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Drug Resistance during Indinavir Therapy Is Caused by Mutations in the Protease Gene and in Its Gag Substrate Cleavage Sites

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Two different responses to the therapy were observed in a group of patients receiving the protease inhibitor Indinavir. In one, suppression of virus replication occurred and has persisted for 90 weeks (bDNA, <500 human immunodeficiency virus type 1 [HIV-1] RNA copies/ml). In the second group, a rebound in virus levels in plasma followed the initial sharp decline observed at the start of therapy. This was associated with the emergence of drug-resistant variants. Sequence analysis of the protease gene during the course of therapy revealed that in this second group there was a sequential acquisition of protease mutations at amino acids 46, 82, 54, 71, 89, and 90. In the six patients in this group, there was also an identical mutation in the *gag* p7/p1 *gag* protease cleavage site. In three of the patients, this change was seen as early as 6 to 10 weeks after the start of therapy. In one patient, a second mutation occurred at the *gag* p1/p6 cleavage site, but it appeared 18 weeks after the time of appearance of the p7/p1 mutation. Recombinant HIV-1 variants containing two or three mutations in the protease gene were constructed either with mutations at the p7/p1 cleavage site or with wild-type (WT) *gag* sequences. When recombinant HIV-1-containing protease mutations at 46 and 82 was grown in MT2 cells, there was a 68% reduction in its rate of replication compared to the WT virus. Introduction of an additional mutation at the *gag* p7/p1 protease cleavage site compensated for the partially defective protease gene. Similarly, rates of replication of viruses with mutations M46L/I, I54V, and V82A in protease were enhanced both in the presence and in the absence of Indinavir when combined with mutations in the *gag* p7/p1 and the *gag* p1/p6 cleavage sites. Optimal rates of virus replication require protease cleavage of precursor polyproteins. A mutation in the cleavage site that enhanced the availability of a protein that was rate limiting for virus maturation would confer on that virus a significant growth advantage and may explain the uniform emergence of viruses with alterations at the p7/p1 cleavage site. This is the first report of the emergence of mutations in the *gag* p7/p1 protease cleavage sites in patients receiving protease therapy and identifies this change as an important determinant of HIV-1 resistance to protease inhibitors in patient populations.

Recent studies of human immunodeficiency virus type 1 (HIV-1)-infected patients have shown that protease inhibitors alone or combined with nucleoside analogs reduce the virus load in blood to levels less than 500 HIV RNA copies per ml. Levels of virus have remained below 500 copies/ml for as long as 2 to 3 years (3, 15, 18, 22, 32). It is not yet established if the high levels of virus that have been demonstrated in lymphoid tissue (10, 14, 28) are also completely cleared during this therapy. While these remarkable therapeutic responses are the most encouraging reports emerging from years of therapeutic trials, drug-resistant variants do emerge within a fraction of the patient population in these studies, as evidenced by an increase in the levels of virus in blood even in the continued presence of virus inhibitors.

Genotypic analyses of the virus populations during the course of protease inhibitor therapy have shown that resistant virus variants are the result of a sequential accumulation of changes that can occur in as many as 20 amino acids within the protease gene (4, 5, 19, 23–25, 29). The most frequent changes occur within a pocket that defines the drug binding site (7, 13, 34), but mutations outside of this domain are also observed and play a significant role in conferring resistance (13, 34). The sites at which drug-induced mutations occur following long-term therapy depend on which protease inhibitors have been used (24), and even within a patient group receiving the same drug, resistance can be affected by the accumulation of different patterns of mutation. While viruses that are selected because they are resistant to one protease inhibitor frequently are cross-resistant to other protease inhibitors (4, 5), this response is variable. The growth of resistant virus selected after therapy with one protease inhibitor may still be partially or completely inhibited by another protease inhibitor (25).

During a clinical trial carried out at the National Institutes of Health with the protease inhibitor Indinavir, two different responses to therapy were observed. In one group, there was a sharp decline in virus levels in plasma that has persisted for 1.5 years. In the second group, there was an immediate decline in virus levels in plasma that was followed by a rise in the titer of virus in blood. In the present study, we have characterized the properties of the virus in the latter category. In this group, which fails in its long-term response to therapy, resistant viruses that contained mutations M46L/I, I54V, A71V/T, V82A/F, and L90M in the protease gene arose. The earliest changes in the virus protease gene occurred at positions 46 and 82, but these changes did not confer significant levels of resistance to Indinavir. However, these mutations did confer a comparative advantage on the mutated viruses compared with wild-type

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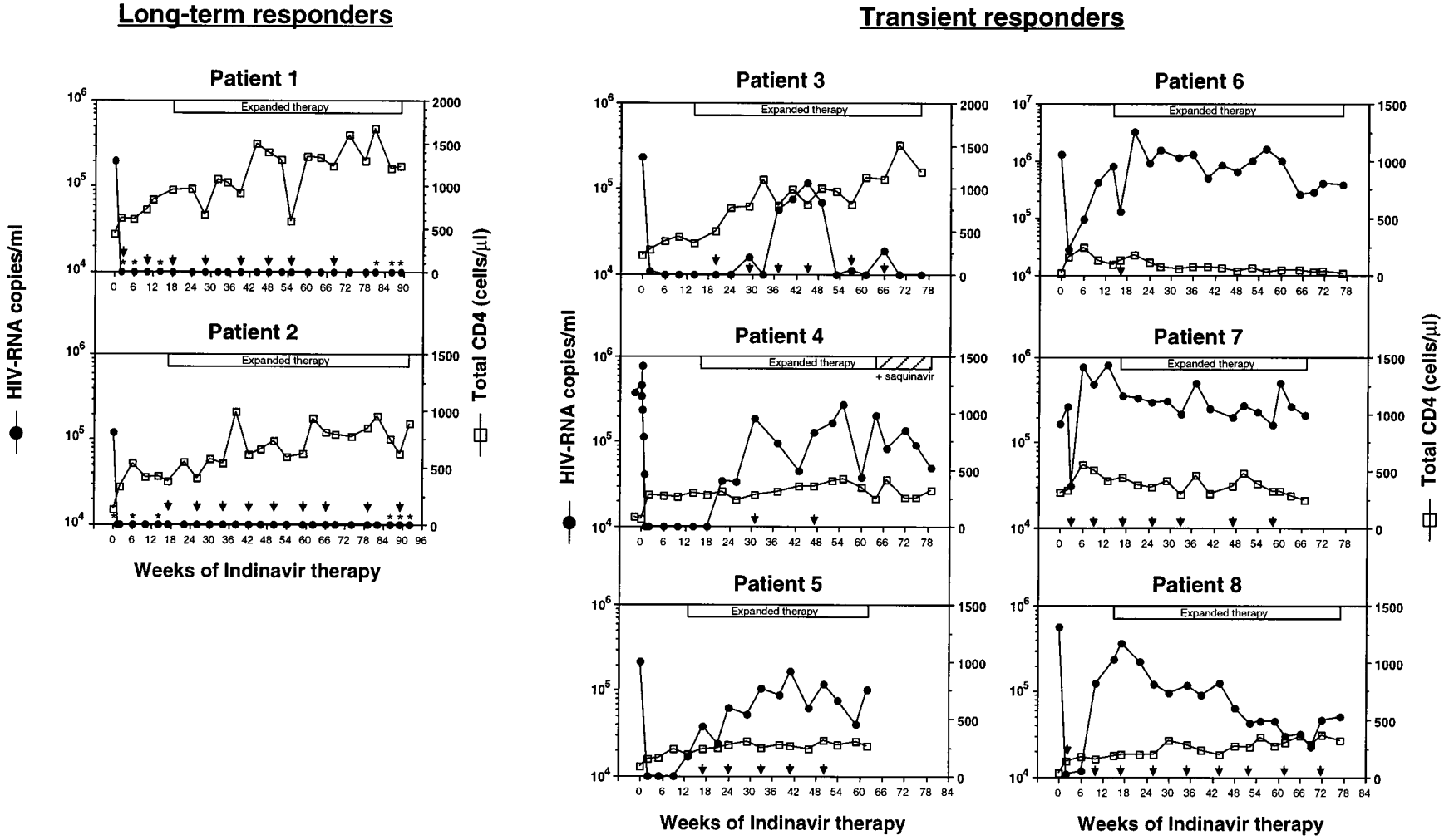

FIG. 1. Virologic and immunologic responses of patients to Indinavir. The patients were classified into two categories; in long-term responders (patients 1 and 2), there was a sharp decline in the virus level in plasma to undetectable levels, which has persisted for 90 weeks; in the transient responders (patients 3 to 8), there was an initial decrease in virus levels in plasma followed by a rebound. While all patients received Indinavir therapy for the first 3 months, after that period all the patients were permitted to add other antiretroviral agents (indicated as "expanded therapy" in the graphs). The times at which interleukin-2 were administered are noted by arrows on the abscissa. Declines in virus levels in plasma that were observed after the expanded therapy was initiated are probably the therapeutic consequence of the reverse transcriptase inhibitors. The virus level in plasma was initially measured by the bDNA assay, which has a detection limit of 10,000 copies of RNA per ml. For several time points, the assays have been repeated with a modified bDNA assay, which has a detection limit of 500 copies of RNA per ml. Asterisks (*) represent the times when in each case the virus was below the detection limit of 500 copies of RNA per ml. Solid circles show bDNA levels; open squares show the number of $CD4^+$ cells.

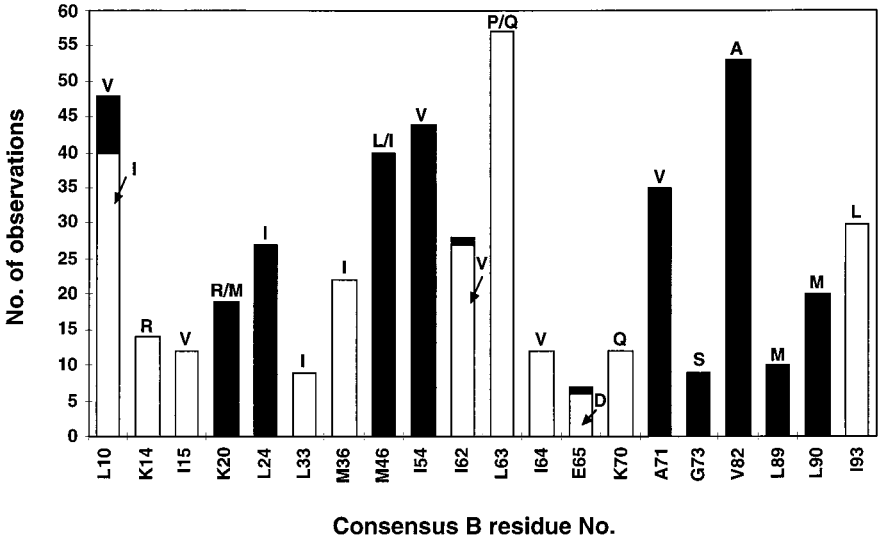

FIG. 2. Variation of HIV-1 protease sequences in patients during Indinavir therapy. In the group of six patients, 59 clones were obtained immediately prior to the start of therapy and 57 clones were analyzed 59 to 75 weeks after therapy. Sequences were compared to the HIV-1 protease consensus B sequence. The changes in the amino acid sequences noted at time zero identify amino acid changes associated with allelic polymorphism. The 57 sequences that were obtained after 59 to 75 weeks of therapy from the transient responders (patients 3 to 8) were compared to the HIV-1 consensus B sequences. Open bars identify amino acid changes at 59 to 75 weeks that were also seen on day 0, and solid bars show amino acid changes that were seen only during Indinavir therapy. The predominant amino acid change is shown at the top of bar, and when more than one amino acid substitution was found at the same position, the predominant change is shown followed by a slash (/) and the minor change. Amino acid changes that appeared with a frequency of less than 10% were omitted from the plot. Variations were found at residues L10, K20, L24, M46, I54, A71, V82, L89, and L90 during Indinavir therapy.

(WT) virus when their growth rates were compared in the presence of 100 nM Indinavir if these viruses also contained an additional mutation in the *gag* p7/p1 protease cleavage site. We have observed an identical A-to-V mutation at position P2 in the *gag* p7/p/1 cleavage site in each of six patients after 1.5 years of Indinavir therapy. In three patients, this mutation could be detected as early as 6 to 10 weeks after the start of protease therapy, and its frequency within the virus population increased almost simultaneously with the progressive introduction of additional changes in the protease gene.

MATERIALS AND METHODS

Samples. Plasma samples were obtained from patients infected with HIV-1 who were treated with Indinavir (600 mg four times a day) (31) alone (patients 2 through 6) or in combination with intermittent administration of interleukin-2 by continuous infusion for 5 days every 2 months (patients 1, 7, and 8) as part of a 12-week protocol. At later times, reverse transcriptase RT inhibitors were also administered in the continued presence of Indinavir (11) and patients were allowed to add interleukin-2 to their treatment regime.

Virologic monitoring. Particle-associated HIV-1 RNA levels in plasma were determined by the version of the standard branched-DNA signal amplification assay (bDNA assay; Chiron) (8) that has a detection limit of 500 copies of HIV-1 RNA per ml.

RNA isolation and cDNA synthesis. HIV-1 RNA was isolated from 130 μl of plasma by using the QIAamp HCV kit (Qiagen Inc.). HIV-1 RNA was reverse transcribed to cDNA with a primer (minus strand), 5′-TTGTTTTACATCAT TAGTGTGGGC-3′ (nucleotides [nt] 3626 to 3649 of HIV-1 HXB2) and avian myeloblastosis virus reverse transcriptase (cDNA cycle kit; Invitrogen Corp.).

PCR. HIV-1 DNA corresponding to the *gag* (p7/p1/p6), protease, and part of reverse transcriptase was amplified by PCR with a mixture of Klentaq1 (Ab Peptides, Inc.) and *Pfu* (Stratagene) with the following primer pair: forward primer (nt 1881 to 1904) 5′-GAAGCAATGAGCCAAGTAACAAAT-3′ and reverse primer (nt 3543 to 3566) 5′-GATATGTCCATTGGCCTTGCCCCT-3′. Nested PCR was carried out with the following primer pair: forward primer (nt 1965 to 1988) 5′-TTCAATTGTGGCAAAGAAGGGCAC-3′ and reverse primer (nt 3500 to 3523) 5′-TAAGTCTTTTGATGGGTCATAATA-3′. The PCR product was purified with the QIAquick spin PCR purification kit (Qiagen Inc.).

Cloning and sequencing. The purified PCR products were ligated using the pCRII vector (Invitrogen Corp.), and the ligated product was used to transform *Escherichia coli* TOP10F′ competent cells. Positive colonies were identified, and the presence of a 1.6-kb insert was confirmed by restriction enzyme digestion with *Eco*RI. Plasmid DNA containing the HIV-1 *gag* p7/p1/p6 and protease insert was purified with a QIAwell eight-plasmid kit (Qiagen Inc.). Dye-Deoxy-labeled sequencing reactions were performed with the ABI PRISM Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase FS (Perkin-Elmer), and the reaction products were resolved by electrophoresis on a 4.75% polyacrylamide gel and analyzed with an Applied Biosystems 377 automated sequencing system.

Genotypic analyses. The nucleotide sequences of the *gag* p7, p1, and p6 regions and the protease gene were translated, aligned with Clustal W (30), and edited with the MASE (multiple-aligned sequence editor) program (12). Changes in HIV protease and *gag* p7/p1/p6 sequences were analyzed with the VESPA (Viral epidemiology signature pattern analysis) program (20), using the HIV-1 consensus B sequence as a reference sequence.

Cells and viruses. MT-2 cells (16, 17) and the HIV-1 pNL4.3 proviral DNA clone (1) were obtained from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, and were contributed by Douglas Richman and Malcom Martin, respectively. RD cells (human embryonal rhabdomyosarcoma cell line) were provided by the American Type Culture Collection. MT-2 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories Inc.) and antibiotics. RD cells were maintained in Eagle's minimal essential medium supplemented with 10% FBS and antibiotics.

Construction of molecular clones. Plasmid NL4.3 containing full-length HIV was modified to delete one *Bal*I restriction site located at nt 4553 by silent mutagenesis (TGGCCA to TGGCCC) by using the Altered Site II in vitro mutagenesis system (Promega). To construct the modified plasmid (called pNL4.3Bal), mutagenesis was performed with a primer, 5′-GTACTGTTT TTACGGGCCATCTTCC-3′, as specified by the manufacturer. Chimeric infectious NL4.3Bal clones containing the mutated *gag* and protease genes were constructed by replacing the *Apa*I-*Bal*I fragment of pNL4.3Bal with the appropriate *Apa*I-*Bal*I fragment from the cloned DNA derived from patient's samples.

Transfection and infection. Transfection was performed with the Perfect Lipid Pfx-3 kit (Invitrogen). Briefly, 3.5×10^5 RD cells in 35-mm culture dishes were used for each transfection with 2 μg of the chimeric pNL4.3Bal DNA. After a 4-h transfection, the medium was replaced with fresh Eagle's minimal essential medium supplemented with 10% FBS and further incubated for 16 h. MT-2 cells (10^6 cells) were added to the transfected RD cells, and they were cocultured for an additional 24 h. The MT-2 cells were then recovered and resuspended in the supplemented RPMI 1640 and cultured for 3 to 5 days when HIV-specific cytopathic effects appeared. The cell-free culture supernatants were used as infectious stocks and stored at −80°C until used.

Protease inhibitor sensitivity of recombinant HIV-1s containing mutant protease genes. The infectivity of virus stocks was determined as follows. Infected cell cultures were set up in triplicate with fourfold dilutions of the HIV-1 stocks. Each well in the 96-well dish contained 4×10^4 MT-2 cells. The plates were incubated for 7 days, the culture supernatants were collected, and p24 assays

TABLE 1. Emergence of HIV-1 variants containing mutations in the protease gene and the *gag* cleavage sites during Indinavir therapy[a]

Patient	Weeks of therapy	Mutations in the protease (relative to consensus B sequence)										Cleavage site sequence (relative to consensus B sequence)																					
												p7/p1										p1/p6											
		L10	K20	L24	M46	G48	I54	A71	G73	V82	L89	L90	E	R	Q	A	N	I	F	L	G	K	I	R	P	G	N	F	I	L	Q	S	R	P
Patient 3	D0	I	-	-	-	-	-	-/T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-/N	-	-
	wk14	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-/G	-	-
	wk29	I	-	-	L	-	-	-	-	-	-	-	-	-	-/R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	wk33	I	-	-	L	-	-/T	-	-	-	-	-	-	-/G	-	-/V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	wk37	I	-	-	L	-	-	-	-	A/-	-	-	-	-	-	V/-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	wk46	I	-	-/I	L	-	V/-	-	-	A	-	-	-	-	-	V	-	-	-	-	-	-	-/V	-	-	-	-	-	-	-	-	-	-	-
	wk53	I	-	-	L	-	V	-	-	A	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	wk66	I	-	-/I	L/I	-	V	-/V	-	A	-	-	-	-	-	V	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-
	wk75	I	-	I	L/I	-	V	V	-	A	-	-	-	-	-	V	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-
Patient 4	D0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-/N	-	-/T
	wk6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	wk18	-	-	-	-	-	-	-	-	A/-	-	-	-	-/G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	wk31	-	-	-	-/L/I	-	-	-/V	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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	wk55	-	-	-	L	-	V/-	V/-	-	A	-	-	-	-	-	V/-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	wk64	F/I/-	-	-	L	-	V	-	-	A	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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Patient 5	D0	-	-	-	-	-	-/V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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	wk29	-	-	-	I/L	-	-	-/V	-	A/T/-	-	-	-	-	-	-/P/V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	wk54	-/I	-	-/I	L/I	-	V	V/T/-	-	A	-	-	-	-	-	V	-	-	-	-	-/T	-	-	-	-	-	-	-	-	-	-	-/N	-	-
	wk59	-/I	-	I/-	I/L	-	V	V	-	A/-	-	-/M	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Patient 6	D0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	wk3	-/I	-	-	-/I	-	-	-	-	-	-/P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	wk6	-	-	-	L/-	-	-	-	-	A/-	-	-	-	-	-	-/V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	wk10	-	-	-	L	-	-	-	-	A	-	-	-	-/K	-	V/I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	wk16	-	-	-	L	-	-	-	-	A	-	-/W	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	wk20	-	-	-	L	-	V/-	-	-	A	-	-/M	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	wk24	-	-	-	L/-	-	V/-	-	-	A/-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-/F	-	-/N	-	-
	wk27	-/I	-	-	L	-	V/-	-	-	A	-	-/M	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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	wk36	I	-	-	L	-	V	-	-	A	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	-	-	-	-
	wk40	I/-	-	-	L	-	V/-	-	-	A	-/M	-/M	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F/-	-	-/N	-	-
	wk44	I	-	-	L	-	V/-	-	-	A	-/M	-/M	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F/-	-	-	-	-
	wk71	I	R/-	I/-	L	-	V	-/V	-	A	M/-	-/M	-	-	-	V/I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	-	-	-	-
Patient 7	D0	-	-	-	-	-	-	T/-/I	-	-	-	-	-	-	-	-	-	-	-	-	-	-/X	-/V	-	-/L	-	-	-	-	-	-	-	-	-
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Patient 8	D0	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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	wk6	I/V	-/R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	wk10	I/V	-/R	-	-/I	-	-	-	-	-	-	-	-	-	-	-/V	-	-	-	-	-	-	-	-/G	-/Q	-	-	-	-	-	-	-	-	-
	wk17	V	-	-	-/I	V/-	-	-/D	-	A/-	-	-	-	-	-	-/V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	wk22	V	-	-	-/I	V/-	V/-	-	-	A/-	-	-/M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	wk26	V/I	-	-	-	V/-	V	-	-	A/F	-	-	N.D.											N.D.										
	wk30	V/I	-/M	-	-/I/R	V/-	V/-	-	-	A	-	-/M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	wk35	I/V	M/-	-	-/I	V/-	-/V	-	-	A/-	-	-/M	-	-	-	-/V	-	-	-	-	-	-	-	-	-	-	-	-	-	-/V	-	-	-	-
	wk39	I/V	M/-	-	-/I	V/-	-/V	-	-	A/-	-	-/M	-	-	-	-/V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	wk44	I/V	M/-	-	-/I	V/-	-/V	-	-	A/-	-	-/M	-	-	-	-/V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	wk48	I/V	M/-	-	-/I	V/-	-/V	-	-	A/F/-	-	-/M	-	-	-	-/V	-	-	-	-	-	-	-	-	-	-	-	-/S	-/I	-	-	-	-	-
	wk56	I/V	-/M	-	-/I	-/V	-/V	-	-	A/F/-	-/M	-/M	-	-	-	V/-	-	-	-	-/R	-	-	-	-	-/R	-	-	-	-	-/N	-/G	-/S		
	wk72	V/I	M/-	-	-/I	-/V	V/-	-	-	A/-	-	-/M	-	-	-	V/-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] Mutations in the protease gene and in the gag cleavage sites were identified during the therapy. All data were obtained from plasma virus isolates (7 to 12 clones at each time point) at different time intervals over the course of therapy (0 to 75 weeks). The major mutations are listed first followed by slashes (/) and minor mutations. Dashes (—) denote identity with the HIV-1 consensus B sequence. X denotes a stop codon which was observed in patient 7 as a minor variant (one clone) at the p7/p1 cleavage site. The single horizontal boxed area that is identified for each patient (▭) indicates the time point when a rebound in the virus level in plasma was detected (Fig. 1).

were performed (p24 antigen capture kit [Immunotech]). Using a cutoff value for p24 of <50 pg/ml, 50% tissue culture infective doses ($TCID_{50}$) were calculated for each stock by the Spearman-Karber method.

The drug susceptibility of each virus was determined as follows. MT-2 cells (4×10^6) were infected with 2,500 $TCID_{50}$ of HIV-1 for 2 h, washed twice, and resuspended in RPMI 1640 to a density of 4×10^5/ml, and 200-μl aliquots were plated in each well in 96-well plates. Each virus was evaluated at each of eight concentrations of Indinavir in quadruplicate. The infected cells were cultured at 37°C for 7 days, virus replication was assessed by measuring the amount of p24 in the culture supernatant, and 50% inhibitory concentrations (IC_{50}) were determined. For the range of drug concentrations, see Table 2.

For studying growth kinetics, MT-2 cells (2×10^6) were infected with 1,250 $TCID_{50}$ and were maintained in 10 ml of RPMI 1640 at 37°C for 7 days. Aliquots of the culture supernatant were harvested every day, and the amount of released virus was measured indirectly by determining the quantity of p24 antigen in the supernatants.

RESULTS

Response of patients to Indinavir therapy. The responses to protease inhibitor therapy are shown in Fig. 1. $CD4^+$ T-lymphocyte counts and virus levels in plasma are plotted during

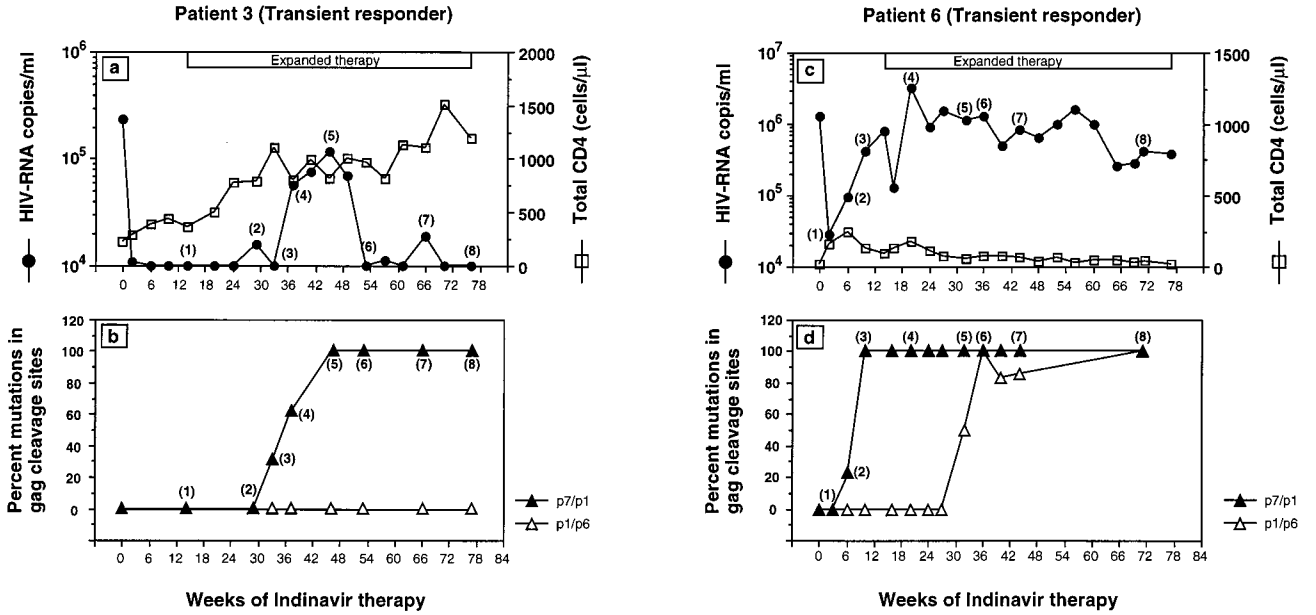

FIG. 3. Correlation between levels of virus in plasma and mutations in the *gag* cleavage sites. Each number in parentheses identifies the time points at which samples were analyzed for bDNA levels, $CD4^+$ cells, and percentage of *gag* mutations for patient 3 (a and b) and patient 6 (c and d). For patient 3, the numbers (1), (2), (3), (4), (5), (6), (7), and (8) correspond to weeks 14, 29, 33, 37, 46, 53, 66, and 75 of therapy; for patient 6, the numbers correspond to weeks 3, 6, 10, 20, 32, 36, 44, and 71. The amino acid changes in the protease gene and the *gag* cleavage sites at these times are shown in Table 1. Solid circles show bDNA levels; open squares show total $CD4^+$ cells; the asterisks represent time points where the detection limit of the bDNA was <500 copies of RNA per ml; solid triangles show the percentage of mutations in the p7/p1 cleavage site; open triangles show the percentage of mutations in the p1/p6 cleavage site.

the course of therapy. Two patterns of response were observed. In patients 1 and 2, a decline in virus levels in plasma to baseline values occurred within weeks of the start of Indinavir therapy and has persisted for 1.5 years. The two patients who responded in this manner (patients 1 and 2) also showed a progressive increase in the $CD4^+$ T-lymphocyte counts.

In the second group of patients (patients 3 to 8), there was biologic evidence suggesting that mutations in protease had occurred, as reflected in an initial decline and a subsequent increase in virus levels in plasma. To identify amino acid positions associated with drug resistance in this group, we examined virus in plasma from patients immediately prior to therapy and after treatment for 59 to 75 weeks where the continued presence of Indinavir did not inhibit virus growth. A PCR product was synthesized by using a cDNA prepared from virus isolated from plasma. The domain that was amplified included (i) the part of the *gag* precursor that included the *gag* p7, p1, and p6 region; (ii) the complete protease gene; and (iii) a part of the reverse transcriptase gene. The PCR product was cloned into pCR II, and 7 to 10 colonies were sequenced from each amplified sample. The amino acid sequences encoded by the protease genes taken from the patients prior to and after 59 to 75 weeks of protease drug therapy were compared with the HIV-1 protease consensus B sequence (26). The data summarized in Fig. 2 display only changes that occurred with a frequency of greater than 10%, in order to exclude minor transient populations that were expected to arise. The changes that occurred during Indinavir therapy were found at residues L10, K20, L24, M46, I54, A71, V82, L89, and L90.

To determine the time when mutations arose and if they emerged in a specified sequential manner, we examined virus in plasma obtained from each patient at multiple times during therapy. The amino acid changes that were found prior to therapy, compared with the consensus B protease sequence, and the changes that occurred subsequently, during the course of protease therapy, are shown in Table 1. A total of 564 clones were examined, and in all of those containing mutations, except for 2 clones, these mutations arose by a single nucleotide change. The five major sites associated with drug resistance are M46, I54, A71, V82, and L90. The most common pathway by which changes occurred was the initial acquisition of changes at positions 46 and 82 followed by changes I54V and/or A71V. While the L90M change was seen frequently, it was not always seen (patient 3) or occurred quite late (patients 4 and 5). In patient 7, the virus evolved in a manner divergent from that seen in the other five patients. A71T changes were present on day 0, and the earliest changes observed during therapy were G73S and L90M. The G73S change was not seen in any of other isolates obtained from the other five patients, and the amino acid sequences in this patient were also unique in that there was no substitution at position 54 at any of the eight time points examined, a change that was seen in the other five patients.

Recently, Doyon et al. described changes in the protease *gag* p1/p6 *gag* cleavage site that they observed during in vitro passage of the virus (9). To determine if a similar phenomenon occurred in vivo, we examined a portion of the *gag* polyprotein gene that included the p7/p1 and the p1/p6 protease cleavage sites in the six patients during the course of Indinavir therapy. The results are shown in Table 1. Each of the six patients displayed an identical change in the p7/p1 *gag* cleavage site that emerged during the course of therapy; in patients 3 and 6, the time of appearance of the p7/p1 mutation coincided with the time at which an increase in the plasma virus load occurred (Fig. 3). In patient 6, a second cleavage site mutation was also seen at the p1/p6 cleavage site, but it occurred 18 weeks later than the time at which the p7/p1 mutation was first identified.

Phenotype analysis of variant HIV-1 proteases. The generation of drug-resistant HIV-1 protease variants has been reported to require the accumulation of multiple mutations

TABLE 2. Growth properties of recombinant viruses

Virus clone	Indinavir concn (nM)	Extent of virus replication[a]	IC_{50} (nM)[b]
WT	0	100	21 ± 3.1
	10	70	
	100	0.13	
46.82 (W/W)	0	32	25 ± 3.0
	10	26	
	100	0.12	
46.82 (M/W)	0	73	28 ± 1.4
	10	70	
	100	1.2	
46.54.82 (M/W)	0	41	197 ± 25.1
	10	39	
	100	23	
46.54.82 (M/M)	0	92	237 ± 32.4
	10	90	
	100	61	

[a] The rates of replication of variant viruses were assessed as described in Materials and Methods. The p24 antigen concentrations were the final values obtained 7 days after infection. Results are expressed as the percentage of growth shown in Fig. 4 compared with WT growth in the absence of Indinavir as the reference. In this experiment, the p24 concentration of the control was 370 ng/ml. The same experiment has been repeated twice, and the mean values for virus replication with standard errors for the three independent studies are as follows: WT HIV-1 in the presence of 10 and 100 nM Indinavir, 70% ± 3.7% and 0.23% ± 0.12% of control values, respectively; 46.82 (W/W), 34% ± 3.0%, 27% ± 0.65%, 0.1% ± 0.027% for 0, 10, and 100 nM Indinavir, respectively; 46.82 (M/W), 76% ± 12%, 65% ± 4.4%, 1.4% ± 0.3% at 0, 10, and 100 nM Indinavir, respectively; 46.54.82 (M/W), 50% ± 11%, 44% ± 1.6%, and 28% ± 1.8% for 0, 10, and 100 nM Indinavir, respectively; 46.54.82 (M/M), 93% ± 13%, 94% ± 1.9%, and 61% ± 1.6% for 0, 10, and 100 nM Indinavir, respectively. The p24 value of controls was 395 ± 61 ng/ml.

[b] The drug susceptibility of each virus was determined as follows. MT-2 cells (4×10^6) were bulk infected with 2,500 $TCID_{50}$ of HIV-1 for 2 h, washed twice, resuspended in RPMI 1640 to a density of 4×10^5/ml, and plated onto 96-well plates in the presence of various concentrations of Indinavir in quadruplicate. The infected cells were cultured at 37°C for 7 days, and virus replication was assessed by measuring the amount of p24 in the four replicate culture supernatant samples, and IC_{50}s were determined. The concentrations of drug added to the growth medium for calculation of the IC_{50} values were 0, 0.01, 0.1, 1, 10, and 100 nM and 1 and 10 μM Indinavir, and the IC_{50}s were derived from plots of percent of inhibition of p24 production in culture supernatant versus Indinavir concentration.

within the protease gene. However, the data in Table 1 suggest a common pathway for emergence of mutations in which the first two mutations that accumulate are M46L/I and V82A and the next is a mutation at either I54V or A71V/T. Addition of a third mutation (I54V) to a background of M46L and V82A is associated with a sharp increase in the IC_{50} (Table 2). To examine how the changes in the proteolytic cleavage sites influence the growth of protease mutant viruses, chimeric HIV variants possessing mutations in protease and in the p7/p1 and p1/p6 cleavage sites were constructed with NL4.3 as the parental HIV-1 strain. The viruses in this series (Table 2) contain an identical backbone, the specified protease mutations, and *gag* cleavage sites either containing mutations or with WT sequences. They were used to infect MT2 cells so that their phenotypic properties could be compared. The viruses were grown in the absence of drug or in the presence of 10 or 100 nM Indinavir. The lower drug concentration is sufficient to partially inhibit WT virus growth, while the higher drug concentration inhibits it more than 99%.

The growth properties of viruses with two mutations in the protease gene at amino acids 46 and 82 were first compared in the absence of Indinavir. A variant HIV-1 strain with the amino acid substitutions M46L and V82A but without mutations in p7/p1 and p1/p6 [46.82 (W/W)] replicated 32% as effectively as did WT HIV-1, while a variant containing the same mutations in protease but containing an additional mutation in the p7/p1 *gag* cleavage site [46.82 (M/W)] replicated 73% as effectively as did WT virus. Viruses with mutations that occur at sites that are associated with protease inhibitor resistance are at a distinct growth disadvantage compared to WT viruses when grown in the absence of drug. Similar findings have also been reported for protease-resistant isolates obtained in vitro (6, 9, 27). The impaired replication of these mutants is consistent with the failure to find these changes in patient isolates prior to receiving protease inhibitors (5, 21, 33). The introduction of a mutation at the p7/p1 cleavage site into the virus that contains a mutated protease gene partially reverses the deleterious effects of the mutations.

The replication of these recombinant viruses was also compared in the presence of 10 and 100 nM of Indinavir (Fig. 4b and c). The extent of growth in 7 days in the presence of 10 nM Indinavir for WT HIV-1, 46.82 (W/W), and 46.82 (M/W) was 70, 26, and 70%, respectively, compared with the growth of WT HIV-1 in the absence of drug (Fig. 4a). In the presence of 100 nM Indinavir, only 46.82 (M/W) showed evidence of growth (Fig. 4c), although it was inhibited 99% compared with the growth of WT virus in the absence of drug.

The growth properties of variants containing three mutations (M46L, I54V, and V82A) in protease were also examined (Fig. 4d to f). In the absence of drug, the variant containing three mutations in protease and an additional mutation in the p7/p1 site [46.54.82 (M/W)] replicated at 41% of the rate of WT virus (Fig. 4d). In contrast to that, once the variant acquired an additional mutation in the p1/p6 cleavage site [46.54.82 (M/M)], it replicated at 92% of the rate of WT HIV-1. When the viruses were grown in the presence of 10 nM Indinavir (Fig. 4e), 46.54.82 (M/W) and 46.54.82 (M/M) grew at the same rate as in the absence of the drug. In the presence of 100 nM Indinavir (Fig. 4f), the variants grew at 23 and 61% of the rate at which WT HIV-1 replicated in the absence of the drug. These results demonstrate that mutations in the cleavage sites of the enzyme restore functionality of the mutated protease.

Table 2 summarizes the IC_{50}s of the drug for the viruses. While the viruses with three amino acid mutations in protease (M46L, I54V, and V82A) and with mutations in the *gag* p7/p1 cleavage site resulted in a 10-fold increase in resistance to Indinavir (Table 2), viruses with two mutations in protease (M46L and V82A) were not significantly resistance to Indinavir compared with WT HIV-1.

DISCUSSION

In the present study, we have monitored the response of a group of HIV-1-infected people who have received the protease inhibitor Indinavir for 59 to 75 weeks and demonstrated that the emergence of clinically significant resistance, as indicated by an increase in the levels of HIV RNA in plasma, requires mutations in both the protease gene and the *gag* p7/p1 protease cleavage site. Studies with infectious clones constructed to contain a similar series of mutations revealed that mutations in both the protease cleavage site and *gag* led to the most replication-competent viruses.

Resistance to Indinavir has been previously shown to require an accumulation of three or more mutations. The mutations that were reported to occur with the highest frequency were L10V, M46I, L63P, A71V, V82T, and I84V (5). We have also

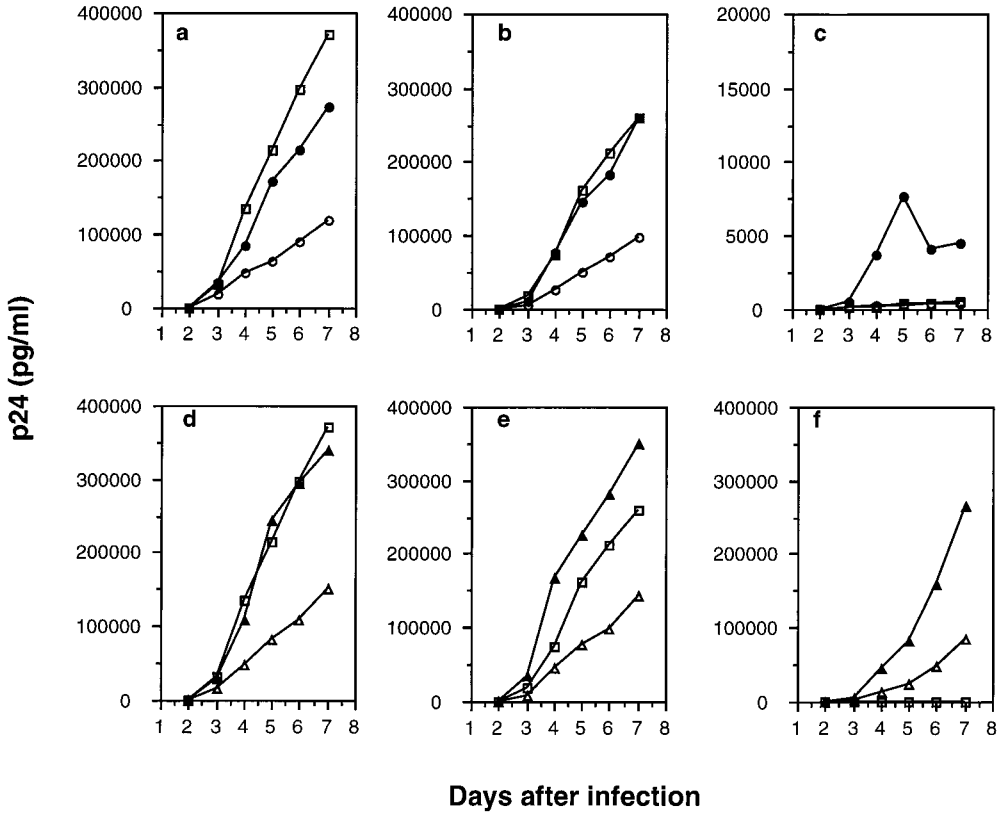

FIG. 4. Growth kinetics of recombinant HIV-1 variants. MT-2 cells were infected with recombinant viruses in the presence of 0 nM (a and d), 10 nM (b and e), and 100 nM (c and f) Indinavir. Virus growth was monitored daily by quantitation of p24 antigen in the culture supernatants. Open squares show the WT protease and WT p7/p1 and p1/p6 cleavage sites [WT (W/W)]. Open circles show the virus containing amino acid substitutions M46L and V82A in the protease and WT at both cleavage sites [46.82 (W/W)]. Solid circles show the variant containing M46L and V82A and A-to-V substitution in the p7/p1 [46.82 (M/W)]. Open triangles show three mutations in the protease M46L, I54V, and V82A and the A-to-V substitution at p7/p1 [46.54.82 (M/W)]. Solid triangles show the same three mutations in the protease and also mutations in both cleavage sites [46.54.82 (M/M)]. The results are representative of at least three independent experiments (see Table 2).

observed a progressive accumulation of mutations within the protease gene, but there appears to be a stable structure that arises when four or five mutations have accumulated, and there does not appear to be a continued strong selective pressure for the accumulation of additional changes. In the present study, the major changes that were observed in the protease gene of viruses characterized during long-term Indinavir therapy were M46L/I, I54V, A71V/T, V82A/F, and L90M. Amino acid substitutions at positions 10 and 63 have also been observed in drug-resistant virus isolates. While changes at these positions may alter the properties of protease, they are also seen in the protease of virus populations that have not been exposed to protease inhibitors (21, 27, 33, 35).

When we monitored the changes in the structure of protease over a period of 59 to 75 weeks, it was clear that there was an ordered accumulation of mutations. Initially, we saw either the M46L or the M46I mutation and the V82A mutation. These were usually followed by the I54V and/or the A71V/T mutation. While the L90M mutation was seen frequently, it was absent in one patient (patient 3) and was observed only very late in therapy in two (patients 4 and 5). An ordered appearance of mutations was also shown to occur during Ritonavir therapy (25). Here, V82A/T/F arose first, followed by I54V, A71V/T, M36I/L, and I84V. With that drug, the M46I/L/V mutation does not occur with either high frequency or early during therapy.

A distinct pattern of mutations was observed in patient 7. In that patient, an A71T change was present at time zero. The predominant changes observed during therapy were G73S and L90M substitutions. The M46I and V82A mutations were not seen until after the time when virus levels has rebounded in the patient. The G73S mutation that was detected early has not been seen in any of the other patients we have monitored. Among the group of six patients whose virus isolates became drug resistant, there were significant differences in the amino acid compositions that were present prior to the start of therapy. When 10 isolates from each of these patients were analyzed prior to therapy and sorted phylogenetically, sequences from each individual were contained in a separate, single clade (18a). These differences that existed prior to therapy were maintained during the 1.5 years of therapy and may be an important determinant of the pathway in which the viruses evolve. The finding that a single change at position 10 in protease, a position that is not associated with conferring drug resistance, changes the properties of a virus that is replication incompetent and enables it to grow highlights the potential biologic importance of even minor changes in the protease gene (27). The mutational changes that confer a significant growth advantage on a given virus are likely to be dependent on the background into which it is introduced. This may explain why alternate resistant forms of the virus evolve.

While the most obvious determining force directing the emergence of new viruses would be the progressive accumulation of mutations that confer successively higher levels of drug resistance (5, 25), the present study suggests that is not a major determinant in the early emergence of the M46L and V82A

changes. The IC_{50}s WT for HIV-1, 46.82 (W/W), and 46.82 (M/W) are all similar. Condra et al. have also shown that mutant viruses constructed with the M46I or the V82T protease substitutions or with the double mutant M46I and V82T were as susceptible to Indinavir as was WT NL4.3 (5). Even a slight enhancement in the growth rate can have a profound effect on the composition of the virus population (2). While viruses with mutations at positions 46 and 82 do not have higher levels of drug resistance, in the presence of 100 nM Indinavir, a concentration that is obtained in patients, if the 46.82 mutant virus also has a second-site mutation at the *gag* p7/p1 cleavage site, it would be rapidly selected (Fig. 4c).

Recently, Doyon et al. have described mutations in the *gag* p1/p6 cleavage site that were observed during in vitro passage of $HIV\text{-}1_{IIIB}$ in the presence of high concentrations of the protease inhibitors BILA 1906 BS and BILA 2185 BS. These mutations in the *gag* cleavage site appeared after the accumulation of multiple mutations in the protease gene (6, 9). In the present study of clinical isolates, the emergence of resistance, as manifested by a rapid increase in the levels of virus, was accompanied by changes in the *gag* p7/p1 cleavage site. These changes were seen in each of the six patients examined, and in contrast to the in vitro results, these changes occurred at approximately the same time as the emergence of mutations in the protease gene. In three of the patients, the mutations arose between 6 and 10 weeks after the start of therapy. It is unlikely that the findings that exactly the same amino acid change occurred in each of the six patients and that during in vivo replication the mutation occurred in the p7/p1 rather than in the p1/p6 *gag* cleavage site were random events. Rather, these findings suggest that this mutation conferred on the virus a significant growth advantage and that the gene products that are generated by cleavage at this site are rate limiting for virus production.

Changes in the protease gene are primarily responsible for the emergence of drug-resistant virus variants during Indinavir therapy. The occurrence of mutations in the *gag* p7/p1 and p1/p6 cleavage sites in patients undergoing protease inhibitor therapy is a second important factor in the emergence of HIV-1-resistant variants in patient populations. While this is still not clearly defined, we believe that differences in the protease gene that preexist among HIV-1 species, prior to the start of therapy, may also play an important role in determining the response to therapy.

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