Cystamine Inhibits Human Immunodeficiency Virus-1 Replication in Cord Blood-Derived Mononuclear Phagocytes and Lymphocytes

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The effects of cystamine on the human immunodeficiency virus (HIV-1) expression in cord blood monocytes-derived macrophages (CBMDM) and lymphocytes were investigated. Cystamine suppressed HIV-1 expression in CBMDM and lymphocytes in a concentration-dependent fashion as determined by HIV-1 reverse transcriptase (RT) activity. This inhibitory effect of cystamine occurred with all five HIV-1 strains (both laboratory adopted and fresh isolates) tested in the study. The addition of cystamine to cultures of HIV-1 chronically infected CBMDM also suppressed 80% to 90% of RT activity in comparison with untreated controls. Cystamine also decreased HIV-1 protein expression in CBMDM as determined by indirect immunofluorescence assay. The inhibitory effects of cystamine on HIV-1 did not appear to be caused by toxicity to CBMDM or lymphocytes because there was no change in cell viability or cellular DNA synthesis as evaluated by trypan blue dye exclusion and 3H-thymidine incorporation at doses of cystamine that inhibit the virus. HIV-1 infected CBMDM or lymphocyte cultures (without cystamine treatment) demonstrated giant syncytium formation or cytopathic effect (CPE), respectively, whereas cystamine-treated cultures lacked the giant syncytia or CPE induced by HIV-1 infection. Thus, these observations indicate that cystamine may have the potential to limit HIV-1 replication in monocytes/macrophages and lymphocytes in vivo and may represent a potentially useful compound in the treatment of pediatric HIV-1 infection and acquired immunodeficiency syndrome.

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TRANSMISSION OF human immunodeficiency virus type 1 (HIV-1) from an infected mother to her offspring is the leading cause of pediatric HIV-1 infection.1,2 In the United States, approximately 90% of cases of pediatric acquired immunodeficiency syndrome (AIDS) reported to the Centers for Disease Control and Prevention occur as a result of maternofetal transmission.1,2 Infection with HIV-1 in children presents unique problems that differ from those that occur in adults. Perinatal HIV-1 infection usually progresses more rapidly than HIV-1 infection in adults. Most perinatally infected infants exhibit a rapid development of symptoms within the first 12 months of life. Clinically, children infected with HIV-1 exhibit a different spectrum of manifestations than similarly infected adult patients. In infants with AIDS, brain involvement (encephalopathy), bacterial infection, and a unique type of lymphocytic interstitial pneumonia occur more frequently than in adults.3,4 In addition, the neonatal immune system differs qualitatively and quantitatively from the adult system. Neonates have an increased number of circulating immature and atypical lymphocytes, and the umbilical cord blood contains stem cells. HIV-1 is capable of infecting a number of types of peripheral blood leukocytes, mainly CD4+ T helper cells and monocytes/macrophages.5 CD4+ T cells carry much of the viral burden in the blood and ultimately are depleted by HIV-1 infection. In contrast, infected macrophages are long-lived and may serve as a viral reservoir. We have demonstrated that human cord blood monocyte-derived macrophages (CBMDM) have increased in vitro susceptibility to infection with certain strains of HIV-1 in comparison with adult MDM.6

Both zidovudine (ZDV) and didanosine (dDI) have been licensed for treatment of HIV-1-infected children and have been shown to be of clinical benefit.7 However, the emergence of ZDV-resistant strains of HIV-1 and the recent description of a primary infection with such a virus10,13 highlights the importance of developing agents that are neither nucleosides nor inhibitors of reverse transcriptase (RT). The Food and Drug Administration has recently approved a protease inhibitor (Saquinavir) for treatment of HIV-1 infection. AZT-resistant strains of HIV-1 can be transmitted to uninfected individuals, including children.14-16 In addition, vertical transmission of AZT-resistant strains of HIV-1 have also been reported. Thus, a search for other antiretroviral agents in infants and small children is required. We7 and others17 recently reported that cystamine potently suppresses in vitro HIV-1 replication in MDM and lymphocytes derived from adult peripheral blood and in the chronically infected mononuclear cell line (U1) and T-cell line (ACH-2). We report in this communication that cystamine suppresses HIV-1 replication in CBMDM and lymphocytes.

MATERIALS AND METHODS

Cord monocyte and lymphocyte preparations. Heparinized cord blood was obtained from the umbilical vein of healthy term newborn infants after uncomplicated pregnancies and deliveries. All cord blood samples were identified as HIV-1 antibody negative by anonymous testing with the enzyme-linked immunosorbent assay method (Coulter Immunology, Hialeah, FL). Monocytes were purified according to our previously described techniques.18,19 In brief, heparinized cord blood was separated by centrifugation over Lymphocyte Separation Medium (Organon Teknika Corporation, Durham, NC) at 400 to 500 g for 45 minutes. The mononuclear layer was collected and incubated with Dulbecco’s modified Eagle’s medium (DMEM; GIBCO, Grand Island, NY) in 2% gelatin-coated flask for 45 minutes at 37°C, followed by removal of the nonadherent cells with DMEM. Cord monocytes were detached by EDTA. Cells were plated in 48-well culture plates at a density of 2.5 x 10^5 cells/well in the DMEM

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containing 20% fetal calf serum (FCS). After the initial purification, >97% of the cells were monocytes, as determined by nonspecific esterase staining and fluorescence-activated cell sorting analysis using monoclonal antibody against CD14 (Leu-M3)* and low-density lipoprotein specific for monocytes and macrophages. Nonadherent lymphocytes were collected from the gelatin-coated flasks and washed three times with phosphate-buffered saline (PBS) and maintained in culture in RPMI 1640 medium containing 10% FCS and 1 μg/mL phytohemagglutinin-P (PHA-P) for 72 hours. The cells were then treated with interleukin-2 (IL-2) (50 ng/mL) and cultivated in a 24-well plate at a density of 5 x 10⁶ cells/well (1 mL total volume). CBMDM viability was monitored by trypan blue exclusion and maintenance of cell adherence. For the lymphocytes, cell viability was also measured by cell proliferation assay (as described below).

In all cases, interleukin amebocyte lysate demonstrated that media and reagents were endotoxin-free.

Cell proliferation assay. PHA-P and IL-2 stimulated cord blood–derived lymphocytes (5 x 10⁶ cells/well) in 96-well microtiter plates were cultivated in the presence or absence of cystamine at different concentration ranging from 12.5 to 400 μmol/L for 96 hours. [3H]thymidine (Amersham Life Science, Arlington Heights, IL) at 1 μCi/well was added to the cells and incubated for a further 18 hours in 5% CO₂. The cells were then harvested onto glass fiber filter paper with an automated cell harvester (M-24 R: Brandel, Gaithersburg, MD), and [3H]thymidine incorporation in dried filters was measured by a liquid scintillation counter (Packard Instrument Inc, Temecula, CA).

Chemical reagents and recombinant cytokines. Cystamine was purchased from Sigma Chemical Co (St Louis, MO) and was dissolved in endotoxin-free media before use.

Virus preparation. HIV-1 Bal, III B, RF, SF-2 strains were provided by the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, National Institutes of Health, Bethesda, MD). HIV-1 HD strain was isolated from an HIV-1–infected woman’s cord blood sample in our laboratory. HIV-1 Bal strain was prepared in adult peripheral blood MDM and titered by 50% tissue culture infective dose (TCID₅₀) triplicate endpoint dilution assay in primary macrophages using HIV-1 RT activity as indicator. The stock of HIV-1 Bal contained 8.0 x 10⁵ cpmp/mL of RT activity. HIV-1 IIIB, RF, SF-2, and HD strains were prepared in peripheral blood mononuclear cells from normal donors and have 5 x 10⁵ cpmp/mL of RT activity. The 50% TCID₅₀ was defined as the amount of virus that resulted in half of the peripheral blood leukocyte cultures demonstrating detectable viral infection (ie, supernatant RT activity greater than 2 SD above background).

HIV-1 RT assay. HIV-1 RT activity assay was carried out based on the technique of Willey et al with modification. Briefly, 10 μL of culture supernatants were added to a cocktail containing poly (A), oligo(dt) (Pharmacia, Inc, Piscataway, NJ), MgCl₂, and 32p dTTP (Amersham Corp) and incubated for 20 hours at 37°C. Then, 30 μL of the cocktail were spotted onto DE81 paper, dried, and washed five times with 2× SSC and once with 95% ethanol. The filter was then air-dried. Radioactivity was counted in a liquid scintillation counter (Packard Instrument Inc). Addition of cystamine to RT assay had no effect on the readout (CPM/5 μL) of results using both positive and negative control samples.

HIV-1 infection. The purified cord blood-derived monocytes were cultured in 48-well plates (2.5 x 10⁶ cells/well) for 10 days and then inoculated with HIV-1 Bal in a volume of 0.1 mL/well with RT activity 8 x 10⁵ cpmp/mL for 2 hours at 37°C. The inoculum was removed, and cells were washed three times with DMEM to remove unabsorbed virus. Fresh medium (DMEM with 10% FCS) was then added. For PHA-P- and IL-2–treated cord blood–derived lymphocyte cultures, the cells were inoculated with HIV-1 lymphocyte-tropic strains (IIIB, RF, SF-2, and HD) at concentration of 2 x 10⁴ cpm RT activity/10⁶ cells for 2 hours, and the cells were washed three times with RPMI-1640 and cultivated in RPMI 1640 media 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.

Cystamine treatment. Ten-day cultured CBMDM or PHA-P and IL-2 stimulated cord lymphocytes were incubated with cystamine (25, 50, 100, and 200 μmol/L) immediately after the infection with HIV-1 either once (single time point) or every 4 days (CBMDM) or every 3 days (cord lymphocytes) postinfection. Approximately 50% of the culture supernatants in each well were collected and replaced with fresh medium every 4 days for 3 to 4 weeks of culture (CBMDM) and every 3 days for 2 weeks (cord lymphocytes). To test effects of cystamine on HIV-1 replication in chronically infected CBMDM, 10-day cultured CBMDM were infected with HIV-1 Bal strain as described above. The HIV-1–infected CBMDM cultures (12 days after the viral challenge) were then treated with cystamine (100 or 200 μμ) either once only or every 4 days in subsequent course of infection. Approximately 50% of the culture supernatants in each well were collected and replaced with fresh medium every 4 days. The collected culture supernatants were stored at −70°C and assessed for the presence of HIV-1 RT activity.

HIV-1 specific immunofluorescence assay. Cord blood–derived monocytes (1.0 x 10⁶ cells/well) were cultivated in eight-chamber slides (Lab-Tek) for 10 days and then infected with HIV-1 Bal strain in a volume of 0.05 mL/well with RT activity 8 x 10⁵ cpmp/mL and treated with cystamine 200 μmol/L (immediately postinfection). Medium and cystamine were replenished every 4 days. Cord blood–derived lymphocytes were infected with HIV-1 (IIIB strain) and treated with cystamine (100 μmol/L) as described above. The cells were fixed 16 days (CBMDM) or 12 days (lymphocytes) postinfection with 100% cold acetone for 20 minutes at room temperature. Immunoglobulin G (IgG) purified from HIV-1–seropositive human serum pool was diluted in PBS (1:100) and added to the slides for 45 minutes at room temperature. The slides were then washed three times with PBS and stained with fluorescein isothiocyanate-conjugated goat antihuman IgG (1:400 dilution in PBS; Chemicon, Temecula, CA). The slides were then examined blindly under a Zeiss fluorescence microscope illuminated with a super pressure mercury lamp (Carl Zeiss, Inc, Thornwood, NY). The number of HIV-1 antigen positive cells in the chamber of slides was counted at x400 magnification.

RESULTS

The cytotoxicity effect of cystamine on CBMDM and lymphocytes. The cytotoxic effect of cystamine on CBMDM and lymphocytes was investigated. Fifty and 100 μmol/L of cystamine show no toxic effects on CBMDM and lymphocytes as determined by a trypan blue dye exclusion and by cell proliferation assay (Fig 1, A and B). Although 200 μmol/L of cystamine showed 25% cytotoxic effect on cord blood–derived lymphocytes, there was little cytotoxic effect on CBMDM with cystamine at a concentration as high as 400 μmol/L (Fig 1). Thus, we selected concentration of cystamine at 50 to 100 μmol/L (for cord lymphocytes) and 100 to 200 μmol/L (for CBMDM) as a standard concentration for the subsequent experiments.

Anti-HIV activity in primary cord blood–derived lymphocytes. Cystamine at nontoxic doses inhibited HIV-1 IIIB replication in cord blood–derived lymphocytes stimulated with PHA and IL-2 in a dose-dependent fashion (Fig 2). Cystamine also showed the inhibitory effect on three other HIV-1 strains (RF, SF-2, and HD) tested, including an...
Lymphocyte proliferation assay and cytotoxic effect of cystamine on CBMDM and cord blood-derived lymphocytes. PHA-P- and IL-2-stimulated cord lymphocytes (5 x 10^4 cells/well) in 96-well microtiter plates were cultivated in the presence or absence of cystamine at concentration indicated above for 96 hours. [³H]thymidine incorporation was measured 18 hours after addition to the cultures (A). CBMDM and cord lymphocytes were treated with cystamine at concentration indicated above every 48 hours at the concentrations indicated above. At the end of the culture period (16 days for lymphocyte cultures and 24 days for CBMDM cultures), CBMDM and lymphocytes were stained with trypan blue and counted (B). The results represent the mean of three experiments carried out in triplicate.

isolate from an HIV-1-infected mother’s cord blood (Fig 3). Additionally, cystamine-treated cord lymphocyte cultures infected with these strains showed absence of cytopathic effect induced by the virus (data not shown). Unstimulated lymphocytes infected with these HIV-1 strains did not produce HIV-1 RT activity and p24 protein (data not shown).

Anti-HIV activity in CBMDM. Cystamine at concentrations of 100 and 200 μmol/L was tested for its effect on acute and chronic HIV-1 infection in CBMDM as measured by RT activity. Immediately after HIV-1 infection, addition of cystamine to the acutely infected CBMDM at concentra-
concentration of 100 or 200 \( \mu \text{mol/L} \) inhibited the HIV-1 RT expression (Fig 4). Twelve days after HIV-1 inoculation (HIV-1 infection was already established), addition of cystamine at a concentration of 100 or 200 \( \mu \text{mol/L} \) to chronically infected CBMDM cultures also suppressed RT activity during the subsequent course of infection (Fig 5). Each experiment was performed on three independent occasions using monocytes from three different cord blood samples and similar results were obtained for each donor's cells. In addition, there was little HIV-1-induced syncytium formation in HIV-1 infected CBMDM treated with cystamine at concentration of 200 \( \mu \text{mol/L} \) (Fig 6).

**Suppression of HIV-1 protein expression in CBMDM and lymphocytes.** Cystamine also decreased HIV-1 protein expression as measured by indirect immunofluorescence assay (IFA) in CBMDM and cord lymphocytes (Fig 7). To identify effect of cystamine on HIV-1 major protein expression, we used IgG purified from HIV-1 positive serum pool as the antibody for the staining in IFA. We observed that cystamine at maximum concentration used (200 \( \mu \text{mol/L} \) for CBMDM and 100 \( \mu \text{mol/L} \) for lymphocytes) inhibited 70% to 80% HIV-1 major protein expression in both cell types (Fig 7).

**DISCUSSION**

This report shows potent antiviral activity of cystamine against HIV-1 replication in CBMDM and lymphocytes, primary target cells for the virus. Considerable progress has been made in developing treatment strategies that have improved the quality and duration of life of infants and children with symptomatic infection due to HIV-1 or AIDS. HIV-1 infection in children differs from that in adults in terms of clinical presentation and rate of disease progression.\(^3\) ZDV, ddI, and saquinavir are approved by the United States Food and Drug Administration for treatment of HIV-1 infection in children.\(^4,5\) Unfortunately, therapy with either ZDV or ddI sometimes is limited by intolerance, toxicity, or HIV-1 disease progression. In addition, HIV-1 resistance to ZDV or ddI can occur in pediatric patients on long-term therapy.\(^14,16,22\) Children with resistant strains have worse clinical outcomes than children whose viruses remained susceptible.\(^15\) Saquinavir is being tested in HIV-1 clinical trials. Thus, the limited efficacy of ZDV and ddI, as well as the poor prognosis observed in pediatric HIV-1 infection, emphasizes the need to develop agents that are better tolerated, safer, and more effective antiretroviral medication for use in children with HIV-1 infection or AIDS. An ideal drug should inhibit or prevent HIV-1 replication in both acutely and chronically infected cells. We and others recently demonstrated that cystamine at nontoxic concentration to cells suppresses HIV-1 replication in both acutely and chronically infected monocytes and lymphocytes.\(^17,18\)

HIV-1 infected macrophages become chronically infected 2 weeks after inoculation.\(^22,25\) To evaluate the effect of cystamine on chronically HIV-1--infected CBMDM, cystamine was added to the CBMDM cultures 12 days after HIV-1 Bal strain infection. As we observed in our previous study\(^17\) using adult peripheral blood MDM, addition of cystamine to CBMDM cultures only once after infection with HIV-1 had no long-term effect on the virus expression (Fig 4). We also treated CBMDM once only with cystamine at different concentrations before HIV-1 Bal infection. As we expected,
there was no long-term inhibitory effect on the virus replication observed (data not shown). However, addition of cystamine at concentration of 100 or 200 μmol/L, every 4 days to acutely or chronically infected CBMDM cultures inhibited 80% to 90% HIV-1 replication (Figs 4 and 5). This observed inhibitory effect of cystamine on HIV-1 is dose-dependent. In this study, we extended our investigation of the inhibitory effect of cystamine to other HIV-1 strains including a primary HIV-1 strain (HD) isolated from infected cord blood mononuclear cells (Fig 3), indicating that cystamine indeed has broad anti–HIV-1 effect and potential for experimental treatment of HIV-1–infected patients.

Further work is required to precisely determine the mechanisms by which cystamine inhibits HIV-1 infection. HIV-1–infected individuals have decreased levels of acid-soluble thiols, in particular cysteine and glutathione (GSH), in their plasma, leukocytes (T-cell subsets), and the bronchoalveolar lavage fluid.29-31 Oxidative stress appears to play an important role in the progression of HIV-1 infection to AIDS.29,30 Because GSH is the major intracellular defense against the production of reactive oxygen intermediate (ROIs), overproduction of such oxidants depletes GSH. Increase GSH levels can not only decrease ROIs but also inhibit stimulation by inflammatory cytokines, such as tumor necrosis factor-α (TNF-α).29,30 Thus, treatment of HIV-1 infection may need to include agents that restore systemic and intracellular GSH.29,30 N-acetyl cysteine (NAC), a cysteine precursor that is converted intracellularly 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 antioxidant by itself.29,30 We have shown that GSH and NAC potently inhibits HIV-1 replication in both adult peripheral blood and CBMDM.31,32

Fig 6. Effect of cystamine on the HIV-induced syncytium formation in CBMDM in vitro. The HIV-1–infected cells were treated with or without cystamine at a concentration of 200 μmol/L, immediately and every 4 days after HIV-1 infection through the entire course of the experiment. The morphology of the uninfected (A), infected and treated (B), and infected and untreated CBMDM (C) shown above was observed under a light microscopy (×250) at day 20 postinfection.
In addition, we recently demonstrated that cystamine inhibits the TNF-α-induced HIV-1 expression in chronically infected U1 and ACH-2 cells and that cystamine inhibits lipo-polsaccharide-stimulated endogenous TNF production in MDM, which may play an important role in the mechanism(s) of cystamine’s anti-HIV-1 effect. In most cell systems, cystamine, the disulfide of cysteamine, is reduced to the thiol on entering a cell. Although it has not yet been tested in human, cystamine has been used as a scavenger for free radicals. Thus, a combination of cystamine with RT inhibitors and other standard therapies may prove to be useful treatment for pediatric HIV-1 infection and AIDS.

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