Single Mutations in HIV Integrase Confer High-Level Resistance to Raltegravir in Primary Human Macrophages[▽]

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CD4⁺ T cells and macrophages are the primary target cells for HIV *in vivo*, and antiretroviral drugs can vary in their ability to inhibit the infection of these different cell types. Resistance pathways to the HIV integrase inhibitor raltegravir have previously been investigated in T cells. Primary raltegravir resistance mutations, most often at integrase amino acid position 148 or 155, afford some resistance to the drug. The acquisition of pathway-specific secondary mutations then provides higher-level resistance to viruses infecting T cells. We show here that during macrophage infection, the presence of a single primary raltegravir resistance mutation (Q148H, Q148R, N155H, or N155S) is sufficient to provide resistance to raltegravir comparable to that seen in viruses expressing both primary and secondary mutations in costimulated CD4⁺ T cells. These data implicate macrophages as a potential *in vivo* reservoir that may facilitate the development of resistance to raltegravir. Notably, the newer integrase inhibitor MK-2048 effectively suppressed the infection of all raltegravir-resistant viruses in both T cells and macrophages, indicating that more recently developed integrase inhibitors are capable of inhibiting infection in both major HIV cellular reservoirs, even in patients harboring raltegravir-resistant viruses.

Stimulated CD4⁺ T helper cells and macrophages are the two principal target cell types for human immunodeficiency virus (HIV) *in vivo* (11, 16, 31, 32, 49). In untreated infected individuals, CD4⁺ T cells are the most abundant infected cell type. These cells are responsible for producing the majority of virus in the body as a whole (22, 38, 39), and their depletion ultimately leads to the clinical decline culminating in AIDS. However, macrophages also play an important role in HIV pathogenesis (17, 24, 47). One of the clearest examples of this is within the central nervous system, where the infection of macrophages and microglial cells can lead to the development of HIV encephalitis and HIV-associated dementia (16, 20, 24). Macrophages also are capable of recruiting nearby CD4⁺ T cells and efficiently transferring virus to them (45), allowing infected macrophages to directly influence the depletion of the T-cell compartment. Evidence from a pathogenic simian immunodeficiency virus/HIV chimeric virus (SHIV) infection of rhesus macaques demonstrates that infected macrophages are capable of maintaining very high viral loads for months after CD4⁺ T cells are almost completely depleted (23). Moreover, while the infection of stimulated CD4⁺ T cells usually results in the death of the host cell within several days, HIV-infected macrophages can in some cases survive for weeks or months

following infection (1, 18, 37). This greater longevity, coupled with the presence of macrophages in tissues, such as the central nervous system, with the reduced penetration of some antiretroviral drugs, such as protease inhibitors (3, 8, 27), provides evidence supporting a role for macrophages in the persistence of HIV during therapy.

The integration of the double-stranded viral DNA into the host cell chromosome is an essential step in the HIV life cycle and is therefore an attractive focus for antiretroviral drug development. This step is catalyzed by the viral integrase protein encoded by the *pol* gene. The antiretroviral drug raltegravir (RAL) is the first integrase inhibitor to be approved for the clinical treatment of HIV-infected individuals (21). RAL specifically inhibits the strand transfer reaction responsible for inserting the viral genome into host cell DNA. While a range of different mutations have been associated with resistance to RAL (reviewed in reference 33), resistance generally evolves in a stepwise process via one of several potential pathways. The mutually exclusive primary resistance mutations N155H, Q148H/R/K, and, more rarely, Y143R/C/H provide some resistance to RAL (9, 14, 15, 28). These primary mutations often are associated with secondary mutations specific to each primary mutation pathway, which increase RAL resistance and may restore fitness defects caused by primary mutations (15). A secondary G140S mutation is most commonly associated with the Q148 pathway, and an E92Q mutation most often accompanies the N155H primary resistance mutation (15). Q148 pathway mutations typically produce viruses that are more resistant to RAL than N155 pathway mutations (15).

The level of resistance provided by these pathways during

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the infection of T cells has been investigated previously (19, 46). However, fundamental differences in cellular composition, metabolism, and viral replication kinetics can lead to differences in the efficacy of antiviral drugs in T cells and macrophages (3). For example, nucleoside analog reverse transcriptase (RT) inhibitors typically work more effectively in macrophages than in T cells (42), whereas protease inhibitors are less effective in chronically infected macrophages than in T cells (40). It therefore is important to assess the resistance profiles of viruses harboring clinically important RAL resistance mutations during macrophage infection. Here, we show that in contrast to the situation with CD4⁺ T cells, individual mutations in HIV integrase can produce viruses with high-level resistance to RAL during the infection of macrophage cells. This single-step pathway to high-level resistance demonstrates a potential role for macrophage infection in the development of RAL-resistant virus *in vivo*.

MATERIALS AND METHODS

Molecular cloning and plasmid construction. The clone *denv*(Wt) is a previously described (30) HIV reporter virus proviral backbone that expresses the enhanced green fluorescent protein fused to firefly luciferase in place of HIV *env*, but it retains the expression of all other viral genes. Integrase mutant versions of this virus were constructed by site-directed mutagenesis using the QuikChange multikit (Stratagene) according to the manufacturer's instructions. Mutations first were introduced into a subclone of NL4-3 containing the integrase coding sequence, and then the modified integrase sequences were inserted into the corresponding region of *denv*(Wt) using the *Sbf*I and *Sal*I restriction enzyme sites.

Virus production. To ensure the efficient infection of primary macrophages, reporter viruses pseudotyped with vesicular stomatitis virus G (VSV-G) protein were produced by the transient transfection of 293FT cells using Lipofectamine 2000 reagent (Invitrogen). Cells were seeded in 10-cm dishes the day before transfection at a concentration of 6.6×10^6 cells/dish in media without antibiotics. To produce infectious virus, each plate was cotransfected with 10 μ g of the relevant virus-encoding plasmid and 2 μ g of pHCMVG. The cells were incubated with Lipofectamine/DNA complexes for 20 h and then incubated with fresh media for a further 30 h before virus harvesting. During harvesting, the medium was removed from cells and centrifuged at $800 \times g$ for 10 min to remove any floating cells and large debris. The supernatant then was passed through a 0.45- μ m filter, and the virus was concentrated by ultracentrifugation at $50,000 \times g$ for 90 min at 4°C. Aliquots were frozen at -80°C and thawed immediately prior to use. Virus was assayed for p24 concentration using an HIV p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (Beckman Coulter) according to the manufacturer's instructions, and titers were determined by the infection of 293FT cells as previously described (7).

Cell culture and isolation procedures. All cells were cultured at 37°C in 5% CO₂. 293FT cells were split every 2 to 3 days as needed and were cultured in Dulbecco's modified Eagle medium (Invitrogen) containing 10% fetal bovine serum (FBS; Omega Scientific), 100 μ g/ml of G418 (Invitrogen), and 100 U/ml of penicillin with 100 μ g/ml of streptomycin (pen/strep; Invitrogen). Peripheral blood mononuclear cells (PBMC) were isolated from healthy human donor blood by Ficoll-Paque Plus separation (GE Healthcare) according to the manufacturer's instructions. CD14⁺ cells were isolated from PBMC by positive selection using CD14 MicroBeads (Miltenyi Biotec). Monocytes were differentiated into macrophage cells by culturing for 7 days in Iscove's modified Dulbecco's medium (IMDM; Irvine Scientific) supplemented with 10% FBS, 5% human AB serum (Sigma), pen/strep, and 10 ng/ml of macrophage colony-stimulating factor (R&D Systems). CD14⁺ cells were cultured in a 96-well plate with 2.5×10^4 cells/well.

Primary CD4⁺ T cells were isolated from PBMC by negative selection using the CD4⁺ T-cell isolation kit II (Miltenyi Biotec). Cells were costimulated by culturing in RPMI 1640 (Invitrogen) containing 10% FBS, pen/strep, and 20 U/ml of interleukin-2 (Roche) in the presence of plate-bound anti-CD3 and soluble anti-CD28 antibodies as previously described (6, 7). CD4⁺ T cells were costimulated in bulk for 2 days and then seeded into a 96-well plate at a concentration of 5×10^4 cells/well without stimulatory antibodies for 1 day

before infection. All cell sorting was performed by using an AutoMACS cell sorter (Miltenyi Biotec) according to the manufacturer's instructions.

Infection procedures. Cells were preincubated with the relevant concentration of drug for 1 day prior to infection, and this concentration was maintained throughout the infection and subsequent culture. All infections were performed by incubating cells for 2 h at 37°C with virus in medium containing 10 μ g/ml of Polybrene, followed by the removal of infection medium, washing, and addition of culture medium containing the appropriate drug. For each donor and cell type, equivalent infectious units of the different mutant viruses were used for infections (based on 293FT titrations). Multiplicities of infection ranged from approximately 0.1 to 0.3 in different experiments. Macrophages were differentiated for 7 days and then pretreated with drug for 1 day before infection. Cells were harvested at day 5 postinfection. CD4⁺ T cells were costimulated for 2 days and then pretreated with drug for 1 day before infection. Infected cells were harvested at 3 days postinfection. All infections were performed independently at least three times on separate occasions using cells obtained from different donors.

Luciferase assays. The expression of firefly luciferase was measured using the Luciferase assay system (Promega) according to the manufacturer's recommendations. Medium was removed from cells, and then 100 μ l of $1 \times$ cell lysis buffer was added to wells. Lysates were stored at -80°C before analysis.

During analysis, cells were thawed and mixed with 100 μ l of Luciferase assay reagent and then analyzed using a FLUOstar Optima luminometer (BMG Labtech). Light unit values for infected cultures treated with drug were normalized to corresponding untreated cultures in the same experiment.

Statistical analysis. For comparisons of mutants between cell types during RAL treatment, the data first were log transformed to meet model assumptions. A two-sided *t* test then was performed to compare differences between the mean 50% effective concentrations (EC₅₀s) for specific mutational pathways in T cells and macrophages. For all other comparisons the Wilcoxon rank sum test was used to compare groups. This is a nonparametric test that is powerful in small samples. All *P* values are two sided.

Ethics statement. Peripheral blood was obtained through the UCLA Blood Bank in an anonymous fashion as approved by the UCLA institutional review board.

RESULTS AND DISCUSSION

A panel of HIV *pol* clones harboring single and double RAL resistance mutations in the integrase catalytic core domain sequence was generated by site-directed mutagenesis (Fig. 1A). These modified sequences were inserted into *denv*(Wt) (30). Infectious virions restricted to a single round of infection were produced by pseudotyping with vesicular stomatitis virus G. Differentiated primary macrophages and costimulated primary CD4⁺ T cells were pretreated with the relevant concentration of drug for 24 h before infection, and the drug was maintained throughout the subsequent culture period.

The nucleoside analogue RT inhibitor zidovudine (AZT) and the nonnucleoside RT inhibitor efavirenz (EFV) were included in these studies for control and comparison purposes. Resistance to these drugs is associated with modifications in the RT gene (4, 26), therefore integrase gene mutations would not be predicted to alter susceptibility to AZT or EFV. Indeed, there were no significant differences in EC₅₀ between RAL-resistant viruses during both T-cell and macrophage infection using these RT inhibitors (Fig. 1B and C). Consistently with prior studies (2, 41), we found that EFV inhibited the infection of T cells and macrophages at equivalent concentrations (mean EC₅₀ of 1.9 nM for T cells and 1.8 nM for macrophages; *P* = 0.3), whereas AZT worked more effectively in macrophages than T cells (mean EC₅₀ of 23.5 nM for T cells and 3.9 nM for macrophages; *P* = 0.004). This likely is because macrophages are nondividing cells and contain smaller nucleotide pools than do stimulated T cells (13). Therefore, the reduced competition for the incorporation of normal thymidine nucleotide triphos-

FIG. 1. Location of mutations within integrase and the reverse transcriptase inhibitor resistance profiles of mutant viruses. (A) The indicated primary and secondary raltegravir resistance mutations were introduced into a luciferase-expressing HIV reporter virus. (B) Reporter viruses were used to infect primary costimulated CD4⁺ T cells (T cells) or primary macrophages (MACS) in the presence of the nucleoside analog reverse transcriptase inhibitor zidovudine (AZT). (C) Primary costimulated CD4⁺ T cells or primary macrophages were infected in the presence of the nonnucleoside reverse transcriptase inhibitor efavirenz (EFV). Each data point is derived from at least three independent experiments using cells from different donors. Mean values $\pm$ standard errors of the means are presented.

phages in macrophages makes the incorporation of the chain-terminating nucleoside analog AZT more likely in this cell type.

When cells were treated with RAL, a different pattern of activity emerged. Virus encoding wild-type integrase was slightly more resistant to RAL during macrophage infection than during corresponding T-cell infection (EC_{50} of 11.35 nM for macrophages and 3.3 nM for T cells) (Fig. 2). For Q148 pathway mutations, the Q148H or Q148R single-mutant viruses showed partial resistance to RAL in T cells (EC_{50} of 41.3 and 158.4 nM, respectively, compared to 3.3 nM for virus with wild-type integrase). Double mutants encoding G140S+Q148H or

G140S+Q148R were more resistant to RAL than the single-mutant viruses during T-cell infection, with EC_{50} s of 1,492 and 1,378 nM, respectively. This stepwise pathway to higher resistance is consistent with previous work demonstrating the ability of secondary mutations to enhance virus infectivity in the presence of drug (12, 19, 43). In contrast, during macrophage infection the presence of a primary Q148 mutation alone provided high-level RAL resistance equivalent to that observed with the corresponding G140S+Q148H or G140S+Q148R double-mutant virus. Single Q148H ($P = 0.028$) and Q148R ($P = 0.021$) mutants were significantly more resistant to RAL in macrophages than in T cells. Moreover, during macrophage

FIG. 2. Q148-associated resistance profiles in T cells and macrophages. (A) Primary costimulated T cells or macrophages were infected with viruses harboring Q148 pathway mutations in the presence of raltegravir. **, $P < 0.05$ for all wild-type viruses versus single mutants and single mutants versus double mutants. *, $P < 0.05$ for wild-type viruses versus all mutants. Each data point is derived from at least three independent experiments using cells from different donors. Mean values $\pm$ standard errors of the means are presented. (B) EC₅₀s for Q148 pathway mutant viruses. MK-2048 is a newer integrase inhibitor.

infection these single-mutant viruses displayed resistance levels that were not significantly different from those of double-mutant virus infection in T cells ($P > 0.5$). EC₅₀s for Q148 pathway mutant viruses are shown for AZT, EFV, RAL, and the newer integrase inhibitor MK-2048 (5, 19) (Fig. 2B). MK-2048 has been demonstrated previously to be active in T cells against viruses that are resistant to RAL (5). Here, we find that the concentration of MK-2048 required to inhibit infection in macrophages was slightly higher than that in T cells, but this drug nevertheless inhibited infection with all of the Q148-associated single- and double-mutant RAL-resistant viruses, with a maximum EC₅₀ of 133 nM for the G140S+Q148H mutant virus in macrophages (Fig. 2B).

Our results are consistent with prior work (15) showing that N155 pathway mutations typically produce lower-level resistance than Q148 pathway mutations. The pattern of RAL resistance activity with single- and double-mutant N155 pathway viruses was similar to that observed with Q148 pathway mutants. Both N155H and N155S single-mutant viruses had intermediate RAL resistance in T cells, with EC₅₀s

between that of the wild-type virus and the double-mutant E92Q+N155H or T97A+N155H virus (Fig. 3). However, in macrophages the N155 pathway single-mutant viruses showed resistance levels similar to those of corresponding viruses harboring double mutations. Moreover, both of the single-mutant viruses were significantly more resistant to RAL in macrophages than in T cells (N155H, $P = 0.00024$; N155S, $P = 0.019$). MK-2048 was effective at inhibiting infection with all of the N155H pathway mutant viruses during both T-cell and macrophage infections, with the highest EC₅₀ being 32.2 nM for T97A+N155H virus infection in macrophages (Fig. 3).

There has been a very limited number of other studies where RAL efficacy was evaluated in macrophage cells (25, 34), but our finding that RAL is slightly less active against HIV with a wild-type integrase coding sequence during macrophage infection than T-cell infection is consistent with those studies. It has been observed that when RAL is included in antiretroviral drug regimens, the viral loads in patients decline with faster kinetics than is the case using an equivalent regimen containing EFV instead (29, 36). One interpretation of this is that HIV

FIG. 3. N155-associated resistance profiles in T cells and macrophages. (A) Primary costimulated CD4⁺ T cells or primary macrophages were infected with N155 pathway mutant viruses in the presence of raltegravir. **, $P < 0.05$ for all wild-type viruses versus single mutants and single mutants versus double mutants. *, $P < 0.05$ for wild-type viruses versus all mutants. Each data point is derived from at least three independent experiments using cells from different donors. Mean values $\pm$ standard errors of the means are presented. (B) EC₅₀s for N155 pathway mutant viruses.

replication in cells contributing to the first- and second-phase viral load decline is affected differently by the two drugs. However, the higher RAL dose required for the inhibition of infection in macrophages than T cells demonstrated here suggests that this more rapid decline in viral load observed in patients receiving RAL is not due to a direct, more potent antiviral activity of RAL in macrophages. This effect may instead be due to other factors, such as the specific pharmacokinetic/pharmacodynamic properties of the individual drugs or the capacity of RAL to inhibit infection at later stages of the virus life cycle than RT inhibitors (44).

The fact that the four different viruses harboring single primary resistance mutations (N155H, N155S, Q148R, and Q148H) all exhibit substantially higher levels of RAL resistance in primary macrophages compared with corresponding CD4⁺ T cells suggests that a common mechanism for enhanced resistance is in action. Limitations on the concentration of drug that can be consistently maintained in macrophages could explain the different resistance patterns in these two cell

types. In HIV-infected patients, the concentration of raltegravir in peripheral blood mononuclear cells is approximately 1/10 of that present in plasma (35), but there are no data available describing the relative intracellular concentrations of integrase inhibitors in T cells and macrophages. It therefore would be interesting to determine whether macrophages indeed contain lower levels of raltegravir than T cells, and if so whether macrophages inhibit the diffusion of raltegravir into cells, degrade the drug intracellularly, or export drug that has already entered the cell.

One clear implication of these results is that during RAL therapy, macrophages are likely to support a robust infection with a virus encoding only a primary resistance mutation at amino acid location 155 or 148 of integrase. This may allow sufficient replication for the development of secondary mutations, generating viruses which can then also efficiently infect T cells in the presence of RAL. The fact that macrophages reside in tissues such as the CNS, which some antiretroviral drugs may not penetrate effectively, further reinforces their potential

role in the development of antiretroviral drug resistance. Raltegravir concentrations in the cerebrospinal fluid (CSF) of HIV-positive patients are more than 10-fold lower than those in plasma, but they generally are above the EC_{50} for wild-type HIV (10, 48). However, the EC_{50} s for single Q148 mutant viruses in macrophages described here are well above the approximately 250 nM maximum concentration of raltegravir detected in CSF. Furthermore, the majority of the patients analyzed in these prior studies had CSF raltegravir concentrations of <100 nM (10, 48), which is below the EC_{50} concentration for macrophages infected with single N155 mutant viruses described here. Chronically infected macrophages continuously producing viruses harboring single RAL resistance mutations also would be likely to significantly outlive infected activated T cells, providing a much greater temporal window for subsequent rounds of infection to produce viruses with secondary mutations. Macrophages therefore may be important intermediary cells in the stepwise process of raltegravir resistance development *in vivo*.

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