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In vitro characterization of Multi-Drug Resistant HIV-1 Isolates from a Recently Infected Patient Associated with Dual Tropism and Rapid Disease Progression

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Abstract

Objective: Multi-drug resistant (MDR)-HIV-1 variants are thought to be less fit than wild type virus. In 2005 we reported a case of transmitted MDR-HIV-1 infection associated with dual tropism and rapid clinical progression. Here, we report the *in vitro* characterization of the virus isolates.

Methods: Replication characteristics of bulk and clonal isolates from this case (MDR-1) were examined and compared with these to a panel of transmitted MDR and wild type viruses (MDR-2~4, WT-1, 2).

Results: Infectivity and frequency of infectious virion of propagated isolates were high in MDR-1 biological clones (mean titer, 3.5×10^5 TCID₅₀/ml; mean frequency of infectious virion, 1/2,444) and its bulk isolate (3.2×10^6 TCID₅₀/ml; 1/301), as compared to the other biological clones (7.3×10^3 TCID₅₀/ml; 1/21,320). Up-slope (log₁₀p24/ml/d) of viral replication in PBMC culture was much higher in MDR-1 clones (1.30 ± 0.30 : mean $\pm$ SD) than those of MDR-2~4 (0.75 ± 0.08) or WT-1, -2 clones (0.82 ± 0.03). The bulk isolate and dual tropic biological clones from MDR-1 depleted CD4⁺ T cells very rapidly in vitro compared to the other viruses tested.

Conclusion: These findings support the hypothesis that multi-drug resistant HIV-1 can effectively evolve and compensate to not only retain high level replication but exhibit virulence associated with rapid disease progression.

Introduction

The course of HIV-1 infection in an infected individual depends on multiple factors, including viral characteristics,¹ host genetics,²⁻⁴ and the innate and adaptive immune responses.⁵⁻⁷ These interactions are complex, and the manner in which they converge to determine clinical outcome remains obscure. That said, there have been viruses described and characterized that are associated with both slow progression⁸ and others with rapid progression- the latter a general consequence of infection with a virus using the CXCR4 coreceptor for entry.⁹⁻¹¹

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Multi-drug resistant (MDR)-HIV-1 variants are generally thought to be less fit than wild type virus.^{12,13} However, transmission of MDR HIV-1 is well documented¹⁴⁻²⁰ and in some cohorts with increasing prevalence.²¹ As transmission efficiency is related to levels of viremia in the donor,²² it is likely that transmissibility is highly related to viral fitness that is, the replication characteristics of a given viral population. It has been demonstrated that replication is impaired in the presence of resistance conferring-mutations,^{12,13} however this impairment may be compensated.²³ Indeed, longitudinal assessments of transmitted MDR HIV-1 show persistence of resistance-conferring mutations despite prolonged viral replication in the absence of therapy.²⁴ It is, nevertheless, unknown to what extent such compensation could restore viral replication capacity, and the mechanisms for the compensation are not fully determined.

In early 2005, we reported a case of rapid clinical progression to symptomatic AIDS in a patient with multidrug resistant, dual-tropic HIV-1 and numerous sexual contacts in the setting of methamphetamine use. As we were unable to identify a known host factor associated with rapid clinical progression,²⁵ we have hypothesized that this individual may have been infected with a particularly virulent HIV-1 variant that may have accounted in large part for the observed clinical course. Though dual tropism was documented and that alone may have accounted for the marked CD4+ T cell depletion, we asked whether this virus exhibited properties that would suggest possible determinants of virulence beyond cell tropism.

Isolates and molecular clones of HIV-1 can be distinguished by differences in replication rates and kinetics, tropism and cytopathicity. Indeed, HIV-1 variants with increased replicative capacity, including those without a change in tropism, have been shown to appear in patients followed longitudinally and are temporally related to a change in clinical course.²⁶⁻²⁹ Fitness and disease progression have been intimately linked. Quinones-Mateu and coworkers showed HIV-1 variants derived from clinical progressors outgrew isolates derived from slow progressors during in vitro competition experiments.¹⁰ More recently enhanced replicative capacity and pathogenicity of HIV-1 isolates from patients with drug resistant virus and declining CD4 cell counts has been reported.³⁰

To achieve a better understanding of the virologic factors which may have contributed to the rapidity of clinical progression we have characterized biological clones and bulk isolates derived from peripheral blood mononuclear cells of this patient and selected a panel of viruses isolated from newly infected individuals for comparison. The control viruses were selected for the presence or absence of multidrug resistance and either R5 or dual tropism. Using a variety of in vitro assays including measurements of infectivity, replication kinetics, and cytopathicity, we conclude that this particular HIV-1 variant is markedly distinct. Further probing into the mechanisms determining this viral phenotype, beyond that of coreceptor usage, will likely advance the understanding of the determinants of HIV-1 virulence.

Methods

Patients

A case of MDR-HIV-1 infection and rapid clinical progression in a patient with dual tropic HIV-1 lacking known host factors for rapid progression has been previously described²⁵ and is referred to as MDR-1. Additionally, we selected 5 subjects newly infected with HIV-1 to serve as controls based on clinical presentation, resistance profiles and tropism of baseline viruses (Table 1).

Sequence analysis for protease and reverse transcriptase genes

To analyze drug resistance-related amino acid changes, viral RNA was extracted and reverse transcribed. The regions covering protease and reverse transcriptase were amplified using primers, PR1: 5'-CTGAGAGACAGGCTAATTTTTAGGG-3' (2069-2094) and PR2: 5'-AATATTGCTGGTGATCCTTTCCATCC-3' (3028-3003) for protease region and RT1: 5'-CACTTTAAATTTTCCMATTAGTCCTATT-3' (2537-2564) and RT2: 5'-AGGAGTCTTTCCCATATTACTATGCTTTC-3' (3710-3681) for reverse transcriptase region. PCR was performed with 1 unit of Phusion DNA polymerase (Finnzymes, Espoo, Finland) and at a hot start condition using AmpliWax PCR Gem 100 (Applied Biosystems, Foster City, CA). The thermal cycle condition was 98°C, 30 sec., 37 cycles of 98°C, 8 sec., 58°C, 30 sec., 72°C, 30 sec., followed by 72°C, 5 min. PCR products were gel-purified and sequenced (Genewitz, South Plainfield, NJ) for both directions. The results were analyzed using a program, Lasergene (DNASTAR, Madison, WI). Consensus sequences of plasma virus were obtained using the commercially available Trugene assay (Siemens Diagnostics, Tarrytown NY).

Phenotype and replication capacity

Susceptibility to a panel of antiretroviral agents and replication capacity were performed using a commercially available recombinant assay, Phenosense (Monogram Biosciences, South San Francisco, CA).

Virus isolation

Biological clones (3~4 clones per case) were isolated from patient's peripheral blood mononuclear cells (PBMC) using the limiting dilution co-culture method.³¹ Briefly, 2×10^6 PHA-stimulated PBMC were plated into each well in 24-well plates. One million patient PBMC underwent 5-fold dilutions added to 4-10 wells for each dilution. The cultures were maintained and p24 concentration in the supernatants was measured by a commercially available ELISA assay (Coulter, Hialeah, FL) at days 7, 14 and 21. The supernatant of the last p24-positive well in each series of dilution was considered a 'biological clone', and the combined supernatants of the first 4 p24 antigen positive wells were collected and designated as a 'bulk isolate'. For expansion each supernatant was inoculated onto 10^8 PHA-stimulated uninfected PBMC in IL-2 containing medium. p24 concentration in the supernatant was monitored and the supernatant was harvested as a viral stock when the p24 value exceeded 20 ng/ml. After centrifugation and filtration, the supernatant was aliquoted and frozen.

Cell tropism assay

Selected isolates were assayed commercially for their cell tropism by Trofile (Monogram Biosciences) and results expressed in relative light units (RLU).³²

GHOST cell assay

GHOST (3) CXCR4, GHOST (3) Hi-5 and GHOST (3) X4/R5 (obtained from NIH AIDS Research & Reference Reagent Program) were maintained in high glucose DMEM supplemented with 10% FCS, 100U/ml penicillin, 100 µg/ml streptomycin, 500 µg/ml G415, 100 µg/ml hygromycin B (Cellgro, Herndon, VA) and 1 µg/ml puromycin (Invitrogen, Carlsbad, CA).³³ On day -1, 2.5×10^4 cells for each cell type were plated into each well of a 12-well plate. On day 0 cells were inoculated with each isolate at an approximate MOI of 0.5. On day 2, cells were detached and fixed with 1% PFA/PBS. GFP positive cells were counted using BD FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). Results are expressed as % GFP positive cells.

Titration of virus stock for infectivity

An aliquot of each viral stock was thawed and titrated for infectivity using the limiting dilution culture method.³¹ The 50% tissue culture infectious dose (TCID₅₀) was calculated by the method of Reed and Muench.³⁴ As such one infectious event follows a Poisson distribution, $f(r; \lambda) = \lambda^r \cdot e^{-\lambda} / r!$, one TCID₅₀, which contains the number of infectious virion, λ , results in negative event ($r = 0$) in 50% of occasions, i.e., $f(0; \lambda) = \lambda^0 \cdot e^{-\lambda} / 0! = 0.5$. Thus, the number of replication competent virions was calculated by multiplying TCID₅₀ value by $\ln 2$.³⁵ Each aliquot of viral stock was freeze-thawed only once for the experiments.

Measurement of viral RNA copy number in virus stock

Viral copy number in each stock virus was measured by the RT-PCR method. Viral RNA was isolated using Qiagen Viral RNA Kit (Qiagen, Valencia, CA), reverse transcribed with Superscript II RT (Invitrogen) to cDNA, which was amplified using AmpliTaq Gold DNA Polymerase (Applied Biosystems). Primers used are: a mixture of forward primers, RF1+2: 5'-CGGCGACTGGTGAGTACG-3' (735 - 752) and 5'-GGCGGCTGGTGAGTACG-3' (736 - 752) and a reverse primer, RR: 5'-GACGCTCTCGCACCCAT-3' (806 - 790). A tag probe, RB: 6-FAM-TTTGACTAGCGGAGGCTAGAAGGAGA-BHQ-1 (761 - 786) (Sigma Genosys, Woodlands, TX) was used to detect the PCR products. A series of 10-fold dilutions of target RNA fragment (532-1419) derived from NL4-3 (0, 1-10⁸ copies) were included in each assay as a standard. Real time PCR was performed using GeneAmp PCR System 9700 (Applied Biosystems) with the cycle condition of 95°C, 10 min, 50 cycles of 95°C, 15 sec and 60°C, 30 sec.

Determinations of *in vitro* replication kinetics

We selected all clones and the bulk isolate from MDR-1 as well as the select clones from MDR-2~4 and WT-1 and -2 with the highest infectious titers for subsequent characterization. 1,000 TCID₅₀ of virus were inoculated onto 2×10^6 PHA-stimulated PBMC from the same donor in 1.5 ml IL-2 medium in 24-well plate. After 6 hours of incubation, infected cells were washed twice and resuspended in 1.5 ml of IL-2 medium. Each day cultures were maintained with one fourth of the culture supernatant was collected and the same volume of new IL-2 medium added back. p24 antigen levels in culture supernatants were measured by ELISA assay (Coulter) and plotted in semi-logarithmic scales. The up-slope (r) of viral replication ($\log_{10} p24/\text{ml}/\text{day}$) was obtained from the best fitting curve of the p24 values during the exponential phase of each culture. *In vitro* replication kinetics comparing MDR-1 bulk and NL4-3 were similarly performed however the inoculums of NL4-3 ranged from 28 to 2,820 TCID₅₀ and cultures were done in duplicate.

In vitro susceptibility to inhibitors of co-receptor binding

A CCR5 inhibitor, TAK-779,³⁶ and a CXCR4 inhibitor, AMD-3100³⁷⁻³⁹ (obtained from NIH AIDS Research & Reference Reagent Program) were added to the culture of 2×10^6 PHA-stimulated donor PBMC at the concentration of 1 μM either as a single or a combination of both prior to the infection with 1,000 TCID₅₀ of each virus isolate. After overnight incubation at 37°C, 5%CO₂, free virions and inhibitors were removed by centrifugation and re-suspension of infected cells in 1.5 ml of IL-2 medium. The supernatant was collected on day 3 of culture. Level of inhibition was estimated by the p24 production relative to that of an infected control culture without inhibitors.

Cytopathicity assay

In vitro cytopathicity kinetics of selected viral isolates was examined. 8×10^6 PHA-stimulated PBMC were infected with 4,000 TCID₅₀ of viral supernatant in 6 replicates.

After overnight culture unattached viruses were removed by centrifugation and cells resuspended. The culture was maintained until day 16. To avoid cellular overgrowth, the total cell number was reduced to one third on day 10 and 13. Cell counting was performed on days 3, 5, 7, 10, 13 and 16. On the day of cell counting, cells were harvested and the cell suspension was centrifuged with the supernatant saved for p24 measurement. The cell pellet was re-suspended in 5% FCS/PBS, of which 100 μ l was added to an Absolute Counting Tube (Becton Dickinson) together with antibodies of CD3-FITC, CD8-PerCP and CD4-APC (Exalpha). Stained cells in the tube were treated and analyzed according to the procedure recommended by the manufacture. In a different experiment, all biological clones were tested for their cytopathicity. 2×10^6 PHA-stimulated PBMC were infected with 1,000 TCID₅₀ of virus isolate in duplicate. After overnight culture, free virions were removed by centrifugation and cells resuspended. The culture was maintained until day 10. To avoid cellular overgrowth, the total cell number was reduced to one third on day 7. On day 10, cells were suspended by gentle and thorough pipetting, and 100 μ l of cell suspension was added to an Absolute Counting Tube (Becton Dickinson) together with antibodies and processed as described above. Uninfected cell cultures were included as controls.

Statistical analysis

All statistical analyses were made using SAS software (SAS Institute, Cary, NC). Mean values were calculated as of geometric means, unless otherwise indicated. Nonparametric variables were analyzed by Mann Whitney U test.

Results

Resistance testing and in vitro replication capacity

Consensus sequencing of patient plasma-derived viruses and select biological clones and the MDR-1 bulk isolate were performed (Table 2). Phenotype tests including replication capacity were performed on all plasma-derived viruses in patients MDR-1-4 (Table 3). Among MDR-1 isolates, the drug resistance-associated amino acid changes in protease, i.e., L10I, L33F, M46I, I54M, L63F, I66F, A71V, G73S, I84V and L90M were shared with all biological clones. In contrast, the amino acid changes in reverse transcriptase showed some differences among isolates. M41L, K101E, V118I and Y181I were conserved, but the changes at D67, L210, T215 and K219 varied. Though T215Y was observed as a composite in the bulk isolate the revertant mutants T215C or T215D were detected. Clonal sequence analysis indicated that one out of 11 clones carried T215Y in the patient's PBMC DNA (data not shown). The genotype predicted consensus phenotypes in all cases except for some discordance in RTI susceptibility in MDR-1 as discussed in the previous report²⁵ (Table 3). Of note the replication capacity as determined commercially was highest for MDR-4-p, followed by MDR-1-p, MDR-2-p, and finally MDR-3-p (Table 3).

Determination of tropism of biological clones and bulk isolates

We determined the coreceptor usage profile of all biological clones and the bulk isolate derived from MDR-1, as well as the select clones from the control subjects using 2 methods as detailed above (Fig. 1). The commercially available pseudotyped virus assay, Trofile (Monogram Biosciences) demonstrated that only the biological clone MDR-1a was R5-tropic, whereas clones MDR-1c-d and the bulk isolate were dual tropic. Clone MDR-1b was not tested. Among isolates in the control group, MDR-2a, MDR-3c and WT-1b were confirmed as R5-tropic, whereas MDR-4c and WT-2b tested dual tropic (Fig. 1a). We confirmed co-receptor usage using a GHOST cell assay (Fig. 1b). All but one isolate, WT-2b, were found to be concordant the results obtained by the commercially available assay. To further characterize this biological clone we inoculated 10^5 MT-2 cells with 1,000 TCID₅₀ of WT-2b and observed robust syncytia formation, consistent with the ability to use

CXCR4 for entry. Co-receptor dependence was examined by the susceptibility assay for TAK-779 (CCR5 inhibitor) and/or AMD-3100 (CXCR4 inhibitor) in PBMC culture (Fig. 1c). The infections with control viruses, NL4-3 (X4) and JRCSF (R5) were blocked by AMD-3100 and TAK-779 respectively, whereas the infection of 89.6 (X4/R5X4) was blocked only by a combination of two inhibitors. Similarly to 89.6, the infection of MDR-1bulk was not blocked by either inhibitors but by the combination of both, confirming MDR-1bulk is dual tropic. A low level inhibition by AMD-3100 indicated some dependence for CXCR4 co-receptor. A further experiment using U87 cell lines with different co-receptors indicated that CCR1, CCR2 and CCR3 were not used by MDR-1 isolates (data not shown).

MDR-1 isolates grew to high titers

Infectious titers for the clonal isolates derived from MDR-1 PBMC ranged from 1×10^5 TCID₅₀/ml to 2.0×10^6 TCID₅₀/ml (mean: 3.5×10^5 TCID₅₀/ml) and for its bulk isolate reaching 3.2×10^6 TCID₅₀/ml were significantly higher than the 16 clones isolated from the PBMC of the 3 additional MDR cases (mean: 7.0×10^3 , range: 2.1×10^3 to 4.5×10^4 TCID₅₀/ml) as well as that from WT-1 and WT-2 (mean: 7.8×10^3 , range: 3.2×10^3 to 3.2×10^4 TCID₅₀/ml) ($p=0.005$ and 0.01). The mean infectivity of the isolates from MDR-1 was on average 45 to 50 fold higher than the isolates from the 5 other cases. There was no difference in infectious titers between MDR-2~4 and WT-1 and WT-2 ($p=0.39$) (Fig. 1a). We measured p24 antigen concentrations and viral RNA copy numbers in each viral stock (data not shown). The ratios of TCID₅₀ to p24 antigen concentration and viral RNA copy number were highest in MDR-1 isolates when compared to the other biological clones ($p=0.0025$ and $p=0.0034$ respectively). The fraction of replication-competent virions was calculated by assuming that each virion contains 2 copies of viral RNA (Fig. 1b). The frequency of infectious virion was 1 out of 778 to 4,610 (mean- 2,444) in MDR-1 clonal isolates or 1 out of 301 in its bulk isolate compared to 1 out of 7,533 to 76,508 (mean- 22,850) in the isolates from MDR-2~4 ($p=0.005$) and 1 out of 3,925 to 118,831 virions (mean- 18,996) in the isolates WT-1 and 2 ($p=0.01$). Again there was no significant difference in the frequency of infectious virions in supernatants containing clones from MDR-2~4 and WT-1 and -2 (Figure 1b).

MDR-1 isolates grew rapidly in PBMC culture

We determined the up-slopes ($\log_{10}$ p24/ml/d) of p24 antigen levels *in vitro*, reflecting the rate of viral replication for MDR-1a to MDR-1d and compared these values to those obtained for MDR-2a, MDR-3c, MDR-4c, WT-1b, and WT-2b (Fig. 3a). Up-slopes were highest in MDR-1 isolates, varying from 0.88 for MDR-1a to 1.57 for MDR-1c. Mean values were statistically greater ($p=0.014$) for MDR-1 isolates (1.30 ± 0.30 : arithmetic mean $\pm$ SD) when compared to the other 5 clones, which were themselves comparable (MDR isolates $[0.75 \pm 0.08]$ or wild type isolates $[0.82 \pm 0.03]$). We then compared the viral kinetics of the bulk isolate from MDR-1 PBMC to that of the laboratory strain, NL4-3 (Fig. 3b). MDR-1 bulk replicated much faster than NL4-3 with up-slopes of 1.45 and 1.09 respectively. Of note, the up-slopes determined for NL4-3 inoculums of 28, 282 and 2,820 TCID₅₀ were 1.07, 1.11, and 1.07- all substantially less than that measured for the MDR-1 bulk isolate. Importantly, when both viruses were co-cultured in the presence of 1 mM TAK-779, a CCR5 antagonist, the observed difference in upslope was maintained, suggesting that differences in coreceptor availability could not explain the difference in growth characteristics observed between MDR-1bulk and NL4-3 (data not shown). In addition MDR-1 bulk grew more rapidly and to higher titers than the R5 tropic JRCSF and dual tropic 89.6 in the presence of 1 mM AMD-3100 and 1 mM AMD-3100 or TAK-779 respectively (data not shown).

Of note, there were differences in the maximum p24 production level among isolates (Fig. 3a). Infection of MDR-1c reached p24 peak level of as high as 6.8×10^5 pg/ml, while other MDR-1 isolates reaching 2.1 to 2.6×10^5 pg/ml. Peak p24 antigen levels for the control isolates varied from 2.4×10^5 pg/ml (WT-2b) to 6.3×10^5 pg/ml (WT-1b).

MDR-1 isolates depleted CD4+ T cells rapidly.

To examine the in vitro cytopathic effects of select viruses, PHA-stimulated PBMC were infected with MDR-1bulk, WT-1b, WT-2b and MDR-4c, and the absolute cell number for each subset was monitored. In the culture infected with MDR-1bulk, CD4+ T cells started decreasing at approximately day 5 to day 7, and continued decreasing until day 16 at which time the experiment was terminated. In contrast, CD4+ T cells in the cultures of WT-2b and MDR-4c began to decrease at day 10, whereas WT-1a cultures demonstrated a marginal decrease in CD4+ T cell count when compared to the uninfected control (Fig 4a-C). On day 10 after infection, the relative CD4+ T cell counts when compared to the uninfected control were 9.6% in the MDR-1 bulk culture, and 45.9%, 118.1% and 64.0% for the MDR-4c, WT-1b and WT-2b cultures respectively. On day 16, the relative CD4+ T cell counts were further reduced to 1.9% in the MDR-1 bulk culture, and 4.4%, 91.2% and 10.6% for MDR-4c, WT-1b and WT-2b respectively (Fig.4a-A). We also measured CD3+ CD8+ T cell counts (Figure 4a-D) and no increased cytopathicity above control could be demonstrated (Fig. 4c) p24 antigen levels in the supernatant are shown in Figure 4a-B as indicators for the virus replication.

To compare the cytopathicity among the isolates tested, PHA-stimulated PBMC were infected with bulk and clonal isolates at the same inoculation dose of 1,000 TCID₅₀. On day 10 after infection, the absolute cell number for each subset was measured (Fig.4b). Consistent with the result of the experiment above, the relative CD4/CD8 ratio of the culture of MDR-1bulk was 0.21, much lower than the values for the control isolates, MDR-2~5, WT-1, 2 of 0.52 to 0.93. Furthermore, biological clones from the case, MDR-1a~d, demonstrated varying cytopathicity. Among them, MDR-1b had the highest level of cytopathicity, as its relative CD4/CD8 ratio was as low as 0.18, followed by MDR-1c (0.25), MDR-1d (0.42) and MDR-1a (0.91). The results of the characterization of these isolates are summarized in Table 4.

Discussion

The relative contributions of viral and host factors in determining the outcome of HIV-1 infection have been difficult to dissect. HIV-1 isolated from one well-described case of rapid progression in the setting of a MDR and dual tropic viral transmission was isolated from the PBMC and extensively characterized and compared to a similarly derived panel of viruses from newly infected individuals that included those with MDR transmission, rapid progression and the transmission of dual tropic virus. Specifically we have studied in vitro infectivity and growth characteristics, tropism, and in vitro cytopathicity.

When inoculated onto identical numbers of PBMC derived from the same donor, MDR-1-derived HIV-1 grew more rapidly, to higher infectious titers, produced more infectious virions relative to the total number of virions per ml culture supernatant, and proved more cytotoxic in vitro when compared to the selected control viruses. We therefore conclude that these findings are in agreement with the hypothesis that in this subject, viral factors contributed substantially to the observed rapid clinical progression.

It is well established that tropism is an important determinant of in vitro growth characteristics⁴⁰⁻⁴² as well as clinical progression.⁹⁻¹¹ Importantly, we compared properties of MDR-1 to 2 dual-tropic transmitted viruses, WT-2b and MDR-4c. Though WT-2

presented with a CD4+ T cell count of 830 cells/mm³ the baseline viral load nearly 5 months from infection was 721,000 copies/ml plasma- at least 2 standard deviations above median HIV-1 RNA levels in newly infected individuals at a similar time point⁴³. MDR-4 progressed rapidly with a CD4 cell count of 233 cells/mm³ within 5 months of diagnosis and was reported to harbor a virus with a replication capacity of >150%, greater than the 136% reported for patient MDR-1. Nevertheless neither WT-2 nor MDR-4 derived-viruses grew to comparable infectious titers or exhibited as rapid in vitro replication as did the dual tropic biological clones or the bulk isolate derived from MDR-1. In addition we compared replication kinetics of the bulk isolate of MDR-1 to that of the laboratory strain NL4-3, a predominantly CXCR-4 using virus that is highly cytopathic and known to replicate to very high titers *in vitro*. Once again, the kinetics of viral replication were substantially faster as shown by the upward slope of the growth curve (Fig. 3b) and this difference persisted in the presence of CCR5 receptor blockade, strong evidence that the difference is not due to increased receptor availability for the dual tropic virus, but to the intrinsic nature of the MDR-1 isolates. However, it is important to emphasize that these data do not provide firm evidence that MDR-1 characteristics are due to determinants outside the viral envelope, only that the virologic characteristics of the MDR-1 derived viruses are likely to be determined by factors beyond simple tropism as determined by the various experiments described.

Of note, there were differences in the maximum p24 production level among isolates as described above. Importantly, we believe peak p24 production to be an intrinsic characteristic of each isolate likely reflecting the combined effects of infectivity, virus burst size, and cytopathicity.

That the clones of MDR-1 isolates contained more infectious virions when compared to the control isolates is an interesting finding. Layne et al estimated the frequency of infectious virions be 1/10⁴ to 1/10⁷.⁴⁴ On average the frequency of infectious virions in MDR-1a~d was 1/2,444, 10-fold higher than the controls and previous estimated. It should be pointed out that we measured the infectivity on the frozen viral stocks, therefore, the frequency of infectious virus in the fresh viral isolates may be even higher. Possible explanations would include more efficient viral packaging and budding, higher burst size, or changes in envelope that could result in more effective entry at the level of either CD4 binding, co receptor binding, or fusion.

The ability of HIV-1 to lyse activated CD4+ T cells is an important determinant of HIV-1 pathogenesis during acute infection.⁴⁵ In addition, in vitro cytopathicity assays^{26,30,46,47} and SCID-hu mouse model^{48,49} have been used to demonstrate the correlation between viral cytopathicity and disease progression or syncytium-inducing (SI) phenotype. Here we have demonstrated that viruses derived from MDR-1 resulted in more cytopathicity in vitro when compared to control viruses. It must be emphasized that this in vitro observation is just that, and the result should not be over interpreted.

In essence, we believe that on all counts we have documented an infection with a unique and highly pathogenic HIV-1 variant. That said, these results do not speak directly to mechanism, but we believe provide a sound basis for subsequent experiments during which we will dissect the relative contributions of the viral envelope, structural proteins, as well as regulatory and accessory proteins to the resulting observed somewhat unique phenotype.

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References

1. Kimata JT. HIV-1 fitness and disease progression: Insights from the SIV-Macaque model. *Current HIV Research*. 2006; 4:65–77. [PubMed: 16454712]
2. Dean M, Carrington M, Winkler C, et al. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science*. 1996; 273:1856–1862. [PubMed: 8791590]
3. Hogan CM, Hammer SM. Host determinants in HIV infection and disease. Part 2: genetic factors and implications for antiretroviral therapeutics. *Ann Intern Med*. 2001; 134:978–996. [PubMed: 11352699]
4. Carrington M, O'Brien SJ. The influence of HLA genotype on AIDS. *Annu Rev Med*. 2003; 54:535–551. [PubMed: 12525683]
5. Klein MR, van Baalen CA, Holwerda AM, et al. Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. *J Exp Med*. 1995; 181:1365–1372. [PubMed: 7699324]
6. Musey L, Hughes J, Schacker T, et al. Cytotoxic-T-cell responses, viral load, and disease progression in early human immunodeficiency virus type 1 infection. *N Engl J Med*. 1997; 337:1267–1274. [PubMed: 9345075]
7. Martin MP, Gao X, Lee JH, et al. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Genet*. 2002; 31:429–434. [PubMed: 12134147]
8. Deacon NJ, Tsykin A, Solomon A, et al. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. *Science*. 1995; 270:988–991. [PubMed: 7481804]
9. Koot M, Keet IP, Vos AH, et al. Prognostic value of HIV-1 syncytium-inducing phenotype for rate of CD4+ cell depletion and progression to AIDS. *Ann Intern Med*. 1993; 118:681–688. [PubMed: 8096374]
10. Quinones-Mateu ME, Ball SC, Marozsan AJ, et al. A dual infection/competition assay shows a correlation between ex vivo human immunodeficiency virus type 1 fitness and disease progression. *J Virol*. 2000; 74:9222–9233. [PubMed: 10982369]
11. Gottlieb GS, Nickle DC, Jensen MA, et al. Dual HIV-1 infection associated with rapid disease progression. *Lancet*. 2004; 363:619–622. [PubMed: 14987889]
12. Back NKT, Nijhuis M, Keulen W, et al. Reduced replication of 3TC-resistant HIV-1 variants in primary cells due to a processivity defect of the reverse transcriptase enzyme. *EMBO J*. 1996; 15:4040–4049. [PubMed: 8670908]
13. Croteau G, Doyon L, Thibeault D, et al. Impaired fitness of human immunodeficiency virus type 1 variants with high-level resistance to protease inhibitors. *J Virol*. 1997; 71:1089–1096. [PubMed: 8995629]
14. Hecht FM, Grant RM, Petropoulos CJ, et al. Sexual transmission of an HIV-1 variant resistant to multiple reverse- transcriptase and protease inhibitors. *N Engl J Med*. 1998; 339:307–311. [PubMed: 9682043]
15. Yerly S, Kaiser L, Race E, et al. Transmission of antiretroviral-drug-resistant HIV-1 variants. *Lancet*. 1999; 354:729–733. [PubMed: 10475184]
16. Boden D, Hurley A, Zhang L, et al. HIV-1 drug resistance in newly infected individuals. *JAMA*. 1999; 282:1135–1141. [PubMed: 10501116]
17. Little SJ, Daar ES, D'Aquila RT, et al. Reduced antiretroviral drug susceptibility among patients with primary HIV infection. *JAMA*. 1999; 282:1142–1149. [PubMed: 10501117]
18. Brenner B, Wainberg MA, Salomon H, et al. Resistance to antiretroviral drugs in patients with primary HIV-1 infection. Investigators of the Quebec Primary Infection Study. *Int J Antimicrob Agents*. 2000; 16:429–434. [PubMed: 11118853]
19. Grant RM, Hecht FM, Warmerdam M, et al. Time trends in primary HIV-1 drug resistance among recently infected persons. *JAMA*. 2002; 288:181–188. [PubMed: 12095382]

20. Little SJ, Holte S, Routy JP, et al. Antiretroviral-drug resistance among patients recently infected with HIV. *N Engl J Med.* 2002; 347:385–394. [PubMed: 12167680]
21. Shet A, Berry L, Mohri H, et al. Tracking the prevalence of transmitted antiretroviral drug-resistant HIV-1: a decade of experience. *J Acquir Immune Defic Syndr.* 2006; 41:439–446. [PubMed: 16652051]
22. Quinn TC, Wawer MJ, Sewankambo N, et al. Viral load and heterosexual transmission of human immunodeficiency virus type 1. Rakai Project Study Group. *N Engl J Med.* 2000; 342:921–929. [PubMed: 10738050]
23. Simon V, Padte N, Murray D, et al. Infectivity and replication capacity of drug-resistant human immunodeficiency virus type 1 variants isolated during primary infection. *J Virol.* 2003; 77:7736–7745. [PubMed: 12829813]
24. Brenner BG, Routy JP, Petrella M, et al. Persistence and fitness of multidrug-resistant human immunodeficiency virus type 1 acquired in primary infection. *J Virol.* 2002; 76:1753–1761. [PubMed: 11799170]
25. Markowitz M, Mohri H, Mehandru S, et al. Infection with multidrug resistant, dual-tropic HIV-1 and rapid progression to AIDS: a case report. *Lancet.* 2005; 365:1031–1038. [PubMed: 15781098]
26. Connor RI, Mohri H, Cao Y, et al. Increased viral burden and cytopathicity correlate temporally with CD4+ T-lymphocyte decline and clinical progression in human immunodeficiency virus type 1-infected individuals. *J Virol.* 1993; 67:1772–1777. [PubMed: 8095306]
27. Connor RI, Ho DD. Human immunodeficiency virus type 1 variants with increased replicative capacity develop during the asymptomatic stage before disease progression. *J Virol.* 1994; 68:4400–4408. [PubMed: 7515972]
28. Tersmette M, Gruters RA, de Wolf F, et al. Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of acquired immunodeficiency syndrome: studies on sequential HIV isolates. *J Virol.* 1989; 63:2118–2125. [PubMed: 2564898]
29. Kwa D, Vingerhoed J, Boeser B, et al. Increased in vitro cytopathicity of CC chemokine receptor 5-restricted human immunodeficiency virus type 1 primary isolates correlates with a progressive clinical course of infection. *J Infect Dis.* 2003; 187:1397–1403. [PubMed: 12717620]
30. Solomon A, Lane N, Wightman F, et al. Enhanced replicative capacity and pathogenicity of HIV-1 isolated from individuals infected with drug-resistant virus and declining CD4+ T-cell counts. *J Acquir Immune Defic Syndr.* 2005; 40:140–148. [PubMed: 16186730]
31. Connor RI, Notermans DW, Mohri H, et al. Biological cloning of functionally diverse quasiespecies of HIV-1. *AIDS Res Hum Retroviruses.* 1993; 9:541–546. [PubMed: 8347398]
32. Petropoulos CJ, Parkin NT, Limoli KL, et al. A novel phenotypic drug susceptibility assay for human immunodeficiency virus type 1. *Antimicrob Agents Chemother.* 2000; 44:920–928. [PubMed: 10722492]
33. Morner A, Bjorndal A, KewalRamani V, et al. Primary human immunodeficiency virus type 2 (HIV-2) isolates, like HIV-1 isolates, frequently use CCR5 but show promiscuity in coreceptor usage. *J Virol.* 1999; 73:2343–2349. [PubMed: 9971817]
34. Reed LJ, Muench H. A Simple Method of Estimating Fifty percent Endpoints. *Am J Epidemiol.* 1938; 27:493–497.
35. Dulbecco, R. The nature of viruses. In: Davis, BD.; Dulbecco, R.; Eisen, HN.; Ginsberg, HS., editors. *Microbiology.* Harper and Row; Hagerstown: 1980. p. 800-883.
36. Baba M, Nishimura O, Kanzaki N, et al. A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity. *PNAS.* 1999; 96:5698–5703. [PubMed: 10318947]
37. Hendrix CW, Flexner C, MacFarland RT, et al. Pharmacokinetics and safety of AMD-3100, a novel antagonist of the CXCR-4 chemokine receptor, in human volunteers. *Antimicrob Agents Chemother.* 2000; 44:1667–1673. [PubMed: 10817726]
38. Bridger GJ, Skerlj RT, Thornton D, et al. Synthesis and structure-activity relationships of phenylenebis(methylene)-linked bis-tetraazamacrocycles that inhibit HIV replication. Effects of macrocyclic ring size and substituents on the aromatic linker. *J Med Chem.* 1995; 38:366–378. [PubMed: 7830280]

39. De Clercq E, Yamamoto N, Pauwels R, et al. Highly potent and selective inhibition of human immunodeficiency virus by the bicyclam derivative JM3100. *Antimicrob Agents Chemother*. 1994; 38:668–674. [PubMed: 7913308]
40. Asjö B, Morfeldt-Månson L, Albert J, et al. Replicative capacity of human immunodeficiency virus from patients with varying severity of HIV infection. *Lancet*. 1986; 2(8508):660–662. [PubMed: 2429124]
41. van 't Wout AB, Blaak H, Ran LJ, et al. Evolution of syncytium-inducing and non-syncytium-inducing biological virus clones in relation to replication kinetics during the course of human immunodeficiency virus type 1 infection. *J Virol*. 1998; 72:5099–5107. [PubMed: 9573281]
42. Troyer RM, Collins KR, Abraha A, et al. Changes in human immunodeficiency virus type 1 fitness and genetic diversity during disease progression. *J Virol*. 2005; 79:9006–9018. [PubMed: 15994794]
43. Lyles RH, Muñoz A, Yamashita TE, et al. Natural history of human immunodeficiency virus type 1 viremia after seroconversion and proximal to AIDS in a large cohort of homosexual men. Multicenter AIDS Cohort Study. *J Infect Dis*. 2000; 181:872–880. [PubMed: 10720507]
44. Layne SP, Merges MJ, Dembo M, et al. Factors underlying spontaneous inactivation and susceptibility to neutralization of human immunodeficiency virus. *Virology*. 1992; 189:695–714. [PubMed: 1386485]
45. Mehandru S, Poles MA, Tenner-Racz K, et al. Mechanisms of gastrointestinal CD4+ T-cell depletion during acute and early human immunodeficiency virus type 1 infection. *J Virol*. 2007; 81:599–612. [PubMed: 17065209]
46. Pal A, Greenough TC, Sullivan JL, et al. In vitro characterization of adult primary human immunodeficiency virus type 1: demonstration of distinctive single-cell killing phenotypes in spite of similar levels of viral replication. *J Infect Dis*. 1997; 176:933–940. [PubMed: 9333151]
47. Penn ML, Grivel JC, Schramm B, et al. CXCR4 utilization is sufficient to trigger CD4+ T cell depletion in HIV-1-infected human lymphoid tissue. *Proc Natl Acad Sci USA*. 1999; 96:663–668. [PubMed: 9892690]
48. Kaneshima H, Su L, Bonyhadi ML, et al. Rapid-high, syncytium-inducing isolates of human immunodeficiency virus type 1 induce cytopathicity in the human thymus of the SCID-hu mouse. *J Virol*. 1994; 68:8188–8192. [PubMed: 7966610]
49. Camerini D, Su HP, Gamez-Torre G, et al. Human immunodeficiency virus type 1 pathogenesis in SCID-hu mice correlates with syncytium-inducing phenotype and viral replication. *J Virol*. 2000; 74:3196–3204. [PubMed: 10708436]

Figure 1. Co-receptor usage

Co-receptor usage of selected isolates (X-axis) was examined by Trofile (Monogram Biosciences) (a); GHOST assay (b); and inhibition assay (c). In the GHOST (3) cell assay, an equal amount of virus was inoculated to 3 cell types, GHOST (3) X4, (3) hiR5 and (3) X4/R5. The percent GFP+ cells after 3 days of infection are indicated on the Y-axis (b). In the inhibition assay, 1 μ M of TAK-779 (CCR5 inhibitor) or/and AMD-3100 (CXCR4 inhibitor) were used to examine functional tropism of MDR-1bulk in parallel with 3 well-characterized viruses, NL4-3 (X4), JRCSF(R5) and 89.6 (R5X4) (c). The %p24 values over control culture (without inhibitor) are indicated.

Figure 2. Infectious titer and reciprocal frequency of infectious virion in propagated virus isolates

Infectivity (TCID₅₀/ml) of propagated virus isolates was measured on PHA-stimulated donor PBMC culture (a). Reciprocal frequency of infectious virion was calculated by dividing total virion number (viral RNA copy number $\times$ 1/2) by infectious virion number (TCID₅₀ $\times$ $\ln 2$) (b). The results are compared among clonal isolates from MDR-1, MDR-2~4 (MDR-control) and WT-1, 2 (WT-control) with the number of clones for each group indicated in the parenthesis. The geometric mean values are indicated by a line symbol for each group and the p value of Man-Whitney U test is indicated along with each

arrow, which indicates two groups to be compared. The values for MDR-1bulk as indicated by symbols, ω , are not included in the mean value calculation, nor the statistical analysis.

Figure 3. Viral kinetics in PHA-stimulated PBMC culture

1,000 TCID₅₀ of each viral isolate was inoculated to 2×10^6 PHA-stimulated donor PBMC, and the p24 concentration in the supernatant was measured daily. 10 isolates, including 9 biological clones and one bulk isolate from PBMC were tested in parallel (a). In a second experiment, MDR-1bulk and NL4-3 were examined in parallel on donor PBMC. The inoculation doses of 1, 10, 100 µl for NL4-3 were equivalent to 28, 282 and 2,820 TCID₅₀ respectively (b). Up-slope of p24 antigen in the supernatant, r ($\log_{10}$ p24/ml/day), was obtained from the best fitting curve during the initial exponential growth phase.

Figure 4. Cytotoxicity in PBMC culture

The kinetics of cytopathicity in PHA-stimulated PBMC coculture was studied for select isolates, MDR-1bulk, WT-1b, WT-2b and MDR-4c (Fig. 4a). The changes of the absolute numbers of CD4⁺ T cells (C) and CD8⁺ T cells (D) were indicated in parallel with uninfected control culture. The reduction of CD4⁺ T cells as compared to the control culture was calculated (A). The p24 concentration in the supernatant was measured as an indicator of viral replication (B). In a different experiment, cytopathicity of all virus isolates was evaluated by the reduction in the ratio of CD4⁺/CD8⁺ T lymphocyte subsets after 10 days of infection in PHA-stimulated PBMC coculture (Fig. 4b). The results are shown as relative CD4/CD8 ratio over the uninfected control culture. The error bar indicates a standard error for duplicate cultures.

Table 1

Patients' Baseline Data

Patient	Duration of infection (months)	CD4 count (/μl)	Viral load (RNA copies/ml)
MDR-1	>4, <18	28	252,000
MDR-2	5.5	254	323,000
MDR-3	2.0	391	1,380,000
MDR-4	5.1	233	135,000
WT-1	1.5	390	77,400
WT-2	4.9	830	721,000

Table 2

Drug Resistance-Associated Genotype of Virus Isolates

PRI Resistance:															
	L10	V32	L33	M36	M46	I47	I54	L63	I66	A71	G73	V82	I84	L90	
MDR-1-p (consensus) [#]	I	-	F	-	I	-	M	P	F	V	S	-	V	M	
MDR-1a ^o	I	-	F	-	I	-	M	P	F	V	S	-	V	M	
MDR-1b ^o	I	-	F	-	I	-	M	P	F	V	S	-	V	M	
MDR-1c ^o	I	-	F	-	I	-	M	P	F	V	S	-	V	M	
MDR-1d ^o	I	-	F	-	I	-	M	P	F	V	S	-	V	M	
MDR-1bulk ^o	I	-	F	-	I	-	M	P	F	V	S	-	V	M	
MDR-2-p (consensus)	I	-	-	I	-	-	-	P	-	T	-	-	-	M	
MDR-2a	I	-	-	I	-	-	-	P	-	T	-	-	-	M	
MDR-3-p (consensus)	V	-	-	-	-	L	-	-	P	-	-	-	-	M	
MDR-3c	V	-	-	-	-	L	-	-	P	-	-	-	-	M	
MDR-4-p (consensus)	I	I	F	-	-	V	M	P	F	V	-	A	V	M	
MDR-4c	I	I	F	-	-	V	M	P	V	-	A	V	V	M	
NRTI,NNRTI Resistance:															
	M41	E44	D67	T69	K70	L100*	K101*	K103*	V108*	V118	Y181*	Y188*	L210	T215	K219
MDR-1-p (consensus) [#]	L	-	N	-	-	-	E	-	-	I	I	-	LGMRVW	CY	E
MDR-1a ^o	L	-	N	-	-	-	E	-	-	I	I	-	G	C	E
MDR-1b ^o	L	-	DN	-	-	-	E	-	-	I	I	-	LG	CD	KE
MDR-1c ^o	L	-	N	-	-	-	E	-	-	I	I	-	G	C	E
MDR-1d ^o	L	-	N	-	-	-	E	-	-	I	I	-	LG	CD	KE
MDR-1bulk ^o	L	-	DN	-	-	-	E	-	-	VI	I	-	LGVW	CY	KE
MDR-2-p (consensus)	L	D	-	D	-	-	-	-	I	I	-	-	W	D	-
MDR-2a	L	D	-	D	-	-	-	-	I	I	-	-	W	D	-

NRTI, NNRTI Resistance:													
	M41	E44	D67	T69	K70	L100*	K101*	K103*	V108*	V118	Y181*	Y188*	K219
MDR-3-p (consensus)	-	-	-	-	R	I	-	N	-	-	-	-	-
MDR-3c	-	-	-	-	R	I	-	N	-	-	-	-	-
MDR-4-p (consensus)	-	-	N	D	-	-	Q	-	-	-	-	L	Q
MDR-4c	-	-	N	D	-	-	Q	-	-	-	-	L	Q

Note:

Blocks with two or more amino acids indicate mixture of sequence.

- either wild type or resistance-unrelated change

* resistance mutation for NNRTI

#The results of consensus are from patients' plasma (Trugene assay). MDR-1-p (consensus) is from the plasma sample (1/13/2005).

° sequence results of virus isolates from MDR-1's PBMC (1/22/2005)

Table 3**Drug Resistance and Replication Capacity**

Drug	MDR-1-p	MDR-2-p	MDR-3-p	MDR-4-p
PI				
ATV	16	n.d.	n.d.	n.d.
AMP	>Max	1.8	2.5	>Max
IDV	70	3.2	3.1	50
LPV/r	299	20.8	1.3	268
NFV	37	6.2	10	43
RTV	>Max	6.8	2.5	>Max
SQV	40	3.6	2.7	54
NRTI				
ABC	0.8	1.2	1.1	0.9
ddl	0.8	1.5	1.0	0.8
FTC	3.8	n.d.	n.d.	n.d.
3TC	3.3	3.1	2.0	1.5
d4T	1.0	1.2	1.3	0.9
ZDV	0.5	0.7	3.6	2.6
TFV	0.4	1.0	1.3	1.0
NNRTI				
DLV	2.1	1.5	>Max	31
EFV	0.6	1.3	>Max	182
NVP	>Max	1.4	67	>Max
Replication Capacity	136%	77%	18%	>150%

* PhenoSense results (fold change) and estimated replication capacity.

Table 4

Summary of Viral Characteristics

Biological clone or bulk isolate	Infectious titer of isolate ($\times 10^3$ TCID ₅₀ /ml)	Fold virion particle over infectious virion	Co-receptor usage	Up-slope p24 antigen (log ₁₀ p24/ml/day)	Peak p24 antigen (pg/ml) $\times 10^5$	Cytotoxicity (relative CD4/CD8 ratio)
MDR-1a	100	2,638	R5	0.88	2.8	0.91
MDR-1b	118	4,610	R5X4	1.42	2.2	0.18
MDR-1c	447	3,770	R5X4	1.57	6.8	0.25
MDR-1d	1,995	778	R5X4	1.32	2.6	0.42
MDR-1bulk	3,162	301	R5X4	1.36	2.1	0.21
MDR-2a	32	7,533	R5	0.68	2.9	0.71
MDR-3c	45	12,463	R5	0.75	5.0	0.52
MDR-4c	32	11,793	R5X4	0.83	3.1	0.52
WT-1b	32	3,925	R5	0.84	6.3	0.93
WT-2b	18	15,138	R5X4	0.80	2.4	0.57