In Vitro Hydroxyurea Decreases Th1 Cell-Mediated Immunity

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Hydroxyurea (HU) is used in the treatment of hematologic disorders and is sometimes added to antiretroviral combination therapy to potentiate human immunodeficiency virus (HIV) suppression. However, HU has toxic effects on rapidly dividing cells, including the effectors of the immune response. To determine whether HU affects specific T-cell responses, we measured lymphocyte proliferation and cytokine production in response to microbial antigen and mitogen stimulation in the presence of added HU (10 to 1,000 μM). HU treatment of peripheral blood mononuclear cells obtained from HIV-infected patients and uninfected controls decreased lymphocyte proliferation and gamma interferon production compared with untreated cells. Interleukin-2 (IL-2) and IL-10 production was not affected by HU. The HU-mediated decrease of lymphocyte proliferation was similar in peripheral blood mononuclear cells from HIV-infected patients and from uninfected controls.

The inhibitory effect of HU required continuous exposure to the drug and could be reverted by washing the drug out of the culture environment. These findings suggest that HU-containing therapeutic regimens might decrease Th1-cell-mediated immune responses in vivo.

The use of hydroxyurea (HU) in antiretroviral combination therapy (ART) increased after encouraging results were reported in the initial HU clinical studies (7, 10, 11, 13–15). In spite of potential added toxicity, HU is an appealing addition to ART because it is cheap and will not induce resistance of human immunodeficiency virus (HIV) to itself or antiretroviral drugs.

HU has no direct interaction with HIV replicative enzymes. Its potentiating effect in ART derives from the inhibition of the mammalian ribonucleotide reductase, a cellular enzyme that transforms ribonucleotides into deoxyribonucleotides. The effect of HU on HIV replication might be mediated by two molecular mechanisms: (i) depletion of intracellular levels of dATP, resulting in a favorable shift of the purine analogue (ddI) triphosphate/dATP ratio; or (ii) enhancement of the mammalian pyrimidine kinase activities in salvage pathways, resulting in increased phosphorylation of the anti-HIV pyrimidine analogues (zidovudine [AZT], 3TC, and ddC). Several in vitro and in vivo studies have demonstrated the benefit of adding HU to anti-HIV therapeutic regimens that contain ddI, adeclovir, d4T, AZT, 3TC and/or ddC (5, 6, 12, 18, 20). HU might also inhibit HIV replication by decreasing T-cell proliferation.

The safety profile of HU has been characterized in patients with myeloproliferative disorders including leukemia, other chronic diseases (e.g., psoriasis and idiopathic thrombocytopenia), and sickle cell anemia, for which HU has been used for many years (2, 3). The main adverse reactions associated with HU therapy result from growth retardation of rapidly dividing cells, resulting in neutropenia, anemia, thrombocytopenia, diarrhea, and delayed wound healing. In HIV-infected patients, the gain in CD4 cell numbers was smaller in patients treated with HU than in patients on non-HU-containing regimens, who achieved similar decreases in HIV viral load (20). It is not clear whether the lower gain of CD4 cells in HU-treated patients is clinically significant.

In vitro measurements of cell-mediated immunity (CMI) have been used as surrogate markers of protection against viral pathogens and other infectious agents whose multiplication is controlled by CMI in vivo. Among different CMI indicators, the presence of antigen-specific in vitro lymphocyte responses, as measured by lymphocyte proliferation assays (LPA) and gamma interferon (IFN-γ) secretion, have been associated with protection against opportunistic viral pathogens such as herpesviruses (4, 16). This study measures the effect of HU on antigen- and mitogen-stimulated T-cell responses.

MATERIALS AND METHODS

Patients. For this study, 13 HIV-infected patients and 10 uninfected controls were enrolled. All the HIV-infected patients were on ART and had CD4 cell numbers of ≥200 cells/μl. Nine of them had met AIDS-defining criteria prior to the study. The HIV viral loads were between <20 and 20,000 copies/ml.

LPA. The LPA was performed as previously described (24). Triplicate wells, each containing 10⁵ cells in RPMI with 10% human AB serum and HU at 0, 10, 100, and 1,000 μM, were incubated for 6 days in the presence of 10 μg of Candida antigen (Greer) per ml; cytomegalovirus, herpes simplex virus, varicella-zoster virus, and control antigen at preestablished optimal concentrations (16); and 10 μg of pokeweed mitogen (PWM) ( Sigma) per ml. Proliferation was measured by counting 6-h [³H] thymidine (Amersham) incorporation in a scintillation counter (Packard). Stimulation indices (SI) were calculated as the ratio between median cpm in controls. Positive responses were defined as SI ≥ 3 for microbial antigens and SI > 5 for PWM.

Cytokine measurement. For the cytokine assay, 10⁶ cells were grown in each well of 24-well plates in RPMI containing 10% human AB serum, HU, mitogen, and antigens at concentrations used for the LPA. Supernatants were harvested at peak production for each cytokine (day 3 for interleukin-2 [IL-2] and IL-10 and day 6 for IFN-γ), as previously determined and published in the Pediatric AIDS Clinical Trials Group Consensus Protocol, and stored at −70°C until assayed. The cytokines were measured as specified by the manufacturer, using Endogen kits for IL-2 and IFN-γ and Immunotech for IL-10. Cytokine production was calculated as the difference between antigen- and mitogen-stimulated cultures and unstimulated controls.

Statistical analysis. Statistical analysis was performed using the appropriate tests for nonparametric variables and software Statview 5.0.1 (SAS).
RESULTS

Effect of HU on LPA responses. HU significantly inhibited in vitro LPA responses, as measured by cpm of samples from 23 HIV-infected patients and controls, to all stimulants in a dose-dependent fashion ($P < 0.0001$, Friedman test) (Fig. 1). In contrast, proliferation in unstimulated wells was not significantly affected by HU, resulting into significant SI differences for all stimulants ($P < 0.0001$, Friedman test). The differences in cpm and SI of the stimulated samples were particularly remarkable at 100 and 1,000 $\mu$M HU compared with untreated wells ($P < 0.001$, Wilcoxon signed rank). This indicated that in vitro HU levels equivalent to those achieved during therapy, such as 100 and 1,000 $\mu$M (21), significantly inhibit antigen-induced LPA responses. However, HU seemed not to affect the survival of unstimulated cells, since the cpm in control wells did not change significantly across all HU concentrations.

To further test the hypothesis that HU does not affect the viability of unstimulated cells, PWM-induced proliferation of peripheral blood mononuclear cells (PBMC) pretreated with HU before stimulation was compared with proliferation of cells under continuous HU treatment and of untreated controls. PBMC obtained from four normal hosts were cultured in 1,000 $\mu$M HU-containing and drug-free medium. After 3 days, the cells were washed and new medium was added such that culture conditions were kept constant for cells originally grown in drug-free medium whereas only half of the original HU-treated wells continued in HU-containing medium and the other half were changed to drug-free culture medium. PWM was added to half of the wells in each treatment group. Thus, quadruplicate unstimulated and quadruplicate PWM-stimulated wells were included in each of the three treatment conditions: HU continuous treatment; HU pretreatment followed by stimulation in drug-free medium; and untreated controls. SI, calculated after 3 days of PWM stimulation, showed that PWM-induced proliferation significantly decreased in the wells that were exposed to HU for the whole duration of the experiment ($P = 0.04$, Wilcoxon signed rank) (Table 1). In contrast, the cells grown in HU-containing medium for the first 3 days and then changed to drug-free medium showed SI similar to untreated controls ($P = 0.5$, Wilcoxon signed rank). These data confirmed that HU treatment did not affect the viability of unstimulated PBMC, because removal of the drug from the culture medium restored the PWM-induced responses to the same level as those in untreated controls. Furthermore, these results indicate that demonstration of the inhibitory effects of HU requires continuous exposure to the drug during in vitro stimulation.

Effect of HU on cytokine production. In vitro treatment of PBMC cultures with HU had a differential effect on cytokine production (Fig. 2). IL-2 and IL-10 levels were not significantly affected by HU ($P = 0.2$ and 0.3, respectively, Friedman test). In contrast, IFN-$\gamma$ levels decreased in a dose-dependent fashion in response to HU ($P = 0.02$, Friedman test).

Comparative effect of HU on lymphocyte proliferation in HIV-infected patients and uninfected controls. LPA results from HIV-infected patients were compared with those from uninfected controls (Table 2). cpm and SI in cultures of PBMC from HIV-infected patients were significantly lower than those

![FIG. 1. In vitro effect of HU on lymphocyte proliferative responses to microbial antigens and mitogens. Data represent medians of cpm measured after a 6-day in vitro stimulation of PBMC from 13 HIV-infected patients and 10 uninfected controls. There was a significant HU dose-dependent decrease in cpm for all stimulants ($P < 0.001$, Friedman test) but not for unstimulated controls.](http://cvi.asm.org/)

![FIG. 2. In vitro effect of HU on microbial antigen and mitogen-induced cytokine secretion. Data represent medians of a composite of experiments that used PBMC from 13 HIV-infected patients and 10 uninfected controls. Cytokine concentrations were measured in culture supernatants of PBMC stimulated with PWM, Candida, and cytomegalovirus antigens. IFN-$\gamma$ synthesis decreased with the HU dose ($P = 0.01$, Friedman test). IL-2 and IL-10 were not significantly affected by HU treatment.](http://cvi.asm.org/)

| Assay condition | Median SI (range) | $P^a$ |
|-----------------|------------------|------|
| Control$^b$     | 339 (278–402)    | NA$^e$ |
| HU continuous$^c$ | 6 (5–8)         | 0.04 |
| HU pretreatment only$^d$ | 332 (297–367) | 0.5  |

$^a$ Value with respect to control, determined by Wilcoxon signed rank.
$^b$ PBMC were grown in culture medium without HU for the duration of the experiment.
$^c$ PBMC were grown in 1,000 $\mu$M HU-containing medium for the duration of the experiment.
$^d$ PBMC were grown in 1,000 $\mu$M HU-containing medium for the first 3 days, washed, and resuspended in culture medium without HU for the next 3 days.
$^e$ ND, not applicable.
in uninfected controls across all HU concentrations (P < 0.05, Mann-Whitney U test). However, inhibition of lymphocyte proliferation elicited with 1,000 μM HU, calculated as the cpm0/cpm1,000 ratio, was similar in HIV-infected patients and uninfected controls (P = 0.96). This indicated that HU was equally inhibitory to in vitro proliferation of PBMC obtained from HIV-infected patients and uninfected controls.

### DISCUSSION

HU inhibits in vitro antigen- and mitogen-stimulated LPA in a dose-dependent fashion over a range of concentrations that includes levels readily achieved in vivo during HU therapy (22). This effect might contribute to the anti-HIV activity of HU, since HIV replication is stimulated by proliferation of CD4 cells (17). However, another consequence of this effect could be impaired antimicrobial defenses in HU recipients. It is known that inhibition of lymphocyte proliferation occurs in vivo during HU therapy since HIV-infected patients on HU have a lower gain in CD4 cells than do patients who achieve the same level of HIV suppression without HU (20). This study demonstrates that the in vitro inhibitory effect of HU is restricted to stimulated cells and spares unstimulated PBMC. Several investigations have shown an association between in vitro microbial antigen-specific lymphocyte proliferation and in vivo resistance to certain microorganisms, such as herpesviruses and Candida albicans (4, 16). Whether lymphocyte proliferation is a surrogate marker of pathogen-specific immunity or a true mediator of protection has not been established. There is inadequate clinical information to indicate whether HIV-infected individuals receiving HU-containing therapeutic regimens experience a higher incidence of opportunistic infections than do those not receiving HU.

Other investigators have demonstrated that HIV-infected patients receiving HU-containing ART exhibited in vitro HIV-specific immunity at least as well as patients receiving HU-sparing regimens (14). These data may seem contradictory to our results that HU at levels commonly achieved in vivo has a profoundly inhibitory effect on lymphocyte proliferation. However, the two findings can be reconciled by the observation that PBMC grown in the presence of HU show recovery of lymphocyte proliferation as soon as HU is washed out of the culture medium. Similarly, in the process of isolating PBMC from HU-treated individuals, the drug is washed off, which might preclude the in vitro demonstration of its inhibitory effect.

The effect of in vitro HU on cytokine secretion further supports the concern that HU treatment might depress CMI. Cytokine production has been traditionally used to characterize T-helper responses (21). Thus, production of IFN-γ among other cytokines, characterizes Th1 responses which promote CMI, such as lymphocyte-mediated cytotoxicity. In contrast, Th2 responses, characterized by IL-10 secretion among others, move the immune response toward antibody production. IL-2, although predominantly a Th1 cytokine, is necessary for initiating both Th1 and Th2 responses. IL-2 levels in culture supernatants were not affected by in vitro HU treatment, indicating that HU does not interfere with antigen recognition and initiation of the response. The production of IFN-γ, however, decreased in HU-treated culture supernatants. In contrast to IFN-γ, secretion of IL-10, a Th2 cytokine, was not significantly affected by HU. The mechanism of the differential HU effect on Th1 and Th2 responses is unclear. It might reflect diverse T-cell susceptibility to HU. An alternative explanation is that HU decreases the proliferation of both Th1 and Th2 cells. However, since IL-10 is constitutively expressed in unstimulated T-cell cultures, the net effect of HU on IL-10 production is attenuated by comparison with IFN-γ. The effect of HU on in vitro cytokine production suggests that HU treatment might create an imbalance between Th1 and Th2 responses. These findings need to be further confirmed by studying Th1 and Th2 frequencies in samples from HU-treated individuals using whole-blood flow-cytometric intracellular cytokine assays (23).

The LPA response to microbial antigens correlates with the degree of immunosuppression of HIV-infected patients in the absence of ART. Highly active ART was shown to restore some of these responses at the same time as it decreases the incidence of opportunistic infections (1, 8, 9, 19). The potential inhibitory effect of HU on CMI needs to be carefully evaluated in patients receiving HU for any therapeutic reasons.

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