Multidrug-Resistant Variants of HIV Type 1 (HIV-1) Can Exist in Cells as Defective Quasispecies and Be Rescued by Superinfection with Other Defective HIV-1 Variants

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A tissue culture cell line infected with multidrug-resistant (MDR) human immunodeficiency virus type 1 (HIV-1) produced only noninfectious particles because of a lethal mutation in env. The defective MDR provirus was rescued by superinfection with either wild-type HIV-1 or a second replication-defective virus lethally mutated in capsid. Drug-resistance phenotyping revealed that the MDR viruses dominated if even single reverse-transcriptase inhibitors were present, reflecting linkage of the various drug resistance mutations on a single viral nucleic acid backbone. These results are most likely attributable to recombination during second rounds of infection and suggest that defective HIV-1 variants may nonetheless constitute part of the HIV-1 reservoir.

Individuals infected with HIV-1 contain diverse viral swarms termed quasispecies that are similar but genetically distinct [1, 2]. Large numbers of mutations, including those responsible for drug resistance, emerge on a daily basis in infected individuals [2, 3]. Among in vivo quasispecies, a large proportion of human immunodeficiency virus (HIV) strains may be defective due to the spontaneous generation of lethal mutations. Thus, it is not surprising that uncultured HIV type 1 (HIV-1) genomes that are present in peripheral blood mononuclear cells of an actively treated patient [3]. Similar findings have also been reported for vif and tat [7].

Despite this, the significance of defective HIV-1 species has not been well investigated. It is not clear whether defective species can play a role in the rapid resurgence of progeny virus that often follows treatment interruption or in the generation of drug-resistant virus that can occur after resumption of therapy. This problem may be further compounded by findings that HIV superinfections may occur more commonly than initially thought.

We now show that defective species can potentially be a part of the HIV-1 reservoir and that defective multidrug-resistant (MDR) HIV-1 infection might contribute over time, through recombination, to fully infectious viral progeny.

Methods. BH10 was used as wild-type virus. CA P357A was a noninfectious proviral construct derived from BH10 that contains a mutation in capsid (CA) [8]. Plasmids gagpol, gp, and gpre were used for cotransfection with CA P357A in an attempt to boost the production of infectious viral particles. Plasmid 83RT was a proviral construct, containing MDR RT mutations. These plasmids and their construction are described in the Appendix, which appears only in the electronic version of the Journal.

To select defective cell lines, 5 × 10^5 MT2 or H9 cells were infected with 1 mL of 83RT or BH10 viruses (inoculum of p24, ∼800 ng/mL) for 2 h at 37°C. The infected cells were washed 4 times, resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium, diluted to a proper concentration, and seeded into 96-well plates. One-half of the medium in each well was changed at 4-day intervals. Clones without cytopathic effect were chosen for evaluation of p24 antigen (bioMérieux) and those that had p24 values >300 ng/mL in supernatants were then subcloned in 96-well plates. Stable cell lines were defined as those that could be recloned at least 3 times and that remained positive for p24. Defectiveness was demonstrated by showing that cells produced p24 but not infectious progeny viruses.

MT2 cells (2 × 10^5) were infected with 0.5 mL of wild-type or mutant viruses and washed as above. Supernatants were monitored for p24 at 4-day intervals for up to 30 days. 83M
cells (5 × 10^3) were infected in the same way but were monitored for cytopathic effect for up to 30 days. See the Appendix, which appears only in the electronic version of the Journal, for more details.

**Results.** We used MT2 and H9 cells to detect defective infections. After exclusion of cultures without viable cells (18% for MT2 cells and 4% for H9 cells), both MT2 and H9 cells had similar infection rates (ie, the numbers of cultures that either demonstrated cytopathic effect or were positive for p24, compared with cultures showing cell growth; 48% and 49%, respectively). A higher proportion of infected MT2 cells (41%) than H9 cells (19%) displayed cytopathic effect. Cultures positive for p24 but without cytopathic effect might represent either abortive or defective infections. The infected MT2 and H9 cells without cytopathic effect had similar strong positive rates for p24 (>300 ng/mL; 24% and 22%, respectively). Cultures that had p24 values >300 ng/mL were subcloned to select cell lines harboring defective proviruses. More than 95% of these became p24 negative or regained virulence during the process of subcloning. However, 2 clones originating from MT2 cells and 4 clones originating from H9 cells were stable after subcloning.

Cell line 83M was selected from infection of MT2 cells with 83RT, a BH10-based HIV-1 proviral construct containing MDR mutations in RT (M41L/E44A/T69N/V118I/M184V/L210W/ T215Y/K219R for nucleoside RT inhibitors and A98G/K101E/ V108I/Y181C/G190A/H208Y for nonnucleoside RT inhibitors; Appendix, which appears only in the electronic version of the Journal). The cells were subcloned several times, and p24 levels in culture supernatants at peak were 5–10-fold lower than those in cultures infected with wild-type viruses. The 83M cell line has been stable for >2 years in culture.

83M cells showed no cytopathic effect and did not produce infectious progeny. However, polymerase chain reaction amplification confirmed that the entire proviral HIV-1 genome was present. Sequencing of the proviral genome revealed several novel mutations in gagpol and the proviral genome carried all of the MDR RT mutations. A E53G(GAG→GGA) mutation was identified in the fingers domain of RT, which should not significantly affect RT structure. Two mutations—N27Y(AAC→TAC) and M50K(ATG→AAG)—were detected in the N-terminal region of integrase, but these also do not appear to be critical for replication. Three mutations—A69T(GCC→GAC), T70A(ACA→GCA), and G367R(GGG→AGG)—were detected in env, among which G367R might be important, because it is located in a highly conserved motif at positions 365–372, which are thought to be a part of the CD4 binding site [9]. Six recombinant clones containing G367R were therefore generated by site-directed mutagenesis. Transfection and infection studies in 293T and MT2 cells confirmed that the env G367R mutation was lethal to HIV-1 replication (data not shown).

Both recombinant wild-type BH10 and isolate IIIb were able to reinfect 83M cells and cause cytopathic effect, although this took longer to appear in 83M than in MT2 cells. The viruses harvested from the superinfected 83M cells were tested for ability to infect MT2 cells in the presence of zidovudine, lamivudine, or nevirapine. The appearance of cytopathic effect was only delayed by 1–2 days in the presence of either zidovudine, lamivudine, or nevirapine, indicating that the viruses used for infection contained MDR viruses.

To test whether the defective proviruses could also be rescued by other defective viruses, we used a noninfectious virus containing a mutation in capsid and used it to infect 83M cells. Although mutations in the C terminus of CA can disrupt viral particle assembly, proviral DNA containing the P357A mutation in CA can only inefficiently generate viral particles that are able to infect MT2 cells, on the basis of low-level detection of p24 in culture fluids (Figure 1A). The infectivity of viruses harvested from the cotransfection of CA P357A virus with either gagpol, gpre, or gp were not increased as tested in MT2 cells.

In contrast, CA P357A viruses were able to infect 83M cells and successfully rescue the provirus harbored in these cells. These results indicate that a small proportion of CA P357A viruses were able to successfully infect 83M cells, and that DNA derived from these viruses was able to integrate into the cellular genome. The results of 4 experiments confirmed that cytopathic effect appeared in all the 83M cultures at days 11–13 after infection but not in the MT2 cells.

We next asked whether wild-type gagpol supplied in trans could increase the efficiency of viral assembly and increase the efficiency of infection of the defective virus in 83M cells. We used the viruses harvested from cotransfection of CA P357A DNA and gagpol, because the latter would supply the gag and pol gene products in trans to be packaged into virions. The viruses that emerged from the CA P357A-gagpol, gpre, or gp transfections were then used to infect 83M and MT2 cells in parallel with CA P357A. Similar to the MT2 infection results, the 83M infections showed that cotransfection of gagpol did not enhance the efficiency of infection, indicating that DNA recombination was unlikely to have occurred during transfection.

The phenotypes of the viruses rescued from 83M cells by CA P357A virus were further analyzed and were resistant to each of zidovudine, lamivudine, and nevirapine, as tested in MT2 cells (Figure 1B). However, the rescued viruses required higher p24 input levels to have comparable growth curves to that of wild-type virus (400 vs 100 ng/10^6 MT2 cells), suggesting that the rescued viruses still contained defective particles. Cytopathic effect appeared in MT2 cells at day 4 in the absence of any drug and at days 4–9 in the presence of lamivudine (1 μM and 10 μM), zidovudine (1 μM and 10 μM), and nevirapine (0.2 μM and 2 μM).
Figure 1. p24 production in MT2 cells after infection. A, MT2 cells infected with p24-positive supernatants harvested from transfected 293T cells. BH10 is wild-type virus, P357A contains a lethal mutation in capsid. gp, gpre, and gagpol were gagpol-expressing plasmids (Appendix, which appears only in the electronic version of the Journal). B, p24 production in MT2 cells after infection by wild-type virus (p24, 100 ng/10^7 cells) or the rescued virus 83M-r (p24, 400 ng/10^7 cells). The infected cells were washed with medium, split into 4 wells for growth in the absence (wild-type and 83M-r) or presence of 10 μM of zidovudine (wt-AZT and 83M-r-AZT), 10 μM of lamivudine (83M-r-3TC), or 2 μM of nevirapine (wt-NVP and 83M-r-NVP).

To confirm the presence of drug-resistance mutations in the rescued viruses, proviral DNA was isolated from the infected MT2 cells cultured in high concentrations of zidovudine, lamivudine, and nevirapine at the beginning of the appearance of cytopathic effect. This DNA was polymerase chain reaction amplified, cloned, and sequenced. As shown in Figure 2A, an analysis of 18 cloned sequences showed that such mutations, regardless of inhibitor, were only retained in 5%–15% of cultures amplified in the absence of drugs (ie, less than the limit of detection on the basis of routine genotyping) [10]. However, drug-resistance mutations were present in most of the cultures amplified in the presence of the inhibitors; 91% of 13 cloned RT sequences derived from MT2 cells cultured in the presence of zidovudine contained the thymidine analogue mutations (TAMs) L210W, T215Y, and K219R, but not M41L, whereas mutations other than TAMs were retained at lower rates (ie, ~10%), except for H208Y, which appeared linked with L210W (Figure 2B). Each of 18 RT clones derived from MT2 cells cultured in the presence of lamivudine retained M184V (Figure 2C). The linked Y181C and L210W/T215Y/K219R mutations were also present at high rates, whereas M41L was not. Similarly, most drug-resistance mutations were retained in each of seven clones derived from cultures grown in the presence of nevirapine (Figure 2D).

**Discussion.** Cells harboring defective HIV-1 in vivo may not die as quickly as those infected with wild-type viruses and might, therefore, be able to multiply and even produce defective viral particles. Such cells might also be prone to superinfection, especially if local viral concentrations are high. For example, it has been reported that most infected spleen cells in vivo have >1 HIV copy; a single cell can contain up to seven different copies of HIV-1 DNA [11]. It has also been shown that cells harboring 5 copies of defective provirus were able to produce highly infectious viral progeny [12].

We identified a cell line containing a novel G367R env mutation that may disrupt HIV env-CD4 binding, because the 365–372 motif in env is highly conserved and participates in CD4 binding [9]. For example, a D368R mutation was reported to disrupt env binding to both CD4 and neutralizing antibodies [13]. We believe that G367R would also disrupt env CD4 binding in a similar way.

Our results indicate that a tissue culture cell, when successively infected with 2 defective viruses, can produce infectious viruses through complementation and recombination. The fact that a second-round infection successfully rescued a defective provirus indicates the potential importance of defective viral species. Moreover, mutations responsible for drug resistance can be preserved as defective forms in infected cells. Lymphocytes that have been infected by defective species may not die quickly and may survive longer than cells infected with wild-type viruses.

This in vitro finding may have clinical significance, especially because the presence of resistance mutations within integrated viral DNA is unlikely to be affected by antiviral drugs, but the potential clinical relevance of these findings can only be inferred at this time. Cells containing such proviral DNA might serve
Figure 2. Percentage of clones containing drug-resistance mutations. MT2 cells were infected by rescued viruses harvested from 83M cells which were superinfected with capsid P357A viruses, a noninfectious quasispecies. Cellular DNA, extracted at the time of cytopathic effect appearance, was polymerase chain reaction amplified and sequenced. The figure shows the percentage of clones observed in the infected cells grown in the absence of any reverse-transcriptase inhibitors (A; n = 18) and presence of zidovudine (ZDV; B; n = 13), lamivudine (3TC; C; n = 18), nevirapine (NVP; D; n = 7).
as reservoirs for drug resistance, if defective species are rescued by superinfection with either wild-type or defective viruses. Such rescue may be a factor in helping to explain the more rapid emergence of drug resistance in patients who underwent treatment interruption as opposed to continuing to receive therapy [14]. Superinfection was also able to rescue defective HIV-1 provirus containing an insertion in gagpol [15].

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**References**