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L-2-Oxothiazolidine-4-Carboxylic Acid Inhibits Human Immunodeficiency Virus Type 1 Replication in Mononuclear Phagocytes and Lymphocytes

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We investigated the effects of L-2-oxothiazolidine-4-carboxylic acid (OTC; Procysteine), a cysteine prodrug, on human immunodeficiency virus type 1 (HIV-1) expression in both adult peripheral and cord blood mononuclear phagocytes and lymphocytes. OTC suppressed HIV-1 expression in monocyte-derived macrophages (MDM) and lymphocytes in a dose-dependent fashion as determined by HIV-1 reverse transcriptase (RT) activity. This inhibitory effect of OTC occurred with three HIV-1 strains (two laboratory-adapted strains and one primary isolate). Addition of OTC to chronically HIV-1-infected MDM cultures also suppressed RT activity by 40 to 50% in comparison to untreated controls. The inhibitory effects of OTC on HIV-1 were not caused by toxicity to MDM or lymphocytes because there was no change in cell viability or cellular DNA synthesis, as evaluated by trypan blue dye exclusion and [^{3}H]thymidine incorporation, at doses of OTC that inhibit virus replication. These observations indicate that OTC has the potential to limit HIV-1 replication in mononuclear phagocytes and lymphocytes and may be useful in the treatment of HIV-1 infection and AIDS.

Considerable progress has been made in developing treatment strategies that have improved the quality and duration of the lives of patients with human immunodeficiency virus type 1 (HIV-1) infection or AIDS. Zidovudine (ZDV), didanosine, ritonavir, and saquinavir are approved by the U.S. Food and Drug Administration for treatment of HIV-1 infection. Unfortunately, therapy with combinations of these agents is occasionally limited by intolerance, toxicity, or HIV-1 disease progression. HIV-1 resistance to ZDV or didanosine can occur in both adult and pediatric patients with HIV-1 infection or AIDS on long-term therapy (7, 9, 10, 29, 32, 36, 37). Children with resistant strains of HIV-1 have worse clinical outcomes than children whose viruses remain susceptible (31). In addition, transmission of HIV-1 with a ZDV resistance mutation from one child to another has been documented (13). Saquinavir and other protease inhibitors have been tested in HIV-1 clinical trials. The limited efficacy of ZDV emphasizes the need to develop agents that are better tolerated, safer, and more effective antiretroviral drugs for use in adults and children with HIV-1 infection or AIDS. An ideal drug or combination of drugs should be able to inhibit or prevent HIV-1 replication in both acutely and chronically infected cells.

The role of oxidative stress in the pathophysiology of many diseases suggests that agents that decrease oxidative stress might have a clinically meaningful impact on disease progression. Glutathione (GSH) is central to the body's antioxidant defense system, as well as to redox-mediated metabolism. Under conditions of oxidative stress, GSH is often depleted and damaged in cells and tissues by reactive oxygen species. Consequently, free-radical-mediated damage to tissue often occurs. HIV-1-infected individuals have decreased levels of acid-soluble thiols, in particular, cysteine and GSH, in their plasma, leukocytes (T-cell subsets), and bronchoalveolar lavage fluid (5, 8, 39, 40). Thus, replenishment of GSH is an attractive therapeutic approach to alleviating oxidative stress caused by HIV-1 infection. In vitro repletion of GSH with *N*-acetyl cysteine (NAC) or GSH ester can inhibit HIV-1 replication (18, 35, 38). We have demonstrated that GSH and NAC inhibit HIV-1 replication in human peripheral blood monocyte-derived macrophages (MDM) (20, 22, 28). In addition, we (21, 22) and others (2) have recently reported that cystamine potently suppresses in vitro HIV-1 replication in MDM and lymphocytes isolated from adult human peripheral and placental cord blood. However, the effectiveness of direct administration of GSH is restricted because GSH is easily oxidized and is hydrolyzed by intestinal and hepatic γ-glutamyltransferase (45). The rate of intracellular GSH synthesis is often limited by the availability of cysteine. However, L-2-oxothiazolidine-4-carboxylic acid (OTC; Procysteine) is able to overcome this limitation (24). Extensive preclinical research has demonstrated that OTC, a cysteine prodrug, increases GSH levels in animal models in which GSH was depleted by oxidative stress or chemical means (24). In the presence of 5-oxoprolinase, OTC is converted to cysteine (43, 44). Since peripheral and cord blood-derived monocytes and lymphocytes are the primary target cells of HIV-1 replication, we investigated whether OTC inhibits HIV-1 replication in both adult and cord blood-derived mononuclear phagocytes and lymphocytes.

MATERIALS AND METHODS

Monocyte and lymphocyte preparations. Peripheral blood was obtained from healthy adult human donors. Heparinized cord blood was obtained from the umbilical veins of healthy full-term newborn infants after uncomplicated pregnancies and deliveries. All adult and cord blood samples were HIV-1 antibody negative as determined by enzyme-linked immunosorbent assay (Coulter Immunology, Hialeah, Fla.). Monocytes were purified as previously described (16, 17). In brief, heparinized adult and cord blood samples were separated by centrifugation over lymphocyte separation medium (Organon Teknika Corporation, Durham, N.C.) at 400 to 500 × *g* for 45 min. The mononuclear layer was collected and incubated with Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, N.Y.) in 2% gelatin-coated flasks for 45 min at 37°C, and

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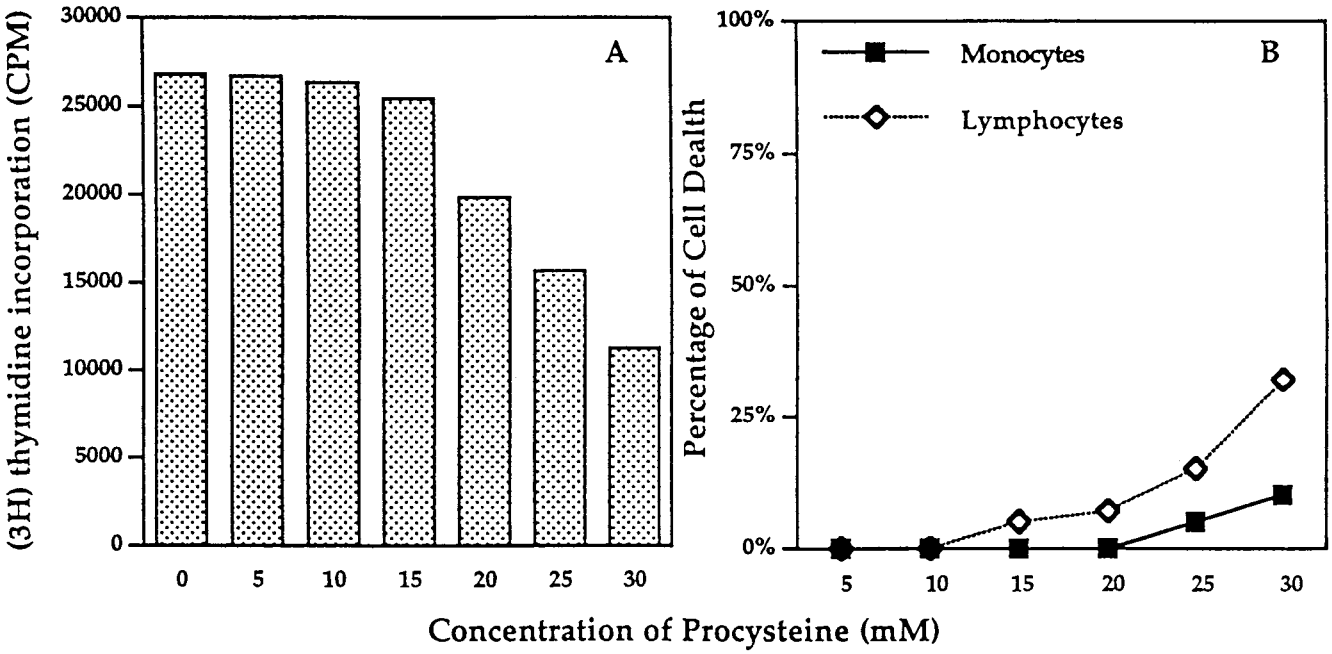

FIG. 1. Cytotoxic effect of OTC on cord blood lymphocytes and MDM. PHA-P- and IL-2-stimulated cord blood lymphocytes (5×10^4 cells/well) were cultivated in 96-well plates in the presence or absence of OTC at the concentrations indicated for 96 h. Incorporation of [^{3}H]thymidine was measured 18 h after its addition to the cultures. MDM and cord lymphocytes were incubated with OTC at the concentrations indicated. OTC was added to the cultures every 48 h. At the end of the culture period (12 days for lymphocyte cultures and 24 days for MDM cultures), MDM and lymphocytes were stained with trypan blue and counted. The results shown are means of three experiments done in triplicate with three adult peripheral blood and cord blood samples. (A) Thymidine incorporation assay. (B) Trypan blue staining assay.

then the nonadherent cells were removed by washing with DMEM. Cord monocytes were detached by incubating with EDTA. Cells were plated in 48-well culture plates at a density of 2.5×10^5/well in DMEM containing 20% fetal calf serum (FCS). Following initial purification, >97% of the cells were monocytes, as determined by nonspecific esterase staining and fluorescence-activated cell sorting analysis with a monoclonal antibody against CD14 (Leu-M3) and low-density lipoprotein specific for monocytes and macrophages. Nonadherent lymphocytes were collected from the gelatin-coated flasks, washed three times with phosphate-buffered saline, and maintained in culture in RPMI 1640 medium containing 10% FCS and 1 μg of phytohemagglutinin P (PHA-P) per ml for 72 h. The cells were then treated with interleukin 2 (IL-2) (50 ng/ml) and cultivated in 24-well plates at a density of 5×10^5 per well (1-ml total volume). Viability of monocytes and macrophages was monitored by trypan blue exclusion and maintenance of cell adherence. For lymphocytes, cell viability was also measured by lymphocyte proliferation assays (as described below). A *Limulus* amebocyte lysate assay demonstrated that all of the media and reagents used were endotoxin free.

Lymphocyte proliferation assay. PHA-P- and IL-2-stimulated adult peripheral blood and cord blood lymphocytes (5×10^4 cells/well) in 96-well microtiter plates were cultivated in the presence or absence of OTC at concentrations ranging from 5 to 30 mM for 96 h. [^{3}H]thymidine (Amersham Life Science, Arlington Heights, Ill.) at 1 μCi/well was added to the cell cultures, which were incubated for a further 18 h in 5% CO_2. The cells were then harvested onto glass fiber filter papers with an automated cell harvester (M-24 R; Brandel, Gaithersburg, Md.), and [^{3}H]thymidine incorporation into the dried filters was measured by a liquid scintillation counter (Packard Instrument Inc.).

Chemical reagents and recombinant cytokines. OTC was generously provided by Transcend Therapeutics, Inc., Cambridge, Mass., and was dissolved in endotoxin-free medium and adjusted to pH 7.0 before use.

Virus preparation. HIV-1 strains Bal and IIIB were provided by the AIDS Research and Reference Reagent Program, Division of AIDS, Allergy and Infectious Diseases, National Institute of Bethesda, Md. HIV-1 strain HD was isolated in our laboratory from a HIV-1-infected woman's placental cord blood sample. HIV-1 strain Bal was prepared in adult peripheral blood MDM, and its titer was determined with a 50% tissue culture-infective dose triplicate endpoint dilution assay in primary macrophages by using HIV-1 reverse transcriptase (RT) activity as the indicator. The stock of HIV-1 strain Bal contained 8.0×10^5 cpm of RT activity per ml. HIV-1 strains IIIB and HD were prepared in peripheral blood mononuclear cells from healthy donors and had 5×10^5 cpm of RT activity per ml. The 50% tissue culture-infective was defined as the amount of virus that resulted in detectable viral infection, i.e., supernatant RT activity greater than 2 standard deviations above the background, in half of the peripheral blood lymphocyte or MDM cultures.

HIV-1 RT assay. The HIV-1 RT activity assay used was based on the technique of Willey et al. (42). Briefly, 10 μl of culture supernatants was added to a cocktail containing poly(A), oligo(dT) (Pharmacia, Inc., Piscataway, N.J.), $MgCl_2$, and [32P]dTTP (Amersham Life Science) and incubated for 20 h at 37°C. Samples (30 μl) of the cocktail were spotted onto DE81 paper, dried, and washed five times with 2× SSC (1× SSC is 0.15 M sodium citrate plus 0.15 M NaCl) and once with 95% ethanol. The filter was then air dried. Radioactivity was counted in a liquid scintillation counter (Packard Instrument Inc.). Addition of OTC (20 mM) to the RT assay had no effect on the results (counts per minute per 5 μl) with positive and negative control samples.

HIV-1 infection. Purified adult peripheral blood and cord blood monocytes were cultured in 48-well plates (2.5×10^5 cells/well) for 10 days, inoculated with HIV-1 strain Bal at a multiplicity of infection of 1 (0.1 ml/well, RT activity, 8×10^5 cpm/ml), and incubated for 2 h at 37°C. The inoculum was removed, and the cells were washed three times with DMEM to remove unabsorbed virus. Fresh medium (DMEM with 10% FCS) was then added. For PHA-P-treated (3 days) adult peripheral blood and cord blood lymphocyte cultures, the cells were inoculated with HIV-1 lymphocyte-tropic strains IIIB and HD at a multiplicity of infection of 1 for 2 h, washed three times with RPMI 1640, and cultivated in RPMI 1640 medium containing 10% FCS and 10% IL-2. In each experiment, the final wash was tested for viral RT activity and shown to be free of residual inoculum. Twelve days after HIV-1 inoculation, chronic infection was established as determined by HIV-1 RT and p24 assays (20).

Assays for antiviral effects of OTC. MDM maintained in cultures for 10 days or PHA-P-stimulated (3 days) lymphocytes were incubated with OTC (5, 10, 15, or 20 mM) or medium alone 24 h before and during a 2-h HIV-1 infection. Immediately after infection with HIV-1, the cells were treated with OTC either once (single time point) or every 2 days postinfection. Approximately 50% of the culture supernatant in each well was collected and replaced with fresh medium every 4 days for 3 to 4 weeks of culture (MDM) or every 3 days for 2 weeks (lymphocytes). To test the effects of OTC on HIV-1 replication in chronically infected MDM, 10-day-cultured MDM isolated from adult peripheral blood and cord blood were infected with HIV-1 strain Bal as described above. The HIV-1-infected MDM cultures (12 days after the viral challenge) were then incubated with OTC (10 and 20 mM) either once only or every 2 days or incubated with medium alone in a subsequent course of infection. Approximately 50% of the culture supernatant in each well was collected and replaced with fresh medium every 4 days. The collected culture supernatants were stored at −70°C and assayed for the presence of HIV-1 RT activity.

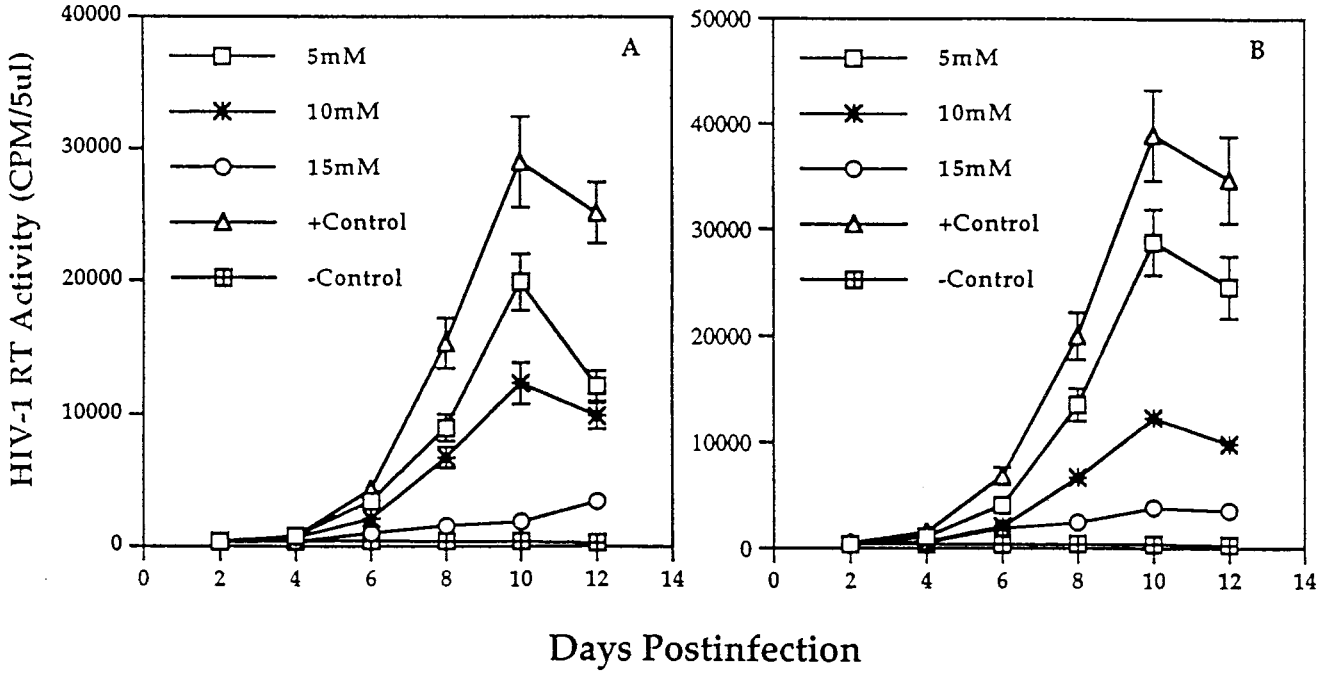

FIG. 2. Inhibition of HIV-1 replication by OTC in both adult peripheral blood- and cord blood-derived lymphocytes. PHA-P-stimulated cord blood-derived lymphocytes were incubated overnight with OTC or medium alone, inoculated with HIV-1 strain, IIIB, and incubated for 2 h at 37°C. OTC also was added to the lymphocyte cultures during infection, immediately postinfection, and every 48 h afterwards at the same concentrations as used prior to infection. Controls included cultures infected with HIV-1 strain IIIB only (+Control) and uninfected adult peripheral blood or cord blood lymphocyte cultures (−Control). HIV-1 RT activity was measured in culture supernatants collected every 48 h. The results shown are representative of three independent experiments. The curves represent mean values for triplicate cultures, and the bar represents 1 standard deviation of the mean. (A) Adult peripheral blood-derived lymphocytes. (B) Cord blood-derived lymphocytes.

RESULTS

Cytotoxic effect of OTC on cord blood MDM and lymphocytes. The cytotoxic effect of OTC on cord blood MDM and lymphocytes was investigated. In the presence of 5 to 10 mM OTC, no toxic effects on MDM and lymphocytes were noted as determined by trypan blue dye exclusion and cell proliferation assays (Fig. 1). Although 15 to 20 mM OTC had a cytotoxic effect (5 to 10%) on cord blood lymphocytes, there was not cytotoxic effect on MDM (Fig. 1). OTC, at the concentrations described above, had similar effects on adult peripheral blood monocytes and lymphocytes (data not shown). Thus, we selected concentrations of OTC equal to or less than 15 mM (for lymphocytes) and 20 mM (for MDM) as the standard concentrations for the subsequent experiments.

Anti-HIV activity of OTC in primary adult peripheral blood- and cord blood-derived lymphocytes. OTC, at noncytotoxic doses, inhibited HIV-1 IIIB replication in a dose-dependent fashion in both adult peripheral blood and cord blood lymphocytes stimulated with PHA-P and IL-2 (Fig. 2A and B). OTC also showed an inhibitory effect on adult peripheral blood monocyte cultures inoculated with a primary isolate (HD) (Fig. 3). Additionally, OTC (10 and 20 mM)-treated adult peripheral blood and cord blood lymphocyte cultures infected with these strains showed no cytopathic effect induced by the virus (data not shown).

Anti-HIV activity of OTC in MDM cultures. OTC was tested at concentrations of 5, 10, and 20 mM for its effect on acute and chronic HIV-1 infection as measured by RT activity in MDM isolated from adult peripheral blood and cord blood. The MDM incubated with OTC before, during, and immediately after infection with the Bal strain showed decreased HIV-1 RT expression (Fig. 4). Addition of OTC at a concentration of 10 or 20 mM to chronically infected cord blood MDM cultures also suppressed RT activity during the subsequent course of infection (Fig. 5).

DISCUSSION

This communication reports the antiviral activity of OTC against HIV-1 replication in both adult peripheral and cord blood-derived monocytes and lymphocytes, primary target cells for the virus. Monocytes and macrophages are a major reservoir of HIV-1 infection and contain much of the virus in the brains of AIDS encephalopathy patients (6, 14, 19, 27, 34). Monocytes and macrophages have powerful antioxidant systems that are supported by rapid and efficient uptake of cysteine (1). Thus, low levels of OTC in vivo may be sufficient to supply significant amounts of cysteine to monocytes and macrophages and thereby influence the course of HIV-1 infection. OTC may have an additional significant benefit via cysteine and GSH replenishment, which is its ability to augment T-cell function and proliferation (11, 12), thereby improving normal immune responses. HIV-1-infected macrophages become chronically infected 2 weeks following inoculation (6, 14, 34). To evaluate the effect of OTC on chronically HIV-1-infected MDM, OTC was added to MDM cultures 12 days after HIV-1 strain Bal infection. Addition of OTC to MDM cultures only once before or after infection with HIV-1 had no long-term effect on virus expression (data not shown). However, addition of OTC at a concentration of 10 or 20 mM every 2 days to acutely or chronically infected MDM cultures significantly inhibited HIV-1 replication. This observed inhibitory effect of OTC on HIV-1 was concentration dependent. OTC also inhibited replication of a primary HIV-1 strain (HD) isolated from infected cord blood mononuclear cells in the lymphocytes

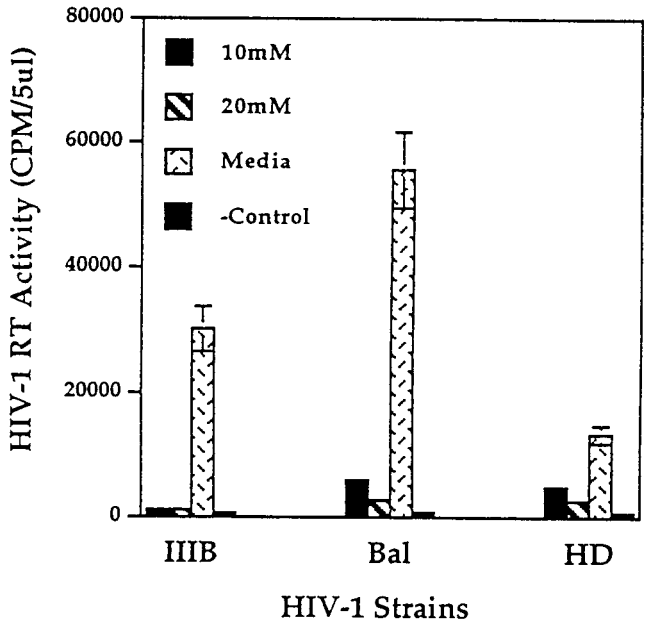

FIG. 3. Effect of OTC on the replication of different HIV-1 strains in adult peripheral blood-derived monocytes and lymphocytes. Ten-day-cultured monocytes or PHA-P-stimulated lymphocytes were incubated overnight with OTC or medium alone, inoculated with different HIV-1 strains (IIIB and HD for lymphocytes and Bal for monocytes), and incubated for 2 h at 37°C. The cells were cultured in the presence or absence of OTC (added to the cultures during infection, immediately postinfection, and every 48 h afterwards) at the concentration indicated. The controls included cultures infected with HIV-1 only (+Control) and uninfected cultures (−Control). HIV-1 RT activity was measured in culture supernatants collected at day 12 postinfection. The results shown are representative of three independent experiments with three adult donor blood specimens. Each column represents the mean value for triplicate cultures, and the bar on each column represents 1 standard deviation of the mean.

(Fig. 3), indicating that OTC indeed has a broad anti-HIV-1 effect and potential for treatment of HIV-1-infected patients. Further work is required to precisely determine the mechanisms by which OTC inhibits HIV-1 infection.

Oxidative stress plays an important role in the progression of HIV-1 infection to AIDS (18, 35, 38). Since GSH is the major intracellular defense against the production of reactive oxygen intermediates, overproduction of such oxidants depletes GSH. Increased GSH levels not only decrease reactive oxygen intermediates but may also inhibit stimulation by inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) (18, 35, 38). GSH, a major intracellular reducing agent may play an important role in the pathogenesis of HIV infection and AIDS (5, 8, 39, 40). Thus, treatment of HIV-1 infection with agents which restore systemic and intracellular GSH may be a valuable adjunct therapy. We have shown that GSH, NAC, and cystamine inhibit HIV-1 replication in both adult peripheral blood and cord blood MDM (20–22, 28). In addition, we recently demonstrated that cystamine inhibits TNF-α-induced HIV-1 expression in chronically infected U1 and ACH-2 cells and that cystamine inhibits lipopolysaccharide-stimulated endogenous TNF-α production in MDM (22), which may contribute to the anti-HIV-1 effect of cystamine.

NAC, a cysteine precursor that is converted to cysteine, which is, in turn, incorporated into GSH, has been proposed as a drug that would be useful in preventing the progression of HIV-1 infection, as it restores GSH levels and may also act as an antioxidant by itself (18, 35, 38). OTC replenishes cysteine, and thus cellular GSH stores, in situations in which GSH is acutely depleted due to the detoxification of a toxic drug metabolite (4, 30, 33). OTC may actually be more effective than NAC in replenishing intracellular GSH stores (43). Experimentally, OTC administration has the potential to increase intracellular concentrations of GSH above physiological concentrations (41, 44). Recent studies have demonstrated that OTC inhibits HIV-1 in three different models: acute infection, chronic infection, and gene expression controlled by the HIV-1 long terminal repeat promoter (15, 26). Our study with primary mononuclear phagocytes and lymphocytes isolated from adult peripheral blood and cord blood has confirmed these observations, indicating that OTC has a potential application in HIV-1-infected patients.

Clinical trials of OTC have shown that OTC treatment alleviated injury to the lungs and other organs in patients with acute respiratory distress syndrome (3), and appeared to have important clinical benefits for patients infected with HIV-1 (15). Extensive preclinical research has demonstrated that OTC increase GSH levels in animal models in which GSH was depleted by oxidative stress or chemical means (24). OTC, when administrated at the highest dosage (100 mg/kg), increased whole-blood GSH levels, although no effect on changes in CD4 cell counts, viral load, or proviral DNA frequency was observed in asymptomatic HIV-infected subjects (23). In a toxicology study, OTC had very low acute toxicity and was well tolerated by patients (24). Of the drugs designed for GSH replenishment, Procysteine is the most advanced in commercial development (25). In addition, only minor adverse experiences have been reported across all clinical trials (24). Thus,

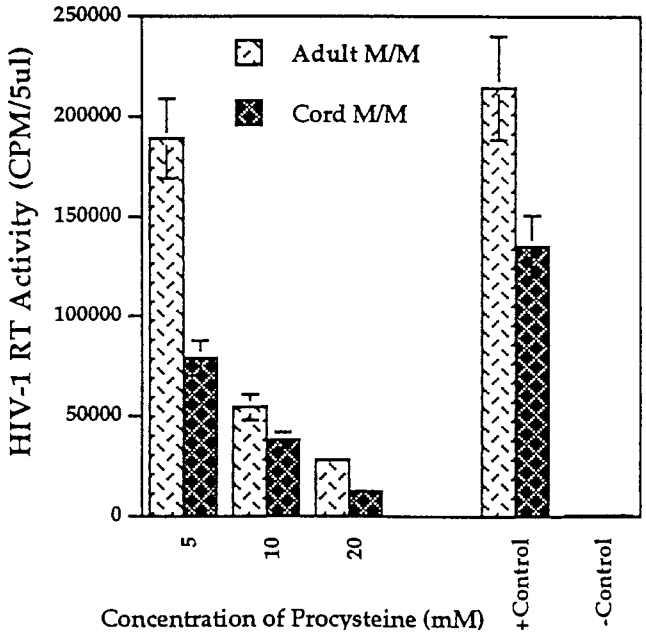

FIG. 4. Effect of OTC on HIV-1 replication in both adult peripheral blood and cord blood MDM. The cells were cultured in vitro for 10 days, incubated overnight with OTC at different concentrations, and then infected with HIV-1 strain Bal for 2 h at 37°C. The cells were incubated with OTC (added to the cultures during infection, immediately postinfection, and every 48 h afterwards) at the concentrations shown. The controls included cultures infected with HIV-1 strain Bal only (+Control) and uninfected MDM cultures (−Control). Culture supernatants were checked for HIV-1 RT activity at day 16 posttreatment. The data shown are representative of three independent experiments with three adult peripheral blood and cord blood samples. Each column represents the mean value for triplicate cultures, and the bar on the column represents 1 standard deviation of the mean.

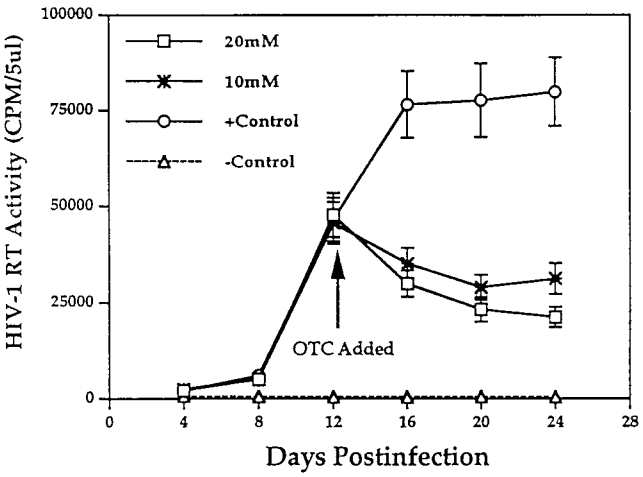

FIG. 5. Suppression of RT activity of HIV-1 (Bal) by OTC in chronically infected cord blood MDM. OTC (10 or 20 mM) was added to infected cultures at day 12 postinfection and every 4 days in the subsequent course of infection. The controls included cultures infected with HIV-1 strain Bal and uninfected MDM cultures. The supernatants from these cultures were collected every 4 days for up to 24 days postinfection and assayed for HIV-1 expression as determined by RT activity. The data shown are representative of three independent experiments with monocytes from three different cord blood samples. The curves represent mean values for triplicate cultures, and each bar represents 1 standard deviation of the mean.

a combination of OTC with other antiretroviral drugs may prove to be useful for treatment of HIV-1 infection and AIDS.

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