

# Comparative study of the anti-HIV activities of ascorbate and thiol-containing reducing agents in chronically HIV-infected cells<sup>1,2</sup>

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**ABSTRACT** To elucidate the action of vitamin C on pathogenic human retroviruses, we investigated and compared the effects of noncytotoxic concentrations of ascorbic acid (AA), its calcium salt (Ca-ascorbate), and two thiol-based reducing agents [glutathione (GSH) and *N*-acetyl-L-cysteine (NAC)] against human immunodeficiency virus (HIV)-1 replication in chronically infected T lymphocytes. Ca-ascorbate reduced extracellular HIV reverse transcriptase (RT) activity by about the same magnitude as the equivalent dose of AA. Long-term experiments showed that continuous presence of ascorbate was necessary for HIV suppression. NAC (10 mmol/L) caused less than twofold inhibition of HIV RT and conferred a synergistic effect (approximately eightfold inhibition) when tested simultaneously with AA (0.426 mmol/L). In contrast, nonesterified GSH ( $\leq 1.838$  mmol/L) had no effect on RT concentrations and did not potentiate the anti-HIV effect of AA. These results further support the potent antiviral activity of ascorbate and suggest its therapeutic value in controlling HIV infection in combination with thiols. *Am J Clin Nutr* 1991;54:1231S–5S.

**KEY WORDS** Human immunodeficiency virus, HIV, vitamin C, antioxidants, *N*-acetylcysteine, glutathione

## Introduction

Ascorbic acid has been shown to inactivate a broad spectrum of viruses as well as to inhibit viral replication in infected cells. Viruses reported to undergo inactivation after exposure to ascorbate include poliovirus, herpesvirus, vaccinia virus, hepatitis virus, and bacteriophages (1, 2). Among the studies on viral replication, Schwerdt and Schwerdt (3) showed that the growth of rhinovirus, after the first replicative cycle, was suppressed by the addition of ascorbic acid (AA) and glutathione (GSH) to human diploid-cell culture. Similar effects were noted by Bissell et al (4), who observed that AA inhibited the replication and infectivity of Rous sarcoma virus in avian tendon cells and chicken embryo fibroblasts. Blakeslee et al (5) reported that ascorbate inhibited the induction of human T-cell leukemia virus in latently infected cells exposed to activating agents. Recently, in laboratory tests of cells infected with human immunodeficiency virus (HIV), we (6) showed that ascorbate significantly suppressed the activity and growth of the acquired immune deficiency syndrome (AIDS) virus.

Clinical improvements were reported after ascorbate administration to patients suffering from various viral diseases, and

large doses of AA are effective in preventing and treating poliomyelitis, hepatitis, infectious mononucleosis, and other herpesvirus infections has been published (7–10). Pauling (2) reviewed the studies evaluating the efficacy of vitamin C against the common cold. He noted that the administration of 1 or 2 g AA resulted in reducing the frequency and severity of the common cold. Other scientists reported a modest reduction in the severity of the infection (11–13). To date, 20 studies have been carried out, all showing that vitamin C has a protective effect, sometimes stopping the development of the cold and sometimes greatly decreasing the symptoms (14).

Since 1984, the Linus Pauling Institute has received reports from several AIDS patients who had voluntarily ingested high doses of vitamin C and reported marked clinical improvement. On the basis of these anecdotal reports and preliminary observations on the beneficial effects of high-dose vitamin C on AIDS patients (15), we undertook a study to evaluate the effects of ascorbate on HIV-1 replication in vitro. In a previous report (6) we described the suppression of HIV replication by ascorbate in chronically and acutely infected cells. In this investigation, we study the long-term effects of ascorbate on HIV-infected cells in culture and determine and compare the effects of AA, its calcium salt, and two thiol-containing reducing agents [*N*-acetyl-L-cysteine (NAC) and GSH] on HIV replication in chronically infected cells. Further, we study the effects of the combination of ascorbate with thiol-containing compounds. In this report, we summarize our earlier observations and present the new findings.

## Materials and methods

### Cell growth and viability

The source and growth conditions of chronically infected H9/HTLV-III<sub>B</sub> cells were described elsewhere (6, 16). Growth rate of cultures in the presence and absence of reducing agent was determined by measuring the number of viable cells at periodic intervals by using the trypan blue dye exclusion test.

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### Ascorbate and other reducing agents

The source and preparation of AA were reported previously (6). Calcium ascorbate (Ca-ascorbate) was purchased from Bronson Pharmaceuticals (La Canada, CA); it contained 88.1% AA net. NAC (Aldrich Milwaukee) was dissolved in RPMI and the pH was adjusted to 7.0 with NaOH. Stock solutions were stored at  $-20^{\circ}\text{C}$ . GSH was obtained from Sigma (St Louis, MO). Stock solutions were made in RPMI and stored at  $-20^{\circ}\text{C}$ .

### Experimental protocol

To study long-term effects of AA, cells were grown in complete RPMI medium with and without supplemental AA for 30 d, with a fresh AA solution added daily to the growth medium. Every 3 d, cells were harvested by centrifugation ( $2400 \times g$ , 10 min,  $4^{\circ}\text{C}$ ), resuspended in fresh growth medium (with or without AA), and reseeded at a cell density  $1 \times 10^9$  cells/L in 25-cm<sup>2</sup> flasks containing 5 mL medium. Culture supernatant collected every 3 d was assayed for HIV reverse transcriptase (RT) activity as described by Hoffman et al (17).

For the evaluation of other reducing agents, H9/HTLV-III<sub>B</sub> cells were grown in 5 mL in 25 cm<sup>2</sup> flask at  $\sim 2.5 \times 10^8$  cells/L in the presence of various concentrations of NAC (0–40 mmol/L) or glutathione (0–1.838 mmol/L). Because of the instability of added compounds at  $37^{\circ}\text{C}$ , cells were centrifuged daily and resuspended in fresh growth medium containing the required concentration of the compound. Culture supernatant was periodically tested for HIV RT activity as described by Hoffman et al (17) and for p24 antigen by the method of Goudsmit et al (18). RT was expressed as the amount of [<sup>3</sup>H]dTMP incorporated into DNA template (Bq per  $10^6$  cells). The p24 value was expressed as ng protein/ $10^6$  cells.

## Results

### Summary of previous findings in chronically and acutely infected cells

We previously reported the effects of noncytotoxic concentrations of ascorbic acid against HIV replication in T-lympho-

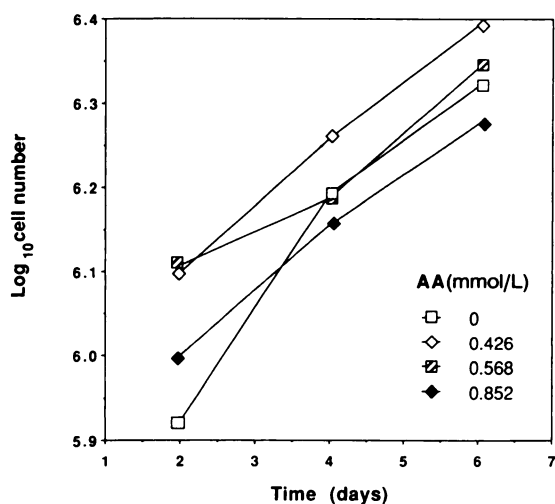


FIG 1. Growth rates of H9/HTLV-III<sub>B</sub> cells with and without ascorbate. Growth conditions and measurement of cellular viability were essentially as described by Harakeh et al (6). Data are taken from Figure 1 of that study and plotted to obtain growth curves shown above.

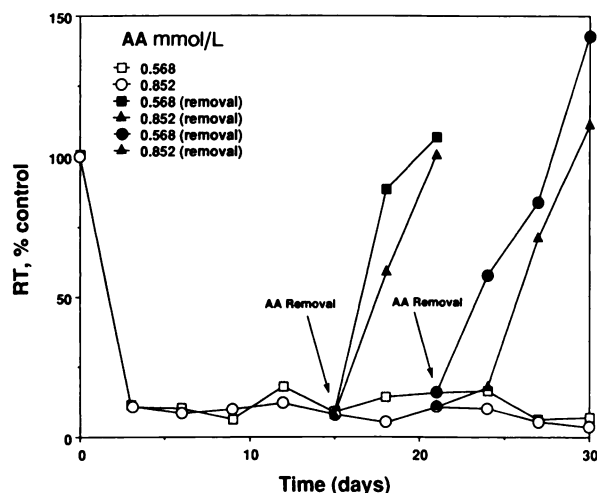


FIG 2. Effect of long-term ascorbate treatment and removal on virus production in H9/HTLV-III<sub>B</sub> cells.

cytes (6). In chronically infected H9/HTLV-III<sub>B</sub> cells exposed to the highest nontoxic dose of AA (0.852 mmol/L) for 4 d at  $37^{\circ}\text{C}$ , extracellular RT activity was reduced by  $> 99\%$  and p24 HIV core antigen was lowered by  $\sim 90\%$ . In acutely infected VB cells, ascorbic acid (0.568 mmol/L) reduced giant-cell syncytia formation by  $\sim 93\%$ . Exposure of cell-free virus to AA for short-term exposure (1 d at  $37^{\circ}\text{C}$ ) had no effect on its RT activity or syncytia-forming capacity. Prolonged exposure of virus to ascorbate (4 d at  $37^{\circ}\text{C}$ ) resulted in a drop in RT activity by a factor of 3–14 compared with a reduction in extracellular RT released from chronically infected cells by a factor of 25–172.

### Effects of AA on cell growth rates

In our previous cytotoxicity analysis, we evaluated the effects of AA on cell viability, host metabolic activity and rate of protein synthesis at concentrations (0.568 and 0.852 mmol/L) at which ascorbate produced significant inhibition of HIV replication. No detectable inhibitory effects were seen on these cellular variables, indicating that the suppression of HIV was not the result of nonspecific effects of the compound. To further look into the actual growth rates of infected cells in the presence and absence of AA, we computed growth curves (Fig 1). The growth rates for ascorbate cultures (at 0.426, 0.568, and 0.852 mmol/L) were very similar to those for the control. These plots provided further evidence that ascorbate did not adversely affect the growth and viability of chronically HIV-infected cells.

### Effect of long-term ascorbate treatment and removal on virus production

To evaluate whether the inhibition of RT activity by ascorbate persisted beyond 4 d of incubation, chronically infected H9/HTLV-III<sub>B</sub> cells were grown in the presence of ascorbic acid for 30 d with daily addition of fresh compound. The data showed that virus suppression, first measured on day 3, was maintained for 30 d in the presence of 0.568 and 0.852 mmol AA/L (Fig 2). To determine if the continuous presence of AA is required for RT inhibition in this 30-d experiment, the AA was removed on days 15 and 21. Cultures were then reseeded in regular growth medium (without added AA) and RT activity was tested at 3-d

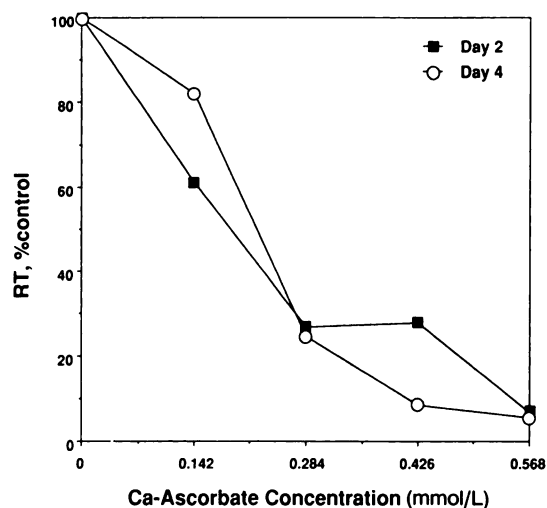


FIG 3. Effect of Ca-ascorbate on extracellular RT activity released from H9/HTLV-III<sub>B</sub> cells.

intervals as shown in Figure 2. Six days after AA removal, RT production in the cell culture returned to the concentration seen in the control. These results indicate that the anti-HIV effect is reversible and that continuous presence of AA is necessary to keep the virus suppressed.

#### Effects of ascorbate salt on RT production

To extend our results to derivatives of AA, we used Ca-ascorbate, a common ascorbate salt. In our previous work with AA (6), 10× stock solutions of AA were made in RPMI and frozen at -20 °C. Just before addition to cell cultures, these solutions were thawed and neutralized (to ~pH 7.0) by mixing with buffered growth medium. A neutral stock solution of Ca-ascorbate was obtained by dissolving the salt directly in RPMI.

H9/HTLV-III<sub>B</sub> cells were exposed to various concentrations of Ca-ascorbate for 4 d and assayed for effects on cell growth and extracellular RT activity. The rate of cell growth in the presence of Ca-ascorbate concentration ≤ 0.568 mmol/L was not significantly different from that in the control culture (data not shown). At a concentration of 0.568 mmol/L, Ca-ascorbate reduced extracellular RT levels by ~94% (Fig 3) compared with ~99% reduction seen with 0.568 mmol AA/L (data not shown).

#### Effects of other reducing agents on HIV production

It was recently reported by Roederer et al (19) that NAC, an antioxidant and an inducer of GSH, was capable of inhibiting HIV expression in lymphocytes stimulated by cytokines. In light of this finding, we investigated the effects of both GSH and NAC on RT and p24 antigen production in the H9/HTLV-III<sub>B</sub> cell line, which constitutively produces virus without supplemented cytokines.

To determine the effect of NAC on HIV replication, H9/HTLV-III<sub>B</sub> cells were grown with various concentrations of NAC (0–40 mmol/L) and assayed for effects on cell growth at periodic intervals. On day 2 there was a 130% increase in cell growth with 10 mmol NAC/L; cell growth was reduced to 77% of the control at 20 mmol/L, with significant cellular cytotoxicity becoming evident at concentrations > 20 mmol/L. After longer incubation times (at days 4 and 6), cell survival was 86% of the

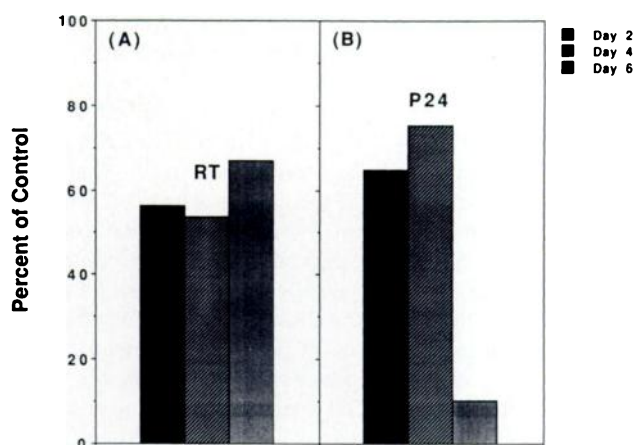


FIG 4. Effect of NAC on HIV replication. Data shown are values for cultures treated with 10 mmol NAC/L, compared as a percentage of control.

control at 10 mmol NAC/L, whereas higher concentrations conferred significant inhibition of cell growth.

On the basis of these cytotoxicity data, the effect of NAC on extracellular RT and p24 protein production was measured in H9/HTLV-III<sub>B</sub> cells at 0–20 mmol NAC/L. On day 2 extracellular RT concentration remaining was 56% of the control with 10 mmol NAC/L (Fig 4, A) and 40% of the control in 20 mmol NAC/L (data not shown). On days 4 and 6, residual RT concentrations in 10 mmol NAC/L were, respectively, 54% and 67% of the control, indicating less than twofold suppression of HIV RT.

For p24, the antigen concentrations in supernatants of cultures treated with 10 and 20 mmol NAC/L were not significantly different from their respective controls after days 2 and 4. However, on day 6, a significant reduction (90% inhibition) of p24 antigen was noted at 10 mmol NAC/L (Fig 4, B).

The effect of supplemental GSH on HIV replication was investigated by growing the H9/HTLV-III<sub>B</sub> line at GSH concentrations ranging from 0 to 1.838 mmol/L. These concentrations were predetermined to produce no adverse effects on cell growth.

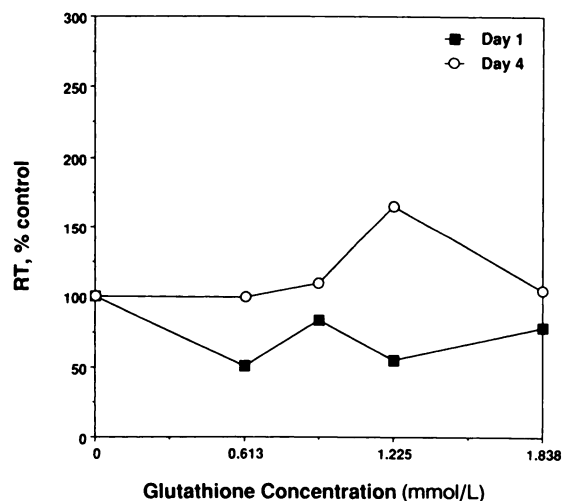


FIG 5. Effect of GSH on HIV replication.



After 4 d the concentrations of RT in cultures treated with 0 to 1.838 mmol GSH/L were not significantly different from those in control culture (Fig 5).

To investigate the combined effect of AA with thiol-containing reagent, we grew H9/HTLV-III<sub>B</sub> cells with NAC or GSH alone or in combination with AA. The results indicated a synergistic effect between AA and NAC (Fig 6). Thus, when 0.426 mmol AA/L was added together with 10 mmol NAC/L, extracellular RT was reduced to 27.7% of the control on day 3, compared with values of 49.5% and 60.4% seen, respectively, with AA alone and NAC alone. On day 6 extracellular RT remaining after AA plus NAC treatment was 12.2% of the control compared with residual RT of 26.8% with ascorbate alone and 63.2% with NAC alone. When ascorbate and GSH were evaluated together, the suppression of RT in the presence of both agents was about the same as that seen with ascorbate alone, indicating that GSH did not potentiate the inhibitory effect of ascorbate.

## Discussion

A comparative *in vitro* evaluation of the anti-HIV activities of three different reducing agents, presented here, shows that, like AA, its neutralized calcium salt inhibited HIV replication to a substantial level (~94% reduction of RT) in chronically infected cells. In contrast, NAC, a thiol-containing substance and inducer of intracellular GSH, conferred at best about a 60% suppression of HIV RT activity in unstimulated cells. Supplemented GSH by itself exerted no inhibitory effects against HIV production *in vitro* when tested at 0–1.838 mmol/L. When tested in combination, NAC, but not GSH, potentiated the anti-HIV effect of ascorbate on RT released from chronically infected cells. These results offer implications for clinical studies aimed at evaluating the effectiveness of ascorbate and other antioxidants in HIV infection and AIDS.

Two thiol-containing antioxidants, NAC and GSH, were evaluated on the basis of recent clinical studies. It was reported that asymptomatic HIV-seropositive individuals exhibit lower intracellular concentrations of GSH in their peripheral blood mononuclear cells and dramatically reduced serum acid-soluble thiol concentrations in blood plasma (20, 21). Of the serum acid-soluble thiols, the concentrations of cysteine in AIDS patients were found to be especially low. Total and reduced GSH concentrations in the plasma of HIV-infected subjects were about 30% of those in normal individuals. The concentrations of these substances in the lung epithelial lining fluid of HIV-infected subjects were ~60% of those in the control subjects.

We found that NAC was cytotoxic for H9/HTLV-III<sub>B</sub> cells at concentrations > 10 mmol/L. When tested at the noncytotoxic concentration, 10 mmol NAC/L did not significantly inhibit RT production but reduced p24 concentrations after 6 d of incubation. These findings are consistent with the observations of Roederer et al (19), who found that in unstimulated cells NAC inhibits viral replication only slightly. They noted high HIV suppression by NAC in cytokine-stimulated lymphocytes. In our unstimulated cells, NAC conferred less than twofold inhibition of HIV RT. However, in the presence of AA, NAC potentiated the anti-HIV effect on RT, indicating a synergistic action. Whereas NAC alone did not significantly affect RT activity, it inhibited extracellular p24 by ~10fold. This differential effect on HIV proteins could be due to the specific interaction of NAC

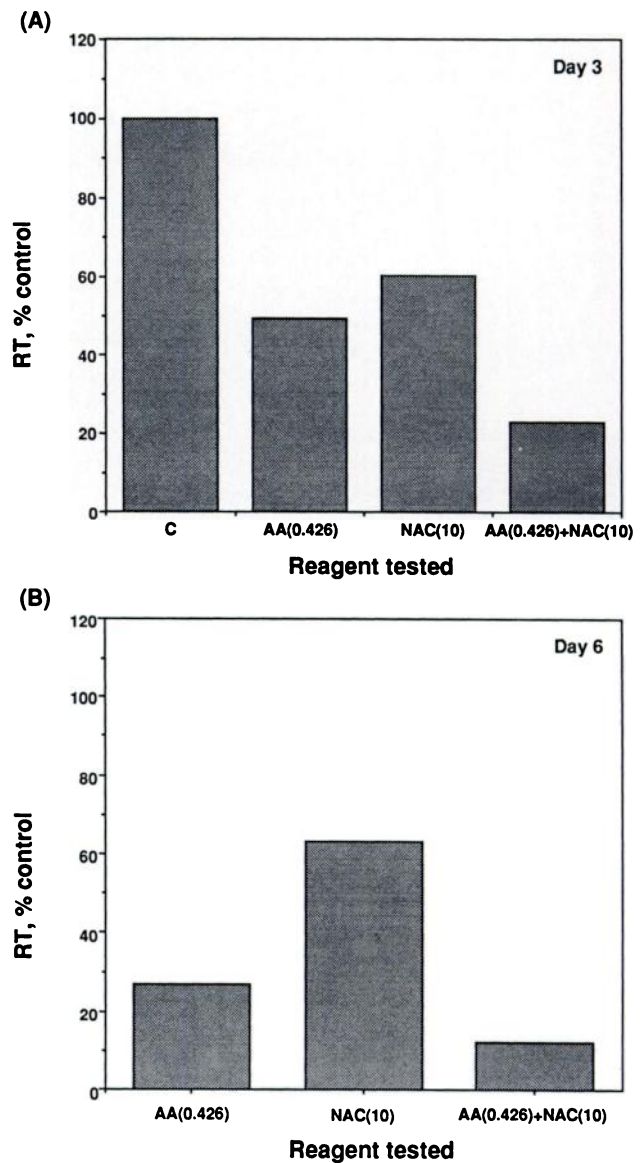


FIG 6. Effect of ascorbate and NAC alone and combined on RT activity in supernatant harvested on days 3 (A) and 6 (B).

with p24, which is a relatively small protein. Further studies can provide more insight into its mechanism of action.

When GSH was tested on unstimulated H9/HTLV-III<sub>B</sub> cells, it did not affect HIV replication at the noncytotoxic concentration range (0–1.838 mmol/L). Further, GSH also did not alter the anti-HIV effect of ascorbate when tested in combination. One explanation for the ineffectiveness of GSH may be its inability to enter cells unless it is degraded or provided in an acetylated form (22). It is known that aerosolized GSH has to be degraded before being absorbed into cells. In this regard, drugs that deliver cysteine or GSH to deficient cells could be potentially useful (22). However, these carriers also have some disadvantages. A simple and straightforward approach to increasing intracellular GSH concentrations is to use inducers of GSH, such as NAC and ascorbate, which combined exerted strong anti-HIV effect in this study (Fig 6).

Data on Ca-ascorbate indicate that the antiviral effects of AA are mediated by the ascorbate ion and are not due to factors such as its acidity or pH of the medium. Further, the antiviral effect was not the result of an apparent decrease in cellular growth. Thus, a plot of relevant growth curves (Fig 1) showed that the growth rate of chronically infected cells in the presence of the antiviral concentrations of ascorbate was not significantly different from that of the control. The index of cellular growth used in our previous study was percent cell recovery, ie, the actual number of viable cells recovered from ascorbate-treated cultures as a percent of those present in control cultures. This is a more stringent criterion of cellular viability than is percent survival, which is simply the percent of viable cells within the same population and is a measure of cell death. The growth rates of ascorbate-treated cultures computed in this study confirms our previous indication that ascorbate did not exert an untoward effect on cellular viability. Taken together with lack of adverse effects of similar ascorbate concentrations on the activity of cellular dehydrogenases and the rate of host protein synthesis (6), the data indicate that HIV suppression by ascorbate was not due to secondary effects resulting from inhibition of cellular growth or metabolic activity.

Another important finding of our current investigation is that HIV suppression by ascorbate persisted in culture as long as vitamin supplementation was maintained in vitro (30 d in this study). Further, the continuous presence of ascorbate was necessary for HIV suppression, because the removal of ascorbate resulted in the resumption of virus replication. These results are relevant to ascorbate dosages necessary for clinical studies. Our previous in vitro study indicated that a minimum of 0.142 mmol ascorbate/L was needed to obtain significant HIV suppression. This translates to ~12 g vitamin C orally in healthy volunteers (23). Further, to obtain greater HIV suppression in vitro, 0.568–0.852 mmol ascorbate/L was effective. One way of achieving high ascorbate concentrations in plasma is to administer oral ascorbate to the bowel tolerance level. Because AIDS patients have a high bowel tolerance (15), it may be possible to attain the desired concentrations. However, oral administration has its limitations because only a certain amount of ascorbate can be absorbed and maintained in plasma because of restrictions imposed by tubular reabsorption (24) and gastrointestinal complications in some patients. In lieu of this, continuous intravenous infusion would be the method of choice for delivering a high dose in the plasma. Once the system is saturated and bowel-tolerance level of the patient has been reduced, the frequency of IV infusion can be cut down or replaced by oral administration of ascorbate. Alternatively, ascorbate can be used combined with another agent. This method has the advantage of permitting a lower dose of ascorbate to be effective, as demonstrated in this study. Our results on synergism between ascorbate and NAC offer a promising strategy for dealing with HIV infection by using ascorbate therapy combined with antioxidants. ■

Since the submission of this manuscript, Kalebic *et al* (25) reported the suppression of cytokine-stimulated HIV expression by GSH, GSH monoester, or NAC at a concentration of 15 mol/L in the chronically infected monocytic U1 cell line. Consistent with our results in T-lymphocytic H9/HTLV-III<sub>B</sub> cells, a lower concentration (1 mmol/L) of GSH did not confer significant inhibition of HIV in U1 cells. GSH, at concentrations >10 mmol/L was cytotoxic to H9/HTLV-III<sub>B</sub> cells.

We thank Dan McWeeny for valuable technical assistance.

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