

Decreased human immunodeficiency virus type 1 plasma viremia during antiretroviral therapy reflects downregulation of viral replication in lymphoid tissue

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ABSTRACT Although several immunologic and virologic markers measured in peripheral blood are useful for predicting accelerated progression of human immunodeficiency virus (HIV) disease, their validity for evaluating the response to antiretroviral therapy and their ability to accurately reflect changes in lymphoid organs remain unclear. In the present study, changes in certain virologic markers have been analyzed in peripheral blood and lymphoid tissue during antiretroviral therapy. Sixteen HIV-infected individuals who were receiving antiretroviral therapy with zidovudine for ≥ 6 months were randomly assigned either to continue on zidovudine alone or to add didanosine for 8 weeks. Lymph node biopsies were performed at baseline and after 8 weeks. Viral burden (i.e., HIV DNA copies per 10^6 mononuclear cells) and virus replication in mononuclear cells isolated from peripheral blood and lymph node and plasma viremia were determined by semiquantitative polymerase chain reaction assays. Virologic and immunologic markers remained unchanged in peripheral blood and lymph node of patients who continued on zidovudine alone. In contrast, a decrease in virus replication in lymph nodes was observed in four of six patients who added didanosine to their regimen, and this was associated with a decrease in plasma viremia. These results indicate that decreases in plasma viremia detected during antiretroviral therapy reflect downregulation of virus replication in lymphoid tissue.

Several immunologic and virologic markers including $CD4^+$ lymphocyte counts, serum β_2 -microglobulin and neopterin, p24 antigenemia, plasma viremia measured by culture or by nucleic acid detection, and quantitation of human immunodeficiency virus (HIV) DNA (viral burden) and HIV RNA in mononuclear cells isolated from peripheral blood are currently used in monitoring the effect of antiretroviral therapy in HIV-infected subjects. A common finding following initiation of antiretroviral therapy with nucleoside reverse transcriptase (RT) inhibitors is a transient improvement in these parameters that is maximal between weeks 4 and 16 of therapy and that is generally more pronounced in patients in advanced stages of disease who are receiving combination therapy (1–10). Most of these markers seem to be valid in predicting progression of HIV disease (11–13); however, their usefulness in assessing the response to antiretroviral therapy is still debated. With regard to the $CD4^+$ lymphocyte count and the serum p24 antigen

level, which are the most widely used surrogate markers in clinical practice, the discrepancy between the transient improvement of these parameters observed following antiretroviral therapy and the absence of demonstrable clinical benefit in early stage disease has further called into question the validity of these markers in the evaluation of response to therapy (refs. 14–16 and 1). Furthermore, a major caveat with regard to the interpretation of changes in surrogate markers in peripheral blood following antiretroviral therapy is the possibility that they may not necessarily reflect the situation in other anatomic sites such as lymphoid organs that are a major reservoir of HIV and primary sites for HIV replication (17–27). Recent data suggest that significant reductions in plasma viremia during antiretroviral therapy predict improvements in $CD4^+$ lymphocyte counts as well as a more favorable clinical outcome;^m however, the relationship between plasma viremia and viral replication in lymphoid tissue remains unknown. This study analyzes the effect of antiretroviral therapy on several virologic parameters simultaneously in peripheral blood and lymph node in order to more accurately assess the utility of peripheral blood virologic markers in the monitoring of antiretroviral therapy.

MATERIALS AND METHODS

Patients. This paper presents data from the larger Division of AIDS Treatment Research Initiative Study 003 examining the effects of antiretroviral therapy on HIV viral burden and virus replication in lymphoid tissue.ⁿ Between June 1992 and March 1993, 16 HIV-1-infected individuals who were receiving antiretroviral therapy with zidovudine for >6 months were enrolled into an 8-week randomized study of either continuing zidovudine alone or adding didanosine to ongoing zidovudine therapy. All patients underwent lymph node biopsy at baseline and after 8 weeks on study. The protocol was approved by the

Abbreviations: HIV, human immunodeficiency virus; RT, reverse transcriptase; ICD, immune complex dissociated.

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^mO'Brien, W., Hartigan, P., McCreedy, B. & Hamilton, J. (1994) Tenth International Conference on AIDS, Yokohama, Japan, abstr. 254B.

ⁿCohen, O., Pantaleo, G., Graziosi, C., Niu, M., Fauci, A. & Division of AIDS Treatment Research Initiative (DATRI) 003 Study Group (1994) Tenth International Conference on AIDS, Yokohama, Japan, abstr. 001B.

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Institutional Review Board of each participating site. Entry criteria included HIV-1 seropositivity without AIDS; continuous zidovudine therapy at 300–600 mg/day for ≥ 6 months and no prior therapy with didanosine; CD4⁺ lymphocyte count of $\geq 250/\mu\text{l}$; and the presence of at least two palpable lymph nodes.

Treatment Regimens. Patients were randomly assigned either to remain on zidovudine (group 1) or to add didanosine (200 mg) twice daily to their zidovudine regimen (group 2) until completion of the week 8 lymph node biopsy. Due to a clerical error, two patients who were randomized to add didanosine to their zidovudine regimens never received didanosine from their clinical site and are included in group 1 for analysis. An intention-to-treat analysis was also done and is not significantly different from the as-treated analysis presented (data not shown).

Characteristics of the Study Population. The clinical characteristics of the study participants at entry are listed in Table 1. The mean baseline CD4⁺ lymphocyte count for the entire group was 406 cells per μl . There were no significant differences in any of the baseline variables between those randomized to continue on zidovudine alone (group 1) versus those who added didanosine to their regimen (group 2).

Evaluation of Patients. Lymphocyte phenotypic analysis was performed by flow cytometry on day 0 (day of the baseline lymph node biopsy) and at weeks 4 and 8. Quantitation of HIV-1 DNA and RNA in mononuclear cells from peripheral blood and lymph node as well as HIV-1 plasma viremia were determined by semiquantitative PCR on day 0 and week 8. These virologic assays were performed by investigators who were blinded with regard to treatment group assignments.

Lymph Node Biopsies. The mean size of the excised lymph nodes was 1.6×0.6 cm. Eighty-eight percent of the excised lymph nodes were axillary and 12% were cervical. The only complication related to the lymph node biopsies was a probable wound infection requiring incision and drainage in one patient after the week 8 biopsy (complication rate, 3.1%).

Determination of p24 Antigenemia. Immune complex-dissociated (ICD) p24 antigen levels were determined from serum by a commercially available ELISA method (Coulter).

Analysis of HIV-1 DNA in Peripheral Blood and Lymph Node Mononuclear Cells by PCR. Mononuclear cells were obtained from peripheral blood and lymph node as described and pelleted cells were frozen at -70°C until use (23). Lysates corresponding to 10^5 cells in $50 \mu\text{l}$ were amplified for 30 cycles using a primer pair (SK 145/101) specific for the HIV-1 *gag* gene. Serial dilutions (1:10) of 8E5 cells, a chronically HIV-1-infected T-cell line containing one proviral copy per cell, were used as positive controls. Negative controls included lysis buffer and Jurkat cell lysates. Amplified products were hybridized to $\gamma\text{-}^{32}\text{P}$ -labeled *gag*-specific probe (SK102) and sep-

arated on 10% polyacrylamide gels by electrophoresis. Visualization was achieved by standard autoradiography and by PhosphorImager analysis (Molecular Dynamics). HIV-1 DNA copy number was calculated by interpolation into the 8E5 serial dilution curve analyzed with ImageQuant software (Molecular Dynamics). Samples were run in at least two separate experiments and the means were reported as HIV-1 DNA copies per 10^6 mononuclear cells.

Analysis of HIV-1 Replication in Peripheral Blood and Lymph Node Mononuclear Cells by RT-PCR. Total RNA was extracted from cells with RNazol (Tel-Test, Friendswood, TX) (28). Aliquots of $1.5 \mu\text{g}$ of total RNA were reverse transcribed using 60 units of avian myeloblastosis virus RT (Life Sciences, St. Petersburg, FL) with random hexamers ($40 \mu\text{g}/\text{ml}$) (Pharmacia) at 45°C for 2 hr. The reaction mixture was then heated to 94°C for 5 min to inactivate RT. One-fifth of each reverse transcription was amplified for 28 cycles with a primer pair (10659/11416) that detects unspliced and single-spliced HIV-1 RNA. The 10659 primer ($5'\text{-gac tca tca agc ttc tct atc aaa-3}'$) corresponds to a region within the first *tat/rev* exon, while the 11416 primer ($5'\text{-ctc att gcc act gtc ttc tgc tct-3}'$) is complementary to a region within the envelope gene. The 10659 primer was $\gamma\text{-}^{32}\text{P}$ -labeled prior to amplification as described (29). Positive controls were serial dilutions of a plasmid cDNA construct containing HIV-1 exons 1 and 4E (30). Negative controls included herring sperm DNA (used as the diluent for the plasmid cDNA) and cDNA obtained from Jurkat cells and normal volunteer peripheral blood mononuclear cell RNA. Amplified products were separated on 6% polyacrylamide gels by electrophoresis. Standard autoradiography and PhosphorImager analysis were used for visualization.

To normalize for RNA input, 1/25th of each reverse transcription was amplified with a primer pair (sense primer, $5'\text{-gaa ccc tga ccc tgc cgt gta cc-3}'$; antisense primer, $5'\text{-agc cgc agc gtc atg agc aga tta aac ccg-3}'$) specific for human T-cell receptor C α mRNA.

All four samples from each patient (peripheral blood and lymph node from weeks 0 and 8) were amplified simultaneously in order to avoid PCR run-to-run variability. Quantitation of HIV-1 RNA was accomplished by normalization of the HIV-1 amplification signal to the C α signal and interpolation into the control cDNA serial dilution curve. Samples were run in at least two separate experiments and the means are reported as fg of HIV-1 RNA per $1.5 \mu\text{g}$ of total RNA.

Determination of Plasma Viremia by RT-PCR. Quantitation of HIV-1 RNA copy number in plasma was determined as described (5). Briefly, duplicate 1-ml aliquots of plasma were centrifuged at $23,000 \times g$ at 4°C for 1 hr to pellet virions after which the pellet was treated with guanidinium thiocyanate and RNA was extracted with phenol/chloroform. Reverse transcription utilized Moloney murine leukemia virus RT (Bethesda Research Laboratories). cDNA was amplified for 30 cycles with unmodified SK38 and biotinylated SK39 (*gag* specific) primers. Amplified products were captured on avidin-coated microtiter plates and hybridized to horseradish peroxidase-labeled SK19 internal probe. Substrate solution was added and the optical density of each well was measured at 490 nm. Copy number per ml of plasma was determined by interpolation into a serial dilution curve of known copy numbers using a *gag* gene cDNA construct. Baseline and week 8 samples were run simultaneously to minimize assay variability.

Statistical Analysis. Mean values ± 1 SD are reported for variables at baseline and week 8; however, paired *t* tests were used to analyze changes from baseline to week 8 within each group, enabling each patient to serve as his or her own control. Comparison of baseline characteristics between groups was performed by using two-sample *t* tests.

Table 1. Baseline characteristics of study participants

	Group 1: Continued zidovudine (<i>n</i> = 10)	Group 2: Added didanosine (<i>n</i> = 6)
Mean age, years	36	38
Mean weight, kg	74	72
Sex		
Male, <i>n</i> (%)	10 (100)	5 (83)
Female, <i>n</i> (%)	0	1 (17)
HIV risk factor*		
Homosexual/bisexual, <i>n</i> (%)	9 (90)	4 (67)
Heterosexual, <i>n</i> (%)	1 (10)	2 (33)
Injection drug use, <i>n</i> (%)	1 (10)	1 (17)
Baseline CD4 ⁺ lymphocyte count, cells/ μl	413	394

*Some patients had more than one risk factor.

RESULTS

CD4⁺ Lymphocyte Counts and ICD p24 Antigenemia. The mean CD4⁺ lymphocyte count remained stable in patients who remained on zidovudine alone (group 1): 413 ± 109 cells per μl at baseline and 408 ± 80 cells per μl at week 8 (Fig. 1A). In those who added didanosine to their ongoing zidovudine therapy (group 2), the mean CD4⁺ lymphocyte count was 394 ± 74 cells per μl at baseline and 436 ± 114 cells per μl at week 8 (*P* = 0.44). One patient in group 2 (Fig. 1B, ○) experienced a significant decline in CD4 count from 509 cells per μl at baseline to 351 cells per μl at week 8, and this was associated with an increase in viral burden in peripheral blood mononuclear cells and viral replication in lymph node mononuclear cells (see below).

ICD p24 antigenemia was detectable in only three patients in group 1. In these patients, the mean p24 antigen level was 677 ± 662 pg/ml at baseline and 643 ± 636 pg/ml at week 8 (*P* = 0.30). In the four patients in group 2 with detectable p24 antigenemia, the mean baseline level was 231 ± 215 pg/ml and 38.6 ± 21.9 pg/ml at week 8 (*P* = 0.17).

Analysis of HIV-1 DNA in Peripheral Blood Mononuclear Cells and Lymph Node Mononuclear Cells by PCR. In patients who remained on zidovudine alone (group 1), the mean viral burden (HIV DNA copies per 10⁶ peripheral blood mononuclear cells) was 1338 ± 1949 at baseline and 1572 ± 2111 at week 8 (Fig. 2A). Although the magnitude of this increase was <20% above baseline, it was statistically significant (*P* = 0.05) because 9 of the 10 patients in this group showed increases in viral burden in peripheral blood mononuclear cells. A slight increase in viral burden was observed in lymph node mononuclear cells (mean HIV DNA copies per 10⁶ cells, 4233 ± 4391 at baseline and 4876 ± 3698 at week 8; Fig. 2A), but this was not significant (*P* = 0.33). In patients who added didanosine to zidovudine (group 2), the decreases in viral burden observed in both peripheral blood mononuclear cells (from 1539 ± 1420 at baseline to 1187 ± 1043 at week 8) and in lymph node mononuclear cells (from 4438 ± 3963 at baseline to 3799 ± 2644 at week 8) were not statistically significant (*P* = 0.40 and 0.34, respectively; Fig. 2B).

Analysis of HIV-1 Replication in Peripheral Blood Mononuclear Cells and Lymph Node Mononuclear Cells by RT-PCR. Levels of virus replication were barely detected or absent in peripheral blood mononuclear cells; low levels of HIV RNA (≤2.2 fg) were found in only 4 of 16 patients (data not shown). In contrast, high levels of virus replication were consistently detected in lymph node mononuclear cells.

No significant changes were observed in HIV-1 RNA levels in lymph node mononuclear cells of patients who remained on zidovudine alone (group 1): mean values were 137 ± 176 fg and 107 ± 207 fg at baseline and week 8, respectively (*P* = 0.52; Fig.

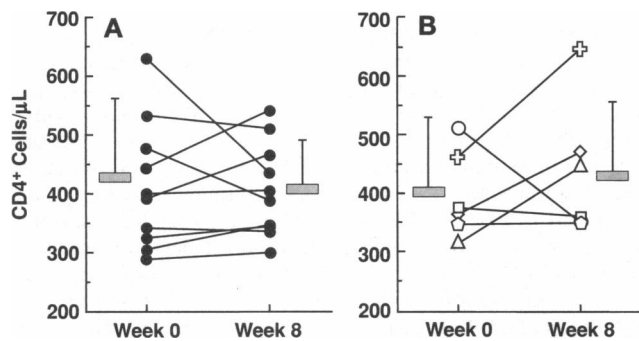


FIG. 1. CD4⁺ lymphocyte counts at baseline and after 8 weeks on study for patients who remained on zidovudine (A) and for those who added didanosine to their zidovudine regimen (B). Shaded bars indicate group mean ± SD of CD4⁺ lymphocytes at each time point. Δ, Patient 1; □, patient 2; +, patient 3; ○, patient 4; ◇, patient 5; □, patient 6.

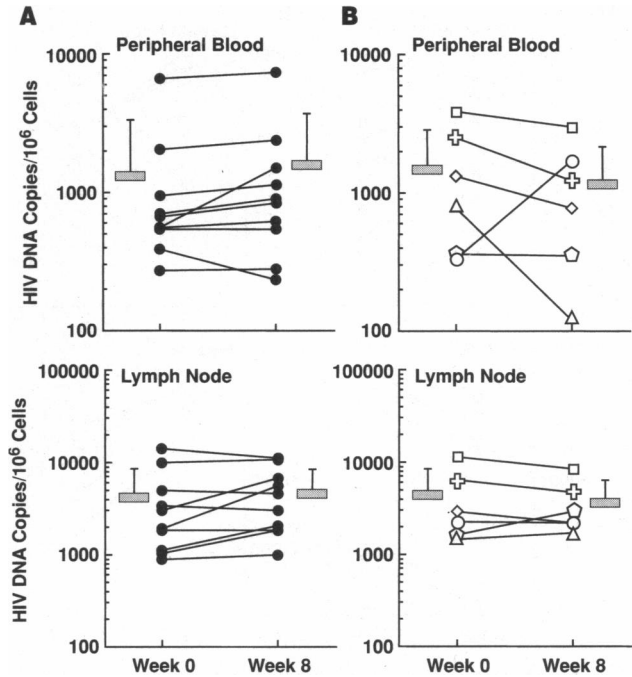


FIG. 2. Frequency of HIV-infected mononuclear cells (viral burden) at baseline and after 8 weeks on study for patients who remained on zidovudine (A) and for those who added didanosine to their zidovudine regimen (B). (Upper) Viral burden in peripheral blood mononuclear cells. (Lower) Viral burden in lymph node mononuclear cells. Shaded bars indicate group mean ± SD of HIV DNA copies per 10⁶ cells at each time point. Δ, Patient 1; □, patient 2; +, patient 3; ○, patient 4; ◇, patient 5; □, patient 6.

3A). In contrast, a sharp decrease (≥0.5 order of magnitude) in the levels of HIV RNA in lymph node mononuclear cells was observed in four of six patients in group 2 (Fig. 3B; Fig. 4, patients 1, 3, 5, and 6). Patients in group 2 overall had a mean decrease of 338 fg in lymph node HIV RNA, which did not reach statistical significance compared with a mean decrease of 30 fg in group 1 (*P* = 0.1). However, four of six patients had substantial decreases in lymph node HIV RNA (mean decrease, 621 fg). HIV-1 RNA levels in lymph node mononuclear cells remained unchanged in patient 2, whereas they increased by 1.3 order of magnitude in patient 4 (Fig. 4).

Determination of Plasma Viremia by RT-PCR. Plasma viremia remained unchanged in patients who remained on zidovudine alone (group 1): the mean HIV-1 RNA copy number per ml of plasma was 40,128 ± 68,100 at baseline and 86,360 ± 163,451 at week 8 (*P* = 0.45; Fig. 3A). In contrast, plasma viremia decreased by >0.5 order of magnitude in four of six patients (patients 1, 3, 5, and 6) who added didanosine to their ongoing zidovudine therapy (Figs. 3B and 4). Plasma viremia decreased by 0.4 order of magnitude in patient 2 and remained unchanged in patient 4 (Fig. 4). The mean HIV-1 RNA copy number per ml of plasma in group 2 was 179,927 ± 85,761 at baseline and 46,730 ± 49,386 at week 8 (*P* = 0.02; Fig. 3B).

Correlation of Changes in Viral Replication in Lymph Node Mononuclear Cells and Plasma Viremia. In all of the patients who had decreases in plasma viremia of >0.5 order of magnitude, there was also a decrease in the levels of HIV-1 replication in lymph node mononuclear cells (Fig. 4, patients 1, 3, 5, and 6). Patient 2 had a moderate decrease (0.4 order of magnitude) in plasma viremia but no significant change in the levels of virus replication in lymph node (Fig. 4). Plasma viremia did not change in patient 4, and this patient had a sharp increase in the levels of virus replication in lymph node (Fig. 4). Only two of the five patients with reductions in plasma

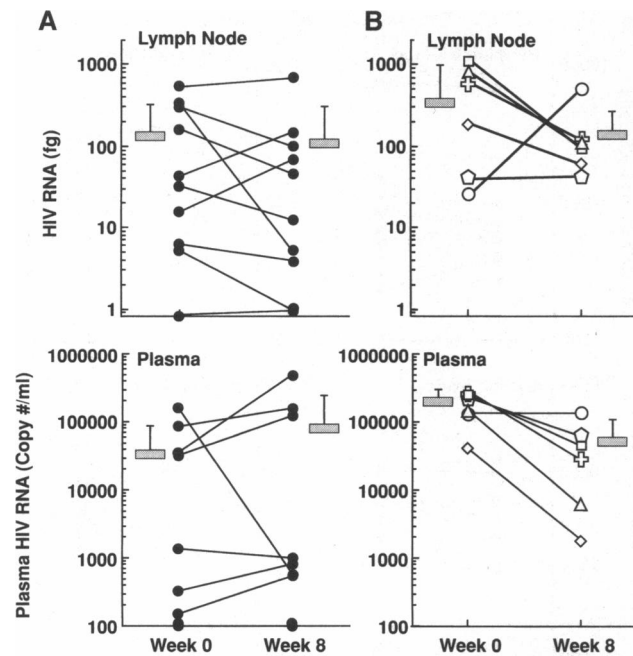


FIG. 3. Measures of virus replication at baseline and after 8 weeks on study for patients who remained on zidovudine (A) and for those who added didanosine to their zidovudine regimen (B). (Upper) HIV RNA in lymph node mononuclear cells. (Lower) Plasma viremia. Shaded bars indicate group mean \pm SD of HIV RNA in lymph node or plasma viremia at each time point. Δ , Patient 1; \square , patient 2; +, patient 3; \circ , patient 4; \diamond , patient 5; \square , patient 6. Plasma was not available from one patient who remained on zidovudine alone (A Lower).

viremia had significant diminution of ICD p24 antigenemia (patients 2 and 3; data not shown).

DISCUSSION

This study examines the effect of antiretroviral therapy on several virologic parameters in peripheral blood and lymph node from the same HIV-infected subjects. Comparison of these measures in peripheral blood and lymph node is important for several reasons. These measures are generally determined by sampling the easily accessible peripheral blood compartment; however, lymphoid organs are the primary sites of virus spreading and replication (23, 25–27, 31). This latter observation may represent a major limitation for the use of surrogate markers from peripheral blood in order to evaluate the response to antiretroviral therapy. In this regard, in the present study we have confirmed that the levels of viral burden (i.e., HIV DNA copies per 10^6 mononuclear cells) and virus replication were greater in lymph nodes compared to peripheral blood by factors of 3 and >200 , respectively (23, 25, 26). It is therefore important to determine whether changes in virologic measures in peripheral blood correlate with those detected in lymphoid tissue during antiretroviral therapy.

In this study, virologic measures including ICD p24 antigenemia, plasma viremia, viral burden, and replication in mononuclear cells isolated from peripheral blood and lymph node remained virtually unchanged over an 8-week period in patients who were receiving antiretroviral therapy (zidovudine) at study entry and who were randomized to continue on zidovudine alone (group 1). In contrast, significant changes in several virologic measures were observed over an 8-week period in patients who added didanosine to their ongoing zidovudine regimen (group 2).

In agreement with previous studies, ICD p24 antigenemia was not an optimal indicator of response to antiretroviral

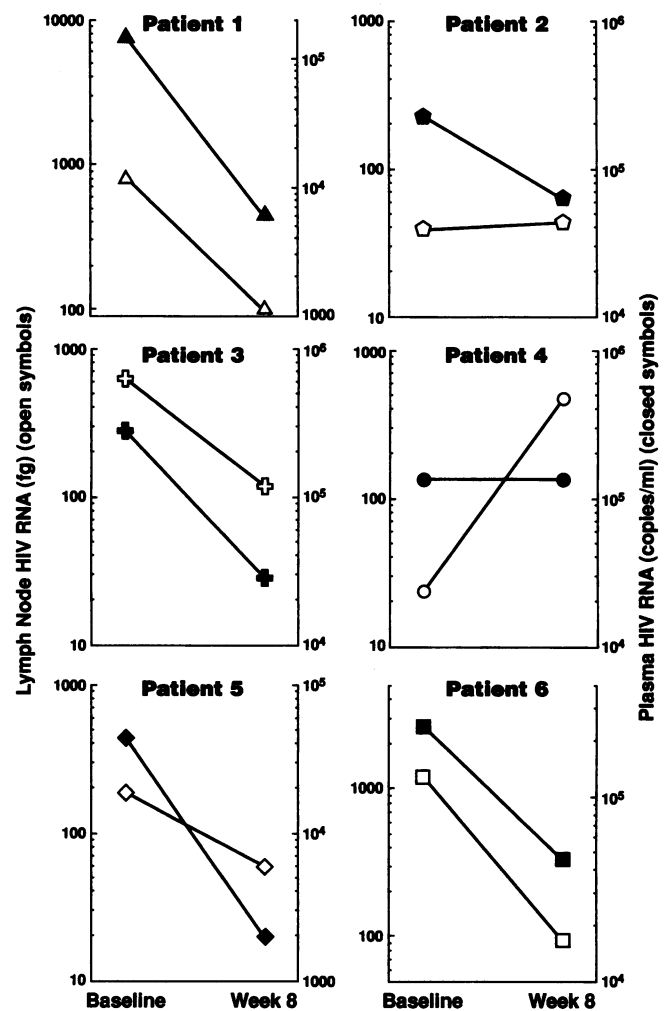


FIG. 4. Measures of virus replication at baseline and after 8 weeks on study for patients who added didanosine to their zidovudine regimen. Open symbols indicate HIV RNA in lymph node mononuclear cells; solid symbols indicate plasma viremia.

therapy (15). ICD p24 antigenemia was undetectable in two of six patients in group 2, and changes in p24 antigen levels on therapy did not correlate with changes in levels of virus replication in lymphoid tissue. With regard to viral burden, although it represents a very sensitive measure of the number of HIV-infected cells, the changes observed after antiretroviral therapy were small and were not consistent with those observed in lymph nodes in two of six patients in group 2 (Fig. 2B, \circ and Δ).

Plasma viremia was detected in all patients in groups 1 and 2; it was greatly (>0.5 order of magnitude) downregulated in four of six patients in group 2 during antiretroviral therapy. More importantly, decreases in the levels of plasma viremia coincided with those of virus replication in lymph nodes. However, in patient 4 an increase (20-fold) in virus replication in lymph nodes was not associated with an increase in plasma viremia. It is possible that this discrepancy may be explained by effective trapping of virions in the follicular dendritic cell network of lymph nodes with preserved architecture, thus at least partially inhibiting the release of newly formed virions into the plasma (24, 25). In this regard, the lymph nodes obtained from patient 4 at baseline and week 8 both showed follicular hyperplasia and extensive virus trapping in the follicular dendritic cell network (data not shown).

These data suggest that decreases in plasma viremia during antiretroviral therapy reflect decreases in viral replication in lymphoid tissues, whereas virologic measures in peripheral

blood mononuclear cells do not accurately reflect the situation in lymphoid tissue. These results are consistent with recent studies showing that zidovudine-resistance mutations occur in plasma prior to peripheral blood mononuclear cells (32) and that HIV-1 sequences in peripheral blood mononuclear cells and lymph node mononuclear cells are different during the long period of clinical latency prior to complete disruption of lymph node architecture (33). Therefore, plasma viremia is a valid indicator of virus replication in lymphoid organs, and its downregulation may reflect a systemic decrease in virus replication. These data add support to the concept that plasma viremia is currently the most reliable virologic marker to monitor the effect of antiretroviral therapy.

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