

BRIEF REPORT

The HBV Drug Entecavir — Effects on HIV-1 Replication and Resistance

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SUMMARY

Entecavir, a drug approved by the Food and Drug Administration for the treatment of chronic hepatitis B virus (HBV) infection, is not believed to inhibit replication of human immunodeficiency virus type 1 (HIV-1) at clinically relevant doses. We observed that entecavir led to a consistent 1-log₁₀ decrease in HIV-1 RNA in three persons with HIV-1 and HBV coinfection, and we obtained supportive in vitro evidence that entecavir is a potent partial inhibitor of HIV-1 replication. Detailed analysis showed that in one of these patients, entecavir monotherapy led to an accumulation of HIV-1 variants with the lamivudine-resistant mutation, M184V. In vitro experiments showed that M184V confers resistance to entecavir. Until more is known about HIV-1–resistance patterns and their selection by entecavir, caution is needed with the use of entecavir in persons with HIV-1 and HBV coinfection who are not receiving fully suppressive antiretroviral regimens.

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N Engl J Med 2007;356:2614-21.

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ENTECAVIR, AN ANALOGUE OF 2'-DEOXYGUANOSINE, IS CONVERTED INTRACELLULARLY to an active 5'-triphosphate form that inhibits hepatitis B virus (HBV) polymerase.¹⁻³ Entecavir is approved by the Food and Drug Administration for the treatment of chronic hepatitis B infection. A previous study¹ indicated that it has no clinically relevant activity against human immunodeficiency virus type 1 (HIV-1), and the package insert states that the concentration needed to inhibit replication by 50% (EC₅₀) for HIV-1 is greater than 1 μM, which is higher than concentrations achieved in vivo with dosages used to treat chronic hepatitis B. Because of this selective activity against HBV,⁴⁻⁷ several treatment guidelines, including the U.S. Public Health Service guidelines, recommend entecavir for hepatitis B treatment in HIV-1–infected persons who do not meet criteria for HIV-1 treatment.⁸⁻¹¹ Here, we report that entecavir has clinically relevant activity against HIV-1 and can select HIV-1 variants resistant to the widely used antiretroviral drugs lamivudine and emtricitabine.

CASE REPORTS

PATIENT 1

Patient 1 is a 31-year-old white man with HIV-1 and HBV coinfection who tested seropositive for HIV-1 in 1990. In 2000, he received zidovudine, lamivudine, and nevirapine for the treatment of HIV-1. He took these drugs intermittently for less than 1 year. In February 2002, his CD4+ T-cell count was 596 cells per cubic millimeter,

Figure 1. Acute Decreases in Plasma Levels of HBV DNA and HIV-1 RNA with Entecavir Therapy.

HIV-1 RNA and HBV DNA levels in Patient 1 (Panel A), Patient 2 (Panel B), and Patient 3 (Panel C) are shown as a function of time relative to the initiation of entecavir therapy. Colored bars indicate treatment with entecavir, tenofovir, emtricitabine, efavirenz, zidovudine, lamivudine, and pegylated interferon alfa-2a. The zidovudine and lamivudine bars are interrupted to indicate the intermittent nature of this treatment. Dotted lines indicate the limits of detection of the relevant viral-load assays. Open symbols indicate assay values above or below the range of the relevant assays. Time-zero values for Patient 2 were estimated from the geometric mean of the three previous values. The hepatitis B DNA value 1.2 months before entecavir therapy was used as the time-zero value for Patient 3.

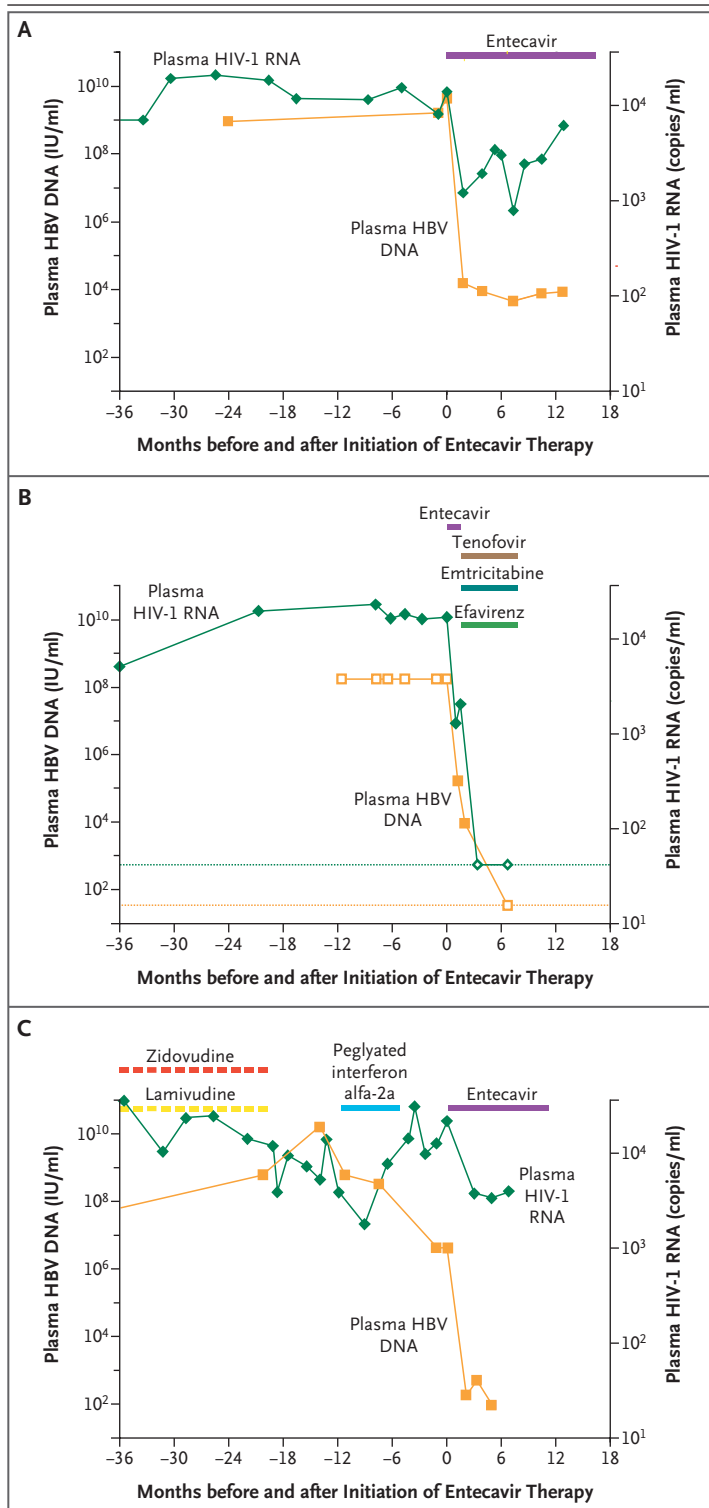
with a plasma HIV-1 RNA level of 14,602 copies per milliliter (Fig. 1A). His CD4+ T-cell counts and HIV-1 RNA levels remained stable for the next 4 years without therapy. In March 2006, he started entecavir therapy for chronic hepatitis B. At that time, his HIV-1 RNA level was 34,088 copies per milliliter and his CD4+ T-cell count was 574 cells per cubic millimeter.

In the first 2 months while receiving entecavir, his HBV DNA level decreased from 9.60 to 3.95 \log_{10} IU per milliliter. Simultaneously, the HIV-1 RNA level decreased by approximately 1 \log_{10} to 2193 copies per milliliter, and his CD4+ T-cell count increased to 634 cells per cubic millimeter. His HIV-1 RNA level has slowly risen from its nadir and is 13,345 copies per milliliter after 12 months of therapy with the CD4+ T-cell count decreasing to 509 cells per cubic millimeter. During this same period, the HBV DNA level remained at its nadir.

PATIENT 2

Patient 2 is a 24-year-old man who received a diagnosis of HIV-1 infection in February 2003. Although acute HBV infection developed in March 2005, his HIV-1 infection did not meet the criteria for antiretroviral therapy. His HIV-1 RNA levels ranged from 40,000 to 60,000 copies per milliliter and his CD4+ T-cell counts were generally greater than 500 cells per cubic millimeter.

In February 2006, he began to receive entecavir for the treatment of chronic hepatitis B. After 1 month, the HIV-1 RNA level decreased 1.23 \log_{10} , from 40,273 to 2347 copies per milliliter (Fig. 1B), and his CD4+ T-cell count rose from 490 to 568 cells per cubic millimeter. While he was receiving entecavir, his HBV DNA level de-



creased from approximately 9 to 5 \log_{10} IU per milliliter. Given the concern for the activity of entecavir against HIV-1 and for the possible generation of drug-resistant HIV-1, his treatment was

switched to tenofovir, emtricitabine, and efavirenz after 45 days of treatment with entecavir.

PATIENT 3

Patient 3 is a 46-year-old man with HIV-1 and HBV coinfection who became HIV-1-seropositive in the 1980s. In 1993, he received zidovudine monotherapy for an undetermined period of time followed by zidovudine plus lamivudine intermittently between 1997 and 2004. He was not fully compliant with this therapy. Before he received zidovudine plus lamivudine in 1996, his HIV-1 RNA level was 30,075 copies per milliliter and his CD4+ T-cell count was 375 cells per cubic millimeter. From September 2005 to February 2006, he received pegylated interferon alfa-2a to treat his chronic hepatitis B and had a poor response.

In August 2006, he began to receive entecavir monotherapy for chronic hepatitis B. When entecavir was initiated, he had an HIV-1 RNA level of 55,451 copies per milliliter and a CD4+ T-cell count of 399 cells per cubic millimeter. After 2 months of receiving entecavir, his HIV-1 RNA level decreased 0.85 log₁₀ to 7797 copies per milliliter with a concomitant rise in his CD4+ T-cell count to 480 cells per cubic millimeter (Fig. 1C). His HIV-1 RNA level has remained below baseline after 7 months of entecavir monotherapy. During this period, his HBV DNA level decreased from 6.63 to 1.99 log₁₀ IU per milliliter.

METHODS

PERIPHERAL-BLOOD SAMPLES

Peripheral blood was obtained from Patients 1 and 3 after they provided written informed consent. HIV-1 RNA was extracted from plasma and a segment of the *pol* gene was amplified by reverse-transcriptase polymerase chain reaction (RT-PCR), as described previously.¹² The products were cloned, and multiple independent clones from each PCR reaction were sequenced. A maximum-likelihood phylogenetic tree was constructed by means of the PAUP program (Sinauer), as described previously.¹³ This study was approved by the institutional review board at Johns Hopkins University.

PHENOTYPIC ANALYSIS

Dose-response curves for inhibition of HIV-1 infection were generated in vitro by means of a previously described phenotypic assay.¹² Briefly, recombinant CXCR4-tropic HIV-1 pseudoviruses were

generated by cotransfecting HEK 293T cells with a plasmid containing the HIV-1 genome with a portion of envelope gene replaced by enhanced green fluorescent protein (pNL4-3-ΔE-GFP) and with a plasmid encoding the HIV-1 envelope (pCXCR4). Culture supernatants were collected, spun at 335×g, and filtered through a 0.22-μm membrane to clear cell debris. Virus was pelleted at 100,000×g for 2 hours at 4°C. Viral supernatants were standardized by means of p24 enzyme-linked immunosorbent assay (Beckman Coulter).

To obtain primary CD4+ T lymphoblasts for infection, peripheral-blood mononuclear cells from healthy donors were activated with phytohemagglutinin for 48 to 72 hours. CD4+ lymphoblasts were isolated with the use of Miltenyi Biotec beads and pretreated for 16 to 19 hours with increasing amounts of the indicated drugs before infection with standardized amounts of virus. The extent of infection was determined by quantifying the number of green fluorescent protein (GFP)-positive cells after 72 hours with the use of fluorescence-activated cell-sorter analysis. These experiments were repeated in triplicate with cells from three different healthy blood donors.

The M184V mutant plasmid was generated by site-directed mutagenesis (Stratagene) of pNL4-3-ΔE-GFP, and virus was made and standardized as above. To test patient-derived *pol* sequences for susceptibility to entecavir, a 1.5-kb *pol* sequence was amplified with the use of RT-PCR from four persons: Patients 1 and 3 in this study and two other HIV-1-infected persons who were not receiving entecavir (Patients 4 and 5, from the study by Bailey et al.).¹³ PCR products were cloned into pNL4-3-ΔE-GFP as previously described.¹² The isolate from Patient 3 had the nonnucleoside reverse-transcriptase inhibitor resistance mutation K103N in HIV-1 reverse transcriptase. The isolate from Patient 4 had no drug-resistance mutations and the isolate from Patient 5 had the V108I and T215D mutations in reverse transcriptase.

EFFECT OF ENTECAVIR ON REVERSE TRANSCRIPTION

CXCR4-tropic recombinant HIV-1 pseudovirus was treated with DNase and used to infect CD4+ lymphoblasts from healthy donors in the presence or absence of entecavir at a concentration of 50 μM. Total DNA was isolated 8 and 24 hours after infection, and the products of reverse transcription were quantified by real-time PCR with the use of three separate sets of primers (see the Supplemen-

tary Appendix, available with the full text of this article at www.nejm.org).

RESULTS

INHIBITION OF HIV-1 REPLICATION IN VITRO

To determine whether the observed *in vivo* reductions in HIV-1 RNA levels were due to the direct effects of entecavir on HIV-1 replication, we used a precise HIV-1 infectivity assay.¹² This assay is related to commercial phenotypic assays,¹⁴ but it has single-cell sensitivity and uses primary CD4+ T cells rather than transformed cell lines. HIV-1 pseudoviruses carrying *pol* gene sequences from a reference HIV-1 isolate or from patient-derived isolates were used to infect CD4+ lymphoblasts in the presence of increasing concentrations of antiviral drugs. Entecavir potently inhibited infection by both the reference strain (a 50% inhibitory concentration [IC₅₀] between 0.1 and 1 nM) and the wild-type pseudoviruses derived from Patient 1 (IC₅₀, approximately 1 nM) (Fig. 2A). Although the pseudovirus derived from Patient 1 had a slightly reduced susceptibility to entecavir as compared with that of the reference strain, the ability of entecavir to inhibit infection at very low concentrations was still apparent. Three additional patient-derived isolates including that from Patient 3 were also tested in this system, and infection by all three was inhibited by entecavir with an IC₅₀ consistently in the low nanomolar range (Fig. 2B). The *in vitro* inhibition is consistent with the 1-log₁₀ decrease in HIV-1 RNA observed in the patients. The IC₅₀ of entecavir for HIV-1 is 100 to 1000 times lower than that of zidovudine in this system (approximately 0.2 μM). The IC₅₀ of entecavir *in vitro* is below the plasma concentrations achieved *in vivo* at doses given for hepatitis B (maximal concentration, 28 nM).¹⁵

For all HIV-1 isolates tested, the dose-response curve for entecavir was atypical in that although the drug partially inhibited HIV-1 infection at very low concentrations, the anti-HIV-1 activity of entecavir reaches a plateau at drug concentrations in the low nanomolar range and concentrations above 10 nM do not cause increased inhibition (Fig. 2A and 2B). In contrast, zidovudine shows higher levels of inhibition of HIV-1 replication with increasing concentrations (Fig. 2A). The fact that the inhibitory activity of entecavir plateaus may explain why anti-HIV-1 activity was not detected with the use of conventional assays.

Because entecavir is a nucleoside analogue, we

hypothesized that the observed anti-HIV-1 activity was due to inhibition of HIV-1 reverse transcriptase. To test this hypothesis, we used real-time PCR to quantify reverse transcripts in cells infected in the presence of entecavir. Entecavir decreased the levels of early, intermediate, and late reverse transcripts (Fig. 3), suggesting that it may inhibit HIV-1 replication at or before the reverse-transcription step.

THE M184V MUTANT VIRUS

To determine whether drug-resistant HIV-1 variants were selected by entecavir, we cloned and sequenced HIV-1 RNA from the plasma of Patients 1 and 3 immediately before the initiation of entecavir therapy and at various time points after initiation of entecavir therapy (Fig. 4A). When entecavir was initiated in Patient 1, none of 19 independent clones had mutations in HIV-1 *pol* known to confer resistance to anti-HIV-1 drugs. At 2, 4, and 6 months after initiation of entecavir therapy, 12%, 61%, and 96% of the clones, respectively, harbored the M184V mutation (Fig. 4B). This mutation confers a high level of resistance to the anti-HIV-1 drugs lamivudine and emtricitabine.¹⁷⁻¹⁹ No other known resistance mutations were observed. The clinical genotype (HIV-1 Genotype, Quest Diagnostics Nichols Institute) obtained 6 months after the initiation of entecavir therapy confirmed the presence of the M184V mutation. By phylogenetic analysis (Fig. 4C), the M184V mutants detected while the patient was receiving entecavir therapy showed greater divergence from the most recent common ancestor than did most of the wild-type sequences present before entecavir therapy. These results are consistent with the selection of M184V viruses by entecavir.

In Patient 3, who had more extensive previous lamivudine therapy than Patient 1, no clones with the M184V mutation were detected at the time entecavir was started. After 7 months of entecavir therapy, only 1 of 175 clones had the M184V mutation. The only other known resistance mutation detected in this patient was the nonnucleoside reverse-transcriptase inhibitor resistance mutation K103N, which was present in all the clones before and after entecavir therapy. Taken together, these results indicated that entecavir can select the M184V mutation in some but not all patients.

Since entecavir can select the M184V mutation *in vivo*, we hypothesized that HIV-1 variants with the M184V mutation would be less susceptible to

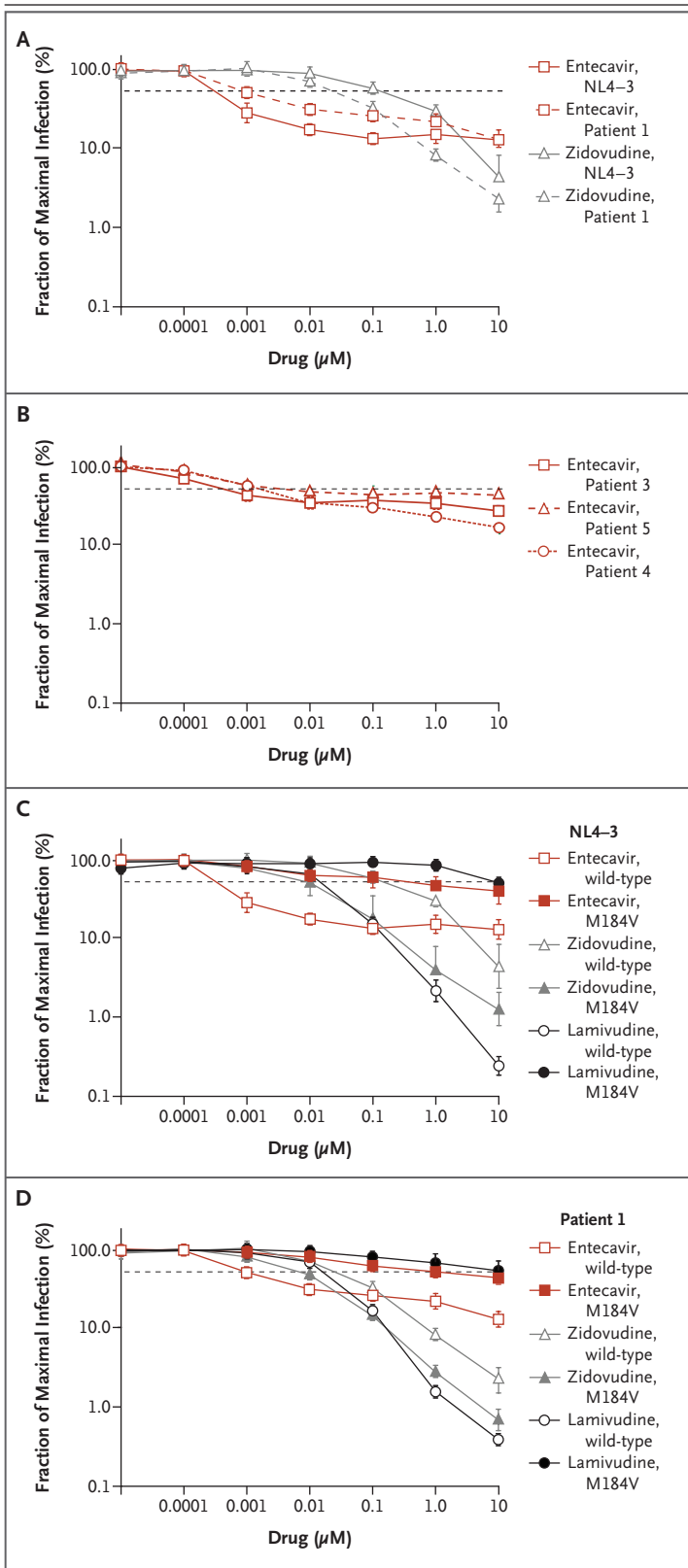


Figure 2. Inhibitory Effects of Entecavir on HIV-1 Infection In Vitro.

Panel A shows the effect of entecavir and zidovudine on infection of primary CD4+ T cells in vitro with pseudoviruses carrying *pol* sequences from the wild-type HIV-1 reference strain NL4-3 or from a wild-type clone from Patient 1. Results are expressed as a percentage of maximal infection equal to the number of infected cells observed in the presence of the indicated concentration of drug divided by the maximal number of infected cells observed in the absence of a drug effect multiplied by 100. The dashed black horizontal line represents a 50% decrease in the number of GFP+ cells. Where they would be obscured by a symbol, error bars are shown at the edge of the symbol. Infection by pseudoviruses carrying the *pol* sequence derived from Patient 1 was inhibited by entecavir with an IC_{50} of approximately 0.001 μM . Inhibition of infection by zidovudine is also shown for purposes of comparison with entecavir. Panel B shows the effect of entecavir on infection of primary CD4+ T cells in vitro with pseudoviruses carrying HIV-1 *pol* sequences isolated from Patients 3, 4, and 5. The dose-response curves were similar for all isolates. The isolate from Patient 3 had the nonnucleoside reverse-transcriptase inhibitor resistance mutation K103N in reverse transcriptase. The isolate from Patient 4 had no drug-resistance mutations. The isolate from Patient 5 had the V108I and T215D mutations in reverse transcriptase. Panel C shows the dose-response curves for wild-type and M184V mutant forms (M184V) of the reference NL4-3 pseudovirus in the presence of increasing concentrations of entecavir, zidovudine, or lamivudine. The data are mean values from three experiments. Panel D shows the dose-response curves for pseudoviruses carrying *pol* sequences from wild-type isolates and M184V mutant isolates from Patient 1. The experiment was repeated three times with very similar results, and representative data from one experiment are shown. The curves shown in Panel A for wild-type viruses in the presence of entecavir and zidovudine are identical to those in Panels C and D.

entecavir. The HIV-1 reference strain carrying the M184V mutation was resistant to entecavir (Fig. 2C). In control experiments (Fig. 2C), we showed that the same M184V mutant virus had markedly decreased susceptibility to lamivudine and the expected hypersusceptibility to zidovudine.^{17,18,20,21} A post-entecavir isolate from Patient 1 containing the M184V mutation behaved similarly (Fig. 2D).

DISCUSSION

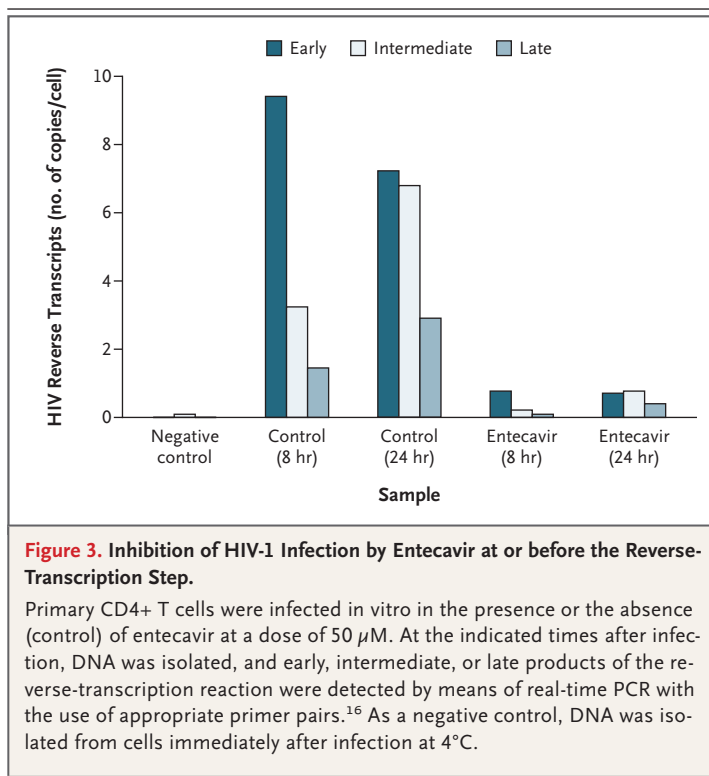
This study shows that entecavir is a potent but partial inhibitor of HIV-1 replication in vitro and in

vivo and that in some patients it can select for viruses bearing the M184V mutation, which confers high-level resistance to entecavir and to the widely used anti-HIV-1 drugs lamivudine and emtricitabine. These conclusions are supported by the temporal association of entecavir therapy with approximately 1-log₁₀ reductions in HIV-1 RNA levels in vivo as well as in vitro studies showing inhibition of HIV-1 infectivity at clinically relevant concentrations. In addition, selection of M184V variants occurred in one patient who was receiving entecavir monotherapy, and in vitro data showed reduced activity of entecavir on M184V variants derived from this patient as well as from a laboratory strain.

These data have important implications for the treatment of hepatitis B in HIV-1-infected patients. Current guidelines recommend entecavir as the first-line treatment in persons with HIV-1 and HBV coinfection who do not require anti-HIV-1 therapy.^{8,9} Since this recommendation is predicated on the assumption that entecavir does not have activity against HIV-1, our data indicate that this recommendation should be reconsidered, especially since entecavir can select for the M184V mutation in some patients.

Our data contradict the findings of a previous report that entecavir does not have anti-HIV-1 activity at clinically relevant concentrations.¹ That report used an assay described in 1989 by Weislow et al., which relies on cytopathic effects resulting from infection.²² We believe the quantitative infectivity assay we used has several advantages over previous assays that permit it to detect subtle anti-HIV-1 effects such as that shown by entecavir. First, the assay in our study permits a more direct measure of drug inhibition of early steps in HIV-1 replication from virus attachment through virus gene expression than older assays that used cell death as a surrogate measure of infection. Second, since the assay relies on a single round of infection, it permits the rapid and precise measurement of individual infection events without the complications introduced by multiple rounds of infection in an extended culture. A third advantage is that drug inhibition of HIV-1 replication is measured in primary CD4+ T lymphoblasts, which are the in vivo target cells of HIV-1, rather than in transformed cell lines, which may metabolize entecavir differently.

Further work is needed to understand why the M184V mutation emerged in one of the two pa-



tients who were studied in detail. It is unlikely that this patient was taking lamivudine, since it was not being prescribed to him at the time or in the recent past, and emergence of this mutation from lamivudine monotherapy typically occurs within weeks.²³ Rather, the appearance of the M184V mutation probably reflects the fact that it decreases the anti-HIV-1 activity of entecavir. Selection of the M184V mutation in HIV-1 reverse transcriptase could be anticipated, since structural models show that the M184V mutation corresponds to the HBV *pol* mutation rtM204V,²⁴ which decreases HBV susceptibility to entecavir.²⁵ In both cases, the targeted methionine is in the YMDD motif at the active site.

The failure of the M184V mutation in HIV-1 reverse transcriptase to become dominant in Patient 3 may reflect the fact that this mutation has a well-known negative effect on viral fitness.²⁶ In some patients, this reduction in fitness may outweigh the modest benefit conferred by entecavir resistance, since entecavir only partially inhibits HIV-1 replication. Thus, in some patients, selection for M184V may not occur or may occur very slowly.

It is also not known whether the M184V vari-

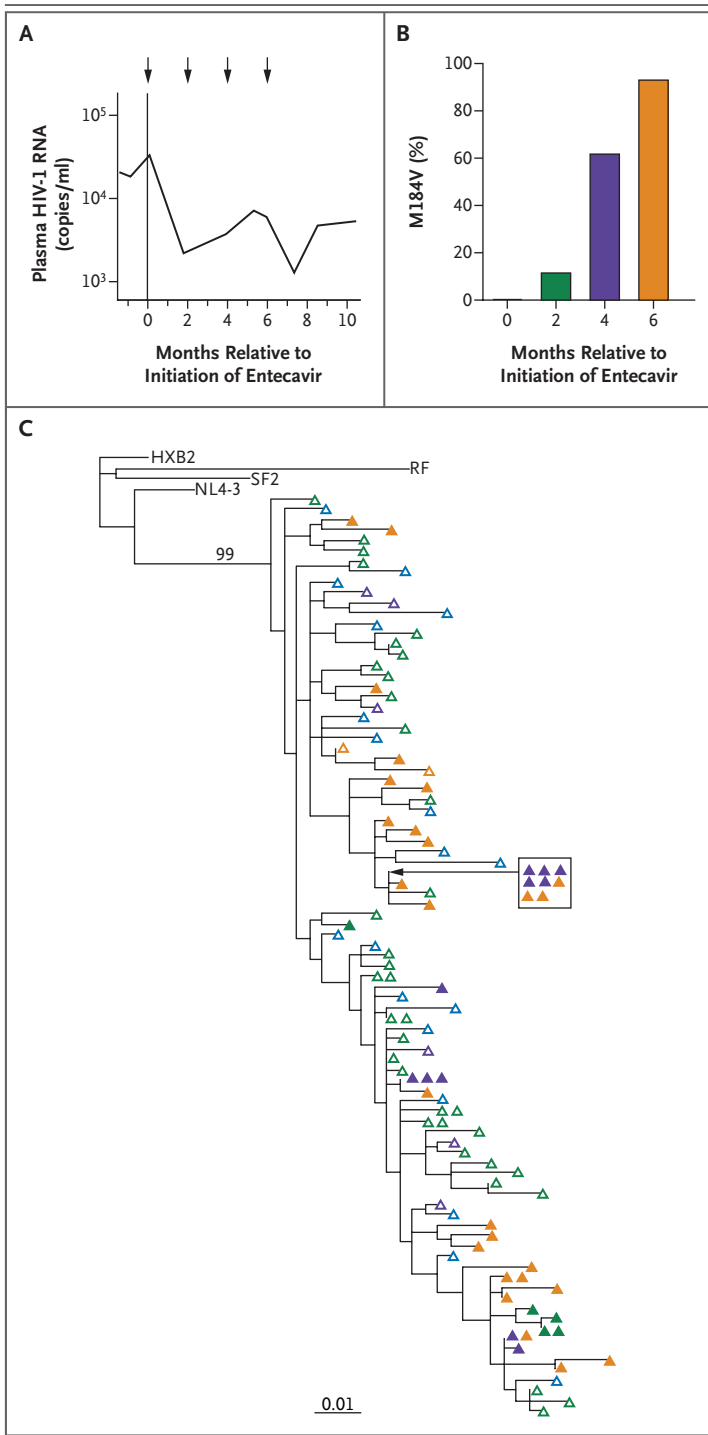


Figure 4. Selection for the M184V Mutation in HIV-1 Reverse Transcriptase in a Patient Receiving Entecavir.

Panel A shows the time course of sampling for Patient 1. Plasma was obtained for genotypic analysis on the day that entecavir was started and after 2, 4, and 6 months of entecavir monotherapy (arrows). The plasma HIV-1 RNA levels during this period are indicated in the graph. Panel B shows the fraction of independent plasma virus isolates carrying the M184V mutation on the day that entecavir was started and at 2, 4, and 6 months. The number of independent clones analyzed was 19, 41, 18, and 27 for these four time points, respectively. No resistant mutations were detected at the first time point. Panel C shows the maximum-likelihood phylogenetic tree of the wild-type forms (open symbols) and M184V mutants (closed symbols) from the four time points. Blue triangles denote the first time point, green triangles the second time point, purple triangles the third time point, and orange triangles the fourth time point. All isolates from Patient 1 cluster together and are clearly distinct (bootstrap value, 99) from reference clade B isolates (HXB2, SF2, RF, and NL4-3) obtained from other patients. The 0.01 scale bar denotes the genetic distance in nucleotide substitutions per site.

vir treatment. The appearance of M184V could also reflect the emergence of an archived variant from the latent reservoir,²⁷ but in either case there is clear evidence that entecavir selected for this variant in Patient 1.

Several other questions are also raised by these observations. One question is whether control of HBV replication led to diminished lymphocyte activation or alterations in cytokine release that affected HIV-1 replication. Although these effects are theoretically possible, treatment of hepatitis B with adefovir dipivoxil in HIV-1-infected persons does not provide support for this hypothesis, since these patients had stable HIV-1 RNA levels despite decreases in HBV DNA levels.²⁸ Although our data show that entecavir affects HIV-1 replication, we cannot rule out that such secondary effects may have contributed to the decrease in the HIV-1 RNA levels. Other questions include why the anti-HIV-1 activity of entecavir plateaus at low nanomolar concentrations, whether modifications of the compound could overcome the plateau effect, whether entecavir could play a clinically meaningful role as an antiretroviral agent for HIV-1, and whether entecavir selects for other HIV-1 drug-resistant mutants.

Entecavir, at doses used to treat chronic hepatitis B, is a potent partial inhibitor of HIV-1 replication and can select for the M184V resistance

ants that appeared in Patient 1 after entecavir treatment were initially generated by exposure to a lamivudine-containing highly active antiretroviral therapy regimen several years earlier. Phylogenetic analysis suggests that the M184V variants evolved recently, most likely as a result of enteca-

mutation in HIV-1 reverse transcriptase. Since the full extent of HIV-1 reverse-transcriptase mutations selected by entecavir monotherapy is not known, caution should be used in treating chronic hepatitis B with entecavir in HIV-1-infected patients who are not receiving fully suppressive antiretroviral regimens. Furthermore, these data underscore the importance of a careful study of agents with potential for anti-HIV-1 activity before licensure.

Supported by grants from the National Institutes of Health (R01AI060449, to Dr. Thio; and A143222 and A151178, to Dr. Siliciano).

No potential conflict of interest relevant to this article was reported.

The views expressed in this article are those of the authors and do not reflect the official policy or position of the Department of the Navy, the Department of Defense, or the U.S. government.

We thank David Thomas for critical reading of this manuscript, Stuart Ray and Meghdad Rahdar for helpful discussions and advice, and our patients for their participation in this study.

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