Altered Drug Sensitivity, Fitness, and Evolution of Human Immunodeficiency Virus Type 1 with pol Gene Mutations Conferring Multi-Dideoxynucleoside Resistance

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Investigations were done to determine whether the replication kinetics of human immunodeficiency virus (HIV)-1 were altered when the virus acquired a set or subsets of five mutations (A62V, V75I, F77L, F116Y, and Q151M) in the pol gene conferring resistance to multiple dideoxynucleosides. In the absence of drugs, the replication rate of all infectious clones generated was comparable to that of wild type HIV-1. However, in the presence of zidovudine or didanosine, the comparative order for replication was HIV-142T75I116F151M > HIV-177V116F151M > HIV-151M, whereas that for drug resistance was HIV-142T75I116F151M > HIV-142T75I116F151M > HIV-151M. The virologic features of these infectious mutants suggest that HIV-1 develops drug resistance through one or more mutations, which, however, sacrifice replicative capability; then it finally acquires optimal replication competence by additional mutations when the multi-dideoxynucleoside-resistant mutant emerges.

Materials and Methods

Reagents and cells. Zidovudine and zalcitabine were purchased from Sigma (St. Louis) and didanosine from Calbiochem (San Diego). H9 and MT-2 cells were used to propagate HIV-1. CD4-LTR/b-gal cells were provided by M. Emerman (AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH). COS-7 cells were purchased from American Type Culture Collection (Rockville, MD). Peripheral blood mononuclear cells (PBMC) were obtained from healthy HIV-1-seronegative donors by use of ficoll-hypaque gradient centrifugation and were stimulated for 2–3 days with 10 μg of phytohemagglutinin M (PHA; Gibco-BRL, Grand Island, NY)/mL before use.

Construction of infectious HIV-1 clones. Infectious molecular clones with mutations of interest were constructed by use of the pHXB2RIP7-based plasmid, pSUM9, as previously described [9, 11]. In brief, the XmaI and NheI sites were generated by introducing four mutations at codons 14, 15, 267, and 268, which did not change the deduced amino acids, into the Apal-SalI fragment of pHXB2RIP7 by site-directed mutagenesis (United States Biochemicals, Cleveland), generating an infectious molecular clone, pSUM9. The XmaI-NheI fragment of pSUM9 was subcloned into pTZT, which originated from pT1Z19R (Pharmacia, Piscataway, NJ), generating pTZNX1. Mutations of interest were introduced.

Human immunodeficiency virus type 1 (HIV-1) has proved to be capable of developing resistance against virtually any currently available therapeutic reverse transcriptase (RT) and protease inhibitors [1–6]. In an attempt to delay or block the development of drug-resistant HIV-1 variants and alleviate drug toxicities, therapy with multiple antiviral drugs has been widely used [7, 8]. However, a set of five mutations in the pol gene, including an Ala Val substitution at codon 62 (A62V), Val Ile at codon 75 (V75I), Phe Leu at codon 77 (F77L), Phe Tyr at codon 116 (F116Y), and Gln Met at codon 151 (Q151M), has been identified in HIV-1 isolated from patients receiving long-term combination chemotherapy with zidovudine plus zalcitabine or zidovudine plus didanosine; these mutations confer on HIV-1 reduced sensitivity to multiple dideoxynucleosides (ddNs) [9–13]. The frequency of the emergence of Q151M and its related mutations has been 3.5% to >19% in patients receiving combination chemotherapy with multiple ddNs for >1 year [9–14] (Kavlick MF, Mitsuya H, unpublished data). We have previously examined enzymatic properties of RT carrying all or a subset of these five mutations [6, 15] and demonstrated that all recombinant RTs carrying a single mutation or combined mutations had comparable catalytic efficiency (kcat/Km) compared with that of wild type RT (RTwt), although a marked difference was noted in inhibition constants (Ki).

There are several reports describing replication kinetics of HIV-1 variants resistant to multiple dideoxynucleosides [13, 14]; however, these works examined HIV-1 variants only in the absence of drugs in vitro. A critical question yet to be answered is whether such drug-resistant HIV-1 variants have replication capacity in the presence of drugs to which those variants are resistant. Therefore, we examined the replication kinetics of HIV-1 variants carrying multidrug resistance-associated mutations in the presence and absence of drugs, which may help us to understand and predict the outcome of infection with mutant HIV-1 and virus population changes after cessation of or change in antiviral therapy.
by oligonucleotide-directed mutagenesis using the plus strand of pTZNX1 as a template as previously described [9, 11]. To construct infectious clones, the Xmnl-NheI fragment with mutations was subsequently transferred to pSUM9, generating infectious HIV-1 molecular clones with the intended mutations.

Production of infectious HIV-1 clones. COS-7 cells were transfected with 15 μg of each molecular clone by the calcium phosphate method (Promega, Madison, WI) according to the manufacturer’s protocol. Infectious virions produced were harvested 48 h after transfection and were propagated in MT-2 cells and subsequently in H9 cells for 7–10 days. Culture supernatants obtained were filtered through a 0.45-μm-pore membrane and stored at −70°C until use. Determination of the nucleotide sequence of the polymerase-encoding region of HIV-1 harbored in H9 cells confirmed that each infectious clone generated had the intended mutation(s) and no additional base changes in the polymerase-encoding region. Each virus preparation was titrated for its infectivity in the multinuclear activation of a galactosidase indicator (MAGI) assay (see below) by formation of blue multinuclear cells as previously described [16].

Determination of drug sensitivity. The sensitivity of infectious clones to various ddNs was determined in the MAGI assay with the virus preparations titrated as described above. Target cells (CD4-LTR/β-gal: 10^5/well) were plated in 96-well flat microtiter culture plates (Costar, Cambridge, MA). On the following day, the medium was aspirated and the cells were incubated at 37°C with various concentrations of drug in fresh medium. In 2 h, an inoculum of each virus preparation (50 μL) that gave 100 blue cells/well was added, and the cells were further cultured in the presence of drugs. Eight to ten different drug concentrations (3-fold serially diluted) were used at concentrations up to 100 μM for zidovudine and zalcitabine and up to 300 μM for didanosine. Forty-eight hours after virus exposure, all blue cells in each well were counted. All experiments were done in triplicate.

Determination of the profile of viral replication. H9 cells (10^5) or PHA-stimulated PBMC (10^6: cultured with 5 ng/mL recombinant interleukin-2) were exposed to each infectious clone for 2 h at 37°C at a virus inoculum 10-fold greater than that used for the determination of drug sensitivity in the MAGI assay, washed twice with PBS, and cultured in 2 mL of complete medium in the presence or absence of 2 μM zidovudine or 10 μM didanosine in 24-well tissue culture plates (Costar). Culture supernatants (200 μL) were removed at various times, followed by the addition of an equal volume of fresh complete medium. On day 5 of culture and beyond, 1 mL of the culture medium was collected, and the equal volume of fresh medium was replenished every 1–2 days to ensure the optimal growth of the cells. Concentrations of each drug were maintained the same throughout the period of the culture. The amount of p24 Gag protein in the supernatants during a 15-day period of culture was determined by the commercially available RIA kit (Du Pont, Boston), which allowed us to determine the replication kinetics during 7–10 rounds of infection. All assays were done two to five times for each infectious clone.

Statistical analysis of p24 Gag production profile. The IC_{50}s of antiviral agents against various HIV-1 clones were compared by the Jonckheere-Terpstra method for trends and by the Tukey Studentized range test in an analysis of variance of the log-transformed values [17].

For the comparison of replication capacity of each HIV-1 infectious clone, a Gompertz growth model was fitted to the log-transformed data collected with each HIV variant propagated in the presence or absence of each antiretroviral agent (zidovudine or didanosine), by use of the nonlinear least squares method. The distribution of the residuals was tested and found to be consistent with normality. The F-like statistics for the comparisons of the two curves were calculated [17]. Because of the nonlinearity of the model, P values derived from the F distribution are only approximate. The Bonferroni correction was then applied to the 21 pairwise tests for each agent. It was assumed that the conservatism of this correction would account for any possible anticonservatism in the P values.

Results

Drug susceptibility of infectious clones in the MAGI assay. We previously determined the susceptibility of HIV-1 variants isolated from patients receiving combination chemotherapy with zidovudine plus zalcitabine or zidovudine plus didanosine to multiple ddNs by use of Molt-4 or H9 cells as target cells [9, 11]. Such culture assay systems, however, involve multiple cycles of viral replication, and the difference in the enzymatic activity of RT could have been unnecessarily amplified. In this work, in an attempt to more directly define the difference in the activity of RT carrying various mutations, we used the MAGI assay, which involves only a single round of viral replication [16]. In the 2-day MAGI assay, the integration of a single HIV-1 genome into the cellular DNA and subsequent Tat protein expression result in the activation of theLTR-Tat—driven reporter gene (β-galactosidase), which is visualized through the formation of blue cells [16]. Soluble CD4 (sCD4: 10 μg/mL) was initially added 16 h after virus exposure to block the reinfection of the cells by newly produced virions; however, the number of blue cells did not significantly differ in the presence or absence of sCD4. Thus, no sCD4 was added in subsequent MAGI assays.

We first determined the drug susceptibility of infectious clones containing various amino acid substitutions. CD4-LTR/β-gal cells were treated with zidovudine, didanosine, or zalcitabine and were exposed to infectious clones at a virus dose that gave 100 blue cells in the MAGI assay (table 1). The infectious clone carrying Q151M (HIV-1_{151}) showed reduced sensitivity to zidovudine (16-fold), didanosine (2-fold), and zalcitabine (7-fold), while the sensitivity of other infectious clones carrying a single mutation (HIV-1_{162}, HIV-1_{75}, HIV-1_{77}, or HIV-1_{116}) were all comparable to that of the wild type infectious clone (HIV-1_{wt}). The virus with three amino acid substitutions (HIV-1_{75/77/116/151}) had a further reduced sensitivity to zidovudine (110-fold), didanosine (9-fold), and zalcitabine (16-fold). The infectious clone with four mutations (HIV-1_{75/77/116/151}) showed the highest level of resistance to zidovudine (420-fold), didanosine (30-fold), and zalcitabine (58-fold). HIV-1 with five mutations (HIV-1_{62/75/77/116/151}) had the next highest levels of resistance to ddNs (table 1). When IC_{50}s were compared, the overall comparative order for drug resistance was as follows: HIV-1_{75/77/116/151} > HIV-1_{62/75/77/116/151} > HIV-1_{77/116/151} >
HIV-1151. Significances of the differences in IC50s compared by the Jonckheere-Terpstra method for trends were P < .0001 for zidovudine, P = .0079 for didanosine, and P = .041 for zalcitabine. Results of comparison of individual IC50s are shown in the legends to table 1.

These data show that the levels of reduced sensitivity of infectious clones to various ddNs are comparable to those determined in the conventional assay [9, 11] and support that the five mutations in the pol gene account for the observed viral resistance to multiple ddNs.

HIV-1 replication in H9 cells in the absence of drugs. We attempted to compare the replication rate of each infectious clone in the absence of drugs. All infectious clones were titrated for their infectivity in the 2-day MAGI assay. H9 cells were exposed to an equal virus inoculum for each infectious clone that gave 1000 blue cells in the MAGI assay and were cultured in the absence of drugs. Since the MAGI assay involves 2 days of culture, then if the cells were infected with HIV-1 at the same infectious dose, the accumulated amount of newly produced virions should be the same at the end of day 2 of culture, assuming that the level of replication of each virus preparation was comparable in CD4-LTR/β-gal cells and H9 cells. The p24 Gag protein production by H9 cells exposed to HIV-1151, HIV-1215, or HIV-1215 was comparable to that of HIV-1wt, although HIV-1116 was slightly delayed in its replication (figure 1A[a]). The p24 Gag protein production by H9 cells exposed to HIV-1 with a single mutation (HIV-162, HIV-1215, HIV-177, HIV-116, HIV-1151) was comparable and also comparable with that of HIV-1wt (figure 1A[b]). The Gag protein production by H9 cells exposed to all or a subset of the five mutations (HIV-177/116/151, HIV-1215/161/151, HIV-1215/161/151) was also comparable to that of HIV-1wt (figure 1A[c]), although HIV-11215 appeared to replicate somewhat more rapidly than other infectious clones. The data from the H9 culture were corroborated when PHA-stimulated PBMC were used as target cells (figure 1B). These data, taken together, agreed with our previous data from steady-state kinetic studies showing comparable catalytic efficiency (kcat/Km) for RTwt and RTs carrying combined mutations [15].

Q151M and T215Y mutations can coexist in RT. HIV-1 variants carrying all or subsets of the five mutations do not have a T215Y or T215F mutation associated with viral resistance to zidovudine, except for a transient or sporadic appearance, despite long-term chemotherapy with zidovudine [9–15]. It is possible that a certain nucleotide or amino acid sequence(s) predisposes HIV-1 to the Q151M substitution and ultimately the rest of the five mutations. It is also possible that once HIV-1 acquires Q151M, T215F or T215F no longer occurs because of their infectivity in the 2-day MAGI assay. H9 cells were infected with HIV-1. We found that an infectious clone carrying both Q151M and T215Y (HIV-1151/215) replicated as efficiently as HIV-1wt (figure 2), and its drug sensitivity profile was comparable to that of HIV-1151 (table 1), suggesting that the two mutations do not impair the replicative ability of HIV-1.

Replication rates of HIV-1 variants in the presence of drug. In the clinical setting, the major driving force for drug-resistant HIV-1 variants to emerge is the selective advantage conferred by high levels of viral drug resistance. We, therefore, exposed H9 cells to each infectious clone and cultured them in the presence of zidovudine or didanosine. To completely inhibit the replication of HIV-1wt, we chose 2 μM zidovudine and 10 μM didanosine, representing the respective IC95 concentrations [18–20]. HIV-1wt failed to produce a significant level of

Table 1. Generation of various infectious HIV-1 clones and their sensitivity to zidovudine, didanosine, and zalcitabine in MAGI assay.

<table>
<thead>
<tr>
<th>Infectious clone</th>
<th>Amino acid</th>
<th>Zidovudine</th>
<th>Didanosine</th>
<th>Zalcitabine</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1wt</td>
<td>Ala Val Phe Phe Gln Leu Thr</td>
<td>0.043 ± 0.020</td>
<td>0.040 ± 0.004</td>
<td>0.054 ± 0.020</td>
</tr>
<tr>
<td>HIV-162</td>
<td>Val Leu Tyr Met</td>
<td>0.054 ± 0.020</td>
<td>0.057 ± 0.042</td>
<td>0.054 ± 0.020</td>
</tr>
<tr>
<td>HIV-175</td>
<td>Val Leu Tyr Met</td>
<td>0.055 ± 0.021</td>
<td>0.29 ± 0.042</td>
<td>0.054 ± 0.020</td>
</tr>
<tr>
<td>HIV-116</td>
<td>Val Leu Tyr Met</td>
<td>0.057 ± 0.021</td>
<td>8.8 ± 3.8</td>
<td>0.067 ± 0.26</td>
</tr>
<tr>
<td>HIV-1151</td>
<td>Val Leu Tyr Met</td>
<td>4.8 ± 3.3</td>
<td>18 ± 3</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>HIV-175/116/151</td>
<td>Val Leu Tyr Met</td>
<td>12 ± 1</td>
<td>19 ± 6</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>HIV-177/116/151</td>
<td>Val Leu Tyr Met</td>
<td>0.075 ± 0.020</td>
<td>9.6 ± 1.2</td>
<td>0.26 ± 0.07</td>
</tr>
</tbody>
</table>

NOTE. Dash denotes wild type amino acid. IC50 values (±SD) of 3 separate experiments are shown.

The Jonckheere-Terpstra method for trends was P < .0001 for zidovudine, P = .0079 for didanosine, and P = .041 for zalcitabine. Results of comparison of individual IC50s are shown in the legends to table 1.

* Mean IC50 values (±SD) of 3 separate experiments are shown.

Mutations at codons 74 and 215 have been associated with viral resistance to didanosine and zidovudine, respectively.
p24 Gag protein in the presence of drugs at the concentrations chosen (figure 3). Four infectious clones with a single mutation (HIV-162, HIV-175, HIV-177, HIV-1116) also failed to propagate in the presence of zidovudine or didanosine (data not shown).

However, there was a significant difference in replication rates of infectious clones carrying certain mutations when propagated in the presence of zidovudine or didanosine (figure 3). All four infectious clones carrying Q151M (HIV-1151 [P < .01], HIV-177/116/151 [P < .01], HIV-175/77/116/151 [P < .01], and HIV-162/75/77/116/151 [P < .01]) replicated significantly faster than did HIV-1wt in the presence of zidovudine. There was also a significant difference in replication rates between HIV-177/116/151 and HIV-175/77/116/151 (P < .01), between HIV-177/116/151 and HIV-162/75/77/116/151 (.01 < P < .05), between HIV-175/77/116/151 and HIV-162/75/77/116/151 (.01 < P < .01), and between HIV-1215 and HIV-177/116/151 (.01 < P < .05) when propagated in the presence of zidovudine, although there was no statistical difference between HIV-1151 and HIV-175/77/116/151 (P = .94). Overall, the comparative order for replication capacity in the presence of zidovudine was as follows: HIV-1151 > HIV-177/116/151 > HIV-175/77/116/151 ≈ HIV-1151. It should be noted that HIV-1215 showed the greatest level of resistance against ddNs in the MAGI assay (table 1) and against ddNTPs in enzymatic assays [6, 15] but propagated less efficiently did than HIV-177/116/151 and HIV-162/75/77/116/151 (figure 3A).

In the presence of didanosine, the three infectious clones producing the most Gag (HIV-177/116/151, HIV-175/77/116/151, and HIV-162/75/77/116/151) replicated significantly faster than did HIV-1215 (P < .01). HIV-177/116/151 and HIV-162/75/77/116/151 replicated faster than did HIV-1wt (P < .05). There was...
Figure 3. Replication profiles of infectious clones carrying various mutations in presence of zidovudine or didanosine. H9 cells were exposed to each infectious clone at infectious dose that gave 1000 blue cells in MAGI assay and cultured in presence of 2 \( \mu M \) zidovudine (A) or 10 \( \mu M \) didanosine (B). Amount of p24 Gag protein released from infected H9 cells into culture medium was determined by RIA at various time points.

It is noteworthy that the amount of Gag protein produced by H9 cells exposed to HIV-1\(_{62/75/77/116/151}\) and cultured in the presence of zidovudine or didanosine reached >300 ng/mL by day 10 of culture. This amount was comparable to that produced by H9 cells exposed to HIV-1\(_{wt}\) and cultured in the absence of drug (figures 1A, 3), suggesting that the replication rate of HIV-1\(_{62/75/77/116/151}\) in the presence of zidovudine or didanosine was roughly comparable to that of HIV-1\(_{wt}\) in the absence of drugs (figures 1A, 3).

Discussion

In the present work, we examine the relative viral replication kinetics of HIV-1 when it acquires a set or subsets of five mutations (A62V, V75I, F77L, F116Y, and Q151M) in the pol gene conferring viral resistance to multiple ddNs [9–14]. It was possible, however, that in the conventional in vitro drug sensitivity assays [7, 18, 20–22], determinants of viral replication kinetics are masked during multiple cycles of viral replication (roughly 7–10). In the present work, therefore, the 2-day MAGI assay using CD4-LTR/\( \beta \)-gal cells, which involves a single cycle of viral infection [16], was used to assess the difference in drug sensitivity. These data show that the levels of reduced sensitivity of infectious clones to various ddNs (table 1) were comparable to those determined in the conventional assay [9, 11], supporting the notion that mutations in the polymerase-encoding region account for the observed viral resistance to RT inhibitors.

The accumulation of multiple mutations associated with drug resistance does not occur at random but follows a certain order in some cases [23, 24]. Among the five mutations, Q151M is thought to be the first mutation to develop [9, 12, 13], followed by V75I, F77L, F116Y, and then A62V during therapy [9, 12]. In this regard, as seen in the case of HIV-1 resistant to a C2-symmetrical protease inhibitor (ABT-77003) [25], once a mutation occurs (in our case, Q151M), without regard to the presence or absence of drugs, HIV-1 may acquire other mutations to improve virus infectivity and drug resistance. It has been noted that there is no viral acquisition of zidovudine-associated mutations except transient or sporadic ones, despite long-term therapy including zidovudine [9–13] (Kavlick MF, Mitsuya H, unpublished data). The present data showed that HIV-1\(_{151/215}\) had a level of resistance to zidovudine comparable with that of HIV-1\(_{215}\) and propagated as efficiently as HIV-1\(_{wt}\) in the absence of drugs, suggesting that the two mutations do not impair the replicative ability of HIV-1 (table 1). This is in agreement with our previous substrate analysis data of RT carrying both mutations showing no significant difference in \( K_{\text{m}}, k_{\text{cat}}, \) and \( k_{\text{cat}}/K_{\text{m}} \) values between wild type and Q151M- or T215Y-carrying RTs [15]. Iversen et al. [13] have also shown that the addition of T215Y to HIV-1\(_{151/215}\) did not change the replication profile of the virus. At present, it still remains to be determined whether HIV-1 carrying Q151M can subsequently acquire the zidovudine-associated amino acid substitutions in patients receiving combination chemotherapy.

In this study, we also attempted to determine the replication profile of each infectious clone carrying a single or combined mutations. When the p24 Gag protein production by H9 cells exposed to the same infectious titer adjusted by MAGI assay and cultured in the absence of drugs was used as an indicator of viral replication, there was no significant difference for all infectious clones examined in H9 cells and PHA-stimulated PBMC (figure 1A[c]). It should be noted that it is difficult to determine the replication kinetics of HIV-1 variants against
that of wild type HIV-1. For example, Kellam et al. [24] have demonstrated that mutations at codons 41, 67, 70, and 215 produced a highly zidovudine-resistant phenotype; however, the addition of the mutations at codon 219 produces no detectable changes in the virus’s sensitivity to zidovudine or in the replication characteristics. On the other hand, Caliendo et al. [26] have observed that HIV-1 carrying four zidovudine-associated mutations (HIV-167/70/215/219) had a replication advantage over wild type HIV-1 when target PBMC were stimulated with PHA after virus exposure and examined in the absence of drugs, but there was no discernable difference when PBMC were stimulated with PHA before virus exposure.

Van Rompay et al. [27] have recently isolated a mutant simian immunodeficiency virus (SIVmac) carrying Q151M from macaques receiving prolonged zidovudine treatment and administered its molecular clone but not zidovudine to newborn macaques. All animals showed persistent viremia with a magnitude comparable to that seen in animals infected with wild type SIV. These results suggest that the Q151M mutation in SIV does not significantly impede the replication competence or pathogenicity and is genetically stable. In this respect, the amino acid at codon 151 (Gln) is located in the highly conserved concatenated motif B, which has been identified in the RT of animal and human retroviruses [28]. In fact, the pol genes from 16 animal and human retroviruses encode a Leu-Pro-Gln-Gly sequence in the corresponding region within motif B [28]. However, the substituted amino acid Met at codon 151 is found in the corresponding region of the RNA-dependent DNA polymerase of three hepadnaviruses examined: human, woodchuck, and duck hepatitis B viruses [28]. These data suggest that the Q151M substitution is not overly detrimental to the function of RT, in agreement with our present and previous data [6, 15].

In this study, the most drug-resistant virus was HIV-157/77/116/151, but the replication rate of this clone was much slower than that of HIV-162/75/77/116/151 and slower even than that of HIV-177/116/151 (figure 3). In this regard, in 5 of 7 patients in our study whose HIV-1 developed the Q151M mutation, all five mutations were identified, while HIV-1 of 2 patients developed all but the A62V mutation (Kavlick MF, Mitsuya H, unpublished data). These data suggest that the mutation at codon 62 is probably the last one to be added among the five mutations. If so, it is likely that the A62V mutation compensates for the impaired replication kinetics of RT, but it does increase the sensitivity to ddNs. It should be of note, however, that the use of a single drug concentration may have skewed the results of viral replication profiles; more effective drug inhibition of less-resistant HIV-1 may have led to an underestimation of their actual replication rates in the presence of drugs.

These viral replication profiles appear to show how HIV-1 can evolve in the face of anti–HIV-1 drugs, by developing drug resistance through certain mutations, although sacrificing some replication ability, then reacquiring optimal replication competence through further mutations (figure 4). Before therapy, wild type HIV-1 is sensitive to ddNs and does not replicate in the presence of drugs. However, with one or three mutations, the virus acquires resistance, represented by higher IC50 values, and acquires partial but reasonable replicative capability in the presence of drugs.

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