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# Clinical Resistance to Enfuvirtide Does Not Affect Susceptibility of Human Immunodeficiency Virus Type 1 to Other Classes of Entry Inhibitors[▽]

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**The clinical use of the human immunodeficiency virus (HIV) fusion inhibitor enfuvirtide (ENF) can select for drug-resistant HIV-1 strains bearing mutations in the HR1 region of the viral envelope (Env) protein. We analyzed the properties of multiple Env proteins isolated from five patients who experienced an initial decline in viral load after ENF therapy followed by subsequent rebound due to emergence of ENF-resistant HIV-1. Prior to ENF therapy, each patient harbored genetically and phenotypically diverse Env proteins that used CCR5 and/or CXCR4 to elicit membrane fusion. Coreceptor usage patterns of the Envs isolated from two patients underwent homogenization following ENF therapy, whereas in the other three patients, recombination appeared to allow the introduction of a single HR1 sequence with ENF resistance mutations into phenotypically distinct Env proteins. Analysis of individual Env clones also revealed that prior to ENF therapy, there was sometimes marked heterogeneity in the susceptibility of individual Env proteins to coreceptor inhibitors. After virologic failure, all Envs acquired resistance to ENF but exhibited no consistent change in their sensitivity to the fusion inhibitor T-1249 or to coreceptor inhibitors. In summary, using patient-derived Env proteins, we found that ENF failure was associated with emergence of high-level resistance to ENF due largely to mutations in HR1 but that susceptibility to other entry inhibitors was unaffected, that in these late-stage patients there was greater clonal variability to coreceptor than to fusion inhibitors, and that recombination events in vivo could sometimes restore Env genotypic and phenotypic heterogeneity by introducing drug-resistant gp41 sequences into heterologous gp120 backgrounds.**

Current therapies for the treatment of human immunodeficiency virus type 1 (HIV-1) infection employ potent antiretroviral drugs that target reverse transcription of the viral RNA genome (RT inhibitors) and virion maturation (protease inhibitors) (44). Despite the potency of these antiretroviral agents, several complications exist that limit their efficacy in the clinic, including viruses resistant to one or more antiviral drugs (30). These issues highlight a need for the development of drugs that target other aspects of the viral life cycle.

Recent advances in the field of viral entry have led to the development of antiviral agents that target several discrete steps in the viral entry process, a number of which are in clinical trials (37). Use of these entry inhibitors will complement and diversify current treatment regimens and increase the prospect for durable treatment of HIV-1 infection. However, the use of these new drugs is complicated by the fact that they target, either directly or indirectly, the highly variable viral envelope (Env) protein. Thus, the efficacies of entry inhibitors are likely to differ considerably within the patient population, depending upon both host and viral factors (24, 48).

Several entry inhibitors are currently in clinical trials, with enfuvirtide (ENF [Fuzeon/T-20]) having been licensed by the FDA in 2003. ENF is a 36-amino-acid synthetic peptide that corresponds to residues 127 to 162 of the HR2 domain in the gp41 subunit of the HIV-1 Env protein. ENF binds to the HR1 domain of gp41, which is exposed following CD4 binding (14, 17, 35). After coreceptor binding, the HR1 and HR2 regions of gp41 interact with each other and form a six-helix bundle structure that is necessary for fusion of the viral and cellular membranes (60). ENF interrupts the fusion process by competitive interaction with the HR1 domain, thereby preventing the formation of the fusogenic six-helix bundle (7, 13).

As with other antiretroviral drugs, entry inhibitor therapy can select for resistant HIV-1 strains. Indeed, viruses resistant to ENF have been selected for in vitro (12, 45) and isolated from patients (16, 25, 31, 34, 36, 52, 59). In these reports, substitutions in the HR1 domain (residues 36 to 45) were typically observed. Since six-helix bundle formation requires interactions between the HR1 and HR2 domains, compensatory ENF resistance-associated mutations might be anticipated in the HR2 domain as well and are in fact sometimes observed (2, 32, 39, 54, 61). In addition, it is possible that new resistance pathways will emerge in vivo that are not observed, or that are rarely observed, in vitro. If so, it will be important to assess the implications of in vivo-derived ENF resistance on viral sensitivity to other classes of entry inhibitors and on viral tropism and pathogenesis.

We analyzed Env proteins isolated from five treatment-experienced patients prior to ENF treatment and at a time after virologic failure. There was considerable phenotypic variability among Envs isolated from each patient prior to ENF therapy with regards to their ability to use CCR5 and/or CXCR4 to elicit membrane fusion. Consistent with this variability in gp120 sequences, there was sometimes considerable variation

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in the sensitivity of individual Envs to coreceptor inhibitors. In contrast, there was little clonal variability in the sensitivity of Envs to fusion inhibitors that target the more highly conserved gp41 region. After virologic failure, all Envs from all patients exhibited resistance to ENF, though this was not associated with any consistent change in sensitivity to coreceptor inhibitors or to the fusion inhibitor T-1249. In two patients, the virus population appeared to pass through an evolutionary bottleneck as all Envs isolated from these individuals were genotypically and phenotypically similar. In three other patients, it appeared that in vivo recombination events restored Env heterogeneity by introducing a single ENF-resistant HR1 genotype into heterologous Env backgrounds.

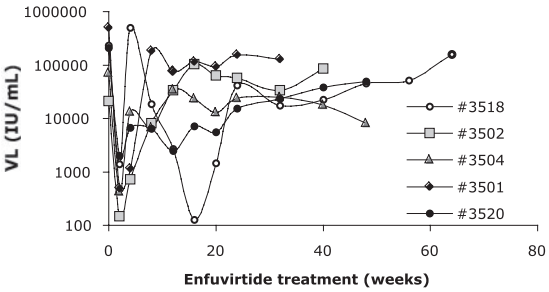


FIG. 1. Viral load profiles of patients under enfuvirtide therapy. All five patients in the study experienced a temporary, though significant, decline in viral load (VL) following ENF therapy, followed by a full rebound presumably owing to the emergence of ENF-resistant virus. Viral load was determined using a branched DNA assay as described previously.

## MATERIALS AND METHODS

**Subject samples.** All subjects participated in a prospective study in which subjects with highly resistant HIV received a regimen containing an optimized background regimen and ENF (3). Eligible subjects had detectable viremia and a screening genotypic/phenotypic resistance assay demonstrating resistance to nucleoside analogues, nonnucleoside reverse transcriptase inhibitors, and protease inhibitors. From this study, we identified subjects who exhibited a potent but transient response to an enfuvirtide-based regimen. Samples obtained before enfuvirtide treatment and during early and late virologic failure were selected for further investigation. All subjects provided written informed consent for participation in this study.

**PCR amplification and cloning full-length *env* genes from patient plasma.** Viral RNA was extracted from 140 μl of blood plasma using the QIAamp viral RNA mini kit (QIAGEN) and recovered in 80 μl. cDNA was synthesized from 9 μl of viral RNA using Thermoscript reverse transcriptase (Invitrogen) as per the manufacturer's directions in a final reaction volume of 20 μl. Full-length *env* genes were amplified by a nested PCR strategy using a thermostable polymerase that possesses proofreading 3′-to-5′ exonuclease activity. The cDNA synthesized from viral RNA (2 μl) was amplified in a reaction mixture containing 0.3 μM each of outer primers (sense, 5′-ATGGCAGGAAGAAGCGRAGACAG-3′; antisense, 5′-KGTGTAGTTMTGCCAATCWGGGAARWAGCCTTGYG-3′), 300 μM each of the four deoxynucleoside triphosphates, buffer containing 1 mM $MgSO_4$, and 2.5 U of Platinum *Pfx* DNA polymerase (Invitrogen). A 5-min hot start at 94°C was performed, followed by 20 cycles of 94°C for 30 s, 50°C for 30 s, and 68°C for 3 min. A final extension was performed for 7 min. One-tenth of the reaction product was used in a second reaction with inner primers (sense, 5′-CACCGAATARBNHAAAGAGCAGAAGACAGTGACCATGAVAGYGA-3′; antisense, 5′-TTTTGACCAYTTGCCACCCAT-3′) with 25 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 2.5 min. Again, a 7-min final extension was performed. The degenerate nested PCR primers used above were designed based on clade B *env* sequences from the HIV Sequence Compendium 2003 to allow amplification from a wide range of primary patient samples.

PCR products were gel purified (Zymoclean; Zymo Research Laboratories) and cloned into pcDNA 3.1D directional TOPO vector (Invitrogen) according to the manufacturer's directions. The resulting constructs were transformed into *Escherichia coli* XL-2 competent bacteria and grown at 30°C to minimize recombination and bacterially induced mutagenesis within *env*.

**Cell-cell fusion assay.** Cell-cell fusion was assayed as described in detail previously (46). QT6 cells cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% L-glutamine were used for this assay. Effector cells, infected with a T7 polymerase-encoding vaccinia virus (vTF1.1) (1) and transfected with Env expression plasmids, were added to target cells cotransfected with CD4, coreceptor expression plasmids, and a luciferase reporter construct under the control of a T7 promoter. Cell-cell fusion of Env and receptor-expressing cells was detected by assaying for T7 polymerase-driven luciferase expression.

**Inhibition assay.** Fusion inhibition assays were performed by applying serial dilutions of the appropriate drug to the target cells prior to addition of effector cells. ENF was obtained from Trimeris. CMPD167 was obtained from Merck, vicriviroc was synthesized, and AMD3100 was obtained from the AIDS Reagent Repository.

**Phylogenetic analysis.** For each patient, the phylogenetic relationships between the pre- and posttreatment *env* clones were studied, to identify the pretreatment clone that was most closely related to the posttreatment *env* clones and which would be used for subsequent mutagenic analyses. The sequence alignments were produced using CLUSTAL_W (56) and checked manually for accuracy. The phylogenetic trees were drawn using the PHYLIP package version 3.64 (11). Briefly, the trees were obtained using parsimony and distance methods. SEQBOOT was used to create a data set containing 1,000 bootstrap replications of the original sequence alignment. This data set was analyzed by the parsimony method (DNAPARS). A consensus tree of the data set was then created using CONSENSE and analyzed by the distance method neighbor joining, as described in the package documentation. The trees were rooted with a closely related HIV-1 strain chosen by performing a BLAST search with one of the pretreatment *env* genes. Trees were visualized using TREEVIEW, version 0.5.0 (38).

**Mutagenesis.** Site-directed mutagenesis was performed using specific oligonucleotides and the Quikchange site-directed mutagenesis kit (Stratagene). The entire *env* gene was sequenced after each round of mutagenesis to ensure the presence of the desired mutations and the absence of any second-site mutations.

## RESULTS

**Isolation and coreceptor usage of full-length *env* clones from before and after the development of ENF resistance.** Five treatment-experienced subjects were identified who exhibited a potent (greater than 1 log) but transient response to an ENF-based regimen (Fig. 1). All subjects had advanced disease at the time ENF was initiated (median CD4 count of 82 [range, 3 to 129] and HIV RNA levels ranging from 20,823 to 500,000). Samples were collected both immediately prior to the start of ENF therapy (termed pretreatment samples) and at a time after drug failure while still under ENF therapy (termed posttreatment samples).

We isolated viral RNA, synthesized cDNA, and amplified *env* genes from the selected samples. Due to the variable nature of the Env glycoprotein, we isolated at least 10 independent clones from each plasma sample, each from an independent PCR. No two *env* genes were identical, and phylogenetic analyses showed that *env* genes from each patient were clearly related (i.e., no contamination). The amplified PCR products were cloned into a pcDNA3.1D-TOPO expression vector via topoisomerase I-mediated cloning, and Env function was assessed by performing cell fusion assays in which target cells expressed CD4 and CXCR4 or CD4 and CCR5. We found that approximately 70% of the cloned Envs were capable of eliciting cell-cell fusion and that considerable diversity was observed in the coreceptor usage patterns of individual clones derived from the pre-ENF treatment samples for all patients (Fig. 2). To compare results between experiments, the fusion activity of each Env was normalized. Since all Envs derived from patients

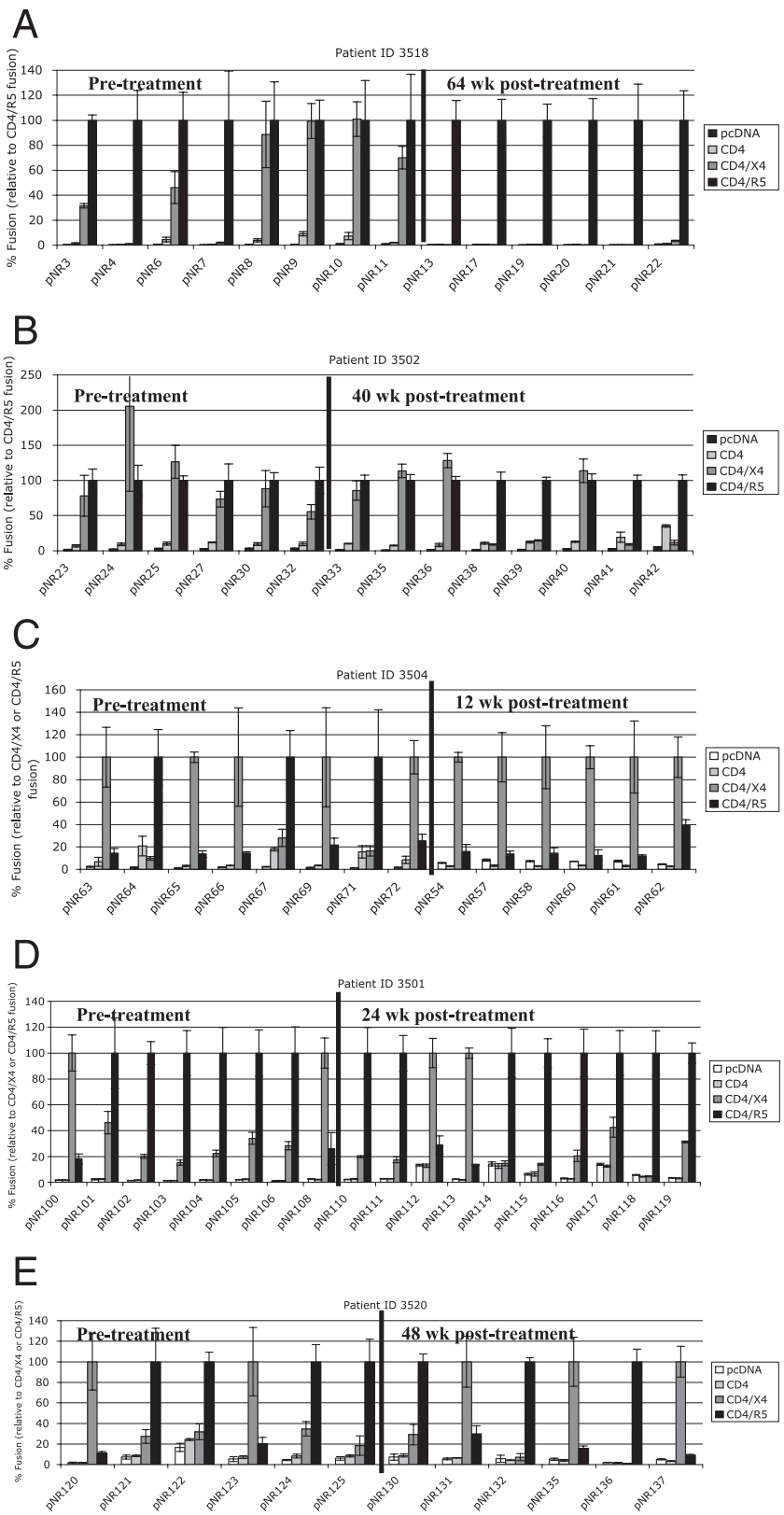


FIG. 2. Fusogenicity and coreceptor usage of patient *envs*. Cell-cell fusion assays were performed with cloned *env* genes from patients 3518 (A), 3502 (B), 3504 (C), 3501 (D), and 3520 (E). Target quail QT6 cells were transfected with empty plasmid (pcDNA) or were transfected with plasmids expressing CD4 alone, CD4 and CXCR4, or CD4 and CCR5 as indicated. In each panel, pretreatment clones are to the left of the vertical black line and posttreatment clones are to the right. The number of weeks following ENF therapy is indicated for the posttreatment clones. For patients 3518 and 3502, all Envs used CCR5, while some also used CXCR4. For these patients, the amount of fusion obtained when cells expressed CD4 and CCR5 was set to 100% for each Env. For the remaining three patients, some Envs used CCR5, some used CXCR4, and some used both coreceptors. Thus, for each Env, we set to 100% the amount of fusion obtained with the coreceptor that was used most efficiently by any given Env. Error bars represent the standard error of the mean of at least three independent experiments.

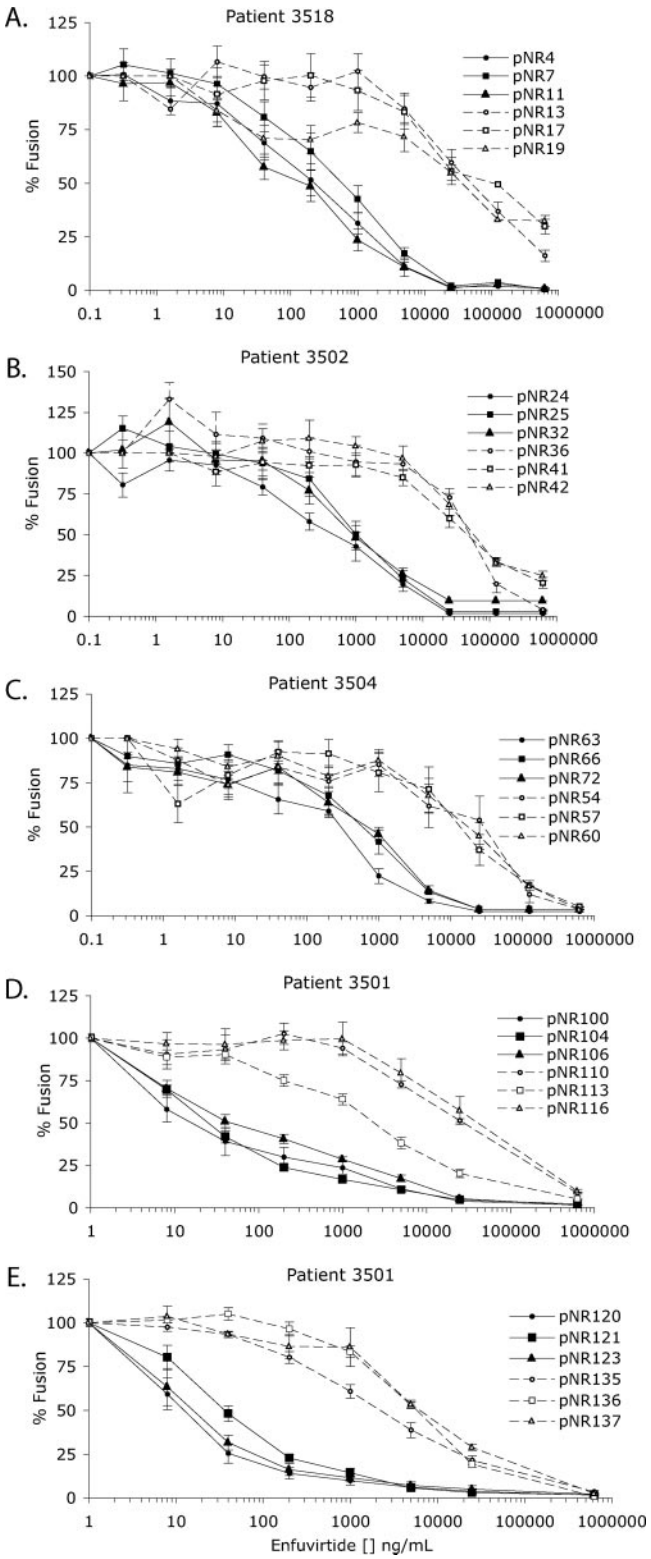


FIG. 3. ENF sensitivity of patient *env* genes. Fusion inhibition assays were performed using serial dilutions of ENF to assess drug sensitivity of pre- and posttreatment *env* genes cloned from patients 3518 (A), 3502 (B), 3504 (C), 3501 (D), and 3520 (E) on QT6 cells expressing CCR5 (for R5 or dualtropic Envs) or CXCR4 (for X4-using Envs). Three pretreatment (solid lines and closed symbols) and posttreatment (dashed lines and open symbols) clones were chosen for each patient to represent the range of ENF sensitivities observed in a given sample. Results are expressed as a percentage of fusion in the absence of ENF and represent the average ± standard error of the mean of at least three independent experiments.

3518 and 3502 used CCR5, the amount of fusion obtained when cells expressed CD4 and CCR5 was set to 100% for each Env. For the remaining three patients, some Envs used CCR5, some used CXCR4, and some used both coreceptors. Thus, for each Env, we set to 100% the amount of fusion obtained with the coreceptor that was used most efficiently by any given Env. Since the ability of an Env protein to mediate fusion with cells expressing a specific coreceptor on cell lines does not necessarily imply that it can use that coreceptor to mediate infection of primary cells (51, 58, 62, 63), the designation of an Env protein as having an R5X4 phenotype is somewhat arbitrary and subject to assay-dependent differences between laboratories. For the purposes of this study, we defined R5 Envs as those that used CXCR4 less than one-third as well as CCR5 in the cell fusion assay and X4 Envs as those that used CCR5 less than one-third as well as CXCR4. Using this definition, then, of the five patient samples obtained prior to ENF therapy, one (patient 3502) harbored only R5X4 Envs, two (patients 3518 and 3504) harbored a mixture of R5 and X4 Envs, and two (patients 3501 and 3520) contained a mixture of R5, R5X4, and X4 Envs (Fig. 2).

We found a marked decline in phenotypic and genotypic diversity in the Env clones obtained from two of the five patients after virologic failure, though ENF therapy was continued due to improved CD4 counts. In patient 3518, the pretreatment quasispecies was comprised of R5X4 and R5 Envs, whereas after treatment, the Envs were all R5. When gp120 sequences from the V1-to-V3 region were compared, Envs prior to treatment were on average 93.2% identical at the nucleotide level, while after treatment, Envs were 99.7% identical in this region. Similarly, for patient 3504 the pretreatment sample contained X4 and R5 Envs, while the posttreatment resistant samples were comprised primarily of X4 Envs. Genetic diversity decreased from 92.4% to 99.2% identity in gp120. In contrast, phenotypic diversity was maintained in patients 3502, 3501, and 3520 following ENF therapy and failure. Likewise, there was little change in genetic diversity in the pre- and posttreatment Envs from these patients (94.4% versus 94.5% posttreatment in patient 3502, 90.8% versus 93.5% in patient 3501, and 92% versus 92% in patient 3520). However, this diversity was not accounted for by differences in ENF sensitivities. As shown in Fig. 3, the posttreatment Envs in all five patients showed similar, high-level resistance to ENF in cell fusion assays using cells expressing CD4 and CCR5, with 50% inhibitory concentration ($IC_{50}$) values 2 to 3 logs higher than those for the baseline pretreatment Envs. Similar results were obtained when cells expressed CD4 and CXCR4 (data not shown). In addition, for R5X4 Envs, the degrees of ENF sensitivity were similar in both CCR5- and CXCR4-expressing target cells (data not shown).

**Consequences of ENF resistance on sensitivity to other entry inhibitors.** To examine the effects of ENF resistance on sensitivity to other entry inhibitors, we performed fusion inhibition assays in the presence of T-1249 (a more potent peptide

fusion inhibitor than ENF that also binds to the gp41 HR1 domain) (15), vicriviroc (SCH-D) (53) and CMPD167 (57) (both CCR5 inhibitors), and AMD3100 (a CXCR4 inhibitor) (10, 49). We have found that the fusion inhibition assay accurately reflects results obtained from virus infection experiments and affords a quantitative and high-throughput approach to study primary Env proteins that are pseudotyped poorly or not at all. As shown in Fig. 4, pre- and posttreatment Envs from all patients tested exhibited similar levels of sensitivity to the fusion inhibitor T-1249, consistent with our previous studies examining the effects of ENF resistance in vitro on sensitivity to T-1249 (42) as well as in vivo data from a short-term phase 1/2 T-1249 study (26). Thus, clinical resistance to ENF was not associated with any significant changes in T-1249 sensitivity.

In assessing the sensitivity of the pre- and posttreatment R5 and R5X4 Envs to the CCR5 inhibitors CMPD167 and vicriviroc, we observed up to a 2-log variability in the amount of these inhibitors needed to prevent fusion mediated by Env clones derived from the same patient sample (Fig. 4). While variability in sensitivity to CCR5 inhibitors has been observed between viruses obtained from different patients (24, 48), we are not aware of studies that have examined the sensitivity of individual Env clones taken from a single patient, at a single time point, to this class of entry inhibitors. This variability in R5 inhibitor sensitivity was observed in both pre- and post-ENF treatment samples, did not correlate with the acquisition of ENF resistance, and stood in marked contrast to the lack of variability seen in ENF and T-1249 sensitivity between Envs obtained from the same patient sample. In addition, in some cases we observed differences in the relative sensitivity of Env clones to vicriviroc and CMPD167. For example, in patient 3502 the pre-ENF treatment clones that were highly sensitive to CMPD167 were often less sensitive to vicriviroc, and vice versa (Fig. 4B and C). These findings are consistent with a report that in at least some instances resistance to one CCR5 inhibitor may not result in resistance to other CCR5 inhibitors (M. Westby, C. Smith-Burchnell, D. Hamilton, J. Mori, M. Macartney, N. Robas, B. Irvine, M. Fidock, F. Peruccio, J. Mills, K. Burt, C. Barber, P. Stephenson, P. Dorr, and M. Perros, presented at the 12th Conference on Retroviruses and Opportunistic Infections, Boston, MA, 22 to 25 February 2005), a result that is perhaps not surprising given the differences in CCR5 conformations as well as the differences in how different Envs engage this coreceptor (4, 23, 29, 33, 47).

We also examined the sensitivities of the various X4 and R5X4 Envs to the CXCR4 inhibitor AMD3100. We found that ENF resistance did not have any significant effects on AMD3100 sensitivity (Fig. 4D). However, as with the CCR5 inhibitors, in some patients we observed clonal variability in AMD3100 sensitivity between Envs derived from the same patient sample. Moreover, in one patient (no. 3501), we observed high-level AMD3100 resistance in some of the pre- and posttreatment X4-using Env clones (pNR100 and pNR113; data not shown). If AMD3100-resistant Env clones are commonly found in late-stage patients, this could lessen the chance that X4 inhibitors will prove to be effective. Together, our results show the value of examining individual Env clones obtained at the same time point: rare clones that exhibit con-

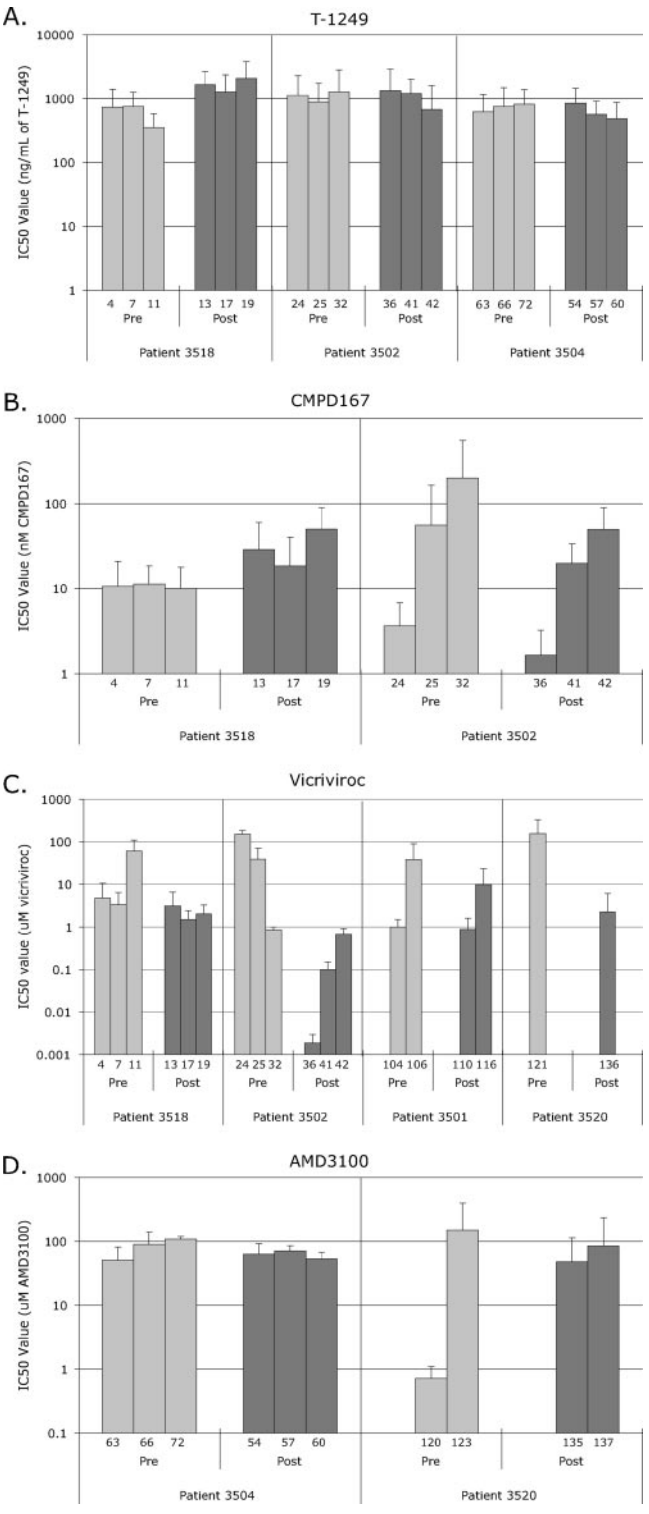


FIG. 4. Sensitivity of cloned *env* genes to other entry inhibitors. Fusion inhibition assays were performed using *env* genes cloned from all five patients to determine $IC_{50}$ values for T-1249 (A), CMPD167 (B), vicriviroc (C), and AMD3100 (D). For each patient, three pre- and three posttreatment clones were chosen to represent the range of ENF sensitivities observed in a given sample. The number under each bar refers to the pNR clone number of the Env being tested. CCR5-expressing QT6 cells were used in panels A, B, and C, and CXCR4-expressing cells were used in panel D. Error bars represent the standard error of the mean of at least three independent experiments.

siderable resistance to coreceptor inhibitors would be missed by assays that examine Envs in bulk.

**Evolution of ENF resistance-associated mutations.** Sequence analysis of ENF-resistant Envs always revealed one or more changes in HR1, while four of the five patients had single mutations in the HR2 region of gp41 as well. To assess the contribution of the HR1 and HR2 regions to ENF resistance, we engineered the HR1 and HR2 mutations from a given patient into a pretreatment clone from the same patient, both singly and in combination. To minimize possible context-dependent differences as well as to more easily identify mutations responsible for imparting ENF resistance, we used phylogenetic analyses to identify the pretreatment clone that was most closely related to the posttreatment *env* clones from patients 3518, 3502, and 3501, since these provided a good sampling of the different mutations observed in the five patients studied here.

Phylogenetic analysis for patient 3518 showed that the ENF-resistant R5 *env* clones clustered with the pretreatment R5 *env* clones derived from this patient, indicating that resistance evolved from circulating virus rather than emerging from a preexisting viral reservoir. In this patient, as in patient 3504, much of the genotypic and phenotypic diversity present prior to ENF treatment was lost, as drug resistance arose in a single Env phenotype (R5 for patient 3518 and X4 in patient 3504). More complex branching patterns were observed for the remaining three patients, in which ENF resistance was associated with at least two Env phenotypes. For illustrative purposes, the bootstrapped maximum parsimony tree from one of these patients (no. 3520) based on gp120 sequences is shown in Fig. 5. This analysis suggests that the ENF-resistant X4 Envs evolved from pretreatment X4 Envs, while the ENF-resistant R5 Envs evolved from pretreatment R5 Envs. Taken at face value, this suggests that the ENF-resistant R5 and X4 lineages evolved independently. Similar branching patterns were observed for patients 3501 and 3502, in both cases suggesting that ENF resistance arose independently in Envs exhibiting different coreceptor usage patterns. However, in patients 3501 and 3520, all ENF-resistant *env* clones shared exactly the same HR1 mutations at both the amino acid and nucleotide levels. While there was some variability observed in the HR1 mutations identified in *env* clones from patient 3502, this variability was not linked to coreceptor usage patterns. Given the diverse array of amino acid and nucleotide changes that can be associated with ENF resistance, we consider it unlikely that precisely the same nucleotide changes would arise on more than one occasion in the face of ENF treatment and failure. An alternative explanation that we favor is that a recombination event occurred between an ENF-resistant and ENF-sensitive virus in these patients, with the crossover event occurring after the V3 region (which largely accounts for coreceptor usage patterns) and prior to the HR1 region in gp41. If so, then Env phenotypic and genotypic diversity can sometimes be rapidly reconstituted following drug failure. Indeed, recombination between viruses resistant to RT and protease inhibitors and viruses that are drug sensitive has been well documented (6, 8, 22; Westby et al., presented at the 12th Conference on Retroviruses and Opportunistic Infections, Boston, MA, 22 to 25 February 2005).

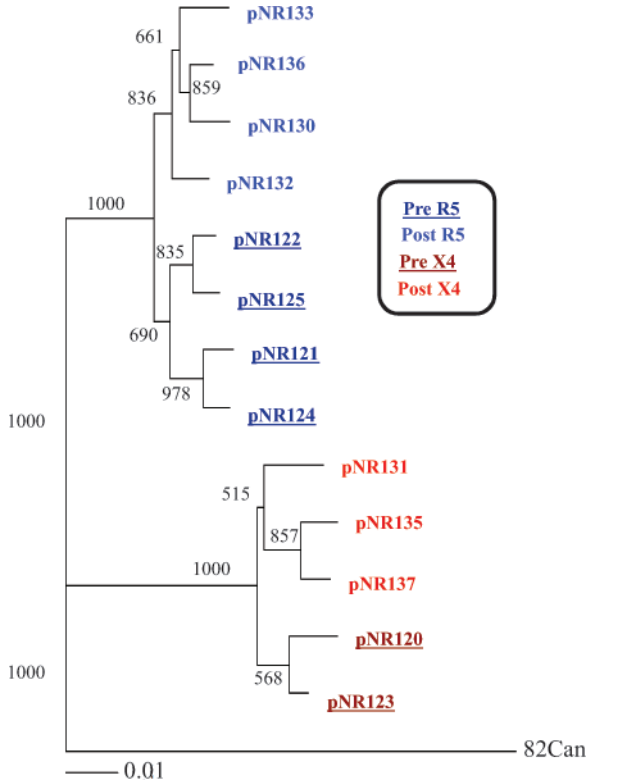


FIG. 5. Phylogenetic tree for patient 3520. A phylogenetic tree was constructed as described in Materials and Methods using parsimony and distance methods and gp120 sequences. The bootstrap values shown are from 1,000 replications of the original data set. The tree was rooted with a closely related HIV-1 strain chosen by performing a BLAST search with one of the pretreatment *env* genes.

**Mutagenesis of the HR region.** The HR1 and HR2 mutations observed in the ENF-resistant clones are shown in Table 1, while the panels of mutants we generated between the Env pairs selected from patients 3518, 3502, and 3501 are listed in Table 2. All mutations were introduced singly into a closely related ENF-sensitive protein isolated from the same patient, and double or triple combinations of these mutations were also generated. The resulting Envs were confirmed to be fusogenic using a cell-cell fusion assay, and their ENF sensitivities were assessed in fusion inhibition assays (Fig. 6).

For patient 3518, the N43D mutation (numbering according to HXB2 gp41 sequence) in HR1, when introduced singly into the pretreatment Env, increased ENF resistance by ~100-fold while the Q66R mutation in HR1 caused a 25-fold increase in $IC_{50}$ (Fig. 6). The N43D Q66R double mutant and the N43D Q66R S138A triple mutant were both almost as resistant to ENF as the posttreatment Env pNR13. This implies that the HR1 region mutations alone, in the absence of any changes in gp120, accounted for ENF resistance in this patient.

As shown in Fig. 2B, all of the pretreatment Envs from patient 3502 were R5X4 using and the posttreatment Envs were a mix of R5X4- and R5-using clones. Phylogenetic analysis of patient 3502 Env sequences showed that the R5X4- and R5-using posttreatment Envs emerged from two different subsets of the pretreatment R5X4 Envs (data not shown). The posttreatment R5X4 clones were most closely related to the

TABLE 1. Enfuvirtide resistance-associated mutations

| Patient | No. of wk of enfuvirtide treatment | gp41 mutation | Frequency of clones (no./total) |
|---|---|---|---|
| 3518 | 72 | N43D | 6/6 |
| | 72 | Q66R | 6/6 |
| | 72 | S138A | 6/6 |
| 3502 | 36 | V38A | 7/8 |
| | 36 | N42T | 3/8 |
| | 36 | N42D | 1/8 |
| | 36 | N43D | 1/8 |
| | 36 | E110H | 2/8 |
| | 36 | N126K | 5/8 |
| | 36 | L130I | 7/8 |
| | 36 | G215E | 3/8 |
| 3504 | 4 | V38A | 3/9 |
| | 4 | V38M | 3/9 |
| | 4 | N43D | 3/9 |
| | 12 | G36S | 1/6 |
| | 12 | N43D | 6/6 |
| 3501 | 24 | Q40H | 5/5 |
| | 24 | N42S | 5/5 |
| | 24 | Q56R | 5/5 |
| | 24 | N125D | 5/5 |
| 3520 | 48 | V38A | 6/6 |
| | 48 | N157D | 6/6 |

TABLE 2. Site-directed mutagenesis of the gp41 HR region

| Mutant plasmid name | Mutation |
|---|---|
| Patient 3518 (pretreatment backbone clone pNR4) | |
| pNR73 | N43D |
| pNR74 | Q66R |
| pNR75 | S138A |
| pNR76 | N43D Q66R |
| pNR77 | N43D S138A |
| pNR78 | Q66R S138A |
| pNR79 | N43D Q66R S138A |
| Patient 3502 | |
| Pretreatment backbone clone pNR25 | |
| pNR80 | V38A |
| pNR81 | N42D |
| pNR82 | E110H |
| pNR83 | N126K |
| pNR84 | L130I |
| pNR85 | G215E |
| pNR86 | V38A N126K |
| pNR87 | V38A L130I |
| pNR88 | V38A G215E |
| pNR89 | V38A N126K G215E |
| Pretreatment backbone clone pNR30 | |
| pNR90 | V38A |
| pNR91 | N42T |
| pNR92 | N126K |
| pNR93 | L130I |
| pNR94 | V38A N42T |
| pNR95 | V38A N126K |
| pNR96 | V38A L130I |
| pNR97 | N42T N126K |
| pNR98 | N42T L130I |
| pNR99 | V38A N126K L130I |
| Patient 3501 (pretreatment backbone clone pNR104) | |
| pNR150 | Q40H |
| pNR151 | N42S |
| pNR152 | Q56R |
| pNR153 | N125D |
| pNR154 | Q40H N125D |
| pNR155 | Q40H Q56R |
| pNR156 | Q56R N125D |
| pNR157 | Q40H Q56R N125D |

pretreatment clone pNR25, whereas the posttreatment R5 clones seemed to have emerged from pNR30. As outlined in Table 2, we made two different panels of mutants based on patient 3502 Envs. The first panel recapitulated the mutations present in the posttreatment Envs of the R5X4 branch, and the second panel represented the R5 branch. In the R5X4 branch mutant panel, the V38A mutation in HR1 elicited a strong increase in ENF resistance, while the N126K mutation conferred a more moderate increase in ENF resistance (Fig. 6B). The V38A N126K double mutant was indistinguishable from the posttreatment Env pNR35. In contrast, the L130I and G215E single mutations did not impact resistance to ENF to any significant level, though in combination with the V38A mutation, G215E appeared to marginally increase ENF resistance.

The R5 branch mutant Envs that we constructed contained the V38A, N42T, N126K, and L130I mutations and various combinations thereof. The V38A mutation again had the greatest impact on ENF resistance. The N42T and N126K single mutations had marginal effects on resistance to ENF, but in combination their effects were additive (approximately eightfold). The V38A N126K double mutation resulted in the highest increase in ENF resistance (500-fold over the pretreatment clone pNR30); however, this mutant was still ~5-fold more sensitive than the posttreatment Env pNR38.

Envs constructed from patient 3501 contained combinations of the Q40H, N42S, Q56R, and N125D mutations. The N42S mutation had no significant effect on ENF sensitivity by itself or in combination with the other mutations (data not shown). As shown in Fig. 6D, the Q40H mutation resulted in the most marked increase in ENF resistance (~50-fold over the pretreatment clone pNR104). The Q56R and N125D single mutations had little to no effect on ENF sensitivity. However, the mutant containing the Q56R mutation in combination with Q40H was as resistant to ENF as the posttreatment clone pNR116. Thus, in the mutant Envs examined here from all three patients, single HR1 mutations typically resulted in significant increases in ENF resistance, though full drug resistance typically required several amino acid changes, and in one instance required a mutation in HR2 (N126K) as well.

## DISCUSSION

The emergence of drug-resistant HIV strains represents a significant clinical problem providing a strong rationale for the development of new classes of antiretroviral drugs such as entry inhibitors (reviewed in reference 43). There are several classes of entry inhibitors, including those that bind to the viral Env protein and prevent CD4 binding, those that bind to CCR5 or CXCR4, and those that bind to Env and prevent membrane fusion (5, 37, 40). While clinical resistance to ENF and in vitro-derived resistance to other classes of entry inhib-

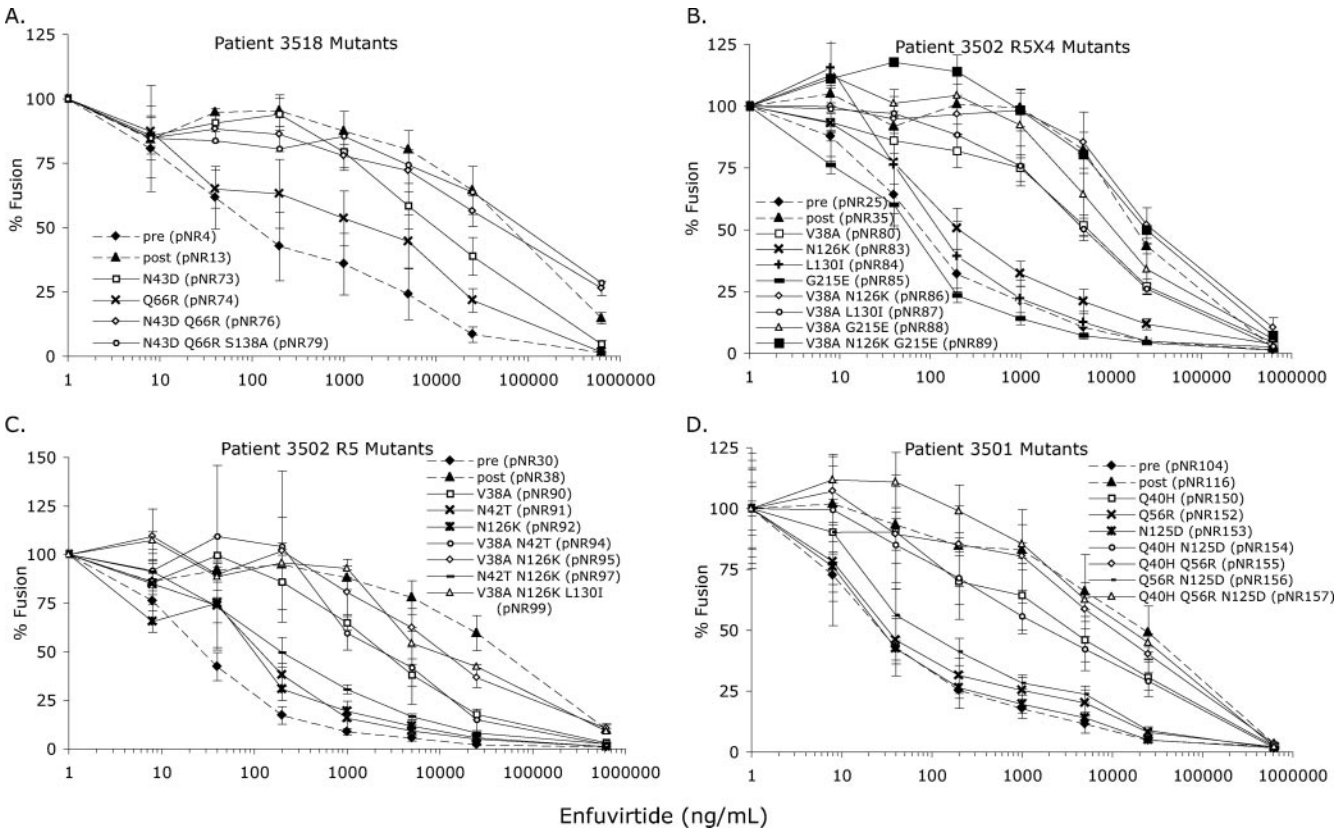


FIG. 6. ENF sensitivity of mutant Envs. Fusion inhibition assays were performed using serial dilutions of ENF to assess drug sensitivity of mutant *env* genes for patients 3518 (A), 3502 R5X4 branch (B), 3502 R5 branch (C), and 3501 (D) on QT6 cells expressing CCR5 (for R5 or dualtropic Envs) or CXCR4 (for X4-using Envs). The broken lines correspond to the inhibition curves for pre- and posttreatment Envs in each case, and solid lines are used for the mutant Envs. Results are expressed as a percentage of fusion in the absence of ENF and represent the average ± standard error of the mean of at least three independent experiments.

itors have been described (reviewed in references 5, 16, and 40), the full consequences of drug resistance are not well understood. Specifically, it is not yet clear if resistance to any given entry inhibitor will influence viral sensitivity to other entry inhibitor classes. In addition, by selecting for changes in the viral Env protein, resistance to entry inhibitors could influence HIV tropism and pathogenesis in ways not associated with resistance to RT and protease inhibitors.

An important finding from our study is that in vivo-derived resistance to ENF did not impact sensitivity to other classes of entry inhibitors. These observations confirm our earlier site-directed mutagenesis experiments which also revealed that ENF-associated mutations in the HR1 region of gp41 do not impact viral sensitivity to coreceptor and CD4 binding inhibitors (42). In addition, in vivo- and in vitro-derived resistance to ENF has not been associated with alterations in viral sensitivity to the fusion inhibitor T-1249, a finding confirmed in our study as well (26, 42). However, while evolution of ENF resistance in vivo is associated with a variety of HR1 mutations, mutations in HR2 and perhaps other regions of Env are also commonly selected, raising the possibility that cross-resistance to other entry inhibitor classes might arise. For example, mutations that enhance Env affinity for coreceptor can accelerate fusion kinetics and decrease susceptibility to both ENF and coreceptor inhibitors (41). However, at least with the Env clones we examined from the patients studied here, high-level resistance to ENF had no discernible effect on sensitivity to coreceptor inhibitors. This, in conjunction with our earlier work, suggests that patients who fail an ENF-containing regimen will remain candidates for other classes of entry inhibitors (42). However, it will be important to extend these findings by studying a larger number of patients.

While mutations that confer ENF resistance have no obvious, direct effect on viral sensitivity to other classes of entry inhibitors, a strong clinical response to ENF followed by virologic failure has the potential to generate a genetic bottleneck, leading to significant changes in viral (and Env) diversity that could indirectly influence subsequent responses to other antiretroviral agents. For example, genetic and phenotypic homogenization resulting from failed antiretroviral therapy could make the resulting dominant viral population more or less susceptible to different types of entry inhibitors by altering the relative proportions of R5 and X4 viral species. This might be an important consideration in the case of ENF, since this drug is most commonly used in a background of optimized highly active antiretroviral therapy for treatment-experienced patients (27, 28). These typically late-stage patients often harbor a mixture of viruses that can use CCR5 and/or CXCR4 (19). Our clonal analyses showed this to be true for the five patients studied here as well. If Envs are classified into R5, R5X4, and

X4 phenotypes based on their abilities to utilize the major coreceptors on cell lines, then the patients in our small cohort always harbored at least two of these Env types. Such phenotypic diversity could be important for viral pathogenesis since it could provide viral quasispecies capable of entering primary cell types that differ in their expression levels of CCR5 and CXCR4.

The five patients studied here initially responded well to ENF, though all subsequently failed therapy, with virus loads returning to near baseline levels (Fig. 1). However, examination of the virus load alone sometimes masked significant changes in viral diversity. In two of the patients, antiretroviral therapy appeared to result in a genetic bottleneck as circulating virus decreased by at least 2 logs before subsequently rebounding. In these individuals, Envs cloned after drug failure were genetically and phenotypically similar, being all R5 in one patient and predominantly X4 in the other (Fig. 2A and C). Thus, while virus load returned to near baseline levels, the predominant circulating type of virus was different. In some instances, such a response might influence subsequent antiretroviral therapy. For example, patient 3518 had an appreciable level of X4 activity prior to ENF therapy, but had only R5 Envs after failure, perhaps making this individual a better candidate for therapy with CCR5 inhibitors.

In cases where we saw heterogeneous coreceptor usage posttreatment (e.g., patients 3502, 3501, and 3520), sequence analysis of the different clones from these samples showed identical ENF-associated mutations in R5- and X4-using Envs, even at the nucleotide level. Moreover, identical silent mutations were present in all of the resistant clones of some patients. This outcome is most consistent with recombination having occurred between drug-sensitive and drug-resistant strains, with the net effect being the restoration of genotypic and phenotypic diversity. While we cannot rule out the possibility that the apparent recombination observed in patients 3502, 3501, and 3520 resulted as an artifact generated during the PCR amplification step, there are several factors that argue against this explanation. First, we used the thermostable *Pfx* polymerase (55), which is similar to *Pfu* polymerase that exhibits significantly lower rates of recombination than *Taq* or *Vent* polymerases (50). Second, our PCR conditions called for long elongation times, which significantly disfavor recombination (21). Third, slow cooling between the denaturation and annealing steps leads to enhanced recombination because this favors annealing of incompletely elongated products to the template and elongation before the annealing temperature of the primer is reached (21), and our PCR conditions were designed to avoid such slow cooling steps. Finally, a number of detailed studies have shown that recombination occurs frequently in vivo and that as a result genetic bottlenecks imposed by antiretroviral therapy can sometimes be restricted to a gene segment—in this case, the HR1 and HR2 regions of gp41—while diversity is maintained in other regions of the viral genome (6, 8, 22; Westby et al., presented at the 12th Conference on Retroviruses and Opportunistic Infections, Boston, MA, 22 to 25 February 2005). It will be interesting to determine how frequently ENF therapy and subsequent resistance results in the loss of phenotypic and genotypic diversity in Env. One factor that may limit recombination frequency is the fact that R5, R5X4, and X4 Envs infect somewhat different cell populations in vivo.

Our clonal analyses of *env* clones from a given patient sample also revealed unexpected variability in baseline sensitivity (up to 2 logs) of *env* clones from a given patient sample to vicriviroc, CMPD167, and AMD3100. In contrast, while differences in ENF sensitivity were seen between patients and between pre- and posttreatment samples, Envs isolated from the same patient at the same point in time exhibited only modest variability in their sensitivity to ENF or to the fusion inhibitor T-1249. This difference may reflect the fact that the ectodomain of gp41 is far more highly conserved than gp120, which is the region of Env primarily responsible for coreceptor interactions. A logical prediction is that clonal variation in coreceptor inhibitor sensitivity will be greatest when Env diversity is greatest. If this in turn increases the likelihood of drug failure due to the presence of minority species that are relatively drug resistant at baseline, this may provide a rationale for using coreceptor inhibitors in patients when viral diversity is low. This highlights the value of clonal analyses in gaining deeper insights into the heterogeneous nature of the viral quasispecies, particularly in the context of the emergence of drug-resistant mutants.

Finally, our mutagenesis studies show that single-amino-acid changes in HR1 and HR2 accounted for ENF resistance in the patients studied here. While in vitro studies have shown that determinants in gp120 can influence viral sensitivity to ENF (9, 18, 41), such variation appeared to play no or a minimal role in the evolution of clinical resistance to ENF. Full resistance to ENF could typically be imparted to closely related pretreatment Envs through the introduction of one to three amino acid changes in HR1 and, sometimes, in HR2. While the role of mutations in HR1 in the development of ENF resistance is well understood, the contribution of changes in HR2 to drug resistance is not clear. Theoretically, these mutations have been predicted to play a compensatory role in the presence of HR1 mutations by enhancing the stability of ENF-resistant gp41 (20). It is possible that HR2 mutations may play a more significant role in the restoration of viral fitness, since uncompensated mutations in HR1 have been shown to slow fusion kinetics and reduce viral fitness, at least in vitro (32). Studies investigating the compensatory role of these HR2 mutations and their effect on fusion kinetics are currently under way.

In summary, by examining *env* clones from patients that have failed ENF therapy, we have gained important insight into the evolution of clinical resistance to ENF. In some patients, there appeared to be an evolutionary bottleneck and a loss in Env phenotypic and genotypic diversity that could impact subsequent therapy with other entry inhibitors. In other patients, recombination events restored Env genetic and phenotypic variability. It will be interesting to determine how frequently, when selective pressure is applied against a region of Env, the emergence of drug resistance mutations is also associated with recombination events that impart resistance to multiple viral types. Our clonal analyses also revealed unexpectedly high variability in sensitivity of individual Envs to entry inhibitors that target gp120, in contrast to ENF and T1249, which target a conserved region in gp41. Such baseline variability could influence resistance pathways in these treatment-experienced individuals. Finally, we have provided evidence that clinical

resistance to ENF does not affect sensitivity to other entry inhibitors and that point mutations in HR1 and HR2 alone are responsible for this resistance. Studies currently under way, investigating the possible compensatory role of HR2 mutations, will shed more light on the precise molecular mechanisms of clinical resistance to ENF.

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