

## Restoration of Blood Total Glutathione Status and Lymphocyte Function Following $\alpha$ -Lipoic Acid Supplementation in Patients with HIV Infection

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### ABSTRACT

**Objectives:** To determine whether supplementation with  $\alpha$ -lipoic acid (ALA), a glutathione-replenishing disulfide, modulates whole blood total glutathione (GSH+GSSG) levels and improves lymphocyte function in human immunodeficiency virus (HIV)-infected subjects with history of unresponsiveness to highly active antiretroviral treatment (HAART).

**Design and setting:** Randomized, double-blinded, placebo-controlled trial conducted at two study sites: an eye clinic at a county hospital in San Jose and a research clinic in San Francisco, California.

**Subjects:** A total of 33 HIV-infected men and women with viral load  $>10,000$  copies/cm<sup>3</sup>, despite HAART, aged 44–47 years, approximately 36% nonwhite, were enrolled.

**Intervention:** Patients were randomly assigned to receive either ALA (300 mg three times a day) or matching placebo for 6 months.

**Main outcome measures:** The change over 6 months in blood total glutathione status, lymphocyte proliferation response to T-cell mitogens, CD4 cell count, and viral load in patients receiving ALA compared to placebo.

**Results:** The mean blood total glutathione level in ALA-supplemented subjects was significantly elevated after 6 months ( $1.34 \pm 0.79$  vs.  $0.81 \pm 0.18$  mmol/L) compared to insignificant change ( $0.76 \pm 0.34$  vs.  $0.76 \pm 0.22$  mmol/L) in the placebo group (ALA vs. placebo:  $p = 0.04$ ). The lymphocyte proliferation response was significantly enhanced or stabilized after 6 months of ALA supplementation compared to progressive decline in the placebo group (ALA vs. placebo:  $p < 0.001$  with phytohemagglutinin;  $p = 0.02$  with anti-CD3 monoclonal antibody). A positive correlation was seen between blood total glutathione level and lymphocyte response to anti-CD3 stimulation ( $R^2 = 0.889$ ). There was no significant change in either HIV RNA level or CD4 count over 6 months in the ALA-supplemented compared to the control group.

**Conclusion:** Supplementation with  $\alpha$ -lipoic acid may positively impact patients with HIV and acquired immune deficiency syndrome by restoring blood total glutathione level and improving functional reactivity of lymphocytes to T-cell mitogens.

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## INTRODUCTION

Acquired immune deficiency syndrome (AIDS) is characterized by infection with human immunodeficiency virus (HIV) and progressive depletion of CD4+ (helper) T cells leading to opportunistic infections and malignancies.<sup>1</sup> In addition to CD4+ cell loss, HIV-infected patients manifest impaired immune responses affecting both CD4+ and CD8+ T-cell subsets that resemble an anergic state of immune dysfunction.<sup>2,3</sup> Although highly active antiretroviral treatment (HAART) can lower viral load in blood, antiretroviral drugs do not fully restore the immune system and patients often fail multidrug treatment, suggesting the need for immunomodulating agents, in addition to antiviral therapy.

In recent years, several reports have suggested that impaired antioxidant defenses, in particular glutathione deficiency, may play a role in the immunopathogenesis of HIV infection.<sup>4-7</sup> Glutathione, a cysteine-containing tripeptide (glutamyl-L-cysteinyl-glycine), is the major redox buffering thiol within cells and a central molecule for lymphocytic function. Glutathione is necessary for T-cell activation, interleukin-2-dependent proliferation, and antibody/cell-mediated cytotoxicity.<sup>8</sup>

Both HIV-infected and AIDS patients manifest depressed, systemic glutathione levels.<sup>9,10</sup> Since several immunologic functions relevant to HIV infection are dependent on adequate intracellular total glutathione balance, glutathione-replenishing compounds have been suggested in the treatment of HIV infection.<sup>11-14</sup>

Thiol replacement with *N*-acetyl-cysteine (NAC) has proven to be beneficial in AIDS patients where the higher glutathione blood level attained was associated with increased survival.<sup>12</sup> However, oral NAC has limited bioavailability *in vivo*.<sup>14</sup> In contrast to NAC, studies with  $\alpha$ -lipoic acid (ALA), a glutathione-augmenting disulfide,<sup>15,16</sup> have shown improved bioavailability, demonstrating 93% absorption from all parts of the gastrointestinal tract in the rat<sup>17</sup> and rapid absorption in human volunteers,<sup>18-20</sup> reaching peak plasma concentrations between 40 and 90 minutes, followed by turnover in the liver. Another advantage over NAC is ALA's redox modulating ability, being recognized as a substrate for bioreduction to dihydrolipoic acid (DHLA), thereby functioning as a metabolic antioxidant.<sup>16</sup>

Both ALA and DHLA have been shown to suppress HIV replication in cultured cells,<sup>21,22</sup> and ALA has been reported to enhance helper T cells in mice.<sup>23</sup> Additionally, ALA was reported to increase cellular glutathione in mitogenically stimulated lymphocytes and to bypass the inhibitory effect of elevated extracellular glutamate.<sup>24</sup> More recently, ALA was modified to a positively charged amide analogue, lipoic acid plus (LA-plus),<sup>25</sup> with a better cellular uptake and retention of the reduced form,<sup>25</sup> that was more potent in protecting neuronal cells from glutamate-mediated cytotoxicity,<sup>26</sup> in inhibiting oxidant or cytokine-stimulated NF- $\kappa$ B

activation,<sup>25</sup> and in protecting rat thymocytes against spontaneous and etoposide-induced apoptosis.<sup>25</sup> No clinical studies heretofore have looked at effects of ALA supplementation on immune function.

We conducted a randomized, placebo-controlled study to evaluate the immunomodulatory effect of ALA in HIV patients with history of HAART unresponsiveness (i.e., viral load above 10,000 copies per cm<sup>3</sup>, despite HAART). The rationale for choosing the latter inclusion criterion was that patients failing HAART therapy have limited treatment options. The study was designed to test the hypothesis whether supplementation with ALA would increase blood total glutathione and improve immune T-cell function in this patient population. Other outcome measures included surrogate markers.

## MATERIALS AND METHODS

### *Patients and Controls*

Subjects were recruited at two study sites: Eye Clinic, Santa Clara Valley Medical Center (SCVMC), San Jose, CA and Quest Clinical Research, San Francisco, CA. Subjects comprised HIV-infected adults (median age 44-47 years), 88% men and 12% women, which included 36% nonwhite participants from minority groups. Clinical inclusion criteria consisted of previous documentation of HIV-positive status and evidence of unresponsiveness to HAART (i.e., prior exposure to at least two protease inhibitors plus nucleoside analogs and viral load >10,000 copies/cm<sup>3</sup> despite HAART). Initial entry criteria also included a CD4+ cell count <50 cells/mm<sup>3</sup>. However, due to scarcity of patients and to avoid an end-stage population that may preclude response to ALA, the initial inclusion criteria were revised upon approval by the Institutional Review Board (IRB) to CD4 cells  $\geq$ 50 cells/mm<sup>3</sup> with viral load above 10,000 copies/cm<sup>3</sup>. Subjects who used excessive amounts (>5 times recommended daily allowance) of NAC, glutathione, and other antioxidant supplements, during 2 months prior to study entry, were excluded from the protocol. To minimize potential risk from ingesting ALA, diabetic individuals, pregnant women, alcoholics, patients with asthma, and those suspected of having a thiamine deficiency (with history of polyneuritis) were also excluded from the study. The protocol was reviewed and approved by the human subjects' research committees (IRB) at SCVMC, San Jose and Saint Francis Memorial Hospital, San Francisco, and all participants gave informed written consent at enrollment.

### *Interventions*

After signing informed consent, 33 eligible subjects were randomized in a double-blind fashion to either treatment or placebo (control) group (Fig. 1). Subjects in the treatment

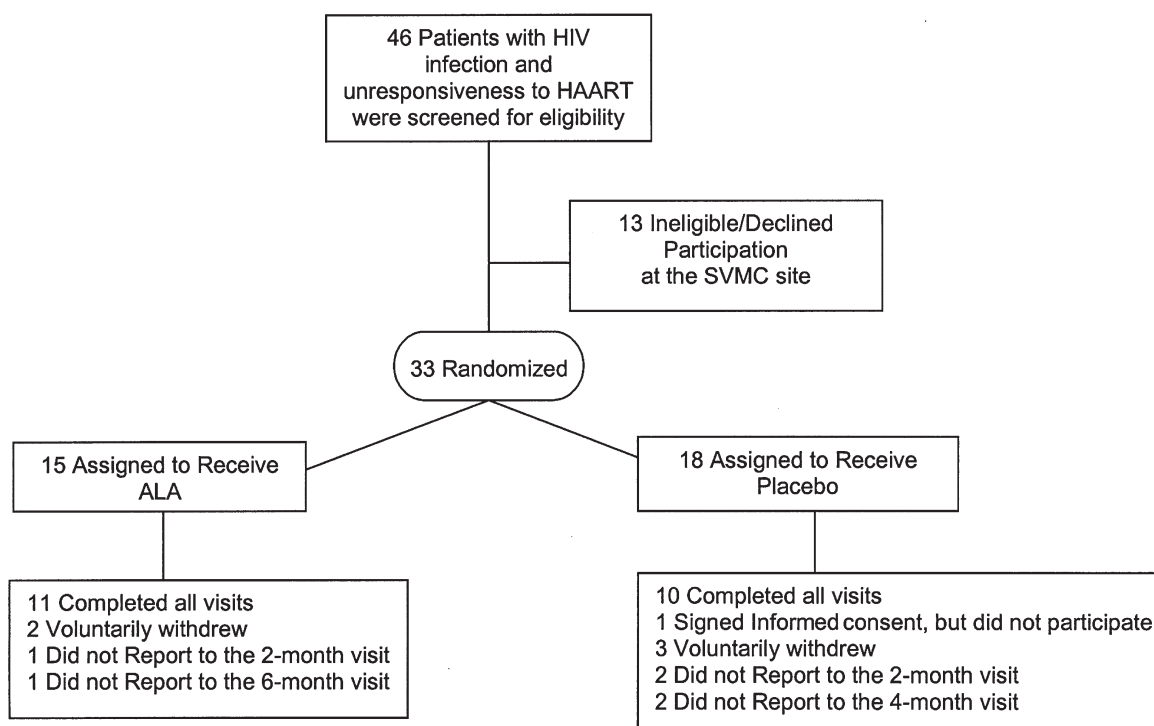


FIG. 1. Patient flow through study.

group were given ALA supplement (ALA 300 sustain, provided by Jarrow Formulas, Los Angeles, CA) at a dose of 300 mg three times daily for 6 months. Patients in the control group received an inert matching placebo containing other ingredients (excipients) present in the ALA supplement (magnesium stearate, stearic acid, silicon dioxide, cellulose, dicalcium phosphate) plus a trace of turmeric and whey powder to match the color. For randomization, a list of sequential numbers (001–050) was randomly assigned to either placebo or ALA based on the random draw of colored chips, and appropriate numbers were affixed on the corresponding placebo or ALA bottles by the clinical research nurse. Patients were assigned the intervention in sequential order according to the list by study coordinator who was blinded to the randomization sequence, which was concealed until data analysis.

### Study evaluations

As part of the study protocol, body weight was recorded, general condition was evaluated, and a complete work-up of blood samples was conducted at baseline and subsequent study visits at 2, 4, and 6 months. Blood was collected in EDTA tubes and processed for tests described below. Subjects were advised to report any unusual symptoms or changes in appetite, weight, or tolerance to ALA supplement. Compliance was verified by interview and counting of leftover supplement.

#### 1. Hematology/viral load analysis.

These tests were done at the SCVMC central laboratory and at Quest Diagnostics, San Francisco, CA. Hematology analysis consisted of complete blood count/differential and T-cell subset panel, which included total T lymphocytes, helper-inducer T (CD4+) and suppressor-cytotoxic T (CD8+), expressed as cells per mm<sup>3</sup>. HIV RNA was quantified using the polymerase chain reaction and expressed as (log<sub>10</sub>) of genomic copies per mL of blood plasma.

#### 2. Assessment of lymphocyte function *in vitro*.

Peripheral blood lymphocytes (PBL) were isolated by centrifugation of whole blood on Ficoll gradients (LSM, BioWhittaker Products Line, Lonza, Walkersville, MD), followed by removal of monocytes through overnight adherence on tissue culture flasks (25 cm<sup>2</sup>). Cells were cultured at 37.0°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air in RPMI 1640 medium supplemented with 10% heat-inactivated serum plus antibiotics and exposed to T-cell mitogens, namely phytohemagglutinin (PHA) or anti-CD3 monoclonal antibody (Sigma-Aldrich, St. Louis, MO). For mitogen stimulation, PBL were seeded in 96-well plates and exposed (in triplicate) to PHA (at 1 µg/mL or 10 µg/mL) or anti-CD3 monoclonal antibody; control (unstimulated) cultures received medium alone. After 48 hours, cells were labeled with bromodeoxyuridine (BrdU) by overnight incubation (for 16–18 hours).

TABLE 1. BASELINE CHARACTERISTICS OF ALL STUDY PARTICIPANTS (N = 33)

	ALA, mean (SD) (n = 15)	Placebo, mean (SD) (n = 18)
Age	47.2 (6.8)	43.7 (7.6)
HIV RNA copies log <sub>10</sub>	4.50 (0.48)	4.87 (0.57)
CD4 <sup>+</sup> lymphocytes (cells/mm <sup>3</sup> )	259.3 (241.8)	184.4 (185.5)
CD8 <sup>+</sup> lymphocytes (cells/mm <sup>3</sup> )	1015.3 (474.0)	821.4 (461.4)

ALA,  $\alpha$ -lipoic acid; HIV, human immunodeficiency virus.

Lymphocyte proliferation was assayed by measuring incorporation of BrdU into DNA at an absorbance of 450 nm ( $A_{450\text{nm}}$ ) using an enzyme-linked immunosorbent assay–based colorimetric assay (Roche Diagnostics, Indianapolis, IN). The stimulatory ratio was determined as  $A_{450\text{nm}}$  (stimulated cells)/ $A_{450\text{nm}}$  (unstimulated cells) and the fold change in lymphocyte proliferation was defined as the mean stimulatory ratio at a given time interval (study visit) over baseline.

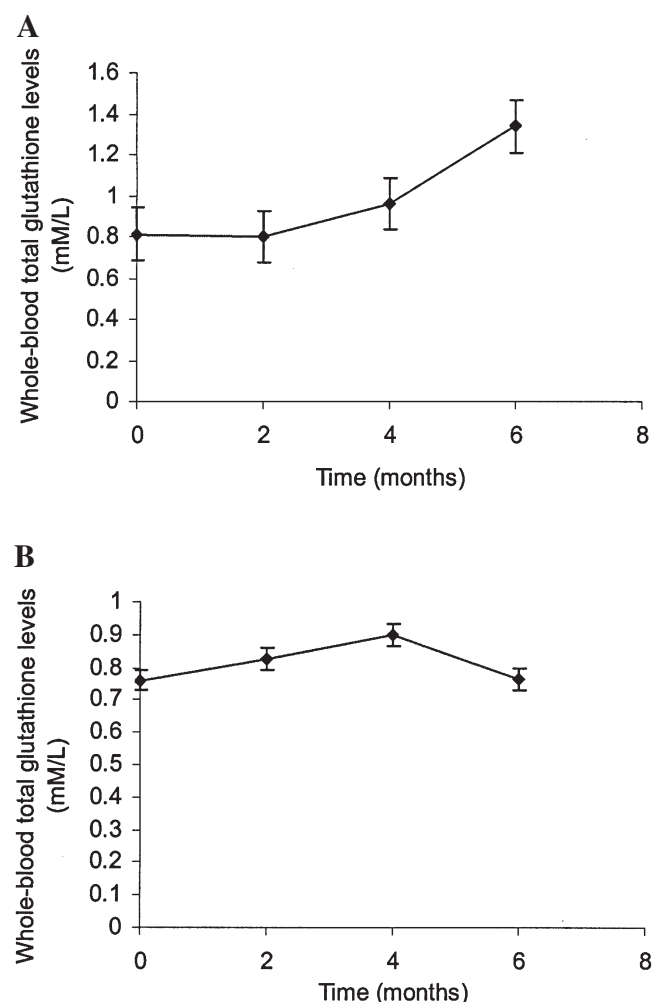
### 3. Estimation of whole blood total glutathione status.

Measurement of total glutathione level in whole blood, an indicator of systemic thiol status in human subjects,<sup>27</sup> was estimated using a microtiter plate method adapted by Vogt and Richie<sup>28</sup> from their modifications of the procedure of Tietze.<sup>29</sup> For sample preparation, an aliquot of whole blood (collected in EDTA tubes) was added to 4 volumes of ice-cold 5% metaphosphoric acid (MPA) to prevent oxidation of reduced glutathione (GSH) *ex vivo*. After quick mixing, precipitated proteins were removed by low-speed centrifugation and the supernatant was stored at  $-70^{\circ}\text{C}$  until assay. Total glutathione (GSH + oxidized glutathione [GSSG] level) was estimated using the GSH oxidoreductase enzymatic recycling reaction<sup>28</sup> in the presence of authentic standards by measuring the rate of color change in a microplate reader (Kinetic-QCL, BioWhittaker) at 410 nm. Readings were corrected for blanks reactions (in the absence of substrate) and total glutathione level was expressed as millimoles (mmol/L) per liter of whole blood.

### Data analysis

Data were analyzed using the Excel (Microsoft Corp., Redmond, WA) data analysis suite. The means and standard deviations of study endpoints, namely, viral load, CD4 and CD8 count, blood total glutathione status, lymphocyte-proliferation response (stimulatory ratio), and body weight at baseline and 6 months were determined for the ALA-supplemented and placebo groups. To assess study outcome, a primary analysis was conducted to compare the change over 6 months between treatment versus placebo in the individual study endpoints, using the unpaired *t*-test. For treatment comparisons, a *p* value of  $<0.05$  was considered significant. For those endpoints

manifesting significant difference, a secondary analysis was done to estimate the change over time in these variables within the respective group and to assess the significance of change from baseline using the paired *t*-test.

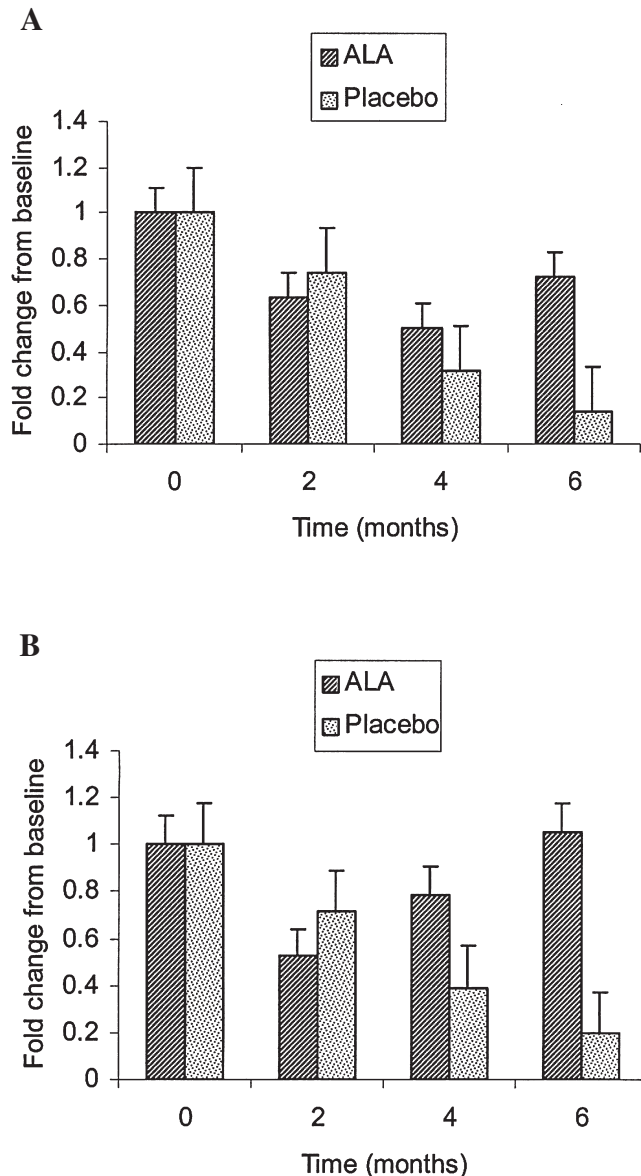


**FIG. 2.** Change in whole-blood total glutathione status over a period of 6 months in human immunodeficiency virus–infected subjects supplemented with (A)  $\alpha$ -lipoic acid or (B) placebo. Data represent mean total glutathione levels  $\pm$  standard error of the mean at the indicated intervals.

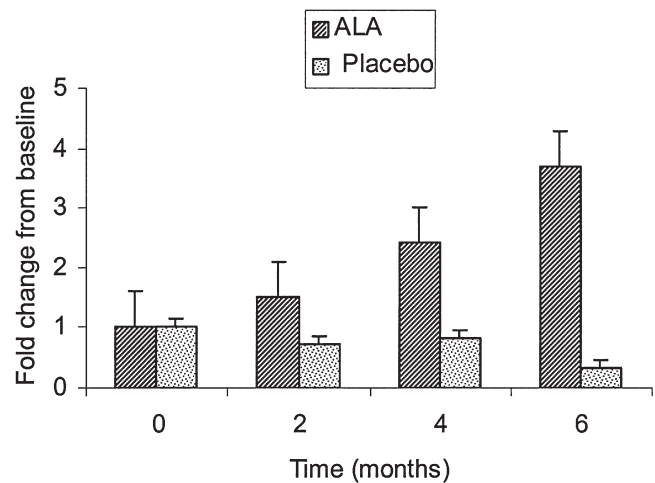
## RESULTS

*Participant characteristics and flow*

The baseline characteristics of the enrolled subjects are shown in Table 1 and the participant flow through the study is presented in Figure 1. Of the 33 patients enrolled into the study, 1 patient signed an informed consent but did not further participate for unknown reasons, 3 patients did not show up at the 2-month visit, 3 did not report at the 4- or 6-month visit, and 5 subjects withdrew from the protocol for personal



**FIG. 3.** Effect of treatment with  $\alpha$ -lipoic acid or placebo in human immunodeficiency virus-infected subjects on proliferative response of isolated lymphocytes following exposure to phytohemagglutinin (PHA) at concentrations of (A) 1.0  $\mu\text{g/mL}$  and (B) 10  $\mu\text{g/mL}$ . Data represent the mean and standard error of the mean of the fold change in PHA-induced proliferative response of lymphocytes relative to baseline.



**FIG. 4.** Effect of treatment with  $\alpha$ -lipoic acid or placebo in human immunodeficiency virus-infected subjects on proliferative response of isolated lymphocytes following exposure to anti-CD3 monoclonal antibody. Data represent the mean and standard error of the mean of the fold change in anti-CD3 proliferative response of lymphocytes relative to baseline.

reasons (Fig. 1). Six-month studies for analysis of the outcome measures were obtained from 21 participants completing the study protocol (11 in the ALA group, 10 in the placebo group).

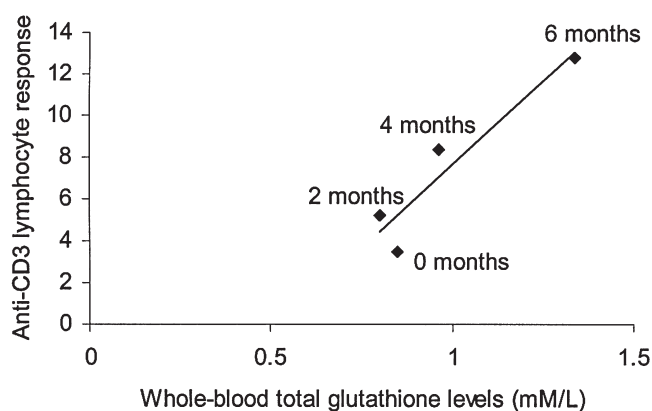
*Whole blood total glutathione levels*

The impact of ALA supplementation on total glutathione (GSH + GSSG) levels in whole blood was evaluated by comparing the change over 6 months between treatment versus control groups using the unpaired *t*-test. By this primary analysis, the 6-month change for whole blood total glutathione level was significant for the ALA-supplemented group compared to placebo ( $\delta = 0.58 \text{ mmol/L}$  in ALA vs. 0 mmol/L in placebo;  $p$  value = 0.04). Accordingly, a secondary analysis was done to estimate the change over time in this endpoint within the respective groups and to assess the significance of change from baseline using the paired *t*-test. By this secondary analysis, the mean whole blood total glutathione level in the lipoic acid-supplemented group increased significantly ( $p$  value = 0.03, by the paired *t*-test) over the 6-month treatment period (Fig. 2A) compared to an insignificant rise in the mean total glutathione level in the placebo group (Fig. 2B) over the same period.

*Lymphocyte function*

The lymphocyte proliferative response (or stimulatory ratio) is an indicator of immune function. The difference in stimulatory ratio ( $A_{450}$  mitogen/ $A_{450}$  unstimulated) from baseline to 6 months between treatment and control groups was significant by the unpaired *t* test for lymphocyte response to both phytohemagglutinin (PHA) ( $p < 0.001$  at 1  $\mu\text{g/mL}$ ;  $< 0.001$  at 10  $\mu\text{g/mL}$ ) and to anti-CD3 monoclonal





**FIG. 5.** Correlation of mean whole-blood total glutathione level with mean lymphocyte proliferative response to anti-CD3 monoclonal antibody for human immunodeficiency virus-infected subjects supplemented with  $\alpha$ -lipoic acid over a period of four visits (6 months).

antibody ( $p = 0.02$ ). The lymphocyte proliferative response was either stabilized or enhanced in the ALA-supplemented group, depending on the mitogen used. Lymphocyte proliferation declined significantly over 6 months in response to PHA (at 1  $\mu\text{g/mL}$  and 10  $\mu\text{g/mL}$ ) in the placebo but was stabilized in the ALA group at 6 months. Figures 3 and 4 show the fold changes in mean stimulatory ratios over baseline for 2-, 4-, and 6-month visits for PHA and anti-CD3 responses. Most importantly, in response to CD3 monoclonal antibody, lymphocyte proliferation decreased by  $\sim 66\%$  in the placebo group over 6 months, whereas there was approximately a 3.7-fold enhancement in the anti-CD3 response in the ALA treatment group over the same period. By secondary analysis, the change in the anti-CD3 activation over 6 months in the ALA group was significant ( $p = 0.03$ ) by the paired  $t$ -test.

#### *Correlation of whole blood total glutathione and lymphocyte proliferation response*

Linear regression analysis showed a positive correlation between blood total glutathione level and lymphocyte pro-

liferative responsiveness to anti-CD3 MoAb ( $R^2 = 0.889$ , Fig. 5).

#### *Viral load and t-cell subsets*

In contrast to the outcome for the preceding endpoints, no significant differences were detected in the 6-month change between the treatment versus control group, for viral load, CD4 count, or CD8 count (Table 2). Subjects were on stable or no antiretroviral regimen at time of study entry, and no major changes in antiretroviral therapy occurred during the duration of the 6-month protocol, except in 1 subject who changed therapy 1 week prior to study termination in the ALA group and in another subject who displayed poor compliance toward the end of the protocol in the placebo group. However, these deviations from compliance did not significantly affect the outcome of the above endpoints when the subjects were factored as outliers into the primary data analysis.

#### *Body weight*

Although subjects in general fared well on the protocol with those on lipoic acid showing a tendency toward weight gain (Table 2), this was not significant compared to the placebo group in the primary analysis.

#### *Adverse events*

There were no adverse events directly related to the study supplement. Subjects on ALA or placebo were also on multiple-drug regimens (including antiretroviral therapy) with their attendant complications. Complaints of skin rash (a minor side effect of ALA) were rare; a couple of patients on ALA experienced some fatigue, but this was transitory and not seen in other subjects on ALA.

## DISCUSSION

The results reported here show that ALA supplementation was associated with enhancement in whole blood total

TABLE 2. VIRAL AND IMMUNE PARAMETERS OF PARTICIPANTS COMPLETING 6-MONTH STUDIES ( $N = 21$ )

	ALA, mean (SD) (n = 11)		Placebo, mean (SD) (n = 10)	
	Baseline	6 months	Baseline	6 months
HIV RNA copies $\log_{10}$	4.53 (0.50)	4.61 (0.55)	5.02 (0.54)	4.78 (0.42)
Body weight (kg)	71.2 (10.0)	73.6 (10.0)	73.4 (9.9)	72.4 (9.2)
CD4 <sup>+</sup> lymphocytes (cells/mm <sup>3</sup> )	300.2 (261.4)	257.8 (248.8)	177.6 (172.6)	171.2 (172.0)
CD8 <sup>+</sup> lymphocytes (cells/mm <sup>3</sup> )	1083.1 (439.4)	973.7 (425.1)	810.1 (458.7)	886.4 (481.8)

ALA,  $\alpha$ -lipoic acid; HIV, human immunodeficiency virus.

glutathione level and recovery of lymphocyte proliferative responsiveness in study subjects who had previously failed HAART. The data indicate for the first time that total glutathione augmentation may positively impact on lymphocyte function. Since HIV-infected persons manifest deficits in both blood total glutathione and lymphocyte function, the study results are significant from the standpoint of immune restoration in that a glutathione-augmenting disulfide can improve lymphocyte reactivity to T-cell mitogens.

The effects of glutathione replacement in patients with HIV infection have been addressed before in other studies with the monothiol NAC with variable results.<sup>12,14,30–34</sup> The variable results with orally administered NAC have been attributed to its limited bioavailability *in vivo*.<sup>14,34</sup> In this regard, disulfides such as ALA have improved bioavailability<sup>17–20</sup> as well as redox-modulating ability<sup>16</sup> and are considered more potent than NAC.<sup>13,16,23,24</sup>

Previous clinical reports of disulfides have involved one pilot study of ALA in HIV infection that reported improvement of antioxidant markers and CD4 cell count.<sup>35</sup> A more recent micronutrient supplementation protocol,<sup>36</sup> which included ALA as an ingredient at a daily total dosage of 400 mg, reported improvement in CD4 count but not viral load in HIV-infected individuals on HAART. However, in both reports, the influence of ALA or micronutrient supplementation on lymphocyte function or reactivity was not evaluated.

Compared to the above studies, patients supplementing with 900 mg/day ALA in the present study showed improvement in both whole blood total glutathione level and lymphocyte reactivity. The lack of a significant effect on CD4 count could be related to the nature of the patient population with respect to their HAART status. In contrast to the present report, which evaluated patients unresponsive to HAART, patients in the ALA pilot study<sup>35</sup> were included before or at the beginning of HAART treatment and those in the micronutrient protocol<sup>36</sup> were HAART responders. It is possible that prior history of HAART nonresponsiveness may diminish the capacity of the immune system to respond to immune modulators. On the other hand, other nutrients in combination with ALA may be required to fully impact the immune system in these patients. Future trials are clearly warranted to evaluate the impact of lipoic acid on various immune parameters in HAART responders as well as the influence of multinutrient supplementation in those for whom HAART fails.

## CONCLUSIONS

Supplementation with  $\alpha$ -lipoic acid may positively impact patients with HIV and AIDS with prior history of HAART unresponsiveness, by restoring whole blood total glutathione level and improving functional reactivity of PBL to T-cell mitogens.

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