

## N-acetylcysteine replenishes glutathione in HIV infection

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See Commentary on page 841 and paper on page 905.

### Abstract

**Background** Glutathione (GSH) deficiency is common in HIV-infected individuals and is associated with impaired T cell function and impaired survival. N-acetylcysteine (NAC) is used to replenish GSH that has been depleted by acetaminophen overdose. Studies here test oral administration of NAC for safe and effective GSH replenishment in HIV infection.

**Design** Oral NAC administration in a randomized, 8-week double-blind, placebo-controlled trial followed by optional open-label drug for up to 24 weeks.

**Subjects** HIV-infected, low GSH, CD4 T cells  $<500 \mu\text{L}^{-1}$ , no active opportunistic infections or other debilitation;  $n=81$ . Study conducted prior to introduction of protease inhibitors.

**Results** Whole blood GSH levels in NAC arm subjects significantly increased from 0.88 mM to 0.98 mM, bringing GSH levels in NAC-treated subjects to 89% of uninfected controls ( $P=0.03$ ). Baseline GSH levels in the placebo group (0.91) remained essentially the same during the 8 week placebo-controlled trial. T cell GSH, adjusted for CD4 T cell count and  $\beta_2$ -microglobulin levels, also increased in the NAC-treated subjects ( $P=0.04$ ). Adverse effects were minimal and not significantly associated with NAC ingestion.

**Conclusion.** NAC treatment for 8 weeks safely replenishes whole blood GSH and T cell GSH in HIV-infected individuals. Thus, NAC offers useful adjunct therapy to increase protection against oxidative stress, improve immune system function and increase detoxification of acetaminophen and other drugs. These findings suggest that NAC therapy could be valuable in other clinical situations in which GSH deficiency or oxidative stress plays a role in disease pathology, e.g. rheumatoid arthritis, Parkinson's disease, hepatitis, liver cirrhosis, septic shock and diabetes.

**Keywords** glutathione, GSH, GSH deficiency, HIV, N-acetylcysteine, NAC, *Eur J Clin Invest* 2000; 30 (10): 915–929

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### Introduction

Glutathione (GSH) is a cysteine-containing tripeptide ( $\gamma$ -glutamylcysteinylglycine) that plays a wide variety of physiological roles, including regulation of signal transduction [1,2], intracellular defense against oxidative stress [3,4], and systemic defense against drug toxicity [5,6]. It is central to the regulation of metabolic and cell cycle related functions in all animal cells [7–11]. In addition, it regulates T and NK cell function, for example: low GSH in T cells impairs interleukin (IL)-2 production, IL-2 responses and cytotoxic T cell activity [12–17]; low GSH in NK cells impairs killing activity [18,19]; and low GSH in antigen presenting cells (APC) impairs IL-12 production and favours T helper (TH)2 over TH1 responses [20].

In the clinical setting, GSH deficiency and oxidative

stress have been associated with a series of debilitating diseases, ranging from Parkinson's disease to HIV infection [21–46]. In HIV infection, GSH levels are low in plasma and other body fluids [32–38,40,41]. Furthermore, GSH levels in erythrocytes and in individual T cell subsets (detected, respectively, by high-pressure liquid chromatography (HPLC) and with the fluorescence activated cell sorter [FACS] as the GSH conjugate glutathione-S-bimane [GSB]), decrease as HIV disease progresses [41,43–46]. The clinical significance of this HIV-associated GSH deficiency is reflected by the strong association demonstrated recently between decreased survival in HIV disease and both low thiol levels in serum [42] and low GSB levels in CD4 T cells [46].

Clinical methods for replenishing GSH are well established [47]. N-acetylcysteine (NAC), a prodrug that supplies bioavailable cysteine necessary for the replenishment, is routinely administered to overcome pharmacologically induced GSH deficiency [5,6,48–51], for example due to acetaminophen or cyclophosphamide overdose. Thus, Droge [12,32,52], ourselves [45,53] and other studies [54–56] have suggested that NAC be evaluated as an effective means for preventing or reversing the GSH deficiency in HIV infection. In addition, Jahoor *et al.* [41] argue directly for treating HIV-infected subjects with a cysteine source (e.g. NAC) based on *in vivo* kinetic evidence demonstrating that GSH is deficient in subjects with AIDS, 'due in part to a reduced synthesis rate secondary to a shortage in cysteine availability'.

Questions have been raised about the bioavailability of NAC in HIV-infected people, based on measurement of NAC blood levels in HIV-infected subjects. However, since NAC is a cysteine prodrug that is rapidly converted to cysteine and GSH in the liver, it is necessary to measure the functional bioavailability of NAC in terms of its ability to replenish GSH. Thus, the placebo-controlled trial reported here was conducted to rigorously evaluate the functional bioavailability of orally administered NAC and the safety of administering it to replenish GSH in HIV-infected individuals.

A limited trial by Akerlund *et al.* [57] recently showed that NAC administration increases plasma cysteine in HIV-infected individuals with CD4 T cell counts above  $200 \mu\text{L}^{-1}$  blood. In our study, in which all subjects [81] were selected to have low GSH levels and half were chosen to have CD4 T cell counts below  $200 \mu\text{L}^{-1}$ , we show that oral administration of NAC significantly restores both T cell GSH (GSB) and total GSH in whole blood.

We also monitored NAC toxicity and survival in an open-label (not placebo-controlled) continuation study in which subjects who completed the 8 week placebo-controlled trial were given the choice of whether they wished to take NAC for a period of 6 months (blinding was maintained until all subjects completed the open-label trial segment). Finally, we determined survival of trial subjects 2 to 3 years after baseline measurements were obtained. Protease inhibitors did not become available until all continuation studies were complete. Findings from these continuation studies, which may be biased by the open-label

design and therefore cannot be taken as conclusive, suggest that long-term high-dose NAC ingestion is safe and is associated with longer survival in HIV-infected individuals.

## Methods

### Study Design

The objective was to test orally administered NAC for efficacy in replenishing GSH and for safety in an 8-week, randomized, double-blind, placebo-controlled trial conducted with HIV-infected subjects who were selected to be relatively healthy according to the criteria listed in the next section. We then proposed to further test NAC safety by monitoring subjects who complete the trial and elect optional open-label NAC for up to 24 weeks and by monitoring survival of all subjects 2–3 years after completion of the trial. (The length of time and the methods used for survival monitoring were not specified in the trial protocol, which did not require long-term survival monitoring as a condition for completion of the trial. However, survival monitoring was part of the original plan as the primary measure of additional benefits of NAC treatment.)

The primary outcome variables, FACS-detected GSH (GSB) in CD4 and CD8 T cell subsets, were measured in peripheral blood samples taken at baseline and at 2-week intervals during the placebo-controlled 8-week trial segment. Total GSH in whole blood, another outcome variable, was measured at baseline and at completion of the 8-week placebo-controlled trial. Survival was not a formal outcome variable; however, survival status was determined for 73/81 subjects within 2–3 years of trial enrollment. The randomization code was not opened until all subjects had completed the open-label trial phase.

The trial protocol was approved by the Human Subjects Committee of the Stanford University School of Medicine. It included a provision for trial termination if the physician monitoring adverse events so decided.

### Trial subjects

HIV-infected subjects were interviewed initially to identify relatively healthy subjects, as judged by the normal mobility, normal activity and absence of active opportunistic infections (see Table 1). From this group, subjects were selected for trial entry if they had essentially normal clinical laboratory values and evidence of GSH deficiency, indicated by FACS-detected GSH (GSB) levels in lymphocytes. The target size of the trial (80 subjects) was determined by power analyses that indicated that completion of the randomized trial by 64 such subjects would give sufficient statistical power to recognize GSH restoration. All subjects gave written informed consent.

Of 828 HIV-infected individuals recruited through newspaper ads and interviewed initially, 251 were selected

**Table 1** Criteria for trial entry

	Criteria
Eligibility criteria	HIV-infected (both ELISA and Western Blot); > 18 years; CD4 T cells < 500 cells $\mu\text{L}^{-1}$ ; and evidence of GSH deficiency, indicated by a CD8 T cell GSB level one standard deviation or more below the mean for healthy uninfected controls [46]. This threshold value corresponds to the median defined in a previous cross-sectional study of HIV-infected subjects [46].
Exclusion criteria	creatinine > 2 mg dL <sup>-1</sup> ; neutrophil count < 500 $\mu\text{L}^{-1}$ ; platelets < 20 000 $\mu\text{L}^{-1}$ ; bright expression of CD45RA on all T cells; active bleeding disorder; haemoglobin < 8.5 g dL <sup>-1</sup> ; AST or ALT > 5 times normal; active opportunistic infections requiring acute treatment; current B cell lymphoma; active peptic ulcer; chronic diarrhoea; Karnofsky scores < 60; change in antiretroviral therapy during the preceding 4 months; significant ingestion of NAC, GSH, pentoxifylline or other GSH-replenishing drugs within the preceding 6 months; daily use of acetaminophen during the preceding 7 days; systemic hydrogen peroxide therapy; immune modulators (including ranitidine and cimetidine); steroids (topical excepted); $\alpha$ -interferon; coenzyme Q-10; daily intake of > 400 I.U. vitamin E; > 2000 mg vitamin C; > 25 000 IU beta-carotene; > 50 $\mu\text{g}$ selenium, or other antioxidant therapy within the preceding month.
Behaviour criteria	Agreement to maintain current vitamin and medication regime and to forego immune modulators, antioxidants, acetaminophen (because it depletes GSH) and more than one alcoholic drink per day. Tetracyclines, ampicillin, erythromycin, amphotericin, trypsin, chymotrypsin and prophylaxis for protozoal, fungal, bacterial or viral infections including acyclovir) were proscribed within two hours of taking NAC.

for screening for GSH levels and 83 individuals were ultimately randomized into the study according to four strata: CD4 T cell count, 200–500 or < 200  $\mu\text{L}^{-1}$  blood; and the 90th percentile for GSB in CD8 T cells, 1.0–1.3 or < 1.0. The basis for choosing the upper GSB threshold for trial entry [1,3] is explained below. Three strata were filled (21–24 subjects); one stratum (high CD4 and low GSB) was partially filled (16 subjects).

The target population size for completion of the randomized, placebo-controlled trial was 64 subjects. Recruitment was terminated when 60–65 of 81 enrolled subjects were considered likely to complete the full 8 weeks.

Acceptance into the trial was based on the disqualifying and qualifying criteria listed in Table 1. Of the 83 initially enrolled subjects, two were mistakenly classified as eligible for enrolment. Thus, data are presented here for 81 correctly enrolled subjects (see Fig. 1). Data for assessing change in GSH levels was lacking for 3 subjects who left the trial after week 0. A total of 61 subjects completed the 8-week trial. The number of subjects who failed to complete the trial (Fig. 1) was not significantly different between the trial arms ( $P=0.26$ , Fisher exact test, 2 sided). The reasons for failure to complete are reported in the footnote to Table 6.

## Randomization

Randomization and blinding were performed by an independent clinical research organization (PharmaQuest, now Inveresk Research, San Rafael, CA) supplied at intervals with lists of eligible subjects by strata. The drug arm to placebo arm ratio was 1 : 1. PharmaQuest held the randomization code until all subjects had completed the full trial, including the open-label segment, and all data had been collected and stored in non-modifiable form, i.e.

blinding was maintained until all subjects completed the open-label trial segment.

## Drug dosage

NAC and placebo were supplied as indistinguishable effervescent tablets to be dissolved in water, juice or soda before ingestion. Subjects were given 10 tablets (8000 mg of NAC) per day in distributed [3,4] doses day<sup>-1</sup>. The drug dose was reduced in accordance with the schedule specified in the protocol if subjects reported an inability to tolerate the highest dose. Median dosage throughout the trial was 6.9 g day<sup>-1</sup>; average dose was 7.0 g day<sup>-1</sup>; median dose for the last two weeks was 6.3 g day<sup>-1</sup>. The dosages recorded for subjects in the trial and placebo arms were essentially identical.

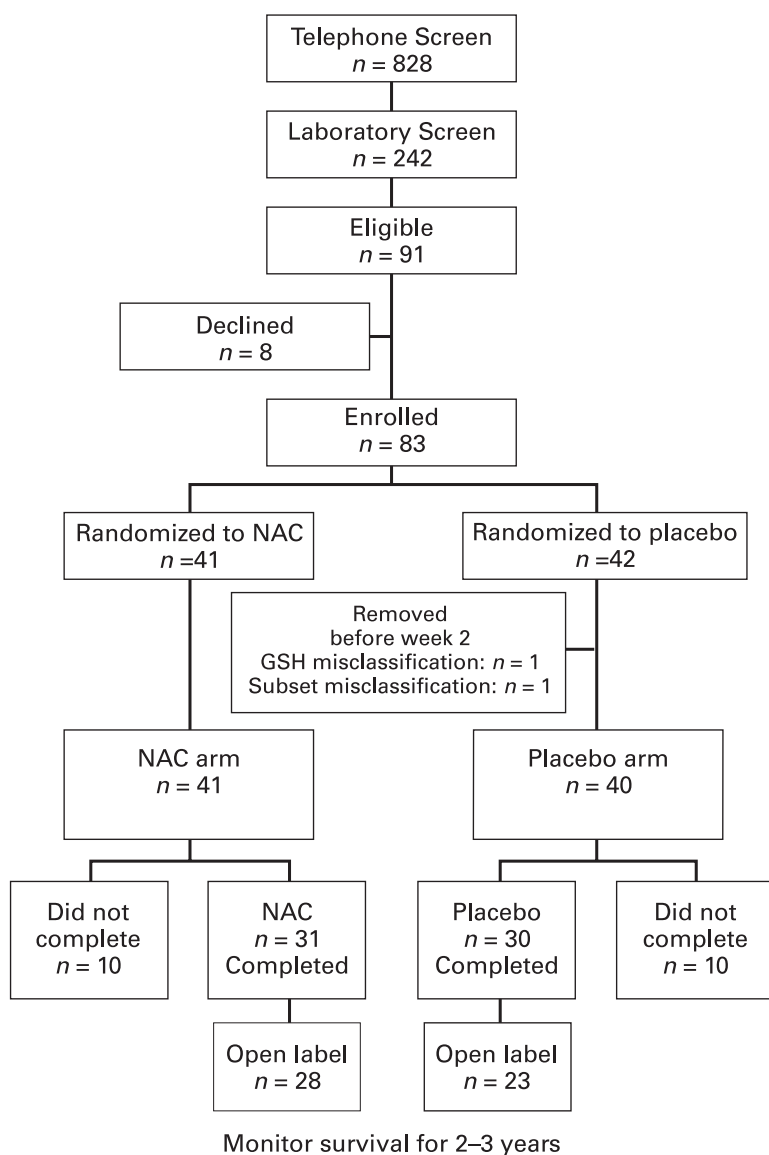
### Open-label (continuation) study

Subjects who completed the trial were allowed to take open-label NAC for up to 24 weeks. 26/31 subjects from the NAC group and 22/30 subjects from the placebo group elected for this option. The drug dosage reduction schedule used during the placebo-controlled trial segment was also applied during the open-label segment. The median NAC dose taken during the trial plus continuation phase was 5.3 g day<sup>-1</sup> and the median time that subjects took NAC was 24 weeks.

## Laboratory Analyses

### Whole blood GSH

One mL of blood was drawn, mixed within 2 min with 1 mL of 10% sulfosalicylic acid (SSA) and frozen immediately on dry ice. Samples stored at  $-70^{\circ}\text{C}$  were later thawed, spun and supernatant GSH measured by HPLC



**Figure 1** Distribution of subjects during the randomized, open-label and survival-monitoring segments of the trial.

as previously described [46]. Because this procedure is highly demanding and expensive, whole blood sample collection and analysis was limited to samples collected at 0 and 8 weeks. GSH in erythrocytes accounts for nearly all of the GSH in 'whole blood' samples.

#### *GSB (FACS GSH) in T cell subsets*

PBMC prepared by Ficoll-hypaque separation were stained initially for 20 min at room temperature with monochlorobimane, which intracellular glutathione-S-transferase couples to GSH to form the fluorescent GSH adduct, glutathione-S-bimane (GSB). At the end of the incubation period, cells were chilled rapidly, aliquoted and stained with fluorochrome-coupled monoclonal antibodies that reveal subset-defining T cell surface markers (BD-Pharmingen, San Diego, CA) [43,46,58]. GSB levels, determined by FACS in the individual T cell

subsets were normalized to the median GSB level of a frozen PBMC standard that was thawed and analyzed in parallel with the samples in the experiment. The normalized GSB fluorescence levels at the 50th and 90th percentile of the GSB distributions for the subsets are, respectively, referred to as CD4/50, CD4/90, CD8/50, and CD8/90.

Intracellular sulfhydryls other than GSH can potentially bind monochlorobimane and generate fluorescent adducts [59]. However, GSB values in individual T cell subsets obtained by monochlorobimane staining under our conditions primarily report reduced intracellular GSH since GSB values for CD4 T cells (and other lymphocytes) correlate with HPLC-measured GSH in whole blood ( $r=0.64$ ,  $P<0.0001$ ) [46]. Furthermore, the low GSB levels that we detect in T cells from HIV-infected subjects [46] are consistent with the increased GSSG:GSH ratios demonstrated by others [60] in bulk assays of T cells [60].

Thus, GSB measurements can also provide an index of oxidative stress.

### Data collection

Data were obtained for 81 subjects at week 0 and, respectively, for 76, 68, 56 and 61 subjects at weeks 2, 4, 6 and 8. Trial visits (5) were scheduled at 2-week intervals during the placebo-controlled segment. Weight, clinical signs and symptoms were recorded; the patients' daily drug diaries were reviewed; pill counts were done on the returned drug vials; and vital signs were taken by the study nurses. Blood samples were drawn and clinical laboratory analyses (liver and kidney function, haematology, CD4 T cell counts) were performed at the Immuno-Diagnostics Laboratories (IDL) San Francisco site. At baseline and at the final visit (week 8), blood samples were also drawn for HPLC analysis of whole blood GSH. Survival, determined 2–3 years after baseline data were collected, was ascertained by direct contact, by contact with physicians or other reliable sources, or by exhaustive search of death records. Methods for measuring whole blood GSH by HPLC and intracellular GSH (GSB) in T cell subsets by FACS have been previously described [43,58,61–63].

### Statistical Analyses

Unless otherwise indicated, all statistical analyses were done on an intent-to-treat basis. All statistical calculations were performed with SAS [64] or SAS JMP software [65,66].

#### *T cell GSB: area under the curve analysis*

For each subject, the average change  $\text{day}^{-1}$  in CD4/50 GSB during pretrial observation (2 measurements taken over a one-month period) was subtracted from the average CD4/50 GSB change  $\text{day}^{-1}$  during the trial (2–5 measurements, mainly 5, taken at 2-week intervals). Trial groups were compared by analysis of covariance with data weighted by the number of trial visits. Five outliers (4 GSB, 1  $\beta_2$ -microglobulin) were excluded from this analysis, which was therefore based on data from 76 subjects).

#### *T cell GSB: regression analysis by baseline GSB tertiles*

For each of the tertiles, the mean least-squared multiple regression slopes of the GSB primary outcome values over the 8-week trial were computed for the NAC and placebo groups and the significance of the differences between the groups determined (*t*-test, 1-sided alternative hypothesis,  $\text{mean}_{\text{NAC}} > \text{mean}_{\text{placebo}}$ ). The regression slopes were adjusted for baseline covariates age; baseline CD4 and CD8 T cell counts; viral load;  $\beta_2$ -microglobulin; use of antiretroviral drugs; completion of trial; and the baseline values of the respective GSB primary outcome variables.

#### *Whole blood GSH*

Treatment effect on whole blood GSH, computed as  $(\text{GSH}_{\text{week8}} - \text{GSH}_{\text{week0}})$ , was examined by analysis of covariance. See 'Missing data and Outliers' (below) for method of estimating values for the intent-to-treat analysis.

#### *Survival*

The Cox proportional hazards model was used to calculate survival functions and relative risks for the treatment groups and explanatory covariates [67]. A simple test in which a time-dependent explanatory variable was introduced into the model and did not have a significant regression coefficient, indicated there was not a significant change of the hazard ratio with time, thereby validating the proportional hazards assumption [68].

### Missing data and outliers

Of the 81 subjects, 3 left the trial at week 0. Therefore, change in GSB level for these subjects could not be calculated. One baseline serum sample was lost, preventing measurement of baseline  $\beta_2$ -microglobulin and viral load for that subject. For intent-to-treat analyses, this sample is assigned the median values of the viral load and  $\beta_2$ -microglobulin distributions for subjects in the same CD4 T cell stratum. Outliers, identified by a JMP procedure [66] that identifies values outside the distance computed as  $(1.5 \times \text{interquartile range})$  from the respective upper or lower quartile, were excluded where indicated.

#### *Whole blood GSH*

Since funding available for this study was limited, the trial protocol was written to require whole blood GSH measurements (which are very expensive) only for subjects who completed the placebo-controlled segment of the trial. Of the 61 subjects in this group, samples from 7 were either not obtained or lost, leaving 54 subjects for whom data were collected. Of these, five additional subjects were classified as having missing data because one or both values were in outlier range. There were no significant differences in baseline data between subjects for whom data was missing or available.

To perform an intent-to-treat GSH analysis, missing values were estimated to favour the null hypothesis [69]. Distributions were computed based on data for all 'non-missing' subjects. A series of 50 independent data sets were then constructed in each, of which data (baseline and change in GSH) for all missing subjects were replaced with randomly selected values from the appropriate distributions. Each constructed data set was then independently analysed for treatment effect by analysis of covariance, and the results were combined to generate median significance values for the input variables and the model as a whole.

**Table 2** Baseline demographic and clinical variables

Variable	Trial Arm		
	NAC	Placebo	Control
Number of subjects	41	40	
Trial completion (% finished)	76%	75%	
Median NAC or placebo day <sup>-1</sup> (g)	6.9*	7.2	
Race (% Caucasian, non-Hispanic)	76%	85%	
Age (years)	38	38	
Sex (% male)	100%	100%	
Taking antiretroviral drugs†	46%	53%	
Haematocrit	40	40	
AST (IU L <sup>-1</sup> )	30	32	
ALT (IU L <sup>-1</sup> )	29	37	
β <sub>2</sub> -microglobulin (μg mL <sup>-1</sup> plasma)	2.0	2.3	
Viral load (x10 <sup>4</sup> mL <sup>-1</sup> , PCR assay)	6.8	8.0	
CD4 T cells μL <sup>-1</sup> blood	203	160	
CD8 T cells μL <sup>-1</sup> blood	648	705	
CD4:CD8 ratio	0.28	0.24	
Subjects with CD4 T cells <200‡	49%	55%	
Primary outcome variables			
GSB§ (CD4/50)	0.77	0.74	1.1
GSB (CD4/90)	1.4	1.3	1.7
GSB (CD8/50)	0.69	0.68	1.1
GSB (CD8/90)	1.1	1.1	1.6
GSH in blood (mM)	0.88	0.91	1.1

\*Except for the number of subjects and for values indicated as percentages, median values are shown for each variable.

†Percentage of subjects taking reverse transcriptase inhibitors. Protease inhibitors were introduced after the survival monitoring phase of this trial was completed.

‡Percentage of subjects in indicated arm.

§FACS measure of intracellular GSH (see methods).

### Survival

Survival data were obtained for 73/81 subjects. Contact was lost with the remaining 8 subjects, for whom we could find no record of death despite extensive searching of death records. To complete the data set for an intent-to-treat analysis: six subjects with baseline CD4 T cells >200 μL<sup>-1</sup> were scored as surviving at the end of the monitoring period, since all other subjects with CD4 T cell counts in this range were documented survivors; of two subjects with CD4 T cells <200 μL<sup>-1</sup>, one who did not take NAC conservatively was scored as surviving until the end of the monitoring period while the other, who took NAC, was scored as deceased at the median survival time for subjects with CD4 T cells <200 μL<sup>-1</sup>.

## Results

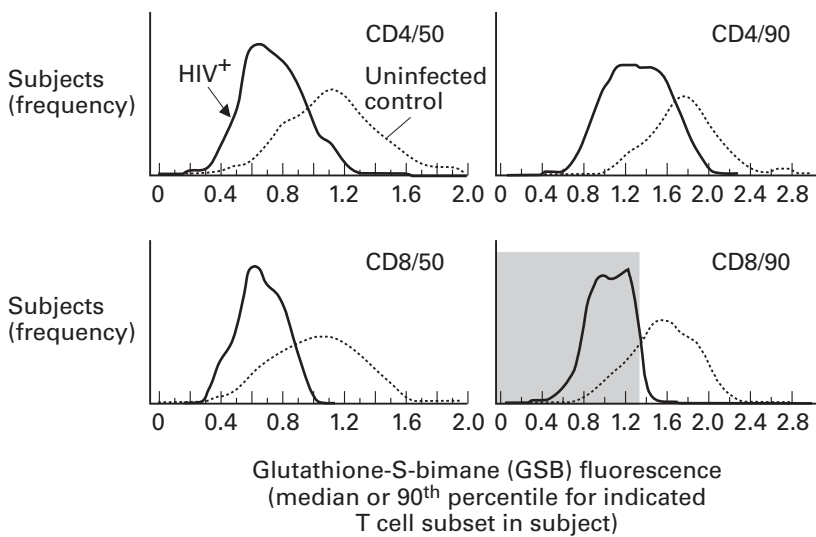
### Baseline demographic and clinical variables

Baseline data, computed as the mean of 2–3 values obtained prior to the beginning of the trial did not differ significantly between the placebo and trial arm groups

(Table 2 and Fig. 2). Anti-retroviral usage was equivalent in the two arms; protease inhibitors were not available until after completion of the full trial, including placebo-controlled and open-label trial segments and the 2–3 years survival monitoring period. The first subject was enrolled in December, 1993 and all survival information was obtained by July, 1996.

### NAC administration increases whole blood GSH

NAC treatment significantly increases GSH levels measured either as whole blood GSH (Table 3 and Fig. 3, this section) or as FACS-measured GSH (GSB) in T cells (Table 4, next section). At the start of the trial, baseline values for whole blood GSH in trial subjects (Fig. 3, top panel) did not differ between NAC and placebo arm subjects. Furthermore, the GSH levels in both treatment groups were significantly below levels in uninfected individuals. By the end of the 8-week trial, GSH levels in the NAC arm rose almost to control level whereas placebo arm GSH levels remained unchanged (Fig. 3). The treatment effect, computed by least squares regression analysis for all subjects for whom valid GSH data were acquired at the beginning and the end of the trial (54 subjects –1 outlier,



**Figure 2** Distributions of GSB levels in T cell subsets in trial and control (HIV-uninfected) subjects. Solid lines show distributions for baseline values (averages of 2–3 measurements) for 81 trial subjects; dotted lines show distributions for 48 HIV-uninfected controls. To be eligible for trial entry, subjects were required to have screening CD8/90 levels below 1.3, i.e. within the shaded area.

defined by standard methods as indicated in the Methods section; Table 3), is significant when treatment is the only explanatory variable in the model ( $P=0.01$ ) and is highly significant when the explanatory variable baseline GSH is included ( $P=0.0002$ ).

To reduce bias due to missing GSH data for subjects who either did not complete the trial or whose samples were lost during processing, we performed an analysis (Table 3) in which missing data values and replacement values for outliers were estimated by a conservative method [69], i.e. values were assigned randomly from distributions of GSH values measured for subjects for whom valid data were available (see Methods section ‘Missing Data and Outliers’). Using this data assignment method, we constructed 50 independent data sets and then computed the significance of the treatment effect for each data set in a least squares linear regression model that included baseline GSH as an explanatory variable. The median significance for the

treatment effect computed in this way is 0.03 (i.q. 0.005–0.07) (Table 3).

Interestingly, the baseline values for GSH were significantly lower among subjects who were taking AZT or other nucleoside reverse transcriptase inhibitors (NRTI) than among subjects not taking these drugs (respectively,  $n=27$  and  $26$ ; median baseline GSH, 0.80 and 0.98;  $P=0.02$ , Wilcoxon one-way  $X^2$ ). NAC administration significantly increased GSH levels in both groups ( $P=0.007$  and  $P=0.03$ , respectively), with the greatest NAC arm increase occurring in the NRTI group (0.17 vs. 0.04). Thus, by the end of the trial, there was no significant GSH difference between subjects taking or not taking NRTI ( $P=0.5$ ). Consistent with this, a least-squares regression model with change in GSH as the dependent variable that includes trial arm ( $P=0.02$ ), with baseline GSH and NRTI as covariates demonstrates that baseline GSH is highly significant ( $P<0.0001$ ) and that NRTI of

**Table 3** NAC administration increases whole blood GSH in HIV-infected subjects

Variable	Subjects with complete data		All subjects* (missing values estimated)			
	$P$ †	Adjusted mean GSH change‡	$P$	Adjusted mean GSH change		
		NAC		NAC	Placebo	
Trial Arm	0.0008§ (0.03)¶	0.101 (0.097)	–0.027 (–0.007)	0.03**	0.072	–0.011
Baseline GSH	<0.0001	–	–	<0.0001	–	–

\*Missing values for GSH measurements were estimated based on values obtained by repeated random selection from distributions of measured values (see methods). Median significance obtained for 50 random selections is shown.

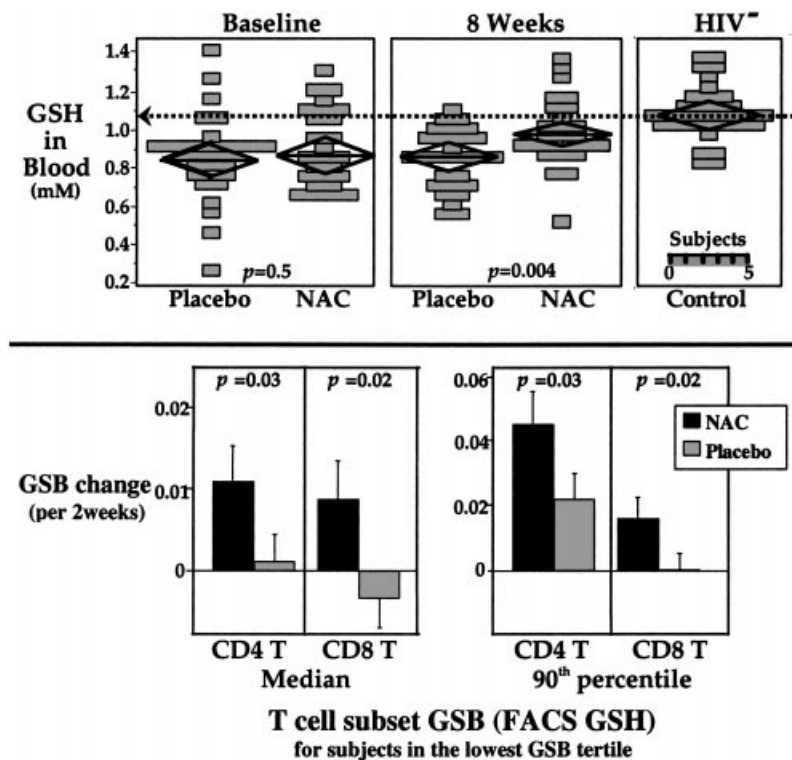
†Standard least squares model:  $P$  = significance of the indicated variable in the model;  $P$  for the entire model <0.0001. Except for baseline GSH, none of the baseline variables measured in this study was a significant covariate in this model.

‡Change in whole blood GSH (mM) from the beginning to the end of the trial.

§Computed for 53/54 subjects for whom data for 0 and 8 week samples for whole blood GSH analysis were available ( $n=54$ ). One outlier whose value was >4sd from the mean for the group was removed; values in parentheses were computed without the outlier removed.

¶Values in parentheses were computed with no outliers removed.

\*\*Interquartile range 0.005–0.07.



**Figure 3** NAC treatment increases whole blood GSH and T cell subset GSB. HIV-infected subjects were treated for 8 weeks with orally administered NAC or placebo. Whole blood GSH was measured by HPLC. GSB (intracellular GSH) was measured by FACS. (a) Whole blood GSH: data are shown for 54 HIV-infected subjects (27 in each arm; 1 GSH outlier in the placebo group excluded) and 34 uninfected male controls. The grey bars show the distribution of subjects in each group as a function of GSH level (ordinate). The lines in the middle of the ‘means diamonds’ show the mean for each group; the vertices at the top and bottom show the 95% confidence interval for the mean. (b) T cell GSB: For this figure, 81 trial subjects were stratified independent of trial arm into three equal-sized groups (tertiles) according to baseline values for the outcome variable being analyzed. Data are shown for the tertile containing subjects with the lowest baseline GSB ( $n = 27$ ; 12 NAC and 15 placebo), for which the adjusted mean slopes for the NAC arm were significantly greater for the primary outcome variables ( $P = 0.02-0.03$ ,  $t$ -test, 1-tailed). Mean increases for NAC arm subjects in this tertile, calculated from the adjusted mean slopes and mean initial values over the 8-week trial period, were 6.6–15.3%. In the two tertiles containing subjects with higher baseline GSB values (not shown), differences between the treatment arms for the four variables were not significant ( $P > 0.4$ ).

**Table 4** NAC administration increases GSB in HIV-infected subjects

Variables	P*	Adjusted mean GSB change†	
		NAC	Placebo
Trial arm	0.04	0.038	0.009
Baseline $\beta_2$ -microglobulin	0.04	–	–
Baseline CD4 T cells	0.02	–	–

\*Standard least squares model fit. GSB data were available for all (81) trial subjects. Four outliers were excluded from the analysis; three on the basis of GSB change and one on the basis of baseline  $\beta_2$ -microglobulin values. P, significance of the indicated variable in the model; P for the entire model  $< 0.0004$ . Except for the two baseline variables shown in the table, none of the baseline variables measured in this study were significant covariates in this model.

†Change in GSB (CD4/50) is computed as the difference between the GSB values (area under the curve) obtained prior to initiation of treatment and GSB values (area under the curve) obtained until the subject completed or left the trial. Statistical computations are weighted according to the number of trial visits (equal to the number of data points acquired).

itself is not significant ( $P = 0.43$ ). There was no significant interaction between trial arm (NAC) and NRTI ( $P = 0.4$ ) with respect to GSH replenishment.

With the exception of the expected perturbation in erythrocyte parameters, we found no baseline variable differences that would indicate that the subjects taking NRTI had more advanced or severe HIV disease. Thus, the findings discussed above suggest (i) that NRTI usage may contribute to lowering GSH levels in HIV-infected subjects and (ii) that NAC reverses this GSH decrease. Consistent with this, we find a trend suggesting that the elevated mean erythrocyte volume (MCV) commonly encountered in subjects taking NRTI is decreased by NAC treatment ( $P = 0.07$  for trial arm; standard least squares model fit;  $n = 30$ , 1 outlier removed). In any event, since entry into the trial required that subjects had adhered to a stable antiretroviral therapy schedule for at least four months prior to entry into the trial, and since subjects were required to maintain that therapy schedule for the duration of the trial, changes in antiretroviral usage during the trial did not contribute to the effect of NAC treatment on whole blood GSH observed here.

**Table 5** Decrease in viral load independent of trial-arm\*

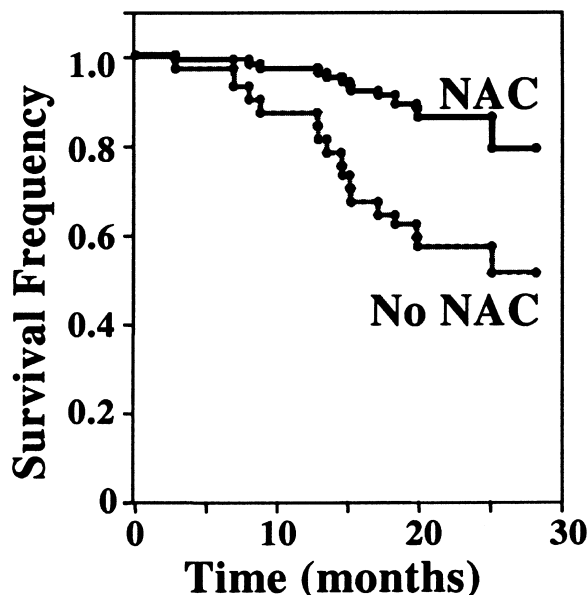
Baseline HIV (copies mL <sup>-1</sup> blood)	n†	Change from baseline HIV copies‡		
		median % change	interquartile range	P§
< 50 000	28	4	- 21-48	0.14
≥ 50 000	32	- 33	- 54-0	0.004

\*There was no significant change ( $P > 0.1$ ) in viral load between the NAC and placebo arms evaluated for all subjects or for subjects in each of the groups shown in the table.

†Number of subjects in each group: < 50,000, 16 NAC arm + 12 placebo arm; ≥ 50,000, 15 NAC arm + 17 placebo arm; data was missing for 1 placebo subject.

‡Viral loads at the beginning and end of the 8-week trial period were compared.

§2-tailed Wilcoxon Signed Rank Test.



**Figure 4** NAC administration is associated with increased survival. Cox Proportional Hazards survival functions comparing the survival of trial subjects who took NAC (randomized to the NAC group and/or elected open-label NAC;  $n = 64$ ) with those who never took NAC ( $n = 17$ ) adjusted for baseline CD4 T-cell count and  $\beta 2$ -microglobulin. Ordinate: proportion of subjects remaining alive. Abscissa: time (months) from the baseline visit. Significance for the Cox model:  $P < 0.0001$  (Wald  $X^2$ ). Survival time is computed from the beginning of the trial for all subjects. 2–3 years survival data were acquired from death records or other reliable sources for 73 of the 81 subjects. For the remaining 8 subjects (2 with CD4 T cells  $< 200 \mu\text{L}^{-1}$ , the group within which all recorded deaths occurred), data were conservatively estimated to avoid bias in favour of a positive association between NAC ingestion and survival (see Methods). For the 73 subjects for whom survival data were obtained (ignoring estimated survival), results for NAC vs. No-NAC were:  $P = 0.0003$ ; risk ratio = 0.3 (0.1–0.6). The median NAC administration time computed for all subjects who took NAC was 24 weeks; the median NAC dose was  $5.3 \text{g day}^{-1}$ . Reverse transcriptase inhibitor usage is not a significant covariate ( $P = 0.9$ ). None of the subjects received protease inhibitors, which were introduced after the end of the 2–3 years monitoring period.

### NAC administration increases T cell GSB

Since relatively small increases (10–30%) in T cell GSB levels are sufficient to bring most HIV-infected subjects into normal range, we tested treatment effect by an ‘area under the curve’ method that minimizes random measurement error. For each subject, we calculated the difference between the average pretrial change in GSB and the average GSB change during the trial and tested for treatment effect in a standard least squares model. We found that the treatment variable (trial arm) was not significant when entered alone ( $P = 0.2$  for all subjects) but was significant ( $P = 0.04$ , Table 4) when four outliers were removed by standard methods and the model adjusted for covariates.

Least squares regression analysis of the rate of GSB change computed over the treatment period also demonstrates a significant NAC treatment effect. Consistent with the importance of baseline GSH on the treatment effect in the whole blood GSH analysis, a significant increase in GSB in the NAC-treated group occurs in subjects whose baseline GSB levels were in the lowest third (tertile) of the placebo-controlled trial group, but was not significant in the upper two tertiles (see Fig. 3(b)).

### NAC administration does not change surrogate markers of HIV disease progression

NAC treatment during the 8-week placebo-controlled trial segment did not result in a significant change in either CD4 T cell count ( $P > 0.9$ ) or viral load ( $P > 0.1$ ). Similarly, it did not significantly affect CD8 T cell counts, CD4/CD8 ratio, haematocrit, other haematological markers or  $\beta 2$ -microglobulin levels ( $P > 0.1$  in all cases). Surprisingly, however, viral load decreased roughly 30% independent of trial arm in subjects with over 50 000 HIV copies mL<sup>-1</sup> at baseline ( $P = 0.004$ , Table 5). This suggests GSH-depleting medications and/or activities proscribed during the trial (e.g. acetaminophen and high alcohol consumption; see Methods) may contribute to maintaining the high viral loads. Alternatively, it may merely reflect regression to the mean.

### NAC treatment is safe for HIV-infected subjects

We found no evidence of toxicity associated with NAC

**Table 6** Safety of NAC treatment

	Trial Arm		<i>P</i>
	NAC ( <i>n</i> = 41*)	Placebo ( <i>n</i> = 40†)	
Symptom reports‡	Median number/subject/visit (i.q. range)		> 0.3
	1.3 (0.9–2)	1.1 (0.5–2)	
	Number (%) of subjects		
Dose reduction§	26 (68%)	29 (69%)	0.6
Election of open label	28 (90%)	22 (73%)	0.08

\*From the NAC arm, 8 subjects were lost by the second week of the trial (2 left the trial voluntarily, 3 developed opportunistic infections (OI) and were removed according to protocol, 1 withdrew with a rash and 2 others withdrew with GI symptoms). One NAC arm subject withdrew after 4 weeks with fever, rash and GI symptoms while another finished nearly 8 weeks and withdrew with a rash.

†From the placebo arm, 5 of the 10 subjects were lost by the second week of the trial: two were removed for non-compliance and three left citing leukoplasia, nasal symptoms and fatigue/GI symptoms, respectively. The remaining five placebo arm subjects, all of whom reported GI symptoms, left after 4 weeks.

‡Symptom reports were mainly gastrointestinal upsets, with diarrhoea and nausea being the most frequent. However, although gastrointestinal symptoms have been claimed to be associated with NAC ingestion, there was no significant difference in the number or severity of these symptoms between the NAC group and the placebo group. Among other complaints, rash and headache were the most numerous. Additional complaints included fever, fatigue, stomatitis, depression and cough. None of the illness reported required hospitalization. Among subjects who reported symptoms 75% of the symptoms reported in both trial arms were minimal (1 on a scale of 1–4). Only 3 patients reported symptoms above 2 on this scale. One of these latter subjects left the trial after 4 weeks (see above).

§The median drug dose during the trial was 6.9 mg NAC and 7.2 mg placebo per day.

administration, either during the 8-week placebo controlled trial segment or the 6-month open-label segment (Table 6). Ten subjects in each arm withdrew or were dropped from the study. There was no significant difference between the trial arms in the number of patients reporting symptoms, the average number of symptoms reported, the severity of symptoms reported or the frequency of dose reduction (Table 6). Thus, NAC ingestion per se was not associated with development of adverse GI or other symptoms, indicating that GI symptoms observed during the trial were either due to ingestion of the dispensed formulation (independent of whether it contained NAC or placebo), i.e. to ingestion of excipient(s), or were unrelated to the treatment. (The dispensed formulation was 8–10 fizzy tabs containing the drug or placebo that subjects consumed per day, dissolved in water or juice, and that contained non-nutritive sweetener and several grams of bicarbonate.)

After completion of the double-blind portion of the trial, subjects in both arms were offered 8 g day<sup>-1</sup> of open-label NAC for up to 24 weeks. A total of 51 of the 61 subjects who completed the trial chose this option; half stayed in the continuation phase for the full 24 weeks; three-quarters stayed at least 14 weeks. The number of subjects electing for open-label NAC was higher in the NAC arm (28) than in the placebo arm (22). As during the placebo-controlled trial segment, there were no serious adverse reactions ascribable to NAC ingestion during the open-label period. Thus, consistent with previous data for uninfected subjects [5,6,70], we found no adverse consequences of

NAC ingestion at high doses even for relatively long periods (> 5 g day<sup>-1</sup> for 14–24 weeks).

### NAC administration and survival

In previous studies, we have shown that low GSB levels are associated with decreased survival in an overall study group that included subjects from this trial [46]. We have shown here that NAC is functionally bioavailable and replenishes GSH in HIV-infected subjects (Tables 3 and 4; Fig. 3). Consistent with these findings, we find a significant association between NAC ingestion and improved 2–3 years survival in a Cox Proportional Hazards analysis comparing the survival (adjusted for baseline CD4 T-cell count and  $\beta$ 2-microglobulin) of trial subjects who took NAC during the placebo-controlled and/or the open label segment of the trial with those who never took NAC ( $P < 0.0001$ ; Fig. 4). Initiation of protease inhibitor therapy does not confound the analysis, since these drugs were introduced after the survival observation period was complete.

Interpretation of this finding is hampered by a potential bias introduced by the self-selection of a proportion of placebo-arm subjects who elected for open-label NAC and by the failure to obtain detailed follow-up information (drug usage, cause of death) for trial subjects once they completed the open-label trial segment. Thus, unlike the findings with respect to GSH replenishment, data from this survival study can only be considered suggestive and is

useful mainly as another indication of the safety of NAC administration to HIV-infected subjects.

## Discussion

A series of studies collectively document GSH deficiency in HIV disease and link this deficiency to HIV disease progression [12,32–34,44,71–73] and decreased survival [42,46]. The mechanism at least partially underlying this deficiency has been shown to involve the decreased availability of cysteine, the limiting precursor required for GSH synthesis [41]. Consistent with this, the randomized, double-blind placebo-controlled trial reported here demonstrates that oral administration of N-acetylcysteine (NAC), the commonly used source of cysteine in clinical settings [5,50], safely replenishes GSH in GSH-deficient HIV-infected subjects.

An independent placebo-controlled trial (45 HIV-infected subjects) has already shown that NAC administration for four months to subjects with CD4 T cell counts above  $200 \mu\text{L}^{-1}$  blood raises plasma cysteine [57], and hence increases the supply of the limiting precursor for GSH. Here, we show directly that NAC replenishes GSH in blood and in CD4 T cells in HIV-infected subjects (whether their CD4 T cell counts are above or below  $200 \mu\text{L}^{-1}$  blood). Further, we show that NAC administration at high doses for up to 8 months has minimal side-effects. Several studies have reported results opposing these findings [74–79]. However, the apparent contradictions are based on misinterpretation of pharmacokinetic data [74–76], inadequate study design [77,78], or the use of GSH measurement or sample storage methods [79] that do not meet current standards [80]. Thus, we conclude that NAC is functionally bioavailable and safely replenishes GSH in HIV-infected subjects, even when they have low CD4 T cell counts.

GSH deficiency is not restricted to the era prior to introduction of protease inhibitors and Highly Active Anti-Retroviral Therapy (HAART). Droge, Breitkreutz and colleagues recently demonstrated a massive sulphur loss in HIV infection that is not ameliorated by HAART [81]. Our preliminary measurements of GSB levels in the HAART-treated subjects (De Rosa *et al.* unpublished) agree with these findings. Importantly, Droge, Breitkreutz and colleagues also report that administration of NAC to HIV-infected subjects in a placebo-controlled trial (32 subjects) improves T and NK cell function in patients under treatment with HAART or other antiretroviral therapy [82]. This improvement following NAC administration most likely reflects GSH replenishment as GSH depletion is known to lead to decreased T and NK cell function [12–20].

Several factors are likely to contribute to the GSH deficiency in HIV disease. Inflammation, oxidative stress and medications that are detoxified through GSH-consuming pathways will all result in GSH loss and excessive cysteine catabolism. Furthermore, HIV viral infection,

through the production and release of TAT protein will create additional oxidative stress and hence increase cysteine loss [83,84]. Indeed, TAT-transgenic mice show very low GSH levels [85]. Studies with these mice indicate that their low GSH levels are due, at least in part, to TAT interference with GSH synthesis. Similar interference may be a feature of HIV disease in man. However, Jahoor and colleagues [41] have shown directly that GSH synthesis rates increase in HIV-infected people when NAC is supplied as a cysteine source. Furthermore, we show here that NAC treatment replenishes GSH in HIV infection. Thus, as a practical matter, the mechanism responsible for GSH deficiency in HIV disease ultimately results in the necessity to increase the cysteine supply in order to restore GSH.

The potential consequences of GSH deficiency in HIV disease should not be ignored. Our previously reported retrospective study of 204 subjects [46] and a smaller study by Marmor *et al.* [42] both demonstrate that survival decreases dramatically at lower baseline CD4 T cell GSB [46] and plasma thiol [42] levels. In addition, a recent experimental study with HIV-infected chimpanzees, who routinely develop high virus titres but do not get sick, links the lack of disease progression in these animals to maintenance of normal GSH levels despite the infection [36]. Furthermore, as indicated above, Droge, Breitkreutz and colleagues detected significant impairment of T cell function in the subjects in their study.

Finally, a recent study [86] has shown that subjects with AIDS respond with significantly increased production of reactive hepatotoxic intermediates ( $P=0.001$ ) when loaded with a modest acetaminophen dose (1.5 g). Thus, acetaminophen metabolism is compromised in subjects with AIDS as it is in alcoholic and other GSH-deficient subjects [5,6,48,49,51].

At a minimum, these findings suggest that it is advisable for HIV-infected individuals to avoid unnecessary consumption of acetaminophen and other drugs that are toxic to GSH-depleted individuals. Further, prudence suggests that they should exercise moderation in activities such as excessive alcohol consumption and UV exposure that contribute to GSH depletion. Administering NAC to replenish GSH in these individuals, however, might be the wisest course of all.

Since NAC effectively replenishes GSH in HIV-infected people, it may also prove to be a useful therapeutic adjunct for replenishing GSH in other clinical situations. GSH deficiency induced by alcohol and certain drugs is well-known. In addition, recent studies have implicated GSH deficiency in acute respiratory distress syndrome (ARDS) and septic shock [31,87–89], hepatitis [29], liver cirrhosis [18,90], hepatorenal failure [31], preeclampsia of pregnancy [91], cardiac failure [92], rheumatoid arthritis [93], neurodegenerative disorders [25,94], aging [27,95], pancreatitis [96], age-related macular degeneration [97] and diabetes [98,99]. Exploration of NAC therapy has already begun in some of these diseases [31]. Although NAC is clearly not a panacea, the success in treating hepatorenal failure with NAC [31] provides good reason to suspect that NAC treatment may support recovery or slow disease

progression in a variety of settings when administered as part of a specific therapeutic regime.

Finally, the potential association we have found between NAC treatment (and hence GSH replenishment) and improved survival in HIV infection argues strongly for a definitive trial designed specifically to test this association. Conduct of such a trial is virtually impossible in this protease inhibitor era. However, there are good reasons to test NAC as an adjunct to these therapies, since NAC has many sites of potential action that could improve health and drug tolerance. Furthermore, because NAC is inexpensive and safe enough to be administered with minimal medical supervision, it could prove useful in developing countries or other situations where protease inhibitors are unavailable or inadvisable. Thus, having shown that NAC effectively replenishes GSH in HIV disease, our data argue both for NAC treatment in suspected cases of GSH depletion due to any cause and for further testing to determine whether other potential benefits of NAC treatment can be identified in HIV disease.

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