line* no change (100%). The effects of NAC were not detectable after 4 weeks but were already visible after 4 months of therapy (not shown)

shown) and reversed the decrease in plasma albumin (PE 14.3, 95% CI 9.15–19.43) and the increase in IL-6 (PE -38.7 , 95% CI -21.8 to -56.3) in the ART-treated patients (Fig. 5, study 1). These effects were not statistically significant in study 2, but the increase in plasma albumin and decrease in IL-6 were significant ($P<0.001$ and

Table 2 Treatment effects in the two trials by Intent to treat analysis (SI stimulation index)

	Study 1				Study 2			
	NAC		Placebo		NAC		Placebo	
	Baseline	Terminal	Baseline	Terminal	Baseline	Terminal	Baseline	Terminal
NK (Lytic units per CD3 ⁺ /16 ⁺ /56 ⁺ cell)	0.22±0.06	1.34±0.34	0.45±0.15	0.37±0.26	0.33±0.13	1.33±0.56	0.52±0.19	0.47±0.11
SI PHA	747±137	1906±355	967±292	759±131	779±164	1267±301	1022±132	906±221
SI TET	25±14	150±128	43±21	37±23	40±31	190±139	46±17	31±15
Virus load	4.10±3.62	4.08±3.55	2.28±0.51	3.53±0.19	4.17±0.24	3.74±0.24	3.82±0.22	3.40±0.25
CD4 ⁺ cells (mm ⁻³)	367±32	432±53	365±39	421±39	503±71	513±77	476±55	437±42

**Fig. 5** Effect of NAC on the relative changes in virus load, plasma albumin, and plasma IL-6 levels. Median and box plots of the measurements. For other details see legend to Fig. 4

$P < 0.05$, respectively), when both studies were taken together.

There were no adverse events except for two NAC-treated subjects with heart burn in study 1. In one case this disappeared spontaneously. The other patient had to be excluded from the study.

Discussion

Because immune reconstitution is a major goal of HIV therapy, the consistent improvement in several immunological functions in our two randomized studies on NAC strongly suggests that HIV-infected patients with or without ART or HAART should receive NAC treatment. There is an increasing awareness that current antiretroviral drugs can decrease HIV replication and virus load considerably but fail to abolish virus replication completely [40, 41]. A proportion of patients show an increase in HIV RNA levels in spite of ART, i.e., a condition that is commonly interpreted as ART failure [40] (see also study 1). The significant beneficial effects of NAC even in the group of patients who already received (HA)ART were in line with a recent study on the daily urinary sulfate excretion which revealed (a) that HIV⁺ patients experience a massive loss of sulfur, and (b) that this loss is *not* ameliorated by (HA)ART [10]. Moreover, in spite of the indisputable merits of HAART it should be noted that ART without protease inhibitors is still the treatment of choice for many HIV⁺ patients who cannot afford or tolerate HAART.

The immune reconstitution by NAC is also in line with a previous nonrandomized investigation which provided suggestive evidence that NAC treatment may increase the 2-year survival rate [14]. Because the dose was not adjusted to individual needs in this earlier study, we have reason to believe that those results could still be improved considerably. Because the patients require on average rather large doses of NAC, we propose that the individual dose of NAC should be decreased whenever the plasma glutamine (or the albumin level) is in the upper normal range. The effect of NAC on the plasma albumin level (Fig. 5) confirmed earlier findings in cancer patients [37] and is best explained by the fact that the oxidized form of albumin has a faster clearance rate [37]. This interpretation is in agreement with earlier reports that the rate of albumin synthesis was *increased* in several conditions of infection and trauma even when albumin concentrations were *decreased* [42, 43]. Earlier studies on smaller numbers of HIV-infected patients did not show significantly decreased plasma albumin concentrations but increased plasma concentrations and synthetic rates of "positive" acute-phase proteins [43, 44]. Wheth-

er NAC treatment may have a general anti-inflammatory effect and reverse the increase in acute-phase proteins remains to be determined.

Because several studies on HIV⁺ patients and simian immunodeficiency virus infected monkeys collectively suggest that HIV/simian immunodeficiency virus infection may increase the cysteine catabolism and deplete the cyst(e)ine and GSH pools (see "Introduction"), we propose tentatively that the therapeutic effect of NAC reflects the reconstitution of the cysteine deficiency rather than a general immunopotentiating effect of NAC. If so, NAC is exceptional among drugs for the acquired immunodeficiency syndrome. There is a strong possibility that the immune system is among the first to suffer from this excessive loss of cysteine. Because GSH and cysteine have been shown to regulate the activation of the transcription factor nuclear factor- κ B and the nuclear factor- κ B-dependent replication of HIV [45, 46], it may be tempting to speculate that the beneficial effects of NAC are due to the inhibition of nuclear factor- κ B and viral replication. However, we did not see a significant effect of NAC treatment on HIV RNA levels. In view of the decreased GSH levels in peripheral blood mononuclear cells of HIV⁺ patients [3, 14], it may be tempting to explain the immunological effects of NAC by its function as a GSH precursor. This question has not been addressed here. NAC has failed, however, in several earlier studies to increase GSH levels of mononuclear cells in vivo [37, 47, 48, 49] but did increase the GSH level in the liver [50, 51] and erythrocytes [14, 18]. Studies on the venous plasma concentrations of NAC have led others to conclude that the oral bioavailability of NAC is low [19, 52], but these authors obviously dismiss the direct uptake and storage of cysteine by the liver from the portal vein. Magnusson and colleagues [53] reported the deacetylation and the subsequent release of cysteine into the blood plasma by the kidneys after intravenous infusion of NAC. Our placebo-controlled trials of NAC treatment clearly indicate that NAC may be beneficial in the treatment of HIV-infection and may reverse the decrease in immunological reactivity. The HIV-induced excessive loss of cysteine and the reconstitution of immunological functions by NAC indicate, finally, that the virus-induced cysteine deficiency may be a novel mechanism by which a virus destroys the immune defense of the host and avoids its elimination by the immune system.

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