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Genotype and Phenotype Patterns of Human Immunodeficiency Virus Type 1 Resistance to Enfuvirtide during Long-Term Treatment

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The human immunodeficiency virus type 1 (HIV-1) fusion inhibitor enfuvirtide has recently been introduced into clinical practice and has exhibited efficient anti-HIV-1 activity in combination with other antiretroviral agents. In the present study, we addressed the effect of long-term treatment with enfuvirtide on the intrahost evolution of HIV-1. The genotype and phenotype patterns and the relative replication capacity (rRC) of enfuvirtide-resistant HIV-1 mutants were evaluated in samples from 11 subjects (7 virological nonresponders and 4 responders) who received the compound for more than 1 year in combination with different regimens. Selection of one or more mutations clustering in a sequence (amino acids 36 to 45) of the gp41 N-terminal heptad repeat was observed in samples from the seven virological nonresponders but not in those from responders. In two subjects who discontinued enfuvirtide, reversion of the resistant genotype was detected within 3 months. Recombinant clones bearing mutated gp41 sequences displayed reduced susceptibilities to enfuvirtide, with the 50% inhibitory concentrations (IC_{50}s) ranging from 0.6 to 12.8 μg/ml, whereas the IC_{50} for isolates with baseline sequences was 0.013 ± 0.010 μg/ml. Interestingly, long-term monitoring of resistant variants provided evidence that ongoing adaptation to the drug is paralleled by phenotypic changes. A limited drop in the rRC in the absence of drug was observed for clones from four of the seven nonresponders bearing mutations associated with resistance. Overall, the data indicate that the different genotype patterns associated with a detectable degree of HIV-1 resistance to enfuvirtide generated during long-term treatments are characterized by a substantially low genetic barrier, possible ongoing adaptation with increased degrees of resistance, and limited influence on the viral rRC.

The envelope gp41 glycoprotein of human immunodeficiency virus type 1 (HIV-1) plays a crucial role in viral entry into target cells. This transmembrane glycoprotein undergoes conformational modification after the interaction of gp120 with the host cell receptors and drives the fusion of viral and cellular membranes. In particular, the N-terminal heptad repeat and the C-terminal heptad repeat (N-HR and C-HR, respectively) of gp41 are involved in the formation of the coiled-coil six-helical-bundle structure (18, 21, 22); this structure allows contact between the viral fusion peptide located upstream from the HRs in gp41 and the cell membrane (1, 13, 20). Inhibition of the gp41 conformational changes prevents virus-cell fusion and, consequently, HIV-1 infection of target cells (9). Enfuvirtide (formerly T-20), the prototype member of a new class of anti-HIV-1 compounds designated fusion inhibitors (FIs), is a 36-amino acid synthetic peptide that binds to a region of the HIV-1 gp41, thus preventing the conformational changes of this HIV-1 glycoprotein (9). During the last few years several FIs have been carefully studied at the preclinical and the clinical levels. Recently, enfuvirtide has exhibited efficient anti-HIV-1 activity in combination with other antiretroviral agents in vivo (2, 3, 7, 8, 11, 12). However, variants resistant to the drug have been seen to arise in vitro (17) and in vivo (15, 19). In this context and in the present perspective of the widespread use of FIs for the treatment of HIV-1-infected patients, a precise understanding of either the dynamic features of the selection of HIV-1 strains resistant to enfuvirtide and the impact of enfuvirtide resistance on viral biopathology not only are of theoretical importance but also are of medical importance.

In the study described here, we investigated the modifications of the genotype and phenotype patterns of HIV-1 susceptibility to enfuvirtide during long-term exposure to selective pressure with this drug. In particular, the selection of viruses with variations in the gp41-coding region, the enfuvirtide resistance phenotype conferred by sequences bearing mutations to recombinant clones (recombinant phenotype), and the analysis of the relative replication capacities (rRCs) of resistant variants selected in vivo in the absence of drug were evaluated. A recombinant assay allowing generation of HIV-1 gp41 chimeric variants was developed and optimized. The data document that (i) single amino acid substitutions in a crucial region of the viral gp41 glycoprotein are able to increase importantly the enfuvirtide 50% inhibitory concentration (IC_{50}) for recombinant clones, (ii) different genotype patterns are associated with a high level of resistance and revert within 3 months after drug discontinuation, (iii) ongoing gp41 adaptation with phenotypic influence occurs under the selective pressure of the compound, and (iv) the mutations for resistance to enfuvirtide have a limited influence on the viral rRCs.

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MATERIALS AND METHODS

Patients. Clinical samples from 11 patients enrolled in the TORO 2 study (12) were used in this study. The patients had received enfuvirtide (90 mg twice daily plus a background antiretroviral regimen optimized with the aid of resistance testing) for at least 1 year (range, 12 to 20 months) and were monitored at the Clinic of Infectious Diseases, San Raffaele Scientific Institute, Milan, Italy. After 54 weeks, four patients (patients 1 to 4) were considered virological responders (less than 400 HIV-1 RNA copy numbers per ml of plasma), and seven patients (patients 5 to 11) were considered nonresponders. Subjects 6, 8, and 11 showed a partial virological response (although HIV-1 viremia was always detectable) and a transient increase in $CD4^+$-T-cell counts 2 months after initiation of the regimen that included enfuvirtide. After 16 to 18 months, they were considered virological nonresponders due to increased plasma viremia levels (41,377, 167,489, and 72,713 molecules per ml, respectively); despite the rebound in the viral load, they maintained increased $CD4^+$-T-cell counts at the time of the last control evaluation. Two of the seven nonresponders (patients 5 and 7) discontinued the treatment with enfuvirtide, but sampling was continued for a few months afterward. The main findings for the two subgroups treated with enfuvirtide, including the levels of HIV-1 viremia (as the number of viral molecules per milliliter of plasma) and $CD4^+$-T-cell counts (as the number of cells per microliter of blood), are shown in Table 1.

Sequencing of gp41. Plasma samples collected at the enfuvirtide treatment baseline (before treatment) and during treatment were used in this study. RNA was purified from plasma samples or the supernatants of cell cultures transfected with replicating viral strains by use of a QIAmp RNA kit (Qiagen GmbH, Hilden, Germany), according to the instructions of the manufacturer. Ten microliters of purified RNA was reverse transcribed in a 25-μl reaction mixture containing 10 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, Calif.), 5 U of RNase inhibitor (Invitrogen), 1 mM deoxynucleoside triphosphates, and antisense primer (GGT GAA TAT CCC TGC CTA ACT CTA TT) in standard PCR buffer. The reaction mixture was incubated for 30 min at 37°C. Subsequently, 75 μl of a mixture containing 2 U of *Taq* polymerase (Finnzymzes, Espoo, Finland) and sense primer (TAG GAG CTT TGT TCC TTG GGT TC) in PCR buffer was added to the reaction mixture, and 45 amplification cycles were performed, with each cycle consisting of 94°C for 20 s, 55°C for 30 s, and 72°C for 90 s. The amplified products were run on a 10% polyacrylamide gel and directly added to 10 μl of the dye terminator cycle sequencing reaction mixtures (Perkin-Elmer, Boston, Mass.) with the same sense and antisense primers. The sequence was determined by capillary electrophoresis on an automated sequencing instrument (ABI 3100; Perkin-Elmer).

Cloning of the N-HR and C-HR regions of gp41. One-microliter aliquots of the amplified products from direct sequencing described above were used in new PCRs with nested primers (primer *gp41s* [TAT GGG CTG CAC GTC AAT TAC] and primer *gp41as* [CAC AGC CAA TTT GTT ATG TTA AAC C]), 1 mM deoxynucleoside triphosphates, and 2 U of a thermostable proofreading polymerase (New England Biolabs, Boston, Mass.) in order to obtain blunt-ended amplification products. The reaction was carried out for 35 cycles, with each cycle consisting of 94°C for 20 s, 55°C for 30 s, and 72°C for 90 s. These patient-derived amplified products were directly added to a ligation reaction containing 10 U of T4 DNA ligase (Promega, Madison, Wis.), 2.5 U of SnaBI enzyme (New England Biolabs), and 25 ng of a vector predigested with SnaBI. The ligation allowed reconstruction of the replication-competent HIV-1 molecular clone. Two microliters of the ligation reaction mixture was used to transform competent cells (JM109 cells; Promega) by the heat shock technique. After transformation, some bacterial colonies were passaged to a new plate, and new single colonies were screened by PCR with primers *gp41s*-gp120 (in the vector) and *gp41s-gp4as* (in the insert) in order to check for both the presence and the directions of the inserts. Positive colonies were grown in 5 ml of Luria-Bertani medium, and plasmids were extracted by use of a Miniprep kit (Promega), according to the instructions of the manufacturer. Correct cloning of the insert was verified by sequencing of the plasmid with the sense and antisense primers used for the amplification, as described above.

Construction of the pNLΔHR HIV-1 molecular clone. A molecular clone from which gp41 was deleted was used to generate recombinant HIV-1. To eliminate the flanking regions of pNL4.3 and render a small number of unique restriction sites within the provirus, the proviral sequence was amplified by a long PCR with a proofreading enzyme (*Pwo*; Roche, Basel, Switzerland) and cloned in the blunt-end SrfI site of the pCRScript vector. This molecular clone was completely sequenced to exclude unwanted mutations and was designated pNL*mod*. The replicative capacity of the molecular clone was also tested by transfection of pNL*mod* onto susceptible U87 cells and propagation (see below). Subsequently, pNL*mod* was further modified by replacing a portion of gp41 with a new unique blunt-end SnaBI site. In particular, to obtain the deletion of a 453-bp fragment (including the fusion peptide–N-HR–C-HR portion of the gp41 ectodomain) and the introduction of a new SnaBI restriction site, two partially overlapping segments of the gp120 and gp41 HIV-1 *env* gene were amplified with four primers: the sequences of the external primers were specific for the upstream BsaBI site (position 7549; BsaBI sense primer TCA TCA AAT ATT ACT GGG CTG CTA) and the HpaI restriction site (position 8650; HpaI antisense primer AAC AGC ACT ATT CTT TAG TTC CTG); the inner primers (SnaBI sense and antisense primers) were designed to be the reverse complement of the other and to contain (i) the original sequence of the virus in the 3′ half, (ii) a blunt-end SnaBI site in the middle, and (iii) again, the viral sequence across the gp41 ectodomain HR region at the 5′ end (sense version, GAG CAG CAG GAA GTA CGT ATA TAA AAT TAT TC). The SnaBI site was introduced by minimally modifying the nucleotide sequence and leaving the amino acid sequence intact. The BsaBI sense primer was used with the SnaBI antisense primer to amplify a portion of *env* upstream from the HR domains, while the SnaBI sense primer was coupled with the HpaI antisense primer to amplify a portion of

TABLE 1. Plasma viremia and $CD4^+$-T-cell counts of HIV-1-infected subjects undergoing combination antiretroviral treatment including enfuvirtide

Subject no.[a]	Sample time (mo)[b]	No. of HIV-1 RNA copies/ml	No. of $CD4^+$ cells/μl
1	0	72,720	110
	12	<50	207
2	0	94,528	146
	12	62	228
3	0	263,913	70
	13	<50	451
4	0	3,806,048	410
	15	127	685
5	0	189,024	17
	2	46,402	65
	9[c]	34,571	53
	17	64,000	45
6	0	1,339,604	149
	2	2,431	367
	16	41,377	337
7	0	554,658	5
	2	322,892	43
	11[d]	174,200	10
	13	140,000	10
8	0	517,247	186
	2	328	532
	18	167,489	614
9	0	50,060	98
	2	32,859	104
	13	59,302	57
10	0	288,430	29
	2	16,612	72
	20	40,800	172
11	0	150,526	109
	2	907	130
	20	72,713	288

[a] Subjects 1 to 4 were virological responders; subjects 5 to 11 were virological nonresponders.
[b] The baseline was time zero.
[c] Enfuvirtide was discontinued at month 11.
[d] Enfuvirtide was discontinued at month 12.

gp41 downstream from the HR domains. The point mutation introduced was conservative. Both amplified products were purified of the primers and used as templates in two different single-primer amplification reactions, each with a specific external primer (BsaBI and HpaI, respectively), in order to obtain single-stranded amplification products. Five microliters each of these reaction mixtures was incubated together in the same tube at 25°C for 1 h of annealing, and then the mixture was added to a standard PCR mixture with the external primers BsaBI and HpaI. Thirty-five amplification cycles were performed. Under these conditions, the separate but partially overlapping amplified products were joined and their products incorporated the deletion of the N-HR–C-HR sequence and introduced a novel SnaBI site in its place. The final amplified product was purified, restricted with BsaBI and HpaI, and ligated into the correspondingly cut pNL*mod* vector. Positive clones were subsequently raised and sequenced bidirectionally. One of them (pNLΔHR) was grown for plasmid purification; the modified HIV-1 molecular clone bore the deletion of the fusion peptide–N-HR–C-HR portion of the gp41 ectodomain, the introduction of a unique SnaBI restriction site across the splice site, and no other modification in the original viral sequence. Once this prototype clone was reconstituted by the insertion of exogenous HR sequences, replication competence was restored.

Recombinant phenotype for resistance to FIs. Five thousand U87 cells transgenic for CD4 and CXCR4 (6) were seeded in each well of round-bottom 96-well plates and cultured for 16 h. For each HIV-1 molecular clone recombinant for the HR domains and the parental NL4/3 clone, 30 ng of the plasmid was transfected onto the cells in each well by using the Lipofectin reagent (Life Technologies, Bethesda, Md.), according to the instructions of the manufacturer. Subsequently, two replicate wells for each recombinant were incubated in normal RPMI medium (to which 10% fetal calf serum was added); the others were incubated in duplicate in the same medium containing eight different concentrations of enfuvirtide and T-1249 obtained from one-half serial dilutions of the most concentrated preparation. For the nonmutated clones, the concentration range of both compounds used was 0.1 to 0.00078 μg of enfuvirtide/ml, while for the mutated clones the concentration range used was 25.6 to 0.2 μg/ml. Two concentrations of T-1249 (0.05 and 0.1 μg/ml) were also tested with the mutated clones. In each session, additional wells (again, in duplicate) were transfected with sensitive and resistant control clones incubated without inhibitors to check for cell-related variables. After 4 days of incubation, p24 antigen (p24Ag) was quantified in the supernatants by an immunoenzymatic assay (Du Pont de Nemours, Wilmington, Del.). Four days of incubation was chosen to satisfy two different criteria under these conditions: (i) the level of p24Ag production due to viral spread, replication, and new infections largely exceeded that due to the transient transfection, and (ii) the cytopathic effect destroyed only 70 to 80% of the cell culture, thus avoiding plateau effects. Susceptibilities were determined by calculating the IC_{50} for each viral recombinant clone by using the Systat computer software program (version 5.1) for the Macintosh personal computer.

rRCs of enfuvirtide-resistant molecular clones. The rRCs of gp41 recombinant viruses bearing sequences with enfuvirtide resistance mutations were evaluated in the absence of enfuvirtide. In particular, the level of p24Ag production by each recombinant clone (clarified cell supernatants) was measured 4 days after cell transfection in the absence of antiviral compounds, and the measurements were carried out under strictly controlled conditions (the viral DNA input was quantified, transfection was performed at 25% confluence to avoid cell overgrowth, experiments were carried out with triplicate samples, and standard deviations were less than 15%). The quantitative values obtained for each clone analyzed were then normalized (as a ratio) to those obtained with the reference NL4.3 sequence in parallel experiments according to the following function: $(\text{p24Ag-gp41}_{\text{sample}}/\text{p24Ag-gp41}_{\text{NL4/3}}) \times 100$. The rRC was expressed as the percent variation of the value obtained with the mutated sequences compared with that given by the baseline gp41 sequence for each subject.

RESULTS

HIV-1 gp41 sequences in samples from patients treated with enfuvirtide. Viral gp41 sequences were amplified from plasma samples collected at the baseline and at different time points during treatment. In the seven virological nonresponders, unlike in the four virological responders, amino acid mutations localized in a hot spot of the N-HR domain were shown to emerge during therapy with enfuvirtide (Table 2). As reported previously (15, 17, 19), the mutations clustered in a region (positions 36 to 45) known to be linked to enfuvirtide resistance. Position 38 was the most frequently mutated; mutations at positions 36, 37, 41, 42, 43, 44, and 45 also occurred in single subjects. In subjects 5 and 7 (Table 2), but not in samples from the other virological nonresponders (data not shown), resistance mutations were already present 2 months after the onset of treatment, although in all cases the mutation pattern later changed. Although the sequence of the C-HR region is usually less conserved than that of the N-HR region, other mutations were observed to appear downstream of the N-HR–C-HR junction and in C-HR in parallel with the resistance mutations described previously. In particular (Table 2), in subject 8, residue 72 changed from methionine (M) to leucine (L), residue 101 changed from threonine (T) to isoleucine (I), and residue 133 changed from asparagine (N) to serine (S). In subject 9, residue 138 changed from S to alanine (A). In subject 10, residue 113 changed from N to aspartic acid (D) and, subsequently, to glutamic acid (E). In subject 11, residue 114 changed from histidine (H) to glutamine (Q) (data not shown) and later to lysine (K). Also in subject 11, residue 119 changed from E to N, and residue 138 changed from S to A (which also occurred in subject 9). In addition to these mutations, a few other residues in the C-HR region not observed in the wild-type sequences also mutated during treatment, whereas no mutations were observed in the fusion peptide sequence. The double mutations detected in the isolates (mutations in the hot-spot region and the N-HR–C-HR junction) arose in close succession; thus, no direct indication of the possible compensatory role of changes in the N-HR–C-HR junction could be obtained. Finally, two patients (patients 5 and 7) discontinued enfuvirtide treatment after the selection of resistant viruses and were monitored for a few months afterward. Resistant variants (Table 2) disappeared from the plasma of these two patients 3 and 2 months after drug discontinuation, respectively.

Validation of recombinant assay. To test the possibility that phenotypic resistance to FI could be transferred by inserting the gp41 HR sequences from resistant strains into the SnaBI restriction site of the pNLΔHR clone, we amplified this portion from culture supernatants of three clones (derived from the HXBII molecular clone), kindly provided by P. R. Sista (Trimeris Inc., Durham, N.C.), two of which had developed resistance to enfuvirtide in vitro and had acquired mutations in the GIV motif (mutations G36S and V38M and mutations G36D and V38M); the amplified sequences were cloned into the replicative backbone. The original sequence of NL4/3 (characterized by G36D, a mutation of the GIV motif not associated with enfuvirtide resistance [17]) was also included. The resulting molecular clones were transfected onto U87 cells. In parallel, each viral isolate from the supernatants was raised and cultured on U87 cells. To establish the phenotypic sensitivity to enfuvirtide and T-1249, assays for determination of IC_{50}s were performed with viruses derived from both the recombinant clones and the corresponding isolates. Of note, the results for the recombinant viruses and their respective isolates completely overlapped (data not shown), indicating that replacement of the HR domains with exogenous sequences reliably reproduces the sensitivity characteristics of the strains from which those sequences were derived.

Recombinant phenotype of resistance to enfuvirtide and T-1249 conferred by patient-derived gp41-coding sequences.

TABLE 2. Sequence analysis of the HIV *env* gp41 region in samples from patients treated with enfuvirtide

Sequence type or subject no.	Samples time (mo)[a]	Sequence at position[b]: 30 • 40 • 50 • 60 • 70 • 80 • 90 • 100 • 110 • 120 • 130 • 140 •
Consensus B		LTVQARQLLS**GIVQQQNNLL**RAIEAQQHLLQLTVWGIKQLQARVLAVERYLKDQQLLGIWGCSGKLICTTAVPWNASWSNKSLDEIWDNMTWMEWEREIDNYTSLIYTLIEESQN
1	0	......L..T..................M.................I.....................S......T.....TQN...N.....Q..K...Y..G..........T
	12	......L..T..................M.................I.....................S......T.....TQN...N.....Q..K...Y..G..........T
2	0	....R.......................D.....N.TQ..NHT...D.........NI..N.L.Q...
	12	....R.......................D.....N.TQ..NHT...Q..K......NI..N.L.....
3	0	..............**H**..............M...Q....G........K......GI..N.L.K...
	13	..........**D**.................M...Q....G..A.....K......GI..N.L.K...
4	0	....R..............................NQ........Q.DK..N..ANT..K...Q...
	15	....R................................Q..N.T...Q.DK..N.H..T....L.Q...
5	0	....M...........................T..N.....Q.........G..........I
	2	............**A**...T..N.....Q.....................I
	9[c]	............**A**.....**M**...T..N.....Q....................I
	17	....F..................T..N.....Q....................I
6	0	.MA...L.............K......R..TYND........Q.........DT....L.K...
	16	............**A**.....**MP**...TYND..N.....Q.........DI..N.L.K...
7	0	................**S**...........M.................I......................N....T.....TQN...N.....Q..K......GV......Q...
	2	............**M**...**S**..**M**........M..N....T.....TQN...N.....Q..K......GV......Q...
	11[d]	................**T**..**M**........M...........................M.............N....T.....TQN...N.....Q..K......G.....L.Q...
	13	.....S......................M........................H................T....T....NTQNQ..N.....Q..K......GV......Q...
8	0	......L.......................................M....R......................T....R...Y........Q.DI..Y...G..HN.L.A..I
	12	......L....**M**.................................L....R............E..........I....R...Y..G.....Q.........G...N.L.....
	18	......L....A.................................L....R.......................I....R...Y..G.....Q..K......G...**S**.L.....
9	0	....S......Q.Q..H...........E...D.......K...
	2	.................**D**...S......Q.Q..H...........E...D.......K...
	10[e]	.................**D**...S......Q.Q..H...........E...D.......K...
	10[e]	...........**M**.....**D**..K..S......QNQ..H...........E...D.......K...
	13	...............**R**.**D**..K..S......Q.Q..H...........E...D.......K**A**..
10	0	............................M.................I....................................ED..N.....Q.DK......GI..N...Q...
	9	..........**D**.....**T**...........M.................I............................T.......N.........Q.DK...K..GI..N...Q...
	20	..........**V**.....**D**...........M.................I............................T...........**E**.....Q..K......GI..N...Q...
11	0	....T...L....W.............I.........T....R...D..NHT.........E.H.......L.Q.HD
	20	....T.......**A**.................................L....W.............I.........T.......ED..N**K**T...**N**.....E.........L.Q**A**HD

[a] The baseline was time zero.

[b] Boldface indicates residues of the N-HR gp41 domain relevant in HIV-1 resistance to enfuvirtide (positions 36–45) and single mutations of the N-HR–C-HR junction and C-HR domain detected in parallel with the primary resistance mutations.

[c] Enfuvirtide was discontinued at month 11.

[d] Enfuvirtide was discontinued at month 12.

[e] Different gp41 clones amplified from a single plasma sample.

TABLE 3. IC_{50}s of enfuvirtide and T-1249 for molecular clones obtained from clinical samples of virological responders and virological nonresponders

Subject no.	Time (mo)[a]	Mutation(s)[b]	Enfuvirtide IC_{50}	T1249 (μg/ml)	rRC[c]
1	0		0.015	<0.05	
	12				
2	0		0.027	<0.05	
	12				
3	0	Q39H	0.002	<0.05	
	13	G36D			
4	0		0.007	<0.05	
	15				
5	0		0.012	<0.05	100 (7.6)
	9[d]	V38A, L44M	3.4	<0.05	107 (15.4)
	17		0.015	ND[e]	ND
6	0		0.033	<0.05	100 (9.4)
	16	V38A, L44M, L45P	8.2	<0.05	23 (4.5)
7	0	N42S	0.027	<0.05	100 (4.6)
	11[f]	N42T, L45M	5.6	<0.05	96 (11.5)
	13		0.008	ND	ND
8	0		0.004	<0.05	100 (8.1)
	12	V38M	3.2	<0.05	ND
	18	V38A	4.1	ND	75 (7.5)
9	0		0.001	<0.05	100 (2.3)
	10	N43D	1.1	ND	98 (12.2)
	10	I37M, N43D	0.6	ND	50 (1.8)
	13	Q41R, N43D	4.2	<0.05	26 (3.1)
10	0		0.007	<0.05	100 (13.9)
	9	G36D, N42T	12.8	<0.05	95 (7.7)
	20	G36V, N42D	12.8	ND	98 (10.4)
11	0		0.018	<0.05	100 (9.2)
	20	V38A	4.6	<0.05	60 (5.9)

[a] From the baseline (time zero).
[b] Amino acid changes from consensus B sequence.
[c] rRC was calculated in the absence of antiviral compounds as the amount of HIV-1 p24Ag produced after 4 days of infection. For each subject, the replicative capacity is shown as a percentage of the p24 values obtained with the patient's own baseline HR sequence (set as rRC equal to 100%; values were calculated as the means of three independent experiments; standard deviations are given in parentheses.
[d] Enfuvirtide was discontinued at month 11.
[e] ND, not determined.
[f] Enfuvirtide was discontinued at month 12.

The HR regions were amplified from the plasma samples of the 11 enfuvirtide-treated subjects. All baseline viral sequences were assayed together with the follow-up sequences of samples from the seven subjects whose viruses had acquired mutations during treatment with enfuvirtide to compare the FI susceptibility of each mutated viral strain with that of the baseline parental strain. The amplified products were cloned into the pNLΔHR vector, and the recombinant clones were assayed for phenotypic resistance, as described above.

At the baseline, the enfuvirtide IC_{50}s for the 11 viral clones ranged from 0.001 to 0.033 μg/ml (mean, 0.013 ± 0.010 μg/ml) (Table 3). By contrast, all the mutated clones from the follow-up samples of the seven nonresponders displayed reduced susceptibilities to the drug, with the IC_{50}s ranging from 0.6 to 12.8 μg/ml. Interestingly, although most of the resistant strains bore mutations in the GIV motif (positions 36 to 38) (Table 2), in subject 9 maximum resistance was associated with Q41R and N43D mutations and in subject 10 maximum resistance was associated with N42T/D mutations. The residues whose mutations were associated with the strongest reductions in susceptibility were V38A, Q41R, N42D/T, N43D, L44M, and L45M ($P < 0.005$). Interestingly, long-term monitoring of three virological nonresponders showed evidence of ongoing adaptation to the drug, which was also reflected by phenotypic changes. In subject 8, V38M, found 12 months into treatment, changed to V38A after 6 months, with a detectable increase in resistance (the enfuvirtide IC_{50} increased from 3.2 to 4.1 μg/ml). For subject 9, two different mutated strains were selected in parallel and coexisted 10 months after the beginning of treatment (Table 2 and Table 3); both bore N43D, but the minor variant also incorporated I37M. An evident increase in resistance was subsequently associated in subject 9 with the selection of Q41R

(which appeared together with S138A), in addition to the mutations of the major variant, while the minor variant disappeared. In subject 10, prolonged treatment (20 months) induced the shift from a 36D/42T genotype (after 9 months) to a 36V/42D genotype 1 year later, without a detectable increase in resistance. Finally, enfuvirtide-resistant clones were also tested with two concentrations of T-1249 (0.1 and 0.05 μg/ml); the T-1249 IC_{50}s for the baseline and follow-up clones were always less than 0.05 μg/ml, thus underlining the lack of cross-resistance between the two inhibitors.

rRCs of recombinant variants bearing mutations for resistance to enfuvirtide. The rRCs of recombinant HIV-1 clones were assayed by analysis of viral growth kinetics in the absence of antiviral compounds by cell transfection under controlled conditions and final quantitative analysis of the level of p24Ag production by each recombinant at 4 days. All experiments were carried out in triplicate with baseline and follow-up samples of the seven virological nonresponders. The rRCs were calculated as a ratio of the amount of HIV-1 p24Ag released after 4 days of infection by each clone and that produced by a recombinant virus reconstituted with the enfuvirtide-sensitive gp41 of NL4.3. Under these assay conditions, the rRCs of recombinant viruses appear to reflect their relative fitness. Changes in rRCs were observed in four of the seven virological nonresponders tested (Table 3; subjects 6, 7, 9, and 11), while no variation was detected in three of the nonresponders. HR sequences bearing the single mutation at position 38 (subject 8, sample obtained at 18 months; subject 11, sample obtained at 20 months; Table 3) determine per se a high degree of resistance to enfuvirtide but only a limited reduction in rRCs.

DISCUSSION

The present study aimed at analyzing the process of selection of HIV-1 strains resistant to enfuvirtide and the impact of enfuvirtide resistance on viral biopathology. A recombinant assay was specifically developed and optimized to address the phenotype of resistance to enfuvirtide and T-1249. Mutations associated with enfuvirtide resistance were observed in samples from the seven virological nonresponders 12 to 20 months into treatment. By contrast, resistance mutations were not detected in the four patients who derived virological benefit from the treatment. The emergence of enfuvirtide resistance in treated subjects was documented phenotypically by variations in the IC_{50}s for recombinant viruses reconstituted with baseline and follow-up sequences. The data indicate that resistance to enfuvirtide is characterized by a low genetic barrier, since full-blown phenotypic resistance arises with single point mutations in a region localized in the first residues of the N-HR domain. A number of other mutations, not previously described, were also observed to appear in parallel with the resistance mutations in different portions of the gp41 HR domains. Further investigation is necessary to establish whether the latter mutations are associated with the recovery of gp41 function (compensatory mutations), since, in theory, mutations in the hinge region or in the C-HR domain of gp41 could compensate for the conformational changes induced by mutations in the N-HR domain. Of note, the study described here also documents ongoing HIV-1 adaptation to the drug during long-term exposure to enfuvirtide in three virological nonresponders. This adaptation was also reflected by phenotypic changes. Finally, reversion of the resistance genotype was observed in two subjects who discontinued the drug.

The recombinant assay developed in this study is based on a modified molecular clone, pNLΔHR. Potentially advantageous features of this recombinant assay are that (i) the short amplification product allows successful reverse transcription-PCR of virus from plasma even in the presence of very low levels of viremia (as documented by using the samples from virological reponders with low levels of viremia), (ii) neither the vector nor the amplification product needs to be purified before blunt-end cloning, and (iii) PCR-driven mutagenesis can easily be applied, when necessary. Several implications of the technical approach used in the present study, however, require specific attention. Recent research has indicated, in fact, that coreceptor specificity (principally, but not exclusively, defined by V3-loop sequences) and receptor density modulate viral sensitivity to enfuvirtide inhibition (16). By using either primary HIV-1 isolates or chimeric constructs, it was observed that higher concentrations of enfuvirtide are necessary to inhibit CCR5-tropic viral variants than CXCR4-tropic viral variants (4, 5). Since the coreceptor usage of the recombinant virus isolates may differ from that of the original viral strains (in particular, the NL4.3 strain used in the present study is CXCR4 tropic, while the viral strains selected during treatments are, in most cases, CCR5 tropic), the use of CXCR4-tropic model systems could theoretically result in overestimation of the sensitivity to enfuvirtide by recombinant methods compared to that of the parental viral isolates, as shown in different studies (4, 5, 10, 16). An additional aspect to be considered is that the single-cell-line system used in vitro cannot account for the complex range of coreceptor concentrations of different cell types and different functional states in vivo (16). In light of the complexity and multifactorial features of the HIV-1–target cell fusion process, these implications indicate that the comparative evaluation of the phenotype of gp41 recombinants generated by using a viral backbone and patient-derived sequences supplies a relative (not absolute) measure of drug sensitivity. However, the influence of coreceptor usage on HIV-1 sensitivity to enfuvirtide is independent from that of (much more relevant) gp41 sequence modifications (5); and comparison of the baseline and posttherapy recombinant viruses (the fold change in sensitivity that maintains the coreceptor used and the cell type throughout all experiments), as performed in the present study, may reliably reflect the variations of the original strains. This point is also confirmed in the present report by comparative analysis of the IC_{50}s for reference resistant mutant viruses and those for the corresponding gp41 recombinant clones.

Finally, a detectable reduction in the rRCs of recombinant viruses was observed in the absence of drugs among the viruses from four of the seven virological nonresponders, while no substantial modification was detected in three of them. Although the findings are limited to a small number of patients, the data suggest that even if enfuvirtide resistance can induce a relative drop in viral fitness in a proportion of patients, this feature is generally of a limited degree. This is particularly evident compared with the great changes in relative fitness observed in viral mutants resistant to protease inhibitors (14). Extensive competitive HIV-1 replication assays, including as-

says that use different cell lines and different viral constructs, could supply a precise understanding of the links between minimal fitness changes and the selection of enfuvirtide resistance mutations.

Overall, in the present perspective of the widespread use of FIs for the treatment of HIV-1-infected patients, the phenotypic analysis of viral resistance to this class of antiviral compounds is necessary not only to understand the role of single amino acid substitutions and those of their possible combinations but also to address the degree of HIV-1 resistance and to analyze the magnitude of the fitness changes (if any) determined by resistance mutations. In this context, the results obtained in the present study may have theoretical and practical implications, since they indicate that (i) development of HIV-1 resistance to enfuvirtide is characterized by a low genetic barrier, since a single point mutation in a crucial region increases significantly the IC_{50} of this compound for HIV-1 isolates; (ii) sequence evolution and adaptation is possible during long-term treatment with enfuvirtide, and this evolution may be paralleled by phenotypic changes; (iii) mutations in the polymorphic region encompassing the N-HR–C-HR junction and C-HR may be selected in parallel with enfuvirtide resistance mutations (it would be of interest to understand whether they are the simple expression of the polymorphic nature of this region or are actually linked to the development of the viral resistance to enfuvirtide); (iv) reversion of the resistant phenotype occurs rapidly after drug discontinuation; and (v) in addition, although substantial drops in the rRCs in the absence of compounds were observed in samples from four of six virological nonreponders, the low degree of change in rRCs detected for only a subset of the resistant clones does not allow us to consider this characteristic as a crucial aspect of the biology of enfuvirtide-resistant variants. Testing of more samples in longitudinal studies is certainly needed to clarify this aspect. Additionally, site-directed mutagenesis of gp41 HR sequences might contribute to gaining insights into the molecular mechanisms underpinning HIV-1 resistance to FIs.

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