

Treatment of Human Immunodeficiency Virus Infection with Hydroxyurea, Didanosine, and a Protease Inhibitor before Seroconversion Is Associated with Normalized Immune Parameters and Limited Viral Reservoir

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Current treatments for human immunodeficiency virus (HIV) require uninterrupted drug administration because they are unable to reconstitute the immune response and do not affect the viral reservoir. Ten patients were treated during acute HIV infection before complete Western blot (WB) seroconversion with the combination of hydroxyurea, didanosine, and indinavir. This treatment was associated with the normalization of some immune parameters and functions. No loss of naive CD4 T lymphocytes was observed, and recovery of up to 35% of naive CD8 T lymphocytes occurred in several weeks. A vigorous HIV-specific T helper response (stimulation index >8) was observed in 7 of 8 patients treated before complete WB seroconversion but in only 1 of 5 controls treated after seroconversion. In addition, a limited latent viral reservoir (<0.02–0.5 infectious units/10⁶ cells) was documented in quiescent peripheral blood lymphocytes after treatment initiated before complete WB seroconversion.

When treatment of human immunodeficiency virus (HIV) infection should be initiated remains an important unresolved question. Reasons to delay treatment include the likelihood of increased long-term toxicity, higher cost, and earlier development of drug resistance, thus narrowing the chances of later treatment options. Reasons to start treatment early are mainly theoretical and rely on the possibility that therapeutic intervention might limit damage to the immune system and confine the spread of HIV.

The immune system is impaired by HIV infection. CD4 T lymphocytes are depleted, the CD4/CD8 ratio is decreased, and HIV-specific T helper cell responses are lost soon after acute infection [1]. Highly active antiretroviral treatment (HAART)

initiated during acute infection appears to normalize CD4/CD8 ratios [2] and HIV-specific T helper responses [1]. Early after infection, renewal of CD4 T lymphocytes is still achievable [3] and T cell receptor damage can be fixed [4]. It is not exactly clear, however, when during acute infection the treatment must be started in order to normalize these parameters.

The spread of HIV throughout the body occurs in the first few weeks [5]. As early as 10 days after the onset of the first symptoms of primary HIV infection (PHI), a reservoir of replication-competent virus, consisting of latent proviral DNA in quiescent T lymphocytes, becomes established [5, 6]. This reservoir does not significantly decrease after years of HAART [6–8]. It remains to be shown whether very early treatment after acute infection might restrict the initial spread of virus and limit the expansion of the pool of latently infected cells.

Because latently infected resting T lymphocytes represent a major viral reservoir [6–8] and because infection of macrophages may be important in initial stages of infection following sexual, parenteral, and vertical transmission [9–12], drugs affecting these compartments are good candidates for early treatment of HIV infection. Hydroxyurea and didanosine are particularly effective antiretroviral drugs in quiescent lymphocytes and macrophages [13, 14]. However, viral replication is very high in activated proliferating lymphocytes early after infection. Therefore, potent antiretroviral drugs, such as protease inhibitors, that inhibit the production of infectious virions should add substantially to the effective treatment of early HIV infection. In this study, we evaluated a drug regimen of hydroxy-

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All patients provided signed informed consent before the start of treatment. The protocol was approved by institutional review boards of the authors' institutions. Human experimentation guidelines of the US Department of Health and Human Services and of the authors' institutions were followed in the conduct of clinical research, as were the principles outlined in the Declaration of Helsinki (1990).

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urea, didanosine, and indinavir as an early treatment for HIV infection.

Methods

Patients. All patients with a documented history of HIV infection of ≤ 1 year were consecutively enrolled, independent of virus load, CD4 cell count, CD4/CD8 ratio, age, and sex. To be eligible for the study, each patient needed to have at least 2 independent consecutive assays (at different time points) that were positive for the HIV in the plasma. All patients had presented with signs or clinical symptoms typical of PHI, most commonly a flu-like syndrome with fever and sore throat. At the time of enrollment, patients were distinguished on the basis of Western blot (WB) analysis. Complete seroconversion was defined as strong WB positivity for antibodies against ≥ 4 of the following viral proteins: gp160, gp120, p65, p55, gp41, p32, p24, and p18. As a result of this screening, 10 patients were enrolled before complete seroconversion and 5 patients (controls) after complete seroconversion (but within 1 year of infection). Enrolled patients were naive to antiretroviral drugs. All 15 patients received didanosine (200 mg twice/day) and indinavir (800 mg 3 times/day); patients weighing < 60 kg were also given hydroxyurea (300 mg 3 times/day), whereas those weighing > 60 kg were given hydroxyurea (400 mg 3 times/day). Plasma viremia was measured by ultrasensitive polymerase chain reaction (sensitivity, 50 equivalent/mL; Roche, Nutley, NJ). CD4 and CD8 T lymphocyte counts/mm³ were done by standard techniques.

Flow cytometry. For detection of naive T lymphocytes, peripheral blood mononuclear cells (PBMC; $2-10 \times 10^5$ /tube), isolated by ficoll-hypaque density centrifugation from heparinized blood, were suspended in 100 μ L of FACS buffer (PBS with 2% fetal calf serum) before the addition of 4 μ L of CD45RA-fluorescein isothiocyanate (FITC) (monoclonal antibody [MAb] ALB11; Immunotech, Hamburg, Germany), 4 μ L of 1 : 10 dilution of CD62L-phycoerythrin (PE) (MAb SCF128T17G6; Coulter, Hialeah, FL), and 4 μ L of either CD8-Cy5 or CD4-Cy5 (MAb 13B8.2; Immunotech). For detection of activated (CD38⁺DR⁺) CD8 T lymphocytes, 4 μ L of CD38-PE (MAb T16; Immunotech), 4 μ L of HLA-DR-FITC (MAb 357; Immunotech), and 4 μ L of CD8-Cy5 or 4 μ L of IgG-FITC, -PE, and -Cy5-conjugated isotype-matched controls (Immunotech) were used. Flow cytometry analysis was done on a tightly gated lymphocyte population, by use of FACscalibur (Becton Dickinson, San Jose, CA).

Proliferation assays. Proliferation assays were performed by resuspension of PBMC in RPMI 1640 medium containing 10% human AB serum, HEPES buffer, L-glutamine, and penicillin-streptomycin. Assays were performed on 23 February 1998, except for patient 1, whose assay was done on 24 June 1998. Cells (10^5 /well) were cultured in 6 replicate wells of 96-well U-bottomed plates in the presence of HIV recombinant p24 protein at a final concentration of 5 μ g/mL. Six days later, the cells were pulsed with [³H]thymidine at 1.0 μ Ci per well, and uptake was measured 6 h later by scintillation counter (Topcount; Packard Instruments, Meriden, CT). The HIV p24 protein (Protein Science, Meriden, CT) is a recombinant protein derived from the *gag* gene of HIV (NY-5 strain) produced in a baculovirus expression system with proved 90%–95% purity. A mixture of baculovirus proteins was used as a

control antigen at a concentration of 1.5 μ g/mL, which is equal to the baculovirus antigen concentration in the recombinant p24 protein. The stimulation index was calculated as the mean counts per minute (cpm) of incorporated [³H]thymidine from cells stimulated with p24, divided by the mean cpm from cells stimulated with baculovirus control proteins.

Statistical methods. Statistical analysis was performed by the Mann-Whitney nonparametric U test (two tailed). *P* values are reported in the text.

Results

Early HIV suppression leads to normalization of immunologic defects. Ten patients were treated with hydroxyurea, didanosine, and indinavir before complete WB seroconversion. The effects of this treatment on virus load have been described previously in this cohort [2]. In brief, plasma viremia ($> 550,000$ copies/mL before treatment) became undetectable (< 50 copies/mL) in all patients. In the present study, we analyzed the effect of this early treatment on immunologic parameters. After 46 ± 21 weeks of therapy, the changes in CD4 and CD8 cell count and CD4/CD8 ratio were +154, -544, and +0.46, respectively. A longitudinal analysis of the changes of naive CD4, naive CD8, and activated CD8 T lymphocytes was performed (figure 1A). An increase of naive CD8 and a decline of activated CD8 T lymphocytes was observed 4 weeks after treatment, whereas the proportion of naive CD4 T lymphocytes remained high throughout.

WB analysis was followed longitudinally in these patients. In most, a complete seroconversion eventually developed, even though HIV was below the level of detection in the plasma. The progression of the seroconversion, however, was very slow. In some patients, complete seroconversion was evident only > 1 year after initiation of treatment. In 1 case, in which only p24 immunoreactivity was detected at the initiation of treatment, no major changes in WB antibodies have yet been observed after 11 months of therapy.

Eight of 10 patients in this cohort were tested for HIV-specific T helper responses (figure 1B). With one exception (patient 7; figure 1B), all patients tested before complete WB seroconversion had a vigorous CD4 T cell proliferative response to p24, with stimulation indices of 8.3–46.1. These results were compared with those of a control group of patients who also were enrolled on the basis of a recent diagnosis of PHI (see Methods) but who presented with complete WB seroconversion. Only 1 (patient C3; figure 1B) of 5 control subjects had a robust CD4 T cell proliferative response to p24. Owing to the lack of baseline (before treatment) samples, we could not establish whether the early treatment rapidly restored or prevented the loss of a CD4 T cell proliferative response. Vigorous T helper responses, however, were detected as early as 34 days after treatment initiation.

Early treatment limits the establishment of HIV reservoirs. We examined whether very early treatment might confine the

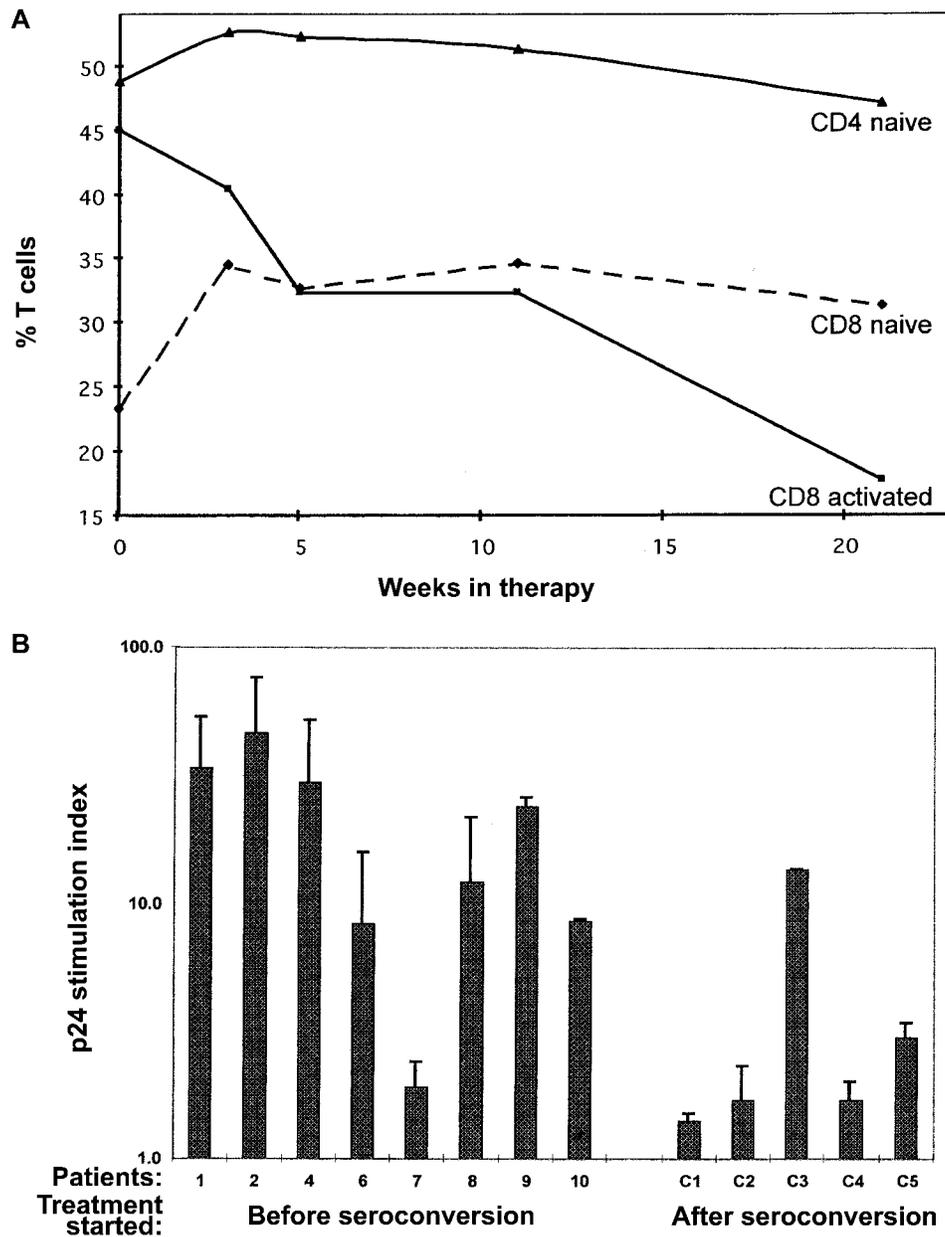


Figure 1. *A*, Longitudinal follow-up of CD4 naive, CD8 naive, and CD8 activated T lymphocytes. Samples were collected at baseline and at regular intervals for 24 weeks after treatment. CD4 naive (% of total CD4 cells), CD4⁺CD62L⁺CD45RA⁺; CD8 naive (% of total CD8 cells), CD8⁺CD62L⁺CD45RA⁺; CD8 activated (% of total CD8 cells), CD8⁺CD38⁺DR⁺. *B*, Human immunodeficiency virus (HIV)-specific T helper proliferative responses in HIV-infected patients treated with hydroxyurea, didanosine, and indinavir before (patients 1–10) and after (patients C1–C5) complete Western blot seroconversion.

size of the HIV reservoir by quantitating the number of latently infected CD4 T lymphocytes in 6 of 10 patients who agreed to donate the necessary amount of blood for this analysis (~200 mL). A previously described assay [6], able to detect latently infected CD4 T lymphocytes in 18 of 18 chronically infected patients on HAART, failed to detect replication-competent HIV in 3 of 4 patients treated before complete seroconversion (stan-

dard assay; table 1). A second attempt with an ultrasensitive assay analyzing a much higher number of cells ($\leq 67 \times 10^6$; table 1) was necessary to recover replication-competent HIV. With this more sensitive assay, virus was recovered from the blood of 5 of 6 patients studied, although at relatively low levels. In 1 case (patient 10; table 1), no HIV could be isolated, even when 37×10^6 cells were analyzed. Rates of latent HIV in rest-

Table 1. Latent human immunodeficiency virus after early treatment.

Patient no.	Time between exposure and treatment, months	Time on drug, months	Assay	10 ⁶ resting CD4 cells screened	Virus isolation	Frequency of latently infected cells (IUPM)
1	2	18	Ultrasensitive	42	+	0.5
2	1	16.5	Standard	5.5	-	<0.2
2	1	17.8	Ultrasensitive	62	+	0.1
3	2	3	ND	ND	ND	ND
4	2	10.6	Standard	8.5	+	0.5
5	2	10	ND	ND	ND	ND
6	2	9	Ultrasensitive	39	+	0.5
7	2	9	ND	ND	ND	ND
8	2	8	ND	ND	ND	ND
9 ^a	1	4	Standard	10.5	-	<0.2
9 ^a	1	9	Ultrasensitive	67	+	0.02
10 ^a	2	5	Standard	15.5	-	<0.2
10 ^a	2	10	Ultrasensitive	37	-	<0.2

Note. Cells from patients 2, 9, and 10 were tested by both standard and ultrasensitive assay. ND, not done; IUPM, infectious units/10⁶ cells.

^a These patients also received delavirdine, 400 mg, 3 times/day.

ing peripheral blood CD4 T cells of our patients were compared with values measured [6] in 18 chronically infected patients who had been aviremic on HAART for up to 2.5 years (average, 1.36 latent HIV/10⁶ cells). The frequency of latently infected CD4 T lymphocytes in our early treated patients was much lower (average, 0.3 latent HIV/10⁶ cells), and the difference was highly significant ($P = .009$).

Although these results confirm recent studies [5, 6] that early treatment does not prevent the establishment of latent cellular reservoirs for HIV, our data show that treatment before seroconversion with hydroxyurea, didanosine, and indinavir is associated with an unusually low frequency of latently infected cells. We could not discriminate the relative contribution of treatment timing from the role of the particular combination of drugs used in our experiments. However, not all patients treated early had the lowest levels of latent HIV. For example, in patient 4 (table 1), HIV was detectable by use of a standard assay with a frequency of latently infected cells of 0.5 infectious units/10⁶ cells. Similar frequencies were found in chronically infected patients on HAART [6].

Discussion

Our results suggest that early treatment may have a major effect on the outcome of HIV infection. Within a few months of infection, the majority of untreated patients have reached a state of equilibrium characterized by high plasma viremia with extensive HIV reservoir, reduced CD4 cell counts, decreased numbers of naive CD4 and CD8 T lymphocytes, and increased activated CD8 T lymphocytes, high antibody levels, and an absence of T helper response. We have shown that if treatment is initiated early, the scenario might be different: Viremia becomes undetectable in the plasma, the HIV reservoir is circumscribed in some patients, CD4 cells and naive CD4 and CD8 T lymphocyte percentages normalize with modest increases of

activated CD8 T lymphocytes, antibodies increase slowly, and the T helper response is vigorous.

Our data showed that the percentages of naive T lymphocytes either never deteriorated or were rapidly restored. These results contrast with a previous report showing that the recovery of naive CD4 and CD8 populations was significant only after 12 months in patients receiving HAART in late stages of chronic HIV infection [15]. HIV infection is characterized by a progressive loss of naive CD4 and CD8 T lymphocytes, possibly because of a limited renewal/production by the bone marrow or thymus [16–19]. If antiretroviral treatment reverts the loss of naive cells, it is conceivable that the time required to replenish the naive T lymphocyte pool will become progressively longer during the progression of the infection. Our findings provide evidence that the earliest treatment leads to the fastest recovery of the naive cell repertoire. Furthermore, early treatment might be the only chance to limit the establishment and expansion of latent HIV reservoirs. In contrast to the lack of decay of viral reservoir observed after treatment of chronic HIV infection [20], the small size of the latent viral reservoir observed in our patients might be due to a limited expansion or to a more rapid decay after early treatment.

Our results provide a more precise timing for the optimal recovery of HIV-specific T helper response previously described during the treatment of acute infection [1]. In fact, treatment before complete WB seroconversion provided the highest chance of normalization of these responses. Starting HAART during chronic progressive infection is usually associated with the failure to normalize HIV T helper responses [15, 21, 22]. A notable exception is represented by a group of patients treated during chronic progressive infection with the combination of only 2 drugs, hydroxyurea and didanosine [23].

This study was not designed to assess the specific contribution of the combination of hydroxyurea, didanosine, and indinavir. The use of hydroxyurea, a cytostatic and potentially immunosuppressive drug, during the course of an infection

causing immunodeficiency has raised theoretical concerns, particularly in view of the lack of CD4 cell increase after treatment with hydroxyurea plus didanosine or didanosine and stavudine, shown in previous trials [24, 25]. In the present study, however, no signs of immunosuppression were detected. All the immune parameters tested showed improvement, and there was a clear increase in CD4 T lymphocytes. Paradoxically, the cytostatic effects of hydroxyurea on CD4 T lymphocytes might be advantageous, since resting cells do not support HIV replication [26–28]. If immunostimulation (primarily represented by CD8 T lymphocyte proliferation [29]) and consequent T cell-mediated immunopathology play a major role in the immunopathogenesis of HIV infection [30, 31], cytostatic treatment is likely to be beneficial.

In conclusion, this study represents a first step toward addressing the important question of when to initiate HIV infection treatment, and controlled studies are required in order to provide a conclusive answer. Treatment before complete seroconversion, however, appears desirable because, although recovery of immune functions and normalization of some immune parameters have been demonstrated when treatment is initiated during chronic HIV infection, recovery was incomplete and did not occur in all patients [15, 21, 22]. Our data suggest that the time before complete seroconversion might provide an important window for therapeutic intervention to prevent irreversible immune damage. Limiting the number of resting infected cells very early after infection might also have implications in the control of HIV replication. Of interest, only examples of complete virus suppression without rebound after drug withdrawal have been reported in patients treated early after infection [2, 32, 33]. Early treatment may therefore be the key to developing functional immunosurveillance to control virus production without permanent drug therapy. However, the immunologic correlates that predict control of viremia after discontinuation of therapy and the relative contribution of the elements required to induce such control need to be analyzed in randomized, controlled clinical studies.

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