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Nevirapine-Resistant Human Immunodeficiency Virus: Kinetics of Replication and Estimated Prevalence in Untreated Patients

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The nonnucleoside reverse transcriptase inhibitor nevirapine rapidly selects for mutant human immunodeficiency virus (HIV) in vivo. The most common mutation occurs at amino acid residue 181 in patients receiving monotherapy. After the initiation of nevirapine therapy, plasma and peripheral blood mononuclear cell samples were collected at frequent intervals and assayed for HIV RNA levels and the proportion of virus containing a mutation at residue 181. HIV RNA levels remained stable for the first 24 h after initiation of therapy and rapidly declined between 1 and 7 days. There was a consistent maximum decrease of 2 $\log_{10}$ HIV RNA copies per ml of plasma (range, 1.96 to 2.43) from baseline after 2 weeks in all monotherapy subjects. The estimated median half-life of HIV RNA was 1.11 days (range, 0.63 to 1.61). After 14 days of therapy, HIV RNA levels began to increase and 181 mutant virus was detected. The estimated doubling time of the emerging virus population ranged from 1.80 to 5.73 days. Viral DNA in peripheral blood mononuclear cells turned over from wild type to the mutant with a mutation at residue 181 significantly more slowly than did HIV RNA in plasma. In two subjects, the calculated prevalence of the 181 mutant virus prior to treatment was 7 and 133 per 10,000 copies of plasma HIV RNA.

The level of human immunodeficiency virus (HIV) RNA circulating in the plasma of an infected individual represents a dynamic equilibrium between the production and clearance of virus. The viral burden, as determined by quantitation of plasma HIV RNA, ranges between 10^2 and 10^8 copies per ml, correlates with the rate of disease progression, and is relatively stable in an individual over a period of months (3, 11, 28). Drug therapy that blocks viral replication produces a rapid decline in the levels of HIV RNA. These decay curves can be used to estimate the rates of viral clearance. For individuals with CD4 cell counts below 500 cells per mm^3, conservative estimates of viral half-life range from 0.8 to 4.0 days (12, 15, 27). With this rapid turnover of virus, it is estimated that at least 30% of the plasma virus population is replaced daily.

The imposition of a selective pressure on virus population rapidly shifts the composition of the most fit quasispecies (2). For example, when drug pressure is applied, resistant virus can emerge within weeks because of the prior existence of drug-resistant mutants (18, 19). In early clinical trials with the nonnucleoside reverse transcriptase inhibitor nevirapine, resistant virus was identified as early as 1 week after the initiation of therapy (23). Point mutations in the region of the reverse transcriptase gene coding for the domains encompassing the binding site of nevirapine produced a greater than 100-fold reduction in drug susceptibility (24). A tyrosine-to-cysteine change in amino acid residue 181 was the most common mutation selected; however, several different mutations were capable of rendering the virus drug resistant. The selection of these mutant populations with nevirapine led to a loss of HIV RNA suppression in most patients (1, 8).

In this report, the application of allele-specific quantitative assays for HIV RNA with either the wild-type or mutant codon at amino acid residue 181 permitted quantification of the diminishing wild-type population and the emerging nevirapine-resistant virus population. The different kinetics of viral turnover in the plasma and peripheral blood mononuclear cells (PBMC) were analyzed, and a mathematical model was used to estimate the prevalence of nevirapine-resistant mutants in the population of HIV before exposure to the selective pressure of drug treatment.

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MATERIALS AND METHODS

Study subjects. HIV-infected subjects with 200 to 500 CD4 cells per mm^3 were enrolled in a double-blind clinical trial of nevirapine versus placebo at the University of California, San Diego. One group of patients had had no previous antiviral therapy; the second group had undergone at least 2 months treatment with zidovudine and continued on zidovudine therapy throughout the study. On the first day of treatment, 200 mg of nevirapine or placebo was administered. The daily dose of nevirapine was increased to 400 mg after 14 days.

All patients signed an informed-consent form which had been approved by the Human Subjects Committee.

Virology. At least two pretreatment samples of plasma were collected for analysis of HIV RNA. Subsequent specimens were obtained at 4- to 12-h intervals over the first 24 h and then on days 1, 2, 5, 6, 7, 14, 15, and 28. HIV RNA levels in plasma were measured by a PCR-based method (Roche Amplicor HIV Monitor Test) as specified by the manufacturer. PBMC were collected at baseline and 2, 7, 14, 21, and 28 days later. Additional plasma and PBMC were collected from some subjects after 2, 3, and 6 months.

Assays for susceptibility were performed with CD4-expressing HeLa (HT4-6C) cell monolayers for syncytium-inducing isolates and by p24 inhibition in PBMC microliter cultures for non-syncytium-inducing isolates (25). The 50% inhibitory concentration (IC_{50}) for pretreatment isolates ranged from <0.01 to 0.075 μM (23).

Cycle sequencing was performed on a DNA thermal cycler with the Prism Ready Reaction DyeDeoxy Terminator cycle-sequencing kit (Perkin-Elmer/Applied Biosystems). Gel-purified double-stranded DNA (50 to 200 ng) obtained from the amplification of RNA extracted from patient plasma was added to a reaction tube containing 9.5 μl of terminator premix and 3.2 pmol of sequencing primer (3RT; 5′ACCCATCCAAAGGAATGGAGGTTCTTTC3′; nucleotides

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2795 to 2768). Thermal cycling was done by the method suggested in the manual provided with the kit. After cycling, the excess dye-labeled terminators were removed from the extension products by CentriSep spin column purification (Princeton Separations) as specified by the manufacturer. Sequenced samples were loaded onto a model 373A DNA sequencer (Perkin-Elmer/Applied Biosystems) for analysis as specified by the manufacturer.

PCR and differential hybridization. Viral RNA was prepared from cryopreserved plasma as previously described (6). Virus was concentrated by centrifugation. The pellet was digested with sodium dodecyl sulfate (SDS) and proteinase K, extracted twice with phenol-chloroform-isoamyl alcohol, and extracted once with chloroform-isoamyl alcohol. RNA was ethanol precipitated, washed with 70% ethanol, and resuspended in diethylpyrocarbonate-treated water. cDNA synthesis and PCR were performed as previously described with primers 5RT and 3RT (6).

Differential hybridization analysis was performed in a chemiluminescent format as a modification of previously described procedures (6). Briefly, the PCR product was dispensed to duplicate streptavidin-coated wells and incubated at 50°C for 30 min. The wells were washed, and the bound PCR product was denatured. Hybridization solution containing 0.3 pmol of either codon 181 WT (5′TRRTYATCTAYCARTACAT3′; nucleotides 2635 to 2653; strain SF2) or MUT (5′TRRTYATCTGYCARTACAT3′) alkaline phosphatase-labeled probes was added to the appropriate wells and incubated for 1 h at 45°C. All the wells were washed three times at 37.5°C for 5 min with 1× SSC (0.15 M NaCl, 0.015 M sodium citrate)–0.1% SDS and subsequently with 1× SSC–0.1% Triton X-100 and then three times at room temperature with 1× SSC. All the wells were incubated with a chemiluminescent substrate (LumiphosTM 530; Lumigen, Detroit, Mich.) at 37°C for 30 min, and hybridization was measured on a luminometer (Chiron, Emeryville, Calif.). To account for the amount of PCR product bound to each well, each well was stripped and rehybridized with a probe to a highly conserved region of the HIV-1 reverse transcriptase, i.e., the generic probe (GNR), and washed at 50°C as described above. The data were analyzed as previously described (6) and are presented as the MUT/GNR ratio. The Y181C MUT control was previously described (24). The WT control consisted of an in vitro transcript of cloned HIV SF2.

Mathematical model. Levels of HIV RNA in plasma are given by $R(t) = R_w(t) + R_m(t)$, where $R(t)$ is the total load at time t and R_m and R_w denote the corresponding 181 mutant and 181 wild-type viral loads. The 181 wild-type population includes virus with mutations at non-181 codon positions. After nevirapine administration, the HIV RNA wild type is modeled as an exponential decline: $R_w(t) = A \exp(-\alpha t)$, where α is the rate of decline and $A_0 = A \exp(-\alpha\delta)$ is the initial wild-type viral load and δ is the "delay" before the drug affects the wild-type viral load. The delay in decline of HIV RNA is due to a pharmacologic delay (the time for the drug to be absorbed, reach the cells, and inhibit replication) and the time for the clearance of virus produced from already infected cells which would not be affected by nevirapine.

As the wild-type viral load decreases, the mutant viral load increases until it reaches a new steady state brought on by competition for resources (17). The growth model $R_m(t) = B/[1 + \exp -\beta(t + \gamma)]$ describes the rise in mutant viral load, where B is the long-run steady-state mutant viral load, β is roughly the rate of exponential increase in the early part of the R_m curve, and γ is the time point where the mutant viral load reaches half its equilibrium value.

Combining these two submodels, $R(t) = A \exp(-\alpha t) + B/[1 + \exp -\beta(t + \gamma)]$ as a model for total viral load at time t. This model assumes that the mutant virus originates from preexisting mutant subpopulations. An approximation of the half-life of the 181 wild-type virus is made by assuming that in the first 5 days, levels of 181 mutant virus are low and the contribution of R_m to R is negligible. Assuming that wild-type viral production has completely ceased and that errors are independent and log-homogeneous, data from days 0 to 5 are fit to the equation to obtain approximate values for A and α. A delay before the drug affects the wild-type viral load is set as 1 day, and the mean of the HIV RNA levels for days 0 to 1 is used for the initial viral load.

Similarly, if the wild-type viral load is negligible after day 10, $R_m(t) = B \exp \beta(t + \gamma)$ is an approximation for the 181 mutant viral load when t is small. Estimates of the doubling time of the mutant population are made by fitting the HIV RNA data, assuming that errors are independent and log-homogeneous. Because the viral load does not increase indefinitely, only data from days 10 to 28 are fit to this equation.

For the two patients (N-06 and N-12) with sufficiently high levels of HIV RNA ($>10^4$ copies per ml) and with photometric data obtained at sufficient frequency, differential hybridization was used to estimate the proportion of mutant and wild-type virus and the data were fit to the corresponding equations described above to estimate the half-life and doubling time. The initial 181 mutant load was estimated by setting $t = 0$ in the growth-constrained model describing the rise in mutant viral load. Confidence intervals were computed with B fixed; the fitting procedure assumes that errors are independent and homogeneous.

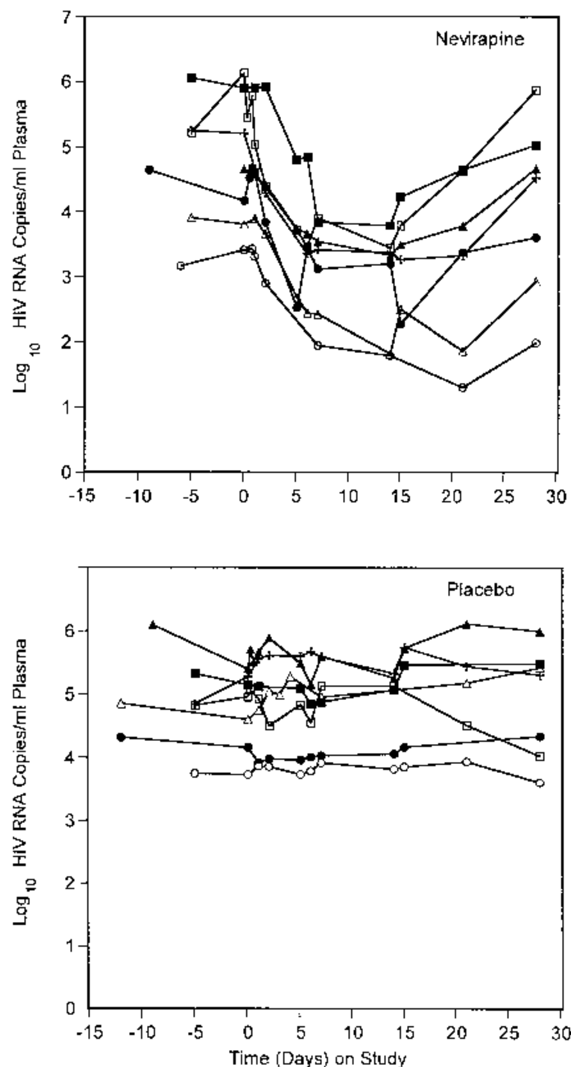

FIG. 1. HIV RNA levels in the plasma before treatment and up to 28 days after receiving either nevirapine or placebo. HIV RNA levels remain stable in patients on placebo and decline 1.27 to 2.43 $\log_{10}$ units in nevirapine-treated subjects. Symbols correspond to individual subjects.

RESULTS

Fourteen subjects participated in the study. Pretreatment CD4 cell counts ranged from 226 to 493 cells per mm^3 (median, 437 cells per mm^3), and pretreatment HIV RNA levels ranged from 2,005 to 956,750 copies per ml. Two subjects had prior zidovudine exposure; 12 were naive to all antiretroviral therapy.

Kinetics of viral clearance. HIV RNA levels remained stable in the placebo-treated patients and rapidly declined in the nevirapine-treated patients (Fig. 1). Of note, even in drug-treated patients, HIV RNA levels remained stable during the first 24 h after nevirapine administration. For example, in subject N-06, HIV RNA levels were 43,516, 32,714, 46,062, 39,044, and 6,811 copies per ml prior to therapy and at 8, 14, 24, and 48 h after dosing, respectively. The nadir of the HIV RNA levels occurred on day 14 or 15 for all subjects except one, for whom the day 21 value represented the nadir. The maximum $\log_{10}$ reduction of HIV RNA levels was 1.96, 2.00, 2.04, 2.19, 2.20, and 2.43 in the monotherapy subjects and 1.27 in the subject on combination therapy. The clearance rates of wild-type virus for each subject given nevirapine are shown in Table 1. The median half-life estimated was 1.11 days (range, 0.63 to 1.61). Incorporating the measurements of wild-type and mu-

TABLE 1. Rates of clearance of wild-type HIV and production of mutant virus in subjects treated with nevirapine

Subject	Baseline no. of HIV RNA copies/ml	Baseline no. of CD4 cells/mm^3	HIV half-life (days) (90% CI)[a]	Doubling time (days) (90% CI)	Mutations emerging with treatment[b]
N-03	7,205	493	1.42 (1.11,1.87)	5.73 (4.63, 7.35)	Y181C, K101E, G190A
N-05	2,005	437	1.11 (0.85,1.60)	ND[c]	K101R, K102Q
N-06	29,068	400	0.63 (0.56,0.70)	4.81 (2.56, 30.24)	Y181C, K102R, K104R
N-07	747,340	449	0.68 (0.54,0.94)	1.80 (1.70, 1.92)	ND
N-12	956,750	226	1.27 (0.97,2.02)	3.84 (3.05, 5.40)	Y181C, Q182R, Y183D, T107A, K102E
N-13	165,929	461	0.66 (0.60,0.73)	3.71 (2.61, 7.77)	Y181C, V108I, K103N
E-01[d]	39,309	342	1.61 (1.41,1.91)	3.35 (2.89, 4.13)	Y181C, K103N

[a] CI, confidence interval.
[b] K103N, V108I, Y181C, and G190A have been documented to confer nevirapine resistance.
[c] ND, not determined because of low RNA levels.
[d] Patient E-01 had prior zidovudine treatment, which was continued during the period of observation. All other subjects were previously untreated with antiretroviral agents.

tant virus into the functions, the half-life of wild-type virus was estimated as 0.81 day (90% confidence interval, 0.63, 1.07) for subject N-12, 0.97 day (90% confidence interval, 0.56, 5.00) for subject N-06, and 2.09 days (90% confidence interval, 1.64, 2.74) for subject E-01. Clearance rates were independent of baseline CD4 cell count and HIV RNA level in plasma.

Production of resistant virus. In all seven nevirapine-treated patients, HIV RNA levels began to increase after the first 14 days of therapy. In two of the seven subjects, the HIV RNA levels returned to baseline values. In the remaining five subjects, the levels remained between 0.71 and 1.32 $\log_{10}$ below baseline at the end of the 28-day observation period.

The detection of the Y181C mutant population in all subjects paralleled a rise in the HIV RNA levels. Sequencing of virus isolated from plasma confirmed the presence of the Y181C mutation; in some cases, additional mutations known to confer nevirapine resistance were detected (Table 1). The virus population became increasingly dominated by the 181 mutant population in the plasma (Fig. 2). Prior to treatment, the proportion of mutant virus in the plasma (calculation of MUT/GNR ratios) was below the measurable threshold of 0.03 for the allele assay for specific assays. At 28 days, the 181 MUT/GNR ratios were 0.16 (N-03), 0.40 (E-01), 0.87 (N-06), 0.55 (N-07), 2.16 (N-12), and 1.18 (N-13). In subject N-03, selection of the 181 mutant did occur but at a much lower rate. At 124 days, the MUT/GNR ratio was 0.41. Quantitation of the 181 mutant was available at later time points for two other subjects (N-05 and N-06), for whom turnover to greater than 90% 181 mutant occurred by 124 and 169 days.

The doubling time of the emerging HIV population in the plasma was estimated by fitting exponential increases in HIV RNA levels occurring after the nadir, as described in the previous section. Estimates of doubling time ranged from 1.80 to 5.73 days (Table 1). Estimates of doubling time incorporate the replication rates of the entire emerging virus population.

The rapid turnover of the viral populations as indicated by the HIV RNA in plasma was not observed in the HIV DNA in PBMC. An increase above the threshold of detection in the 181 MUT/GNR ratio from HIV DNA in PBMC was not observed until day 28 (Fig. 2). In one of the three subjects in whom the Y181C mutant was detected in the PBMC DNA (N-06), the baseline MUT/GNR ratio was below 0.03 and remained so until day 28, when it was noted to be 0.23. By 169 days, it had further increased to 1.03. Resistance to nevirapine was not observed in isolates obtained from PBMC during the first 28 days of therapy (data not shown). In four subjects in whom later isolates were assayed, resistance to nevirapine was observed in two at 12 weeks and in the remaining two at 24 weeks.

Prevalence of mutant population prior to treatment. The prevalence of the Y181C mutant population in the HIV RNA in plasma before drug treatment was calculated by fitting a logistic function to the number of Y181C copies detected after the initiation of nevirapine (Fig. 3). In the two patients (N-06 and N-12) for whom the data permitted the most precise use of the mathematical model, the Y181C mutant was estimated to be present at a prevalence of 133 and 7 per 10,000 copies of HIV RNA prior to treatment (Table 2).

DISCUSSION

This study confirms that the introduction of antiretroviral therapy produces a rapid decline in the HIV RNA level in plasma that is remarkably uniform among patients. Both the magnitude of reduction of the HIV RNA level from baseline (2 $\log_{10}$ copies per ml of plasma) and time to the nadir (2 weeks) were consistent among patients, independent of pretreatment HIV RNA levels and CD4 cell counts. The selective pressure induced by drug therapy rapidly selected for a resistant population in the plasma and correlated with a loss of viral suppression. The doubling times of the emerging virus population varied widely among individuals and resulted in differing levels of virus suppression at 28 days.

Estimates of viral half-life in these patients (1.1 days) are slightly shorter than the initial reports in the literature (12, 15, 27). In three independent studies in which specimens were obtained at intervals of 5 to 10 days, the mean half-lives were estimated as 1.9, 2.0, and 2.1 days. We attribute this discrepancy in part to the fact that we use earlier and more frequent time points to model decay curves. We also observed a delay of 1 to 2 days before the HIV RNA levels in plasma began to decay. This delay was probably the result of viral production by cells already containing reverse transcripts at the initiation of drug therapy. This would indicate a viral replication cycle and the longevity of most infected cells of between 1 and 2 days. Our estimates of clearance rates are probably conservative and viral turnover is actually more rapid because our calculations assume that viral inhibition is complete and that the effect of the drug is immediate. Errors in either of these assumptions would yield estimates of viral half-life that are longer than the true values. In addition, our calculations represent a composite half-life of infected cells and cell-free virions. Recent work by Perelson et al. suggests that plasma virions in fact have a half-life of approximately 6 h and productively infected cells have a half-life of approximately 2 days (22). In this study, insufficient samples of lymphocytes after day 28 precluded the determination of the turnover rate of viral DNA in these cells, which was uniformly greater than 1 month.

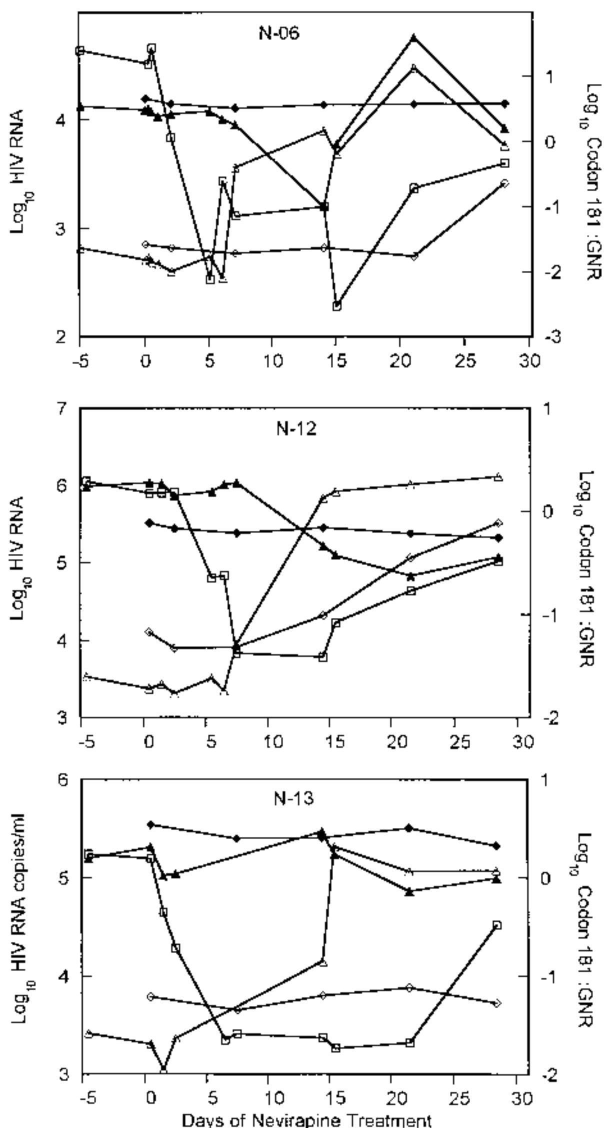

FIG. 2. The HIV RNA levels and the ratio of Y181C or wild-type virus to generic probe by using differential hybridization in the plasma and PBMC are shown for three subjects receiving nevirapine. The HIV RNA levels in the plasma begin to decline after 24 to 48 h. The subsequent increase in RNA levels in plasma is accompanied by a rise in the Y181C mutant population in the plasma. Turnover to the Y181C mutant is significantly delayed in the PBMC DNA. Symbols: □, HIV RNA equivalents per ml (left-hand *y* axis); ▲, codon 181 wild type in plasma; △, codon 181 mutant in plasma; ◆, codon 181 wild type in PBMC; ◇, codon 181 mutant in PBMC (right-hand *y* axis).

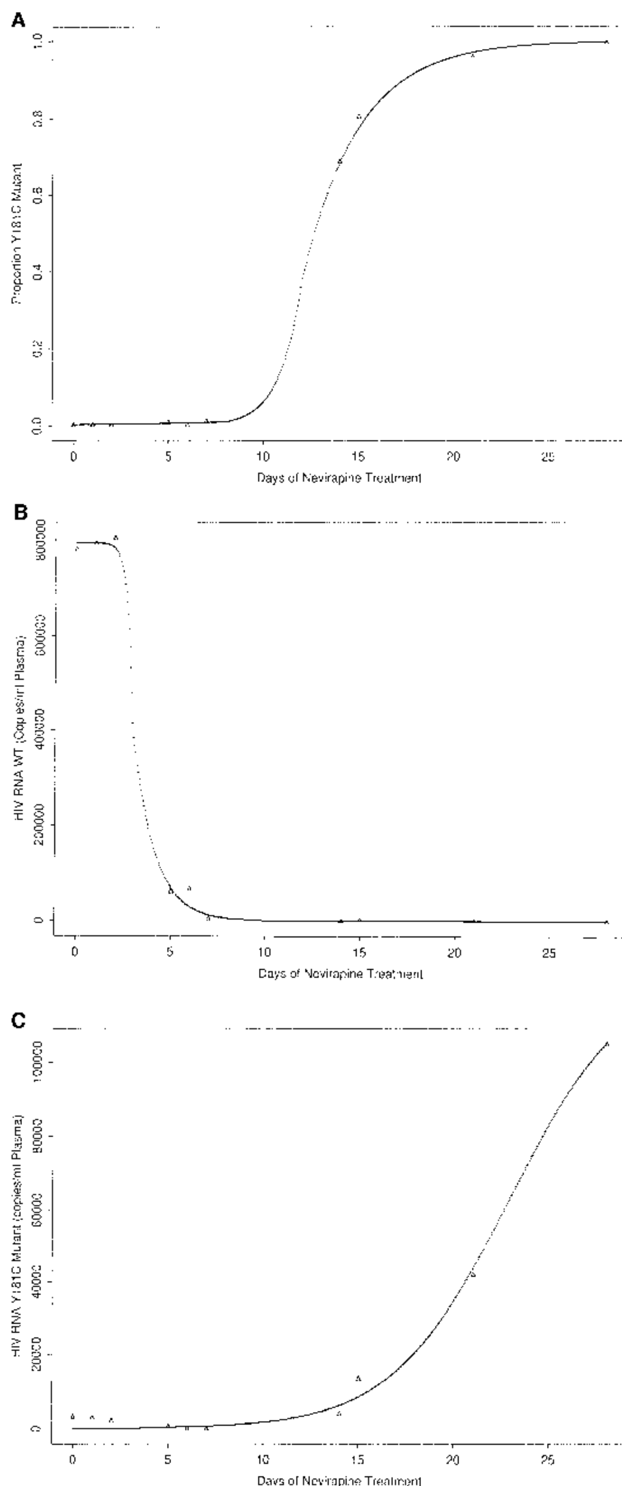

FIG. 3. Proportion of Y181C mutant (A); logistic curve fitted to 181 wild-type virus population $R_w(t) = A \exp -\alpha(t - 1)$ (B), and 181 mutant $R_m(t) = B/[1 + \exp -\beta(t + \gamma)]$ (C) for subject N-12. The half-life of the wild-type virus was estimated as 0.81, and the doubling time of the Y181C mutant was estimated at 2.1 days.

The appearance of the Y181C mutant as early as 1 week after nevirapine initiation suggests that a reduction in the HIV RNA level may be limited at least in part to the rapid selection of resistant virus. Complete turnover to the 181 mutant occurred within the 28-day study period in subject N-12, but turnover occurred over a period of months in the remainder. In previous studies, we demonstrated that complex mixtures of mutant viruses resistant to nevirapine are generated after nevirapine administration (23). When HIV RNA levels are increasing, resistant virus not detected with the Y181C probe is also emerging in most patients (Table 1). Unlike the rise in HIV RNA levels which occurs after 4 weeks of zidovudine administration and has been attributed to replication of wild-

TABLE 2. Prevalence of Y181C mutant population of HIV RNA in plasma at baseline and its estimated doubling time during treatment with nevirapine

Subject	Pretreatment plasma RNA level (Y181C copies/ml) (90% CI)[a]	Prevalence of Y181C mutant (copies/10^4 HIV RNA) (90% CI)	Doubling time of Y181C population (days) (90% CI)
N-06	38.6 (15.8, 88.5)	132.8 (54.4, 304.5)	4.9 (4.4, 5.9)
N-12	65.1 (23.5, 180.8)	6.8 (2.5, 18.9)	2.1 (1.8, 2.4)

[a] 90% CI, 90% confidence interval.

type virus in the rising CD4 cell population, it is more likely that the rise in HIV RNA levels observed in the nevirapine-treated patients is due to replication of a mixture of resistant viruses, a proportion of which contain the 181 mutation (5, 23). The gradual turnover of the population to the Y181C mutant, however, suggests that this is the most fit population under the selective pressure of nevirapine monotherapy.

The doubling time of the resistant virus varied widely among individuals. Longer doubling times resulted in HIV RNA suppression beyond 2 weeks, while shorter doubling times resulted in the return of HIV RNA levels to pretreatment values. The longer doubling times observed in some patients could be attributed either to residual but diminished inhibition of partially resistant virus replication by continuing nevirapine therapy or to the impaired replicative capacity of the resistant virus or both. Variations in nevirapine levels in plasma and variations in the susceptibility of resistant virus have been shown to contribute to sustained suppression of resistant virus by nevirapine treatment (8, 9). The contribution of the baseline sequence of the reverse transcriptase gene to the resistance mutations that emerge with treatment warrants further investigation.

Use of the appearance of the mutation at the Y181C codon as a marker of viral turnover confirmed previous reports that viral DNA in PBMC turns over less rapidly and completely than does HIV RNA in plasma. The persistence of wild-type DNA in these cells suggests that these sequences represent to some extent a viral archive and that virus replication is proceeding in at most a small minority of infected cells (14, 26, 27, 30). These observations are most consistent with a long-lived population of lymphocytes containing wild-type sequence, either because the lymphocytes are not activated or because they contain replication-incompetent virus. Direct sequencing of reverse transcriptase in various tissues obtained at autopsy and from clinical specimens may also exhibit discordant resistance mutations, suggesting that viral turnover varies not only between the plasma and PBMC but also between tissue compartments (13, 29).

The rapid appearance of drug-resistant mutants would indicate that they existed prior to the initiation of therapy. Nevirapine-resistant mutants have been identified in clinical isolates from two untreated patients. In one subject, the tyrosine-to-cysteine mutation at codon 181 was observed after serial passage in cell culture in the absence of drug (19). In the second subject, the same mutant was identified as a small subpopulation of virus prior to treatment (4). In the latter patient, 92% of the virus in serum was the mutant 7 days after the initiation of nevirapine.

In this study, a mathematical model was used on data from two subjects to estimate that the Y181C mutant preexisted in the plasma before the selective pressure of drug treatment in the range of 7 to 133 per 10,000 HIV RNA copies. Mansky and Temin have determined a mutation rate of 3×10^{-5} error per nucleotide per replication cycle for HIV (16). Population genetics would suggest that the smallest selective advantage is likely to rapidly select for a mutant existing at this prevalence when the number of replication cycles is large (2). The varying prevalence of Y181C among patients may be attributable to the differential fitness of the Y181C mutation in different background reverse transcriptase sequences. This method of back calculation of the prevalence of other mutations in reverse transcriptase or protease may provide an approach to assess fitness of a mutation in the absence of selective pressure of drug therapy.

While active antiretroviral agents reduce HIV RNA levels in the plasma rapidly as a result of the dynamic turnover, the extraordinary levels of virus and viral replication ultimately favor survival of the virus by enabling minority resistant populations to survive and proliferate. Even at the earliest stages of HIV disease, when virus is actively replicating in tissues and is in the range of 10^4 to 10^6 equivalents per ml in the plasma, resistant virus could rapidly emerge (7, 20, 21). This hypothesis has recently gained support from studies of administration of nevirapine even to patients with more than 500 CD4 cells per mm^3 (10). Therapeutic and drug development strategies will need to find drug combinations which either effectively suppress these populations or select for mutants with impaired replicative capacity.

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