Oxidative Stress in Cancer, AIDS, and Neurodegenerative Diseases

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Low Glutathione Levels in CD4 T Cells Predict Poor Survival in AIDS; *N*-Acetylcysteine May Improve Survival

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INTRODUCTION

Glutathione (GSH) depletion impairs T cell function (1) and promotes cytokine-stimulated HIV expression (2,3). *N*-Acetylcysteine (NAC), which provides the cysteine necessary to replenish GSH (4), improves T cell function and blocks HIV expression (2,3,5–7). Since GSH levels are lower in HIV-infected individuals, particularly at later stages of HIV/AIDS (8–13), we and others (notably Droge) have suggested that replenishing GSH by administration of NAC or other nontoxic GSH prodrugs could slow the progress of HIV disease (5,14–17). However, despite the strong in vitro data supporting the importance of GSH replenishment in HIV disease, there has been no evidence to date directly linking the low GSH levels in HIV-infected individuals with the pathogenesis of HIV disease in these individuals.

Data summarized here, derived from a GSH monitoring study and an associated clinical trial testing oral NAC for GSH replenishment in subjects with AIDS, provide the first clear demonstration of the importance of GSH depletion in AIDS pathogenesis. In essence, findings for subjects with AIDS, defined by having CD4 T cell counts below 200/μl in this study, show that (i) the probability of surviving for 2 years is dramatically lower in subjects with low GSH levels, particularly in CD4 T cells; (ii) oral administration of NAC replenishes GSH, particularly in individuals with low GSH levels; and (iii) taking NAC for 8–32 weeks is associated with substantially improved survival for individuals with AIDS. Full reports of these studies will be published shortly (18; Dubs et al., unpublished findings).

This work was conducted in collaboration with Drs. J. Gregson Dubs, Mario Roederer, Stanley Deresinski, Michael Anderson, Stephen W. Ela, and Malcolm Zaretsky.

METHODS

GSH Measurements

We used HPLC analyses to measure GSH levels in rapidly processed whole blood samples (19; M.T. Anderson, unpublished findings) and multiparameter fluorescence activated cell sorter (FACS) analyses to measure intracellular GSH levels in T cell subsets in peripheral blood mononuclear cells (PBMCs) reacted with monochlorobimane to form the fluorescent glutathione-*S*-bimane (GSB) conjugate (20). We determined the median GSB level for each PBMC subset for each subject and used these median values in subsequent analyses, for example, to compute means for groups of subjects, to display distributions, to group subjects, and so on. GSB levels are expressed relative to the lymphocyte GSB level in a frozen PBMC standard measured in parallel with the PBMC samples.

Bivariate analyses demonstrate a significant correlation between the FACS measurements of GSH levels (i.e., GSB levels) and HPLC-measured whole blood GSH levels, which mainly reflect GSH levels in erythrocytes. For example, comparison of CD4 T cell GSB levels against HPLC-measured whole blood GSH for 47 subjects in a bivariate analysis generates an r-value of 0.53 and p-value of 0.0001. In a least-squares model, CD4 GSB levels significantly predict whole blood GSH; however, the model is greatly improved by including hematocrit level, which corrects for variation due to the volume of erythrocytes in the blood sample (adjusted R2 = 0.4; $p = 0.004$ for CD4 GSB and 0.002 for hematocrit).

Survival Analyses

We collected baseline data, including GSH levels, T cell subset counts, and clinical laboratory measurements for over 200 HIV-infected subjects, 83 of whom were enrolled into a double-blind, placebo-controlled trial testing the functional bioavailability of NAC. One to two years later, we surveyed the survival status of all subjects and evaluated the relationships between survival and GSH levels. Since all but 2 of the deaths we recorded occurred in subjects with a diagnosis of AIDS (defined as having CD4 T cell counts below 200/μl), we restrict analysis here to the 96 subjects in this group.

GSH Replenishment Following Oral Administration of NAC

Subjects were enrolled in a randomized, double-blind, placebo-controlled trial and given either NAC (6.9 ± 1.1 g/day on average) or placebo for 8 weeks. All subjects who qualified for enrollment had low GSB levels, were free of active opportunistic infections, and were otherwise relatively healthy, as judged by Karnofsky score and professional assessment. Subjects were also required to have maintained a stable reverse-transcriptase inhibitor regimen for the previous 4 months and were limited with respect to the taking of drugs that deplete GSH (e.g., acetaminophen) or diminish oxidative stress (e.g., high doses of vitamins C or E).

Statistical Analyses

We used the JMP Macintosh statistical package produced by the SAS Institute (Carey, NC) for all statistical analyses.

RESULTS

Survival Is Dramatically Lower in Subjects with Low CD4 T Cell GSH (GSB) Levels

CD4 T cell GSH levels, referred to hereafter simply as GSB levels, and measured at time 0 or baseline, tend to be lower in subjects with AIDS (defined for this study as subjects with CD4 T cell counts below 200/μl) (see Table 1). The mean GSB level for these subjects is below the GSB levels observed in over 80% of uninfected control subjects. In addition, because trial enrollment criteria so specified, GSB levels for subjects with AIDS who qualified for the NAC trial were substantially lower than the group with AIDS as a whole. The full report of this study (18) presents data for additional groups of subjects and detailed data for the AIDS subjects shown here.

The low GSB levels in subjects with AIDS were associated with poor survival. Roughly 40% (37/96) of these subjects died within 2 years of baseline data collection. The great majority of these deaths occurred among subjects with GSB levels below the mean for the group as a whole; very few deaths occurred among subjects with the highest GSB levels. Furthermore, the frequency of deaths was substantially higher in subjects with the lowest GSB levels.

Logistic regression analysis in Figure 1 shows the sharp increase in survival as a function of increasing baseline GSB levels in subjects with AIDS. Since, as we show below, subjects with AIDS who took NAC for more than 8 weeks survived longer than comparable subjects who did not take NAC, logistic regression analysis for the subgroup of monitored subjects who were not enrolled in the NAC trial results in an even greater survival differential ($p < 0.0001$, curve not shown). In essence, only 27% of subjects in the lowest quartile of this GSB distribution survived the 2-year

Table 1. CD4 GSB Levels are Lower in Subjects with AIDS

Subjects[a]		n[b]	CD4 GSB[c] (Mean±SD)
Uninfected		47	1.14±0.28
All HIV$^+$		203	0.97±0.28
CD4>200	All	107	1.05±0.25
CD4≤200 (AIDS)	All	96	0.88±0.29
	Trial subjects[d]	37	0.72±0.16

[a]Groups of subjects studied. CD4 ≤200 = subjects with CD4 T cell counts less than or equal to 200/μl, used in this study as synonymous with subjects with AIDS. Percentage of survivors at the end of the 2-year observation period: CD4 >200 = 97%; CD4 ≤200 = 65%.

[b]Number of subjects for whom CD4 GSB, absolute CD4 T cell counts, and survival status were recorded and on which computations are based. Overall study group composition: total, 203; male, 194; Caucasian, 151; mean age, 40.4±7.8 years, range 23–68 years.

[c]CD4 GSB values were normally distributed for all groups and differed significantly for all combinations of distinct groups (Anova t-test): $p \leq 0.0001$ for uninfected vs. all HIV$^+$, CD4>200 vs. CD4≤200 and No-TS vs Trial subjects; $p=0.002$ for No-TS vs. CD4>200. Standard error of the mean (SEM) for CD4 GSB means for the groups shown = 0.02–0.04. SD=standard deviation.

[d]Trial subject group includes all NAC and placebo arm subjects with CD4 T cell counts below 200/μl (37/55 in the trial as a whole). Low CD4 GSB levels for these subjects reflect the trial enrollment requirement for low GSB levels.

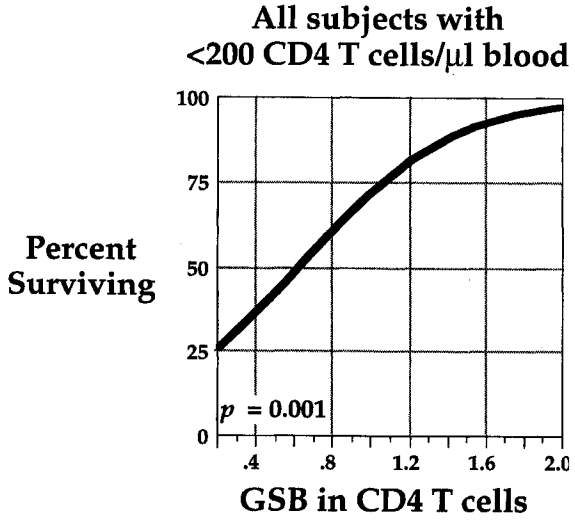

Figure 1. Probability of surviving for 2 years increases with increasing CD4 GSB levels. Logistic regression analysis for all subjects with CD4 counts below 200/μl (AIDS), including those in the NAC trial (most of whom took NAC). Survival status of subjects was determined 2 years after baseline data collection.

observation period of this study, whereas 87% of those in the highest quartile survived.

Logistic regression analyses demonstrate the importance of baseline GSB levels for survival by reporting the effect of baseline GSB levels on the survival status of subjects 2 years after baseline data collection. Kaplan–Meier analyses, which report survival as a

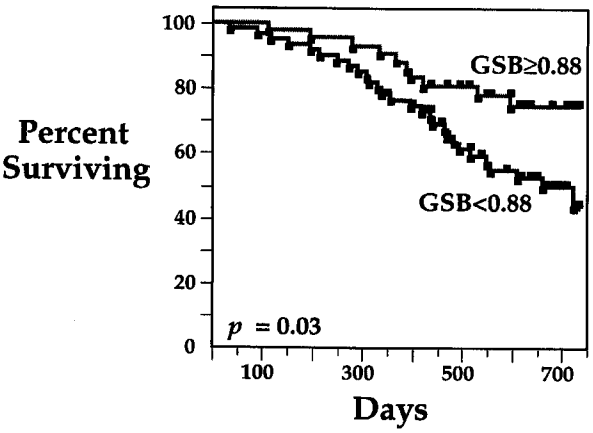

Figure 2. Low CD4 GSB levels are associated with poor survival in AIDS. Kaplan–Meier analysis for all subjects with CD4 counts below 200/μl (AIDS), including those in the NAC trial (most of whom took NAC). Subjects are divided at the mean baseline CD4 GSB level (0.88) for the this group of subjects. Survival times are computed from the date of the baseline visit.

function of time for subjects grouped according to GSB level (above or below the mean for subjects with AIDS), similarly show that higher GSB levels are associated with longer survival (Figure 2). Finally, proportional hazard analyses, which take both survival time and baseline GSB levels into account, show that GSB levels predict survival for the subjects with AIDS cohort (p = 0.0002) and report a 2-fold increase in survival for a difference of 0.3 GSB units (the standard deviation of the GSB levels in the cohort).

GSB Levels Versus CD4 T Cell Counts

CD4 T cell counts show a significant ability to predict survival in subjects with AIDS (p = 0.001); however, the significance of this prediction in proportional hazard analyses is greatly diminished when GSB levels are added to the model (p = 0.01 for CD4 T cell count; p = 0.003 for GSB). Since CD4 T cell counts are loosely correlated with GSB levels (Pearson's r = 0.33), this loss of significance suggests that for relatively healthy subjects with AIDS (i.e., with CD4 T cell counts below 200 μl), GSB levels are as good or a better predictor of survival than CD4 T cell counts. In addition, it also suggests that low GSB levels are an important contributor to the poor survival of HIV-infected individuals with low CD4 T cell counts.

Although the AIDS CD4 T cell count threshold (200/μl blood) provides a strong predictor of survival in overall HIV-infected populations, data presented above indicate that the prognostic value of counts under this threshold is more questionable. Combining GSB levels and CD4 T cell counts improves the accuracy of this survival prediction and thus is potentially of greater value for the group of HIV-infected subjects with low CD4 T cell counts. The combined measurement may also provide a better method for subject selection or stratification in AIDS clinical trials, since low GSB levels identify a high-risk

Table 2. NAC-dependent Increase in Whole Blood GSH

Combination of variables tested	Contribution to prediction of whole blood GSH levels at the end of an 8-week trial[a]		
	p-value	R^{2b}	RMSE[c]
0 week GSH	<0.0001	0.45	0.14
NAC vs. placebo	0.0008		
0 week GSH	0.0005		
NAC vs. placebo	0.0003	0.53	0.13
0 week CD4 GSB[d]	0.004		

[a]Whole-blood GSH principally reports GSH levels in erythrocytes in blood. Blood samples from 47 subjects were tested at the beginning and end of an 8-week randomized double-blind placebo- controlled trial testing the ability of orally administered NAC to raise GSH levels. One outlier was excluded. Data show the standard least-squares model fit. Subjects took 3200–8000 mg of NAC per day (median 5400 mg) for up to 8 months, supplied as 800 mg effervescent tablets.

[b]R^2 adjusted for number of variables added.

[c]Root mean square error.

[d]The significant contribution of initial CD4 GSB values, which are loosely correlated with initial whole-blood GSH levels, reflects the tendency for NAC ingestion to result in a greater increase in GSH levels in subjects with low initial GSB values (Dubs et al., unpublished findings).

subgroup of subjects, only a small percentage of whom are likely to survive longer than 2 years in the absence of intervention.

Oral Administration of NAC Replenishes GSH, Particularly in Individuals with Low GSB Levels

Data from the randomized, double-blind, placebo-controlled trial that we conducted show an average dose of that administration of NAC at 6900 mg/day for 8 weeks significantly elevates whole blood GSH (p = 0.0008) (Dubs et al., unpublished findings). Covariate analyses demonstrate that NAC increases whole-blood GSH levels more effectively in the subjects who had the lowest GSB levels at the start of the trial (Table 2). Thus, as might be expected, NAC is most effective in raising GSH levels when those levels are substantially depleted.

NAC Ingestion is Associated with Improved Survival in Subjects with AIDS

The NAC trial discussed above was not designed to test NAC efficacy in prolonging survival. However, after the initial double-blind placebo-controlled phase of the trial (8 weeks duration), all subjects were offered open-label NAC for up to 6 months during the continuation phase of the trial. As part of our overall monitoring study, we compared the fate of these subjects over the next 2 years with the fate of otherwise similar subjects who were not enrolled in the trial and did not have an opportunity to take NAC.* To our surprise, given the relatively short time (8–32 weeks) that NAC was administered, we found that NAC ingestion was associated with substantially longer survival (Table 3 and Ref. 18).

Since the enrollment criteria for the trial specifically excluded subjects with higher GSB levels, GSB levels in subjects in the NAC and No-NAC groups were amongst the

Table 3. Taking NAC is Associated With Better Survival

NAC history of subjects[a]	n[b]	Survival at 2.5-years[c] %	CD4 GSB (Mean at baseline ± SD)
NAC (8–32 weeks)	25	76	0.73±0.14
No-NAC (matched to NAC group)[d]	19	42	0.72±0.17

[a]All subjects had CD4 T cell counts below 200/μl and GSB levels low enough to qualify for entry into the NAC trial (18). All subjects who took NAC were enrolled in the NAC trial. NAC subjects took NAC for 8–32 weeks. Some were randomized to the NAC arm of the trial; others to open-label NAC during the continuation phase.

[b]Number of subjects in each group.

[c]Proportional hazard calculation for NAC: No-NAC survival yields a survival risk ratio of 1.8 (1.1–3.0, 95% confidence interval), p=0.018. Survival time in the model is computed from the time each subject began taking NAC. Table shows percentage surviving at the end of the 2.5 year observation period. Kaplan–Meier analyses comparing survival in the NAC and No-NAC groups is shown in Figure 3.

[d]There were no significant differences (p>0.1) in baseline measurements between the NAC and the No-NAC group for all parameters tested, including the following: absolute CD4 and CD8 counts; naive and memory T cell subset counts; hematocrit and other clinical laboratory tests; Karnofsky score; age; weight; GSB levels in B cells, monocytes, NK cells and all T cell subsets

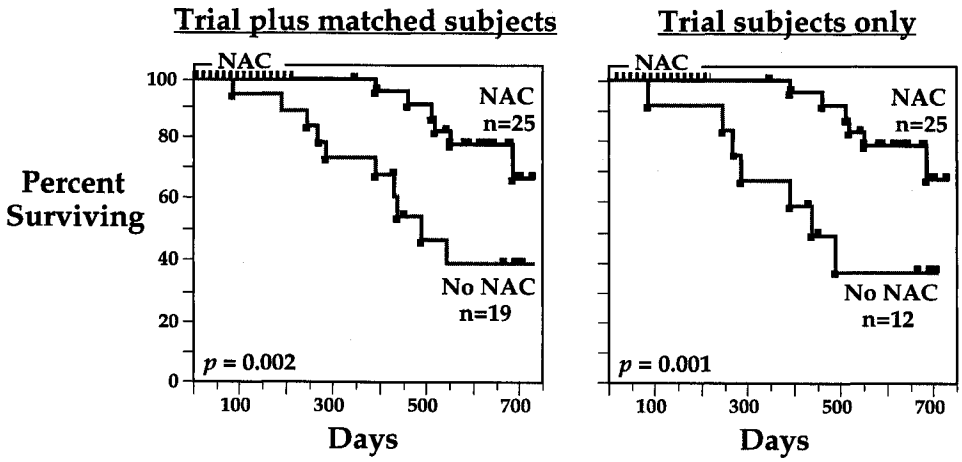

Figure 3. Taking NAC is associated with increased survival. Kaplan–Meier survival analyses compare survival of subjects who took NAC (NAC group) with survival of similar subjects who did not take NAC (No-NAC groups). The NAC group (both panels) includes 25 subjects who took NAC for 8–32 weeks (median 24 weeks; interquartile range 12–27 weeks), initiated either during the randomized, double-blind phase of the NAC replenishment trial (13 subjects) or during the open-label trial phase (12 subjects). The No-NAC group shown in the left panel includes all screened subjects (19) who met the basic criteria for entry into the NAC trial. The No-NAC group shown in the right panel includes only those subjects (12) who were actually enrolled in trial. Survival times for subjects who took NAC are computed from initiation of NAC ingestion (0 week for NAC arm; 8 weeks after the trial began for placebo arm). Survival times for subjects who did not take NAC are computed from the trial entry or screening date.

lowest in the study. Therefore, based on data presented above (Figures 1 and 2), the probability that these subjects would survive the 2-year observation period in our study was very low. Consistent with this, 11 of the 19 subjects in the No-NAC group died before the end of the observation period. In contrast, however, only 6 of the 25 subjects in the NAC group succumbed. Furthermore, half of the deaths in the No-NAC group occurred before the first death in the NAC group (Figure 3).

Proportional hazard analyses show the significant improvement of survival associated with oral administration of NAC. In addition, these analyses demonstrate that recruitment and trial management factors that contributed to determining whether and for how long NAC was taken did not significantly influence survival outcomes. In essence, there was no significant effect attributable to the reasons subjects took NAC (randomized to the NAC arm, elected NAC during open-label; $p > 0.2$) or the reasons subjects did not take NAC (not enrolled in the trial, left the trial, refused open-label; $p > 0.2$). However,

*In essence, although we lacked a proper placebo control group for survival comparison, we had a total of 19 subjects who did not take NAC but whose history indicated that they were very similar to those who did. We confirmed the comparability of these two groups (NAC and "No-Nac" groups) in analyses that failed to reveal any significant differences between them for a wide variety of clinical and FACS measurements, including GSB levels and CD4 T cell counts (18).

NAC ingestion significantly improved survival ($p = 0.019$) and indicated a roughly 2-fold survival advantage for subjects in the NAC group, i.e., NAC:No-NAC risk ratio = 1.8, 95% confidence interval = 1.1–3.0.

The association of prolonged survival with oral administration of NAC in this study is very dramatic. However, it is suspect because NAC was not administered in the context of a prospective trial in which survival was an endpoint. Since subjects in both the NAC and placebo arms of the trial in which NAC was administered were offered open-label NAC, the increased survival associated with taking NAC could be explained by factors associated with whether subjects took NAC rather than with the ingestion of the NAC itself. Although we found no indication of such bias, it cannot be excluded. Therefore, our findings basically argue for the initiation of a prospective placebo-controlled trial designed specifically to determine the therapeutic value of NAC in AIDS (14,16) and/or that of other GSH-replenishing drugs (21–25). Since NAC is nontoxic and could be used where medical services are limited, our findings indicate that such a trial should be initiated as rapidly as possible. Other pharmaceuticals that replenish GSH should also be tried for the same purpose. In any event, the poor survival that we have demonstrated in GSH-depleted subjects with AIDS underscores the importance of finding ways to replenish GSH in these individuals and ways to prevent this GSH depletion earlier in the disease.

DISCUSSION

We have shown that GSH depletion is associated with impaired survival: the greater the depletion, the worse the prospects for survival (Ref. 18, summarized here). These findings complement earlier preclinical data indicating that GSH depletion may play important roles in AIDS pathogenesis, for example, impairment of T cell function, facilitation of NFκB activation and HIV replication (1–3). By replenishing GSH, NAC or other agents may be able to modulate such adverse effects of GSH depletion. However, HIV-infected individuals would be better served if we could identify the mechanisms that underlie the GSH depletion and intervene, if possible, to prevent its occurrence. If ways could be found to do this on a long-term basis, HIV disease progression might be controlled in a way that would prevent the worst aspects of the disease.

Is such intervention possible? We have been struck by recent data indicating that HIV-TAT induces oxidative stress (Chapter 1; 26–29). We wonder whether the production and release of TAT could play a major role in the progressive depletion of GSH in HIV disease. If so, then targeting TAT for intervention (e.g., production of an anti-TAT vaccine) could be an important new strategy for controlling HIV disease.

At a more immediate level, certain rather simple precautions might help to slow the progress of HIV disease. Since studies presented here associate GSH depletion and oxidative stress with poor survival in AIDS, we believe that HIV-infected individuals should avoid excessive exposure to sun and UV irradiation and excessive use of drugs such as acetaminophen (Tylenol) that are known to deplete GSH. Physicians treating HIV-infected individuals should similarly consider exercising caution in prescribing formulations or recommending over-the-counter preparations containing such GSH-depleting drugs. These conservative measures could eliminate some of the more accessible causes of GSH depletion and thus could potentially prevent decrease of GSH to the level that predicts death within the following 2 years.

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