VIROLOGIC AND IMMUNOLOGIC CONSEQUENCES OF DISCONTINUING COMBINATION ANTIRETROVIRAL-DRUG THERAPY IN HIV-INFECTED PATIENTS WITH DETECTABLE VIREMIA

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ABSTRACT

Background In many patients with human immunodeficiency virus (HIV) infection, therapy with potent antiretroviral drugs does not result in complete suppression of HIV replication. The effect of cessation of therapy in these patients is unknown.

Methods Sixteen patients who had a plasma HIV RNA level of more than 2500 copies per milliliter during combination antiretroviral-drug therapy were randomly assigned, in a 2:1 ratio, to discontinue or continue therapy. Plasma HIV RNA levels, CD4 cell counts, and drug susceptibility were measured weekly. Viral replicative capacity was measured at base line and at week 12.

Results Discontinuation of therapy for 12 weeks was associated with a median decrease in the CD4 cell count of 128 cells per cubic millimeter and an increase in the plasma HIV RNA level of 0.84 log copies per milliliter. Virus from all patients with detectable resistance at entry became susceptible to HIV-protease inhibitors within 16 weeks after the discontinuation of therapy. Drug susceptibility began to increase a median of six weeks after the discontinuation of therapy and was temporally associated with increases in plasma HIV RNA levels and decreases in CD4 cell counts. Viral replicative capacity, measured by means of a recombinant-virus assay, was low at entry into the study and increased after therapy was discontinued. Despite the loss of detectable resistance in plasma, resistant virus was cultured from peripheral-blood mononuclear cells in five of nine patients who could be evaluated. Plasma HIV RNA levels, CD4 cell counts, and drug susceptibility remained stable in the patients who continued therapy.

Conclusions Despite the presence of reduced drug susceptibility, antiretroviral-drug therapy can provide immunologic and virologic benefit. This benefit reflects continued antiviral-drug activity and the maintenance of a viral population with a reduced replicative capacity. (N Engl J Med 2001;344:472-80.)

THE goal of antiretroviral-drug therapy in patients with human immunodeficiency virus (HIV) infection is the complete suppression of viral replication.[1,2] Failure to achieve this goal is common in clinical practice, occurring at a rate of 40 to 70 percent.[3-5] Although failure to achieve complete viral suppression is common, failure in broader immunologic and clinical terms is uncommon, at least during the first 24 to 30 months of follow-up.[3,4] These observations suggest that the ability of the virus to deplete CD4 cells may be diminished despite ongoing viral replication.

We studied the consequences of discontinuing therapy in patients who had continued a regimen containing HIV-protease inhibitors despite persistent viremia. Our primary objective was to determine whether antiretroviral-drug therapy provides continued benefit despite large reductions in drug susceptibility and to identify the virologic mechanisms responsible for any continued benefit. As a secondary objective, we studied the persistence of resistant virus in cellular reservoirs after the discontinuation of antiretroviral-drug therapy.

METHODS

Study Design

This study had two components. The first was a nonblinded, prospective study of 16 patients who were randomly assigned in a 2:1 ratio to discontinue or continue antiretroviral-drug therapy. The second was a nonrandomized, prospective, observational study in which all patients discontinued therapy. All patients were enrolled and followed in an identical manner.

The randomized study was undertaken to determine whether patients with persistent viremia require continued antiretroviral therapy in order to maintain the CD4 cell count above the level measured before therapy with HIV-protease inhibitors. To be eligible for this study, patients had to meet the following criteria: receipt of therapy with an HIV-protease inhibitor for at least 12 months with no change in therapy during the preceding 4 months, a documented plasma HIV RNA level of more than 2500 copies per milliliter during the preceding 6 months, and a CD4 cell count at least 100 cells per cubic millimeter higher than the level measured before protease-inhibitor therapy began. Patients who met the first two criteria but who had an increase in the CD4 cell count of less than 100 cells per cubic millimeter from their pretherapy levels were enrolled in the observational study.

Each patient had one screening visit and one base-line visit. At the base-line visit, the patient either continued or discontinued all antiretroviral-drug therapy, after which evaluations were performed weekly for 12 weeks. Patients who discontinued therapy were then assessed every four weeks until therapy was resumed, and every three months thereafter.

This study was approved by the Committee on Human Research of the University of California, San Francisco, and each patient gave written informed consent. Each patient's primary health care provider was contacted before the patient was enrolled in the study and was kept informed of plasma HIV RNA levels and CD4 cell counts throughout the study. The primary care provider was allowed

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to resume the patient's therapy at any time and was encouraged to do so if the plasma HIV RNA level increased by a factor of more than 10 or the CD4 cell count decreased by more than 50 percent.

Measurements of Virus

Plasma HIV RNA levels were measured at each visit with use of a branched-chain DNA assay (Quantiplex 3.0, Bayer Diagnostics, Emeryville, Calif.) whose range of quantification was 50 to 500,000 copies of RNA per milliliter. RNA levels were measured on a logarithmic (base 10) scale. Testing of drug susceptibility was performed on stored, frozen plasma samples by means of a rapid recombinant virus assay (PhenoSense HIV, ViroLogic, South San Francisco, Calif.).[6]

The replicative capacity of HIV was measured with the use of a modified version of this rapid assay. We constructed test vectors by inserting patient-derived reverse-transcriptase and protease sequences into a modified retroviral vector containing a luciferase indicator gene that allows quantification of viral replication.[6] After normalizing the output of the assay on the basis of the viral inoculum, we measured the ability of the vector to replicate in the absence of an antiretroviral drug. The relative replicative capacity of the virus from each patient was calculated as the ratio of the luciferase activity from vectors containing patient-derived sequences to the luciferase activity from vectors containing wild-type sequences. A ratio of less than 1 reflects a reduced replicative capacity as compared with that of the wild-type control.

Cultures of Peripheral-Blood Mononuclear Cells

Peripheral-blood mononuclear cells were isolated from the blood of seronegative donors by means of density–gradient centrifugation and stimulated with phytohemagglutinin A (Sigma, St. Louis) at a concentration of 5 μg per milliliter. Peripheral-blood mononuclear cells from the patients were isolated similarly, processed within six hours, and cultivated with equal numbers of cells from seronegative donors (2×10^6 cells per milliliter). At each time point, parallel cultures were established with and without the protease inhibitor that the patient had taken most recently. We chose drug concentrations at or near the level required for 90 percent inhibition of drug-sensitive strains of viruses but below that required for 50 percent inhibition of resistant strains of virus (indinavir and nelfinavir concentrations were 50 nM; ritonavir concentrations were 50 to 500 nM). The cultures were maintained by regular additions of uninfected, stimulated peripheral-blood mononuclear cells. Viral replication was measured by means of an enzyme-linked immunosorbent assay (ELISA) for HIV p24 antigen. Sequence analysis was performed on virus isolated from plasma and peripheral-blood mononuclear cells by means of two population-based methods (PE Biosystems, Foster City, Calif., and Visible Genetics, Toronto).

Statistical Analysis

We compared the effect of discontinuing and continuing therapy in the patients in the randomized study. A repeated-measures, mixed-model regression analysis was used to compare the changes in CD4 cell counts and plasma HIV RNA levels in the two groups. We analyzed the effect of discontinuing therapy on viral evolution using data from both the randomized and nonrandomized components of the study.

We used Kaplan–Meier analysis to estimate the time it took for a drug-susceptible virus to emerge after the discontinuation of therapy. Base-line plasma HIV RNA levels, base-line CD4 cell counts, duration of previous therapy, the extent of the change from base line in drug susceptibility, and the extent of the change in plasma HIV RNA levels and CD4 cell counts from levels measured before therapy with protease inhibitors were all considered as potential predictors in a Cox proportional-hazards regression analysis. All statistical analyses were performed with SAS statistical software (version 6.12, SAS Institute, Cary, N.C.) or SPSS software (version 8.0, SPSS, Chicago). All P values are two-sided.

RESULTS

Study Subjects

Twenty-three patients, all men, were enrolled: 16 in the randomized component of the study (11 assigned to discontinue therapy and 5 assigned to continue therapy) and 7 in the nonrandomized component in which therapy was discontinued. At base line, most patients in the randomized component had higher CD4 cell counts and lower plasma HIV RNA levels than the patients in the nonrandomized component (Table 1).

TABLE 1. BASE-LINE CHARACTERISTICS OF THE PATIENTS.*

BASE-LINE CHARACTERISTIC	RANDOMIZED COMPONENT		NONRANDOMIZED COMPONENT
	THERAPY CONTINUED (N=5)	THERAPY DISCONTINUED (N=10)	THERAPY DISCONTINUED (N=7)
	median (interquartile range)		
Age (yr)	38 (35 to 43)	40 (37 to 45)	45 (39 to 47)
CD4 cell count (cells/mm^3)	311 (245 to 350)	302 (258 to 355)	78 (44 to 141)
Plasma HIV RNA level (log copies/ml)	5.1 (4.4 to 5.1)	4.5 (4.0 to 4.6)	5.4 (4.1 to 5.6)
Degree of resistance to protease inhibitors (patient IC_{50}:reference IC_{50})	58 (40 to 228)	44 (23 to 73)	72 (36 to 143)
Degree of resistance to abacavir (patient IC_{50}:reference IC_{50})	6 (6 to 10)	9 (5 to 17)	10 (6 to 13)
Duration of protease-inhibitor therapy (mo)	32 (29 to 37)	35 (29 to 39)	38 (34 to 40)
Duration of combination therapy without complete suppression of viral replication (mo)	28 (25 to 34)	26 (25 to 31)	32 (28 to 33)
Change in CD4 cell count after initiation of protease-inhibitor therapy (cells/mm^3)	97 (75 to 139)	178 (123 to 237)	12 (−19 to 27)
Change in plasma HIV RNA after initiation of protease-inhibitor therapy (log copies/ml)	0.2 (−0.2 to 0.7)	−0.4 (−0.6 to −0.3)	−0.1 (−0.6 to 0.1)

*All the patients were men. One patient who was randomly assigned to discontinue therapy was excluded because he stopped taking indinavir but continued to take zidovudine and lamivudine. IC_{50} denotes the concentration required for 50 percent inhibition of viral replication.

One patient who was randomly assigned to discontinue antiretroviral-drug therapy stopped taking indinavir but continued to take two nucleoside analogues (zidovudine and lamivudine). At entry into the study, this patient had a plasma HIV RNA level of 4.02 log copies per milliliter and a CD4 cell count of 104 cells per cubic millimeter. During 12 weeks of observation, his plasma HIV RNA levels, CD4 cell counts, and drug susceptibility remained stable (these data were excluded from further analysis). Two patients resumed therapy before week 12 because of rapid declines in CD4 cell counts (decreases of 102 and 95 cells per cubic millimeter). Data from these patients were censored when antiretroviral-drug therapy was resumed (week 5 and week 10, respectively).

Three additional patients had important adverse events that may have been related to discontinuing therapy. These included *Pneumocystis carinii* pneumonia in one patient, disabling peripheral neuropathy in the second, and severe thrombocytopenia in the third. All three patients resumed therapy at or after week 12 of the study.

Plasma HIV RNA Levels and CD4 Cell Counts

In the randomized component of the study, discontinuation of therapy for 12 weeks resulted in a median

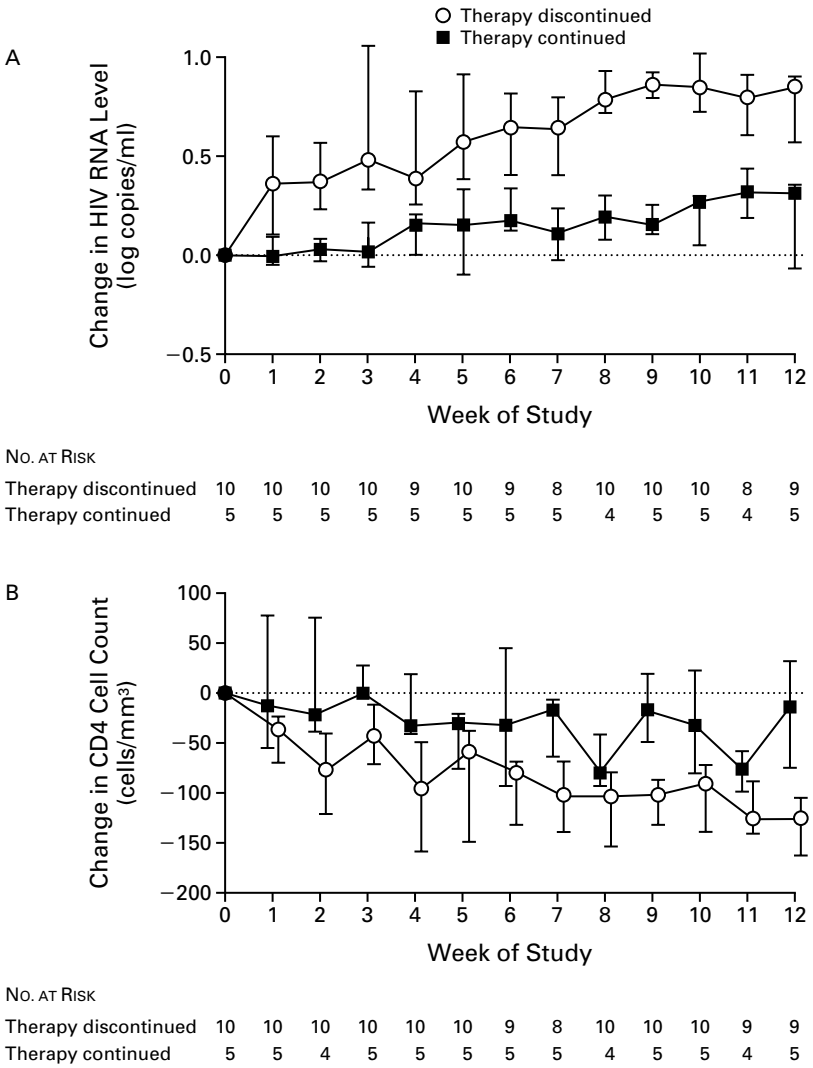


Figure 1. Median Changes in Plasma HIV RNA Levels (Panel A) and CD4 Cell Counts (Panel B) in 5 Patients with HIV Infection Who Were Randomly Assigned to Continue Therapy and 10 Who Were Assigned to Discontinue Therapy.

Bars represent the interquartile ranges.

decrease in the CD4 cell count of 128 cells per cubic millimeter (range, 22 to 231 cells per cubic millimeter) and a median increase in the plasma HIV RNA level of 0.84 log copies per milliliter (range, 0.27 to 1.07 log copies per milliliter) (Fig. 1). Continued treatment for 12 weeks was associated with a median decrease in the CD4 cell count of 15 cells per cubic millimeter (range, −116 to 119 cells per cubic millimeter) and a median increase in the plasma HIV RNA level of 0.31 log copies per milliliter (range, −0.09 to 0.65 log copies per milliliter). When all available data from base line to week 12 were considered in a repeated-measures regression analysis, the difference in the change in plasma HIV RNA levels between patients who continued therapy and those who discontinued therapy was significant ($P<0.001$), as was the difference in the change in CD4 cell counts ($P=0.005$).

Drug Susceptibility

Drug resistance remained stable during 12 weeks of observation in the five patients who continued antiretroviral-drug therapy (median level of resistance to protease inhibitors, 58 times that of the reference virus at base line and 57 times that of the reference virus at week 12). In contrast, protease-inhibitor susceptibility shifted to wild-type levels within 16 weeks in 9 of the 10 patients in the randomized component of the study who discontinued therapy; the other patient in this group had no detectable resistance to protease inhibitors at study entry and was not included in this analysis. The trend was similar among patients in the nonrandomized component of the study who discontinued therapy (susceptibility to protease inhibitors shifted to wild-type levels in six of the seven patients).

The shift in drug susceptibility was often abrupt,

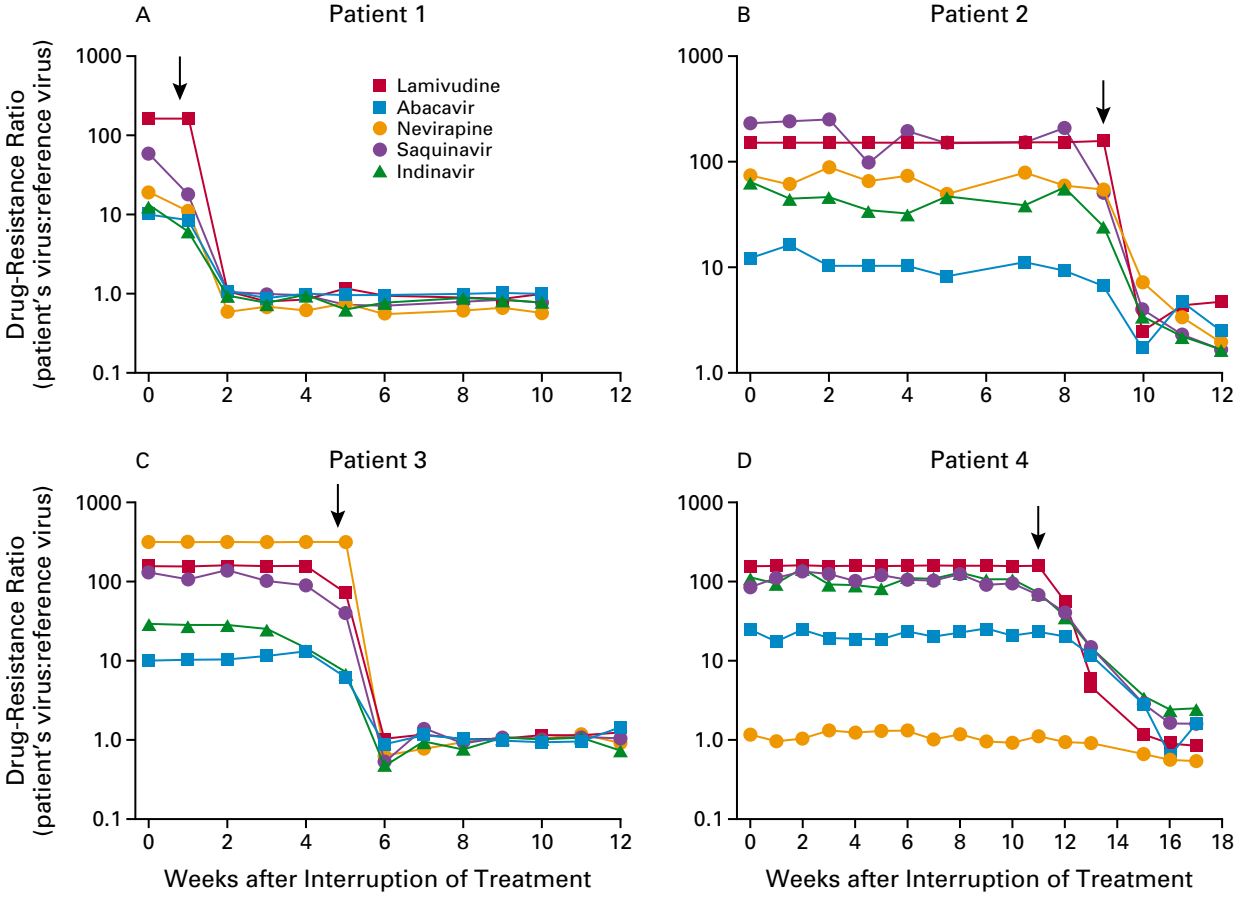


Figure 2. Changes in Drug Resistance after the Discontinuation of Antiretroviral-Drug Therapy in Four Representative Patients with HIV Infection.

Data, expressed as the ratio of the concentration required for 50 percent inhibition of replication of the patient-derived virus to that required for 50 percent inhibition of replication of the reference virus, are presented on a logarithmic scale. As indicated by the arrows, drug resistance began to wane at various times after therapy was discontinued (weeks 2, 9, 5, and 11 in Panels A, B, C, and D, respectively). Only one of the study patients had a gradual decline in drug resistance (not shown).

occurred at various times after therapy was discontinued, and usually occurred for all drugs simultaneously (Fig. 2). Among all 15 patients who discontinued therapy and had a shift in drug susceptibility, the median time from the discontinuation of therapy to the initial waning of protease-inhibitor resistance was six weeks (interquartile range, four to seven weeks). Once resistance began to wane, it disappeared completely after a median of two weeks (interquartile range, one to four weeks).

Resistance to lamivudine, the nonnucleoside reverse-transcriptase inhibitors, and the protease inhibitors generally waned simultaneously. Loss of resistance to other nucleoside analogues, particularly zidovudine, was delayed in five patients, all of whom had received prolonged nucleoside-analogue therapy before beginning therapy with a protease inhibitor.

Multivariate proportional-hazards analysis was used to determine the factors that predicted a shift to virus that was susceptible to protease inhibitors. All 17 pa-

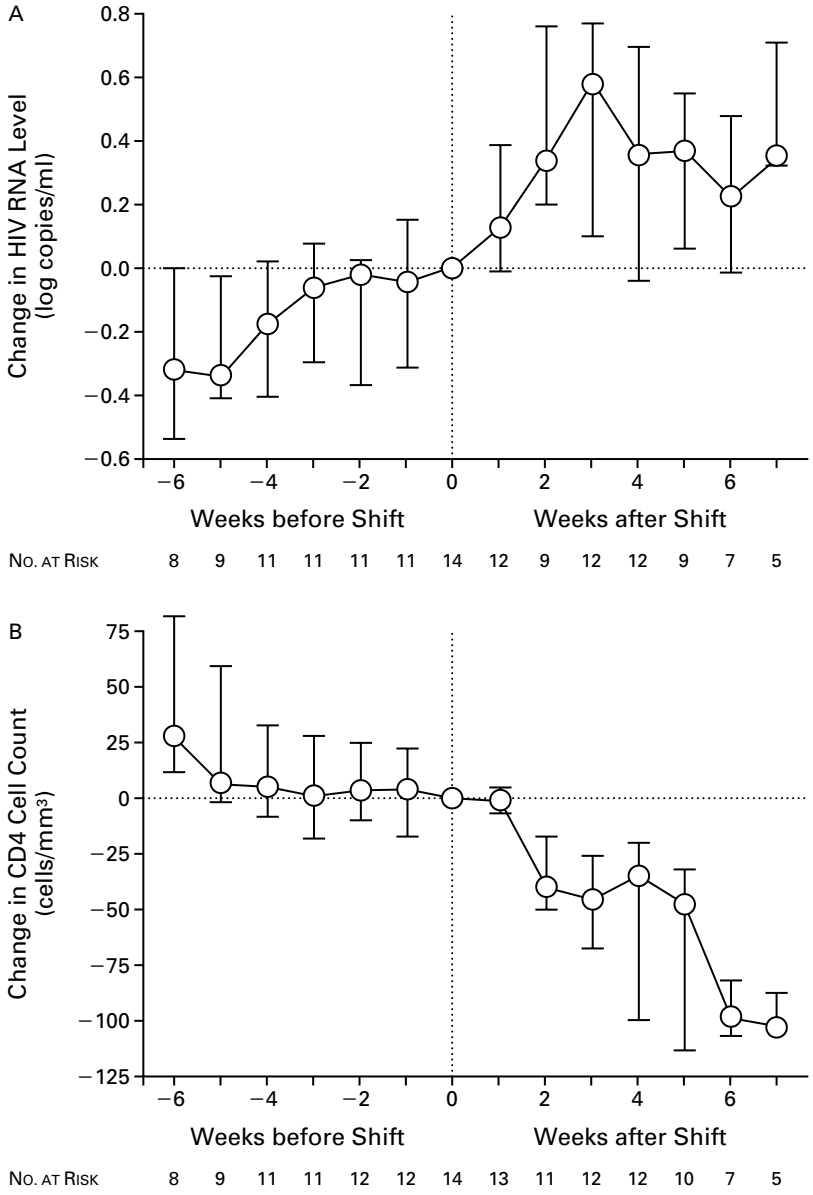


Figure 3. Median Changes in Plasma HIV RNA Levels and CD4 Cell Counts before and after the Predominant Virus Became Susceptible to Protease Inhibitors.

Data from all 14 patients who had a rapid loss of drug resistance are included. Week 0 is defined as the time when resistance to protease inhibitors began to wane (indicated by an arrow in Figure 2). Median values and interquartile ranges are shown.

tients who discontinued therapy were included in this analysis. A greater increase in viral suppression between the level measured before protease-inhibitor therapy and that measured at study entry was associated with a more rapid loss of drug resistance (risk ratio for loss of drug resistance, 1.4 for each 0.10 log decrease in plasma HIV RNA; 95 percent confidence interval, 1.1 to 1.8; $P=0.003$). A lower level of protease-inhibitor resistance at study entry was not associated with a more rapid loss of drug resistance.

Effects of Waning Drug Resistance

We hypothesized that the emergence of wild-type virus might be associated with increased viral replication. Therefore, we analyzed the changes in plasma HIV RNA levels during the time drug resistance began to wane. All 14 patients who discontinued therapy and had an abrupt shift in protease-inhibitor susceptibility were included in this analysis (3 patients were excluded from the analysis because 1 had no detectable protease-inhibitor resistance at study entry, 1 had no change in protease-inhibitor resistance, and 1 had only a gradual loss of protease-inhibitor resistance). Plasma HIV RNA levels and CD4 cell counts were stable immediately before the onset of the shift to a drug-susceptible virus and changed rapidly as the level of drug resistance waned (Fig. 3). The plasma HIV RNA levels at most time points after the shift were significantly higher than those measured when the shift began (week 0 in Fig. 3) ($P<0.05$ for each pairwise comparison between week 0 and weeks 1, 2, 3, 4, 5, and 7). Similarly, CD4 cell counts decreased significantly after the shift to drug-susceptible virus ($P<0.05$ for each pairwise comparison between week 0 and weeks 2, 3, 4, 5, 6, and 7).

Viral Replicative Capacity

Relative to a wild-type reference virus (replicative capacity, 1), the median replicative capacity of vectors derived from 22 viral samples obtained at base line was 0.2 (interquartile range, 0.1 to 0.3). In most samples, the replicative capacity increased between base line and week 12. This increase was greater in the patients who were randomly assigned to discontinue therapy than in those assigned to continue therapy (median increase in replicative capacity, 0.5 and 0.2, respectively; $P=0.01$). There was a significant correlation between the change in plasma HIV RNA levels and the change in replicative capacity during the 12 weeks of evaluation (Spearman's rho = 0.68, $P=0.001$) (Fig. 4).

Persistence of Resistant HIV-1 Peripheral-Blood Mononuclear Cells

At study entry, viral isolates containing mutations associated with resistance were readily cultured from peripheral-blood mononuclear cells both in the presence of a protease inhibitor and in its absence. These isolates were genotypically identical to the predominant plasma virus. In the nine patients with data that could be evaluated, peripheral-blood mononuclear cells were cocultivated with equal numbers of cells from seronegative donors after drugs were discontinued and after the plasma virus had shifted to a drug-sensitive phenotype. In each patient, drug-susceptible

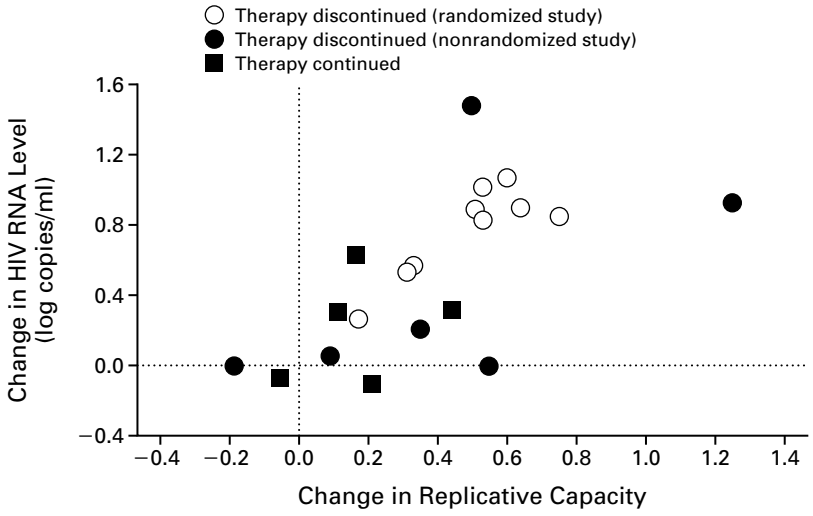


Figure 4. Change in Plasma HIV RNA Levels Compared with the Change in Relative Replicative Capacity between Base Line and Week 12.

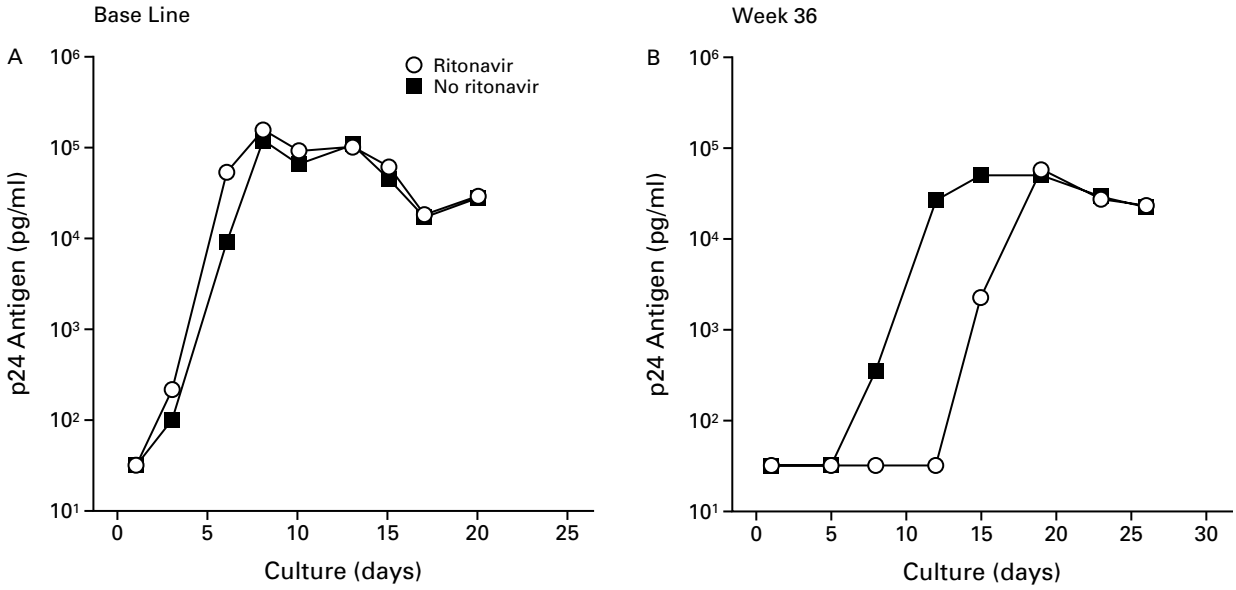
Replicative capacity was calculated as the ratio of the luciferase activity from vectors containing patient-derived sequences to the luciferase activity from vectors containing reference sequences. Two patients who initially discontinued therapy but resumed therapy before week 12 were not included in this analysis.

virus was isolated from cultures in the absence of a protease inhibitor. When the cultures were incubated with a protease inhibitor, there was no viral growth in the samples from four patients; however, drug-resistant virus emerged in the samples from the remaining five patients. This virus was genotypically identical to the resistant virus present in plasma at the time therapy was discontinued. The kinetics of viral replication and the results of a genotypic analysis of viral isolates from one representative patient are shown in Figure 5.

Response to Subsequent Therapy

Fifteen of the 17 patients who discontinued therapy subsequently resumed therapy and had at least 24 weeks of follow-up. Virus from all 15 patients was susceptible to protease inhibitors at the time therapy was resumed. Most patients received a regimen that included at least two nucleoside analogues, one nonnucleoside reverse-transcriptase inhibitor, and two protease inhibitors.

The median decreases in plasma HIV RNA levels between the time treatment was resumed and 12 and 24 weeks later were 2.3 and 1.6 log copies per milliliter, respectively, and the median increases in CD4 cell counts were 102 and 77 cells per cubic millimeter, respectively. After 24 weeks of therapy, 6 of 15 patients (40 percent) had plasma HIV RNA levels of less than 200 copies per milliliter. Patients who had not received a nonnucleoside reverse-transcriptase inhibitor before they discontinued therapy but who received one of these drugs as part of their regimen when they resumed therapy were more likely to have a plasma HIV



C Mutations That Confer Resistance against Ritonavir

	Marker						
	10	20	36	54	71	82	90
Clade B consensus sequence	L	K	M	I	A	V	L
Base line							
Plasma virus	I	R	I	V	V	T	M
Viral isolate with ritonavir	I	R	I	V	V	T	M
Viral isolate without ritonavir	I	R	I	V	V	T	M
Week 36							
Plasma virus	L	K	M	I	A	V	L
Viral isolate with ritonavir	I	R	I	V	V	T	M
Viral isolate without ritonavir	L	K	M	I	A	V	L

Figure 5. Effect of Protease Inhibitors on HIV Replication in Cultures of Peripheral-Blood Mononuclear Cells from One Patient.

The kinetics of viral replication were assessed by measuring the level of p24 antigen in culture supernatants. Panel A shows viral replication at base line (before therapy was discontinued), and Panel B shows viral replication 36 weeks later. Cocultures were maintained in the absence (squares) or presence (circles) of 500 nM ritonavir. Resistance began to wane in this patient at week 11 and had disappeared completely by week 16. Panel C shows the amino acid sequence at positions known to confer resistance to protease inhibitors at which the patient had mutations at base line. At week 36 the sequence of the viral isolate from the patient matched the consensus sequence. L denotes leucine, K lysine, M methionine, I isoleucine, A alanine, V valine, R arginine, and T threonine.

RNA level of less than 200 copies per milliliter 24 weeks after the resumption of therapy.

DISCUSSION

Discontinuation of antiretroviral-drug therapy in patients in whom complete viral suppression had not been achieved resulted in increased plasma HIV RNA levels, decreased CD4 cell counts, and decreased viral drug resistance. As the virus population shifted from drug-resistant to drug-sensitive, plasma HIV RNA levels increased and CD4 cell counts decreased. These observations suggest the emergence of a drug-susceptible wild-type virus with an increased ability to replicate and to deplete peripheral-blood CD4 cells.

Plasma HIV RNA levels increased immediately after therapy was discontinued, suggesting that antiretroviral-drug therapy in fact had some degree of antiviral activity despite viral drug resistance. Plasma HIV RNA levels also increased as drug-sensitive virus emerged, suggesting that drug-sensitive variants of HIV type 1 had a greater capacity to establish productive infection in target cells. Collectively, these data suggest that continued drug activity against "resistant" virus and decreased viral replicative capacity both contribute to partial suppression of viral replication.

The term "viral fitness" refers to the ability of a virus to replicate in a given environment.[7,8] Mutations that confer resistance to protease inhibitors allow virus to replicate in the presence of such drugs, but these mutations may decrease viral replicative capacity.[9,10] We used recombinant vectors containing patient-derived viral protease and reverse-transcriptase sequences to measure replicative capacity before and after therapy was discontinued. At study entry, when there were high levels of drug resistance, replicative capacity was markedly diminished. After antiretroviral-drug therapy was discontinued, drug-sensitive virus emerged and viral replicative capacity increased. Although recombinant-virus assays cannot measure all aspects of viral fitness, these in vitro measurements of replicative capacity support our in vivo observations that in the absence of therapy, drug-sensitive virus has a greater ability to replicate than does drug-resistant virus.

The virologic response to combination antiretroviral-drug therapy is often limited in patients in whom durable viral suppression has not been achieved with other regimens. This lack of efficacy is due in part to broad cross-resistance that is commonly observed within classes of antiretroviral drugs.[1,2] Consequently, there is growing interest in the potential therapeutic benefits of an interruption of treatment in patients who have not had complete viral suppression.[11] Hypothetically, multidrug-resistant virus might be so unfit that it would not persist indefinitely in the absence of therapy and would thus give patients a renewed opportunity for durable viral suppression. Our observation that drug resistance waned rapidly in the absence of therapy may support this hypothesis. However, the persistence of low levels of drug-resistant virus in long-lived cellular reservoirs remains a concern.[12-14] In several of our patients, drug-resistant virus was detected in peripheral-blood mononuclear cells several weeks after the plasma virus had shifted to a drug-sensitive phenotype.

In conclusion, among patients with persistent viremia, antiretroviral-drug therapy is associated with continued immunologic and virologic benefit. This benefit reflects a combination of continued antiviral activity and the maintenance of a viral population with reduced replicative capacity. Although the long-term clinical implications of our findings remain to be determined, continued treatment with a regimen containing protease inhibitors in patients with limited therapeutic options may be associated with sustained clinical benefit.

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