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The Viral Envelope is a Major Determinant of Enhanced Fitness of a Multidrug-Resistant HIV-1 Variant

Hiroshi Mohri, MD, Nicole Prada, PhD^a, and Martin Markowitz, MD

The Aaron Diamond AIDS Research Center, an Affiliate of The Rockefeller University, 448 First Avenue, 7th Floor, New York, NY10016, USA

Abstract

Multi-drug resistant (MDR) HIV-1 viruses are thought to be less pathogenic than wild type viruses due to the fitness costs of drug-resistance mutations. However, we identified an individual infected with MDR virus associated with rapid disease progression referred to as MDR-1. To study the contribution of virologic factors to rapid disease progression, we constructed molecular clones that demonstrated high replication fitness and cytopathicity. To dissect determinants of enhanced fitness of a cytopathic clone, pMDR-1c, we divided its genome into two parts, the envelope (gp160) and the remaining backbone genome and constructed mutual chimeric viruses with a reference, wild type virus clone, pNL4-3. The growth competition assay indicated that pMDR-1c has high fitness (1.62), while its envelope confers remarkably enhanced fitness (2.29), and its backbone confers reduced fitness (0.56) as compared to pNL4-3. We also performed a similar study with a less cytopathic pMDR-5a, a molecular clone derived from another subject MDR-5, infected with MDR HIV-1 and associated with slower clinical progression. The results indicated that pMDR-5a has reduced fitness (0.82), while its envelope confers enhanced fitness (1.64), and its backbone confers reduced fitness (0.49), a fitness pattern compatible with envelope-mediated fitness compensation. These results suggest that the viral envelope may be a major determinant of the enhanced fitness of the MDR HIV-1 variant isolated from a patient with rapid disease progression. Furthermore we speculate that compensation conferred by envelope may be a mechanism by which MDR-HIV-1 maintains overall fitness despite the presence of changes in pol, which reduce replication capacity.

Address correspondence to: Martin Markowitz, MD, The Aaron Diamond AIDS Research Center, 448 First Ave., 7th Floor, New York, NY10016, USA, mmarkowitz@adarc.org, Tel.: 212-448-5020, Fax: 212-725-1126.

^aCurrent address: Antiviral immunity, Biotherapy and Vaccine Unit, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris cedex 15, France
hmohri@adarc.org (H. Mohri), nicoleprada@hotmail.com (N. Prada)

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Conflict of interest: The authors do not have a commercial or other association that might pose a conflict of interest.

Nucleotide Sequences of MDR HIV-1 Clones

The nucleotide sequences of 4 clones have been submitted to GenBank with the accession numbers of KF990605 (pMDR-1c), KF990606 (pMDR-1b), KF990607 (pMDR-1a) and KF990608 (pMDR-5a).

Keywords

determinant of enhanced fitness; multidrug-resistant HIV-1; envelope-mediated fitness compensation; molecular clone; envelope-chimeric virus; growth competition assay

INTRODUCTION

In 2005 we reported a case of dual-tropic multidrug-resistant (MDR) HIV-1 infection associated with rapid disease progression.¹ In general, MDR viruses are considered less fit and less pathogenic than drug-sensitive wild type viruses due to the fitness costs conferred by the emergence of drug-resistance associated mutations.²⁻⁵ Along similar lines, MDR viruses are believed to be less transmissible than wild type viruses.^{6,7} Nonetheless, this individual (MDR-1) was infected with an MDR virus, and progressed to AIDS within as few as 4 and up to 20 months post-infection. Subsequently, we reported that select biologically clonal isolates and a bulk viral isolate demonstrated very high replication rates, syncytium-forming activity and enhanced cytopathicity in vitro¹ suggesting that viral factors were at least in part responsible for the observed rapid disease progression.

To study the potential viral determinants of the observed phenotype, we developed a strategy to generate biologically relevant, full-length molecular clones. We constructed three infectious molecular clones from this case, pMDR-1a an R5-tropic variant, pMDR-1b a dual-tropic variant, and pMDR-1c a second dual-tropic variant with very rapid replication kinetics. In addition, we constructed a less cytopathic MDR virus clone, pMDR-5a, an R5-tropic variant with slow replication kinetics and multidrug resistance isolated from a patient (MDR-5) with slower disease progression. To determine the gene(s) responsible for the enhanced replication fitness of the MDR variant, despite the presence of drug resistance mutations, we constructed chimeric viruses of a rapidly replicating and cytopathic clone, pMDR-1c, with a drug sensitive, wild type pNL4-3⁸ and with a slower replicating pMDR-5a. We characterized these chimeras and performed growth competition assays to understand determinants underlying the replication advantage of the MDR virus variants isolated from the HIV-1 infected individual with rapid disease progression.

METHODS

Patients infected with MDR HIV-1 associated with rapid and slow disease progression

Individuals infected with HIV-1 were identified and evaluated by the Clinical Program of the Aaron Diamond AIDS Research Center and gave written informed consent for study under a Rockefeller University Hospital Institutional Review Board approved observational protocol. The rapidly progressing individual (MDR-1) from whom pMDR-1 clones were constructed has been well described.¹ By contrast, subject MDR-5 presented in April 2006, approximately 10 months post-infection was found to be infected with MDR-HIV-1 with triple class resistance (Table 1). He exhibited slower disease progression when compared to subject MDR-1 (Table 2) and elected to initiate therapy approximately 3.5 years post infection. He was treated with fixed dose combination tenofovir disoproxil fumarate/

emtricitabine, efavirenz, ritonavir-enhanced darunavir and raltegravir, and an excellent response to therapy was observed (Table 2).

Construction of molecular clones

A strategy to construct relevant molecular clones was developed (Suppl. Fig. 1). The supernatant of the primary end-point culture, i.e., a biologically clonal isolate, was inoculated onto PHA-stimulated HIV-1 uninfected peripheral blood mononuclear cells (PBMC), from which genomic DNA was purified and used for the molecular cloning. A complete LTR fragment was amplified and cloned into a vector (designated as pLTR). Then, the remaining HIV-1 genome with the length of ~9.1 kb was amplified and cloned into a corresponding pLTR. Resulted clones with full-length HIV-1 genome were screened for the same infectivity and replication rates as of their parental isolates using TZM-bl cell⁹ and PBMC cultures. The detailed procedure is described in Suppl. text 1.

Sequence analysis

Sequencing reactions were performed by Genewiz (South Plainfield, NJ). Multiple, bidirectional sequences were aligned to obtain a full-length genome sequence using MegAlign within Lasergene V6 (DNASTAR, Madison, WI). The sequences of clones were analyzed by RIP¹⁰ for HIV-1 subtype determination and recombination detection, N-Glycosite¹¹ for putative N-glycosylation sites in envelope (<http://www.hiv.lanl.gov>). Drug resistance mutations were detected by utilizing Stanford University HIV Drug Resistance Database (<http://hivdb.stanford.edu>).¹² For phylogenetic analysis, the full-length genome sequences were aligned with subtype reference sequences using ClustalW, and the phylogenetic tree was constructed by Maximum Likelihood Method in MEGA5.¹³

Construction of envelope chimeras

We divided the HIV-1 genome into two parts, the envelope (gp160) gene and the remaining backbone genome, and constructed envelope chimeras (Fig. 2A). Chimeras were designated as follows; the backbone strain followed by the gene replaced and its origin in parentheses. For example, pNL4-3(envMDR-1c) indicates a chimeric virus with the backbone of pNL4-3 and the env gene derived from pMDR-1c. Note that the envelope gene (6225-8795, in HXB2) also codes a part of vpu (6225-6310), the second exons of tat (8379-8469), rev (8379-8653) and rev response element (RRE). The detailed procedures for constructing each env-chimera are indicated in Suppl. Text 1.

Replication kinetics assay

Viruses and chimeric viruses were produced upon transfection of 293T cells with plasmid DNA and their infectivity was measured by end-point dilution culture with PHA-stimulated, uninfected donor-derived PBMC as described earlier.¹⁴ To determine the replication kinetics 1,000 TCID₅₀ of each virus was used to infect 2×10^6 PHA-stimulated PBMC unless otherwise indicated. Cultures were washed after 6 hours, and then maintained for 7-8 days. The supernatant was collected daily for p24 antigen determinations with Coulter HIV-1 p24 antigen assay kit (Beckman-Coulter, Jersey City, NJ). At the end of culture, infected PBMC

were stained for CD3, CD4 and CD8, and the CD4+/CD8+T cell ratio was used as an indicator of the cytopathicity of each virus.¹⁴

Growth competition assay

The growth competition assay is a sensitive, quantitative and reliable assay that provides a relative fitness value.¹⁵⁻¹⁷ Growth competition cultures between 2 viruses were performed in 2 wells; one well with PBMC from one donor and the other with PBMC from a second donor to ensure that results were not dependent on a unique source of PBMC. The same 2 sources of PBMC were used for all competition experiments. Combinations of two viruses, each at 1,000 TCID₅₀ were inoculated to 2×10^6 PHA-stimulated PBMC in 1.5 ml of RPMI1640/10%FCS supplemented with 20U/ml of IL-2 (Roche Applied Science, Indianapolis, IN). On day 1, infected PBMC were washed twice by low-speed centrifugation and resuspended in prewarmed new culture media. The culture was kept until day 4, when 50 µl of the supernatant was transferred to a newly prepared PHA-stimulated PBMC culture. The competition culture was maintained until day 16 with the virus mixture passaged every 4 days. Culture supernatants were harvested every 2 days for viral RNA concentration determinations.

Virion-RNA copy numbers of two viruses in the culture supernatant were determined by the gene-specific real-time PCR assays, which targeted either V1 region of envelope gene or nef gene. The detailed procedures are described in Suppl. text 1. The ratios of HIV-1 RNA copy numbers of virus 1 over virus 2 were converted into natural log (ln) and used to determine the best-fit slope with 95% confidence intervals using Prism 4 (GraphPad Software, Inc. La Jolla, CA). This slope was defined as 'log relative fitness (LRF)'¹⁸ and used for the calculation of $w_2/w_1 = e^{\text{slope}}$, where 'w1' and 'w2' were net growth rates (or the fitness) for virus 1 and virus 2, respectively. To compare fitness among different viruses, we determined fitness value relative to a reference, wild type virus clone pNL4-3. Thus, a relative fitness value indicates the change in the ratio of copy numbers of a virus over NL4-3 in each day of the culture. Relative fitness was also defined for HIV-1 subgenomes, where we assumed that the relative fitness of a whole virus is a product of the relative fitness of subgenomes.

RESULTS

Construction of infectious molecular clones

According to our strategy (Suppl. Fig. 1), we have constructed three full-length infectious molecular clones, pMDR-1a, -1b and -1c from the MDR HIV-1 isolates (MDR-1a, -1b, -1c),¹⁴ and one infectious molecular clone, pMDR-5a, from the less cytopathic MDR HIV-1 isolate (MDR-5a) (Table 2, Suppl. Fig. 2A&B). The replication kinetics and cytopathicity of the molecular clones were equivalent to their parental isolates (Fig. 1A-E). Co-receptor tropisms of the molecular clones were identical to their parental isolates¹⁴ (Table 1, data not shown).

Sequence characteristics of MDR-1 clones

The nucleotide sequence alignment and the construction of a phylogenetic tree of the molecular clones along with subtype reference sequences indicated that all three MDR-1

clones were of subtype B (Suppl. Fig. 3), and their mean sequence distance was 0.8%, which is consistent with a minimum genetic diversity shortly after transmission (83 days).

All open reading frames are intact in the clones constructed. Some of their genetic characteristics are indicated in Table 1. Drug resistant mutations of the molecular clones are identical with their parental isolates,¹⁴ except for pMDR-1b, which was derived from one of two variants within the isolate, MDR-1b. Accordingly, pMDR-1b carries the same PR mutations as of the original isolate MDR-1b, but only M41L and T215D in the reverse transcriptase gene. All MDR-1 clones shared N348I in RT, which confers a low-level reduction in susceptibility to zidovudine (AZT) and nevirapine (NVP).¹⁹ Deduced Gag amino acid sequences revealed changes at the cleavage sites including A431V at p7/p1^{20,21} and P453L at p1/p6²² in all three clones. Mutations within matrix protein including R76K and Y79F are reported to reduce the susceptibility to PI and improve viral replication fitness.²³

The differences in deduced amino acid sequences in gp120 were primarily within the variable regions. In the V3 loop, pMDR-1a had a glycine whereas both pMDR-1b and -1c had an arginine at position 11, being consistent with their coreceptor usage.²⁴ In gp41, the N651F/I mutation was observed within the HR-2 region and has been reported to be associated with enhanced fusion and increased viral pathogenicity.²⁵ Enhanced fitness has been thought to be associated with increased positive charges in gp120.²⁶ However, the net charge of gp120 did not directly correlate with the fitness values among these envelopes (Table 1).

Construction of envelope chimeras

We used pMDR-1c, a molecular clone with robust replication dynamics, dual tropism and enhanced cytopathicity, to construct envelope-chimeras with wild type virus, pNL4-3, and a less-cytopathic MDR variant, pMDR-5a (Fig. 2A, Suppl. text 1). The env-chimeras were all infectious, except for one chimera, pMDR-1c(envMDR-5a), which showed limited infectivity in TZM-bl cell culture (data not shown), but no infectivity in PBMC culture (Fig. 2B).

We determined the ratio of the infectious titer in PBMC culture (TCID₅₀/ml) over the p24 concentration (pg/ml) in each virus stock (Fig. 2C). TCID₅₀/p24 ratios of the original virus clones, pMDR-1c, pNL4-3 and pMDR-5a were 0.93, 0.66 and 0.36, respectively. TCID₅₀/p24 was reduced in the NL4-3 envelope chimera, pMDR-1c(envNL4-3) by 0.19-fold (0.18), while enhanced in two MDR-1c envelope chimeras, pNL4-3(envMDR-1c) by 2.4-fold (1.61) and pMDR-5a(envMDR-1c) by 2.8-fold (1.01) (Fig. 2C, Suppl. Table 2). These results suggested that infectivity per virion in the respective viral stock was in large part determined by envelope, and that the envelope of pMDR-1c gave the highest infectivity/p24 ratio when inserted into all three viral backbones. Note that all virus stocks were produced, stored and titrated for infectivity simultaneously thereby controlling experimental conditions and minimizing procedure-induced variation in determining infectious titers.

Growth competition assays

To determine the relative fitness of viruses/chimeras and their subgenomes, we set up 8 different growth competition cultures with various combinations of paired viruses (Fig. 3A). Changes in the fractional viral RNA copy numbers (%) over days of culture are shown for each competition culture. Apparently, pMDR-1c grew much faster than pNL4-3 (#1) or pMDR-5a (#2). The env-chimera, pNL4-3(envMDR-1c) grew faster than pNL4-3 (#3) or pMDR-1c (#4), whereas the env-chimera, pMDR-5a(envMDR-1c) grew faster than pMDR-5a (#5), but slower than MDR-1c (#6). In contrast, the env-chimera, pMDR-1c(envNL4-3) grew slower than pMDR-1c (#7), or pNL4-3 (#8). These results suggested that the fitness of envelope of pMDR-1c is higher than the envelope of pNL4-3 (#3, #7), or the envelope of pMDR-5a (#5). The fitness of backbone of pMDR-1c is lower than the backbone of pNL4-3 (#4, #8), but higher than the backbone of pMDR-5a (#6).

Estimating relative fitness of envelope and backbone genome in pMDR-1c and pMDR-5a

For the quantitative comparison, the relative fitness value for each virus was estimated as described in Methods (Suppl. Tables 3 and 4A, Fig. 3B). Relative fitness value was high in pMDR-1 (1.62 with 95% confidence intervals [1.42, 1.85]) and low in pMDR-5a (0.82 [0.69, 0.97]) as compared to the reference virus, pNL4-3 (1.00). For the env-chimeras, relative fitness value was very high in pNL4-3(envMDR-1c) (2.17 [1.94, 2.43]), low in pMDR-1c(envNL4-3) (0.56 [0.39, 0.81]), or equivalent to pNL4-3 in pMDR-5a(envMDR-1c) (1.06 [0.94, 1.19]).

To determine the contribution of subgenomes to fitness, we estimated relative fitness values for envelope and backbone in each virus (Suppl. Table 4B). The relative fitness of pMDR-1c envelope was calculated as high as 2.29[2.05, 2.56], whereas the relative fitness of its backbone was 0.57 [0.39, 0.81]. Similarly, the relative fitness of pMDR-5a envelope was as high as 1.64 [1.42, 1.90], and the relative fitness of its backbone was as low as 0.49 [0.46, 0.53] (Fig. 3C). Given the reduced fitness of the backbone of pMDR-1c, which is composed of all genes except for envelope gene, the envelope is considered as the major determinant of the enhanced fitness of the MDR virus. Similarly, the envelope of pMDR-5a contributed to the fitness of the virus, yet the fitness of pMDR-5a did not exceed the fitness of pNL4-3.

DISCUSSION

We used viruses derived from two patients with contrasting clinical profiles both infected with MDR HIV-1 to probe the issue of viral fitness in the context of multi-drug resistance. Critical to our studies were the construction of biologically relevant molecular clones of the MDR-HIV variants. By selecting biologically clonal isolates that are reflecting the growth characteristics of bulk isolates, and subsequently demonstrating comparable replication characteristics of molecular clones to their source clonal isolates we believe our findings to be relevant. In our experiments we separated the HIV-1 genome into two parts, the envelope and the remaining backbone. By using envelope chimeras and growth competition assay, we could estimate the relative fitness of each segment independently. This allowed us to determine the contribution of each subgenome to the overall fitness of the virus. The backbone genomes of two MDR viruses had reduced fitness, likely due to the presence of

the resistant conferring mutations that in turn affect the activity of RT and protease. The relative fitness of the backbone of pMDR-5a was lower than that of pMDR-1c, perhaps due to its mutations, M184V and T215Y, which are known to exert a substantial impact on viral fitness.²⁷ In contrast, the envelope of pMDR-1c was found to have a relative fitness of 229% as compared to the envelope of pNL4-3.

We believe this compensated for the reduced fitness of the backbone genome, calculated to be 56%, and actually resulted in an increase in the overall fitness of the complete virus to 162%, which far exceeded that of the reference laboratory strain, pNL4-3. Thus, it would appear that the enhancing effect of the envelope of the cytopathic virus, pMDR-1c, more than compensates for the fitness loss conferred by the emergence of drug resistance mutations in constitutive enzyme coding regions. This is in contrast with the less cytopathic MDR virus, pMDR-5a, in which an enhanced fitness of envelope (164%) compensated for the reduced fitness of the backbone genome (49%), but the overall fitness of the virus (82%) did not exceed the wild type virus. Given the dramatic differences in clinical presentation seen between the 2 cases, this observation does support the contention that viral factors may play a significant role in the course of HIV-1 infection in vivo.

Note that we constructed envelope-chimeras by replacing the entire gp160 region, which also encodes the 2nd exons of tat and rev, 3'half of vpu and the RRE (Fig. 2A), thus, it is conceivable that the observed enhanced fitness may result from these gene products rather than envelope. However, we believe the following observations support the major role of the envelope protein in conferring increased fitness. First, the TCID₅₀/p24 ratio in the transfection-derived virus stock of pMDR-1c or two chimeras with MDR-1c envelope was 2- to 5-fold higher than the virus/chimeras with NL4-3 envelope or MDR-5a envelope (Fig. 2C). The ratio of TCID₅₀ over p24 in virus stock or "infectivity per particle" is a reflection of the initial steps of infection. This ratio has been used by others as a measurement of env function.²⁸ Though Tat, Rev and Vpu have critical roles in the viral life cycle, their activity resides more in levels of virus production as opposed to these initial steps in the viral life cycle.²⁸⁻³¹ Second, the virus production upon transfection of pMDR-1c plasmid DNA was not different from that of pNL4-3 (289 ng/ml vs. 292 ng/ml), which does not support a significant effect of Tat, Rev or Vpu. Nonetheless, further studies are needed to conclusively prove that the envelope protein is conferring the observed replication advantage. This could be accomplished with the use of site directed mutagenesis of the env-coding region.

The viral envelope is known to play crucial roles during early steps of viral life cycle; (1) a surface protein, gp120 mediates viral binding to the receptors, the CD4 molecule and coreceptor, CCR5 or CXCR4 or other receptors, and (2) a transmembrane protein, gp41 mediates membrane fusion/semifusion between virion and target cell, or the infected cell and uninfected cell.³²⁻³⁴ Thus, envelope is critical in viral entry and determines the infectivity, cell tropism and cytopathicity.³⁵⁻³⁷ The comparison of envelope amino acid sequences among three clones, especially with pMDR-1a (R5, intermediate growing virus) indicated the difference within V3 loop, which explains cell tropism as described earlier. There are amino acid differences between less cytopathic pMDR-1a and cytopathic pMDR-1b and -1c in gp41, especially within HR-2 region. Given the critical role of gp41 in membrane fusion activity, these changes may be associated with the enhanced pathogenicity

in vivo.²⁵ However, at this time we have yet to determine which functions of envelope are the most critical for the enhanced fitness and cytopathicity we have observed. Measuring the affinity of the envelope to the CD4 molecule and its coreceptors as well as determining its membrane fusion activity may provide some insights into the mechanisms whereby the envelope confers fitness advantage.

We compared the effects of envelope on the backbone genome in two MDR HIV-1 variants to determine whether the observations seen in pMDR-1c were unique. Though pMDR-5a was constructed from a patient with slow disease progression, a similar profile was observed (Fig. 3C). The backbone genome was defective compared to NL4-3, however this was compensated by a more fit envelope, although the degree of fitness enhancement was less than that observed for pMDR-1c. This suggests that MDR viruses may use envelope as a compensatory mechanism for the reduced fitness. This is consistent with our earlier study,³⁸ in which the replication capacity of MDR viruses was higher than drug-sensitive, wild-type viruses when measured by GHOST cell assay, a single cycle infectivity assay where the viral input was normalized by p24, suggesting that a higher TCID₅₀/p24 ratio may have been responsible for the observed increased fitness in vitro. The fitness compensation by envelope may not be unique to MDR viruses. For example, Das et al., reported that the reduced viral replication with impaired translation could be rescued by adaptive mutations in env.³⁹

How did this MDR virus with enhanced fitness arise? We speculate that in the treated patient failing therapy, the MDR virus first compensated for reduced fitness by the selection for secondary mutations within the targeted molecules,⁴⁰ i.e., protease and RT proteins as well as by the mutations within Gag cleavage sites and perhaps by the mutations within MA protein as well with these changes being maintained by drug pressure. However, further compensation occurred by the changes in the envelope glycoprotein via step-wise mutations. On transmission, this MDR virus started reverting towards wild type, or recombined with a co-infected wild type virus and gained further fitness. In fact, pMDR-1c had lost M184V, T215Y and L210W, which were detected in the earliest sample as a mixture.¹ Thus, the enhanced fitness of RT, together with the pre-existed enhanced fitness of envelope could have generated a highly pathogenic virus. Additionally, though not defined here, it is likely that host factors may also contribute to viral evolution in vivo and the emergence of HIV-1 viral variants.

A number of studies have concluded that MDR viruses demonstrate impaired replication capacity or fitness. However, this conclusion was based on the determination of RC values of a recombinant virus derived from the patient pol gene products being inserted into a cassette. As we have demonstrated in this study, drug-resistant virus may use envelope, an area outside of the product of the pol gene, to compensate for apparent reduced fitness. Thus, we believe that full understanding of the fitness and cytopathicity of MDR viruses requires study of the intact virus, and not chimeric viruses with only portions of the viral genome included.

In summary, we have demonstrated that the envelope is a major determinant of enhanced fitness of a cytopathic MDR virus isolated from an HIV-1 infected patient with rapid

progression. Furthermore, in the 2 viruses studied, the enhanced fitness conferred by envelope compensated for the reduced fitness conferred by the backbone genome of MDR viruses. Further studies are needed to understand whether this phenomenon is restricted to the 2 viruses studied here, or perhaps is a more global mechanism of viral evolution to compensate for deleterious mutations that arise as a consequence of drug resistance that reduce fitness in the absence of drug selective pressure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of molecular clones

Replication kinetics of molecular clone-derived viruses and their parental isolates were tested in parallel using PHA-stimulated PBMC. The p24 concentrations in the culture supernatant are indicated for cell cultures with (A) pMDR-1c and MDR-1c, (B) pMDR-1b and MDR-1b, (C) pMDR-1a and MDR-1a, and (D) pMDR-5a and MDR-5a with empty symbols for the molecular clones and filled diamonds for their parental isolates. Inoculation was 1,000 TCID₅₀ for all viruses, except for pMDR-1c and pMDR-1b, for which 3 µl and 30 µl or 30 µl and 300 µl of transfection culture supernatant were used respectively. (E) In a different assay, viral cytopathicity was evaluated by the CD4/CD8+T cell ratios (mean value

$\pm$ s.d.) over the uninfected control on day 8 after the inoculation of 1,000 TCID₅₀ of each virus in culture with 2×10^6 PHA-stimulated PBMC. The levels of cytopathicity of four molecular clones were equivalent with their parental isolates. Indicated along was the result of a reference virus, pNL4-3.

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A

B

C

Figure 2. Construction of env-chimeras

(A) Constructions of four env-chimeras are illustrated. HIV-1 genome map is shown at the top. The constructions of four chimera are indicated at the bottom with the regions derived from pMDR-1c, pNL4-3 and pMDR-5a indicated by dark gray, light gray and white bars, respectively.

(B) Infectious titer of the stock of each virus, or chimera was determined by the end-point dilution culture method using PHA-stimulated PBMC. (C) Ratios of infectious titers over the amount of p24 (TCID₅₀/p24(pg)) are indicated with light gray bars for the original clone-derived viruses and with dark gray bars for the env-chimeras. See Suppl. Fig. 2 for the estimation procedure of TCID₅₀.

Figure 3. Growth competition assays and relative fitness values of viruses and their subgenomes (A) Fractional viral RNA copy numbers (%) of each virus over days of culture (top panels) are indicated for growth competition cultures #1 to #8. Symbols used are: △ pMDR-1c; □ pNL4-2; ◇ pMDR-5a; ⊗ pMDR-1c(envNL4-3); ■ pNL4-3(envMDR-1c); ◆ pMDR-5a(envMDR-1c). Ratios of RNA copy numbers of two viruses in logarithmic scale over days of culture (bottom panels) are indicated with a symbol, ○, for each competition culture. Each value indicates the mean value of 4 measurements: 2 viral RNA measurements in each supernatant of 2 different donor PBMC culture. Slope was estimated from the best-

fit curve (as indicated by a straight line) to the ratios of two viruses. (B) The relative fitness values with 95% confidence intervals for viruses and env-chimeras over pNL4-3, and (C) the relative fitness values with 95% confidence intervals for envelopes and backbone genomes over NL4-3 envelope or subgenome are indicated in box-and-whiskers plots.

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Table 1

Characteristics of molecular clones.

Molecular Clone	pMDR -1a	pMDR -1b	pMDR -1c	pMDR -5a	pNL 4-3
PI	L10I	L10I	L10I	L10I	None
Resistance	L33F	L33F	L33F	V11I	
Mutations	M46I	M46I	M46I	L33F	
	I54M	I54M	I54M	K43T	
	A71V	A71V	A71V	M46L	
	G73S	G73S	G73S	I54V	
	L76V	L76V	L76V	A71I	
	I84V	I84V	I84V	G73S	
	L89V	L89V	L89V	V82A	
	L90M	L90M	L90M	L90M	
NRTI	M41L	M41L	M41L	M41L	None
Resistance	D67N	T215D	D67N	D67N	
Mutations	T215C		T215C	L74I	
	K219E		K219E	M184V	
				L210W	
				T215Y	
NNRTI	K101E	K101E	K101E	K103N	None
Resistance	Y181I	Y181I	Y181I		
Mutations	N348I	N348I	N348I		
Coreceptor Usage	R5	R5X4	R5X4	R5	X4
Proviral Genome Size (bp)	9,746	9,746	9,746	9,723	9,709
Deduced Envelope (amino acids)	862	862	862	853	855
N-linked Glycosylation Sites in Envelope	30	28	29	32	31
Net Charge in gp120	12.7	14.7	11.7	18.8	17.7

Table 2

Clinical course of subject MDR-5. The changes in CD4 count and viral load are indicated. A star symbol (*) indicates the time when combination antiretroviral therapy was initiated.

Approximate duration of infection (days)	CD4 count (/mm ³)	HIV-1 RNA (copies/ml)
307	982	3,250
321	865	661
407	988	1,690
559	1,143	417
874	577	5,870
1,079	552	25,500
1,196	814	22,700
1,268*	633	16,200
1,406	707	<50
1,504	612	<50