BRIEF REPORT

Variant of Hepatitis B Virus with Primary Resistance to Adefovir

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SUMMARY

The reverse-transcriptase inhibitor lamivudine (Zeffix, GlaxoSmithKline) is often used to treat chronic infection with hepatitis B virus (HBV) until resistance develops. Treatment may then be switched to the reverse-transcriptase inhibitor adefovir (Hepsera, Gilead), which has a lower frequency of resistance. Here, we describe three cases of primary adefovir resistance that were sensitive to tenofovir (Viread, Gilead). All three cases involved a rare HBV variant with a valine at position 233 of the reverse-transcriptase domain instead of isoleucine (rtI233V), as in the wild-type virus. This HBV variant also displayed resistance to adefovir and sensitivity to tenofovir in vitro.

ADEFOVIR, AN INHIBITOR OF HBV REVERSE TRANSCRIPTASE, CAN BE USED as a licensed drug for the treatment of chronic HBV infection.[1-3] Virtually 100 percent efficacy has been suggested in lamivudine-resistant HBV infections,[4] but in two reports[5,6] only 39 of 46 patients and 13 of 18 patients, respectively, had a marked treatment response. Resistance to lamivudine developed in all three of our patients with chronic hepatitis B, and all three had a response to tenofovir but not to adefovir. HBV isolated from all three patients before adefovir therapy had the same rtI233V mutation in the reverse-transcriptase domain.

CASE REPORTS

PATIENT 1

Patient 1, a 52-year-old man, had had chronic hepatitis B for more than five years. He had undergone unsuccessful therapy with pegylated interferon. Subsequent treatment with lamivudine (100 mg per day) had initially suppressed the viremia, resulting in fewer than 200 molecules of HBV DNA per milliliter. However, within three months, the level of viremia increased again, to 2.5×10^7 molecules of HBV DNA per milliliter (Fig. 1), and a mutation conferring resistance to lamivudine[9] — namely, the substitution of isoleucine for methionine at position 204 in the reverse-transcriptase domain (rtM204I) — was detected by direct sequencing of the polymerase-chain-reaction (PCR) product in the first available sample from September 9, 2002. After an interval without therapy, during which this mutation reverted to wild-type, the reverse-transcriptase inhibitor tenofovir was administered for nine months (300 mg per day) as an experimental drug for HBV. Tenofovir is licensed for treatment of the human immunodeficiency virus (HIV) and has been used successfully to reduce
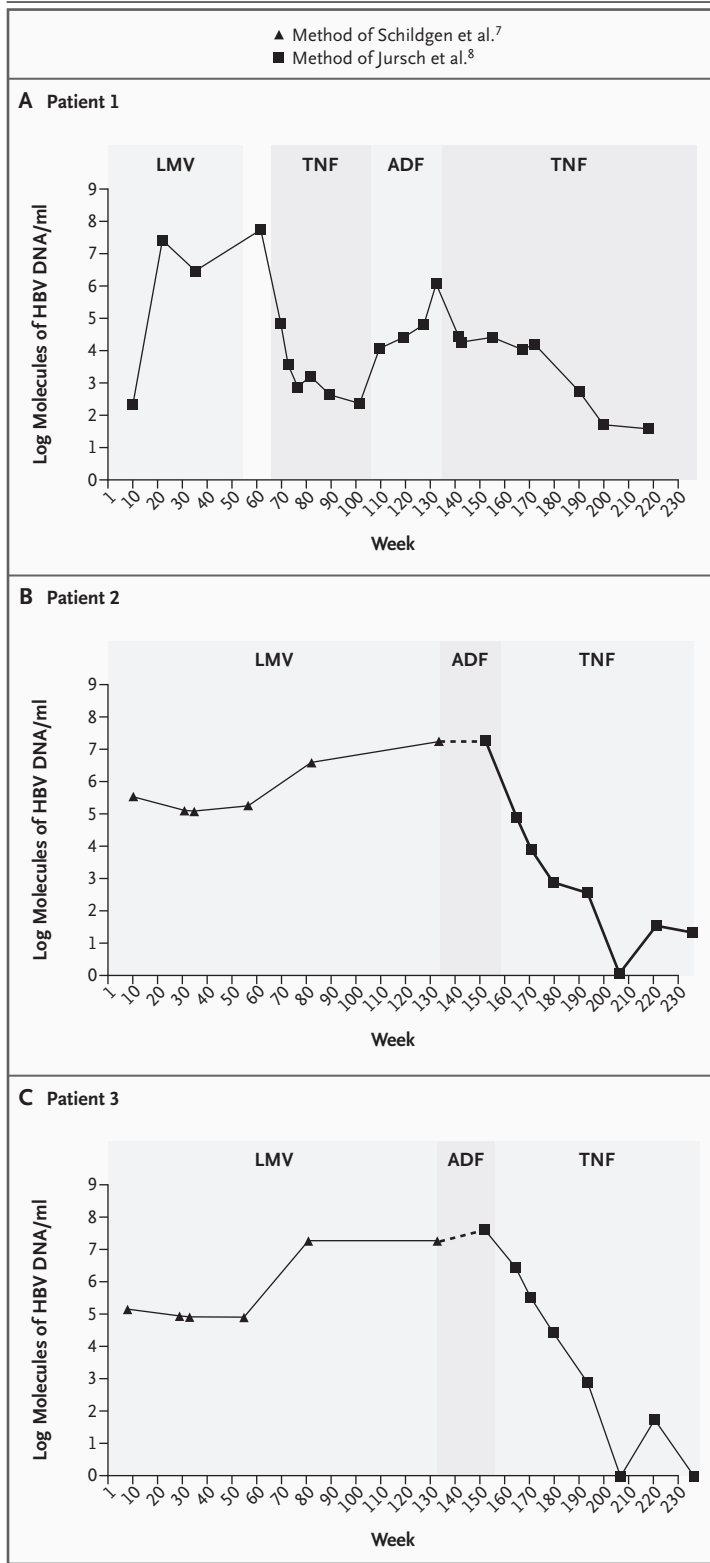
Figure 1. Levels of Viremia in Three Patients Receiving Lamivudine (LMV), Adefovir (ADF), and Tenofovir (TNF).

The levels of viremia were measured by real-time PCR assay as described by Schildgen et al.[7] and Jursch et al.[8] The limit of detection of each assay is 30 molecules of HBV DNA per milliliter. Dashed lines indicate transition from one method to another. Values shown at zero were negative in the assay for HBV DNA (<30 molecules per milliliter).

levels of both HIV and HBV in patients infected with both viruses.[7,10,11] Within the first three months, the level of viremia decreased from 5.5×10^7 molecules of HBV DNA to 740 molecules of HBV DNA per milliliter and remained at similar levels for another six months. Thereafter, tenofovir was replaced by adefovir (10 mg per day) because this drug had become licensed for therapy of chronic HBV. However, during the therapy with adefovir, the level of viremia increased immediately and within seven months had reached 1.5×10^6 molecules of HBV DNA per milliliter. Replacement of adefovir with tenofovir again resulted in a substantial decrease in the level of viremia. The decrease was not as rapid as it had been before, but after 100 weeks of the second course of tenofovir therapy, the level of viremia was down to the detection limit of the PCR assay — at about 30 molecules of HBV DNA per milliliter. Throughout the observation period, the patient had normal or slightly elevated levels of alanine aminotransferase. He was positive for antibody against hepatitis Be antigen (HBeAg); sequencing showed that the virus had the normal precore sequence as well as the core promoter mutations T1753C, A1762T, and G1764A known to be associated with HBeAg negativity.[12]

PATIENTS 2 AND 3

Patients 2 and 3 were a married couple. Patient 2, a 56-year-old man, had received a liver transplant in 1993 because of alcoholic liver disease and turned out to be infected with HBV during follow-up. He had had continuous therapy with immunosuppressive agents since his liver transplantation. Patient 3, his wife, 52 years of age, was found to have chronic hepatitis B in 1994. The genomic sequences of their viruses were very similar, but in Patient 2, the predominant viral variant had a normal precore sequence and the stop codon mu-

tation (G1896A), whereas Patient 3 had only wild-type virus. Both patients were HBeAg-positive. Both husband and wife received lamivudine, which initially reduced their levels of viremia from 10^7 molecules of HBV DNA per milliliter to less than 10^5 molecules of HBV DNA per milliliter. Resistance subsequently developed, and the level of viremia increased again (Fig. 1). This increase was presumably caused by the rtM204I mutation associated with resistance to lamivudine, which had been identified in the viral population of both patients. Patient 3, in addition, had a virus variant containing the mutation rtV173L (Fig. 2). Lamivudine therapy was replaced by adefovir therapy, but the level of viremia did not change significantly in either patient. The substitution of tenofovir therapy for adefovir therapy precipitated a rapid decrease in HBV DNA levels, from greater than 5×10^8 molecules of HBV DNA per milliliter in Patient 2 and from 4.5×10^7 molecules of HBV DNA per milliliter in Patient 3 to fewer than 100 molecules per milliliter in both patients at the end of the study (November 24, 2005).

All three patients were infected by a virus with the rtI233V mutation. The functional significance of this mutation was initially unknown, but the nearby mutation rtN236T had been observed in cases in which resistance to adefovir had developed during therapy.[13,14]

METHODS AND RESULTS

All three patients provided written informed consent for testing. To determine whether the rtI233V mutation alone is necessary and sufficient to render HBV resistant to adefovir, we employed site-directed mutagenesis to replace the isoleucine at position 233 with valine in the wild-type HBV genotype D[15] using the plasmid vector pTHBV1.3 and the primer pair 5′CTTTTGTCTTTGGGTgTACATTTAAACCCTAAC3′ (sense) and 5′GTTAGGGTTTAAATGTAcACCCAAAGACAAAAG3′ antisense; the lowercase letter indicates the mutated nucleotide. The successful introduction of the mutation was confirmed by sequencing. The HepG2 subclonal cell line C3A (American Type Culture Collection) was plated in six-well cell culture plates at a confluence of 50 percent (approximately 1.5×10^6 cells) and then transfected with 12 μg of wild-type or mutated plasmid. On the next day, the cells were passaged into 12-well cell-culture plates. To eliminate input DNA, cells were incubated with 100 μg per milliliter of DNaseI (Roche) at 37°C for two hours. Two days after transfection, cells were treated with the free, unmodified drugs adefovir, tenofovir (Moravek), or lamivudine at concentrations ranging from 0.1 to 3 μM. The medium used to supplement the drugs was renewed daily. Cell-culture supernatants and cells were harvested on day 6. Replicative intermediates of HBV DNA were extracted as described previously,[16] subjected to Southern blotting,[17] and hybridized with a phosphorus 32–labeled probe generated with the Rediprime random prime labeling system (Amersham) from full-length HBV DNA. The quantity of HBV DNA was significantly decreased in treated cells at each concentration of adefovir (Fig. 3A). With the wild-type plasmid, a concentration of adefovir of approximately 0.5 μM resulted in 50 percent inhibition, a value slightly higher than that described by Yang et al.[18] but lower than that found by Brunelle et al.[19] The rtI233V variant required concentrations of adefovir that were about six times as high for a similar level of inhibition (Fig. 3A). This factor exceeds that of previously described variations between naturally occurring strains.[18] In contrast, the inhibition efficacy of tenofovir or lamivudine did not differ significantly between the wild-type and the variant HBV DNA. Lamivudine was by far the most efficacious drug in this in vitro system. The inhibitory effect of all three drugs was most clearly demonstrated by the amount of open circular HBV DNA (Fig. 3A); this is the only form leading to secreted infectious virus particles.

In order to study the effect of adefovir on the production of secreted virus particles, the cell-culture medium was first treated with DNaseI and then digested with proteinase K, and finally, the extracted HBV DNA was subjected to PCR. The amount of released wild-type HBV DNA was decreased by about 80 percent, in response to 1.0 μM of adefovir, whereas the amount of the variant released decreased only by about 15 percent (Fig. 3B). In this assay, the variant required concentrations of adefovir that were about 10 times as high as those for the wild-type virus for the same degree of inhibition. These in vitro data confirm that the rtI233V mutation caused the adefovir resistance in vivo.
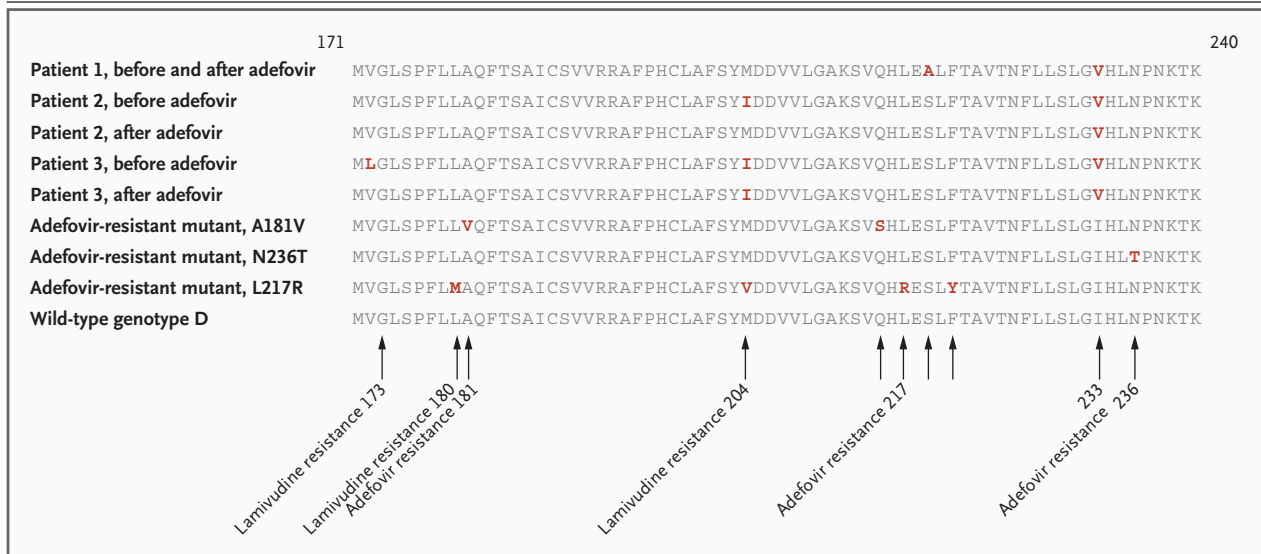
Figure 2. Sequence of Amino Acids 171 to 240 in the Reverse-Transcriptase Domain of HBV Polymerase in Three Patients.

The sequence of amino acids is shown before and after adefovir therapy and compared with three adefovir-resistant HBV isolates selected during adefovir treatment.[13,14] The mutations that are associated with lamivudine or adefovir resistance are in red bold type. The wild-type refers to a central European isolate of genotype D (European Molecular Biology Laboratory accession no. Y07587). The HBV DNA was extracted from serum and amplified in the reverse-transcriptase domain with the use of sense primer 374-390 TGGATGTGTCTGCGGC and antisense primer 995-973 CKTTGACADACTTTCCAATCAATAG. The gel-purified PCR products were sequenced in both directions by MWG Biotech. Alternatively, sequencing was also performed as described by Schildgen et al.[7]

DISCUSSION

In a large clinical study of the efficacy of adefovir as a treatment for chronic hepatitis B, the rtI233V mutation was not observed, but the rtN236T or rtA181V mutation developed in 5.9 percent of the patients within three years. The in vitro efficacy[14] against these adefovir-induced mutants decreased by a factor of 2.5 to 13.8. Thus, the decrease of in vitro efficacy by a factor of 6 to 10 caused by the naturally occurring variant at position rt233 may indicate that this variation in vivo is even more relevant than previously identified adefovir-induced resistance mutations.

The rtI233V mutation was already present before adefovir therapy was initiated. It was independent of lamivudine resistance; as was seen in Patient 1, no lamivudine resistance mutation was detected at the beginning of adefovir therapy. Neither HBeAg status nor the immune status seems to be important for adefovir resistance of this variant, since these two factors differed in the three patients. Furthermore, the variant rtI233V was stable during an observation period of up to 220 weeks in these patients, even without selective pressure from adefovir therapy.

We identified these three rtI233V strains among the strains isolated from 80 patients whose HBV had been sequenced in past years at the Institute of Medical Virology in Giessen, Germany. Using the S gene sequence for genotyping, we identified 41 patients with genotype D (which has a worldwide distribution), 30 with A2, 2 with A1, 5 with C, 1 with E, and 1 with G plus A2. Our three patients had genotype D (subgroup D3)[20] and had closely related but not identical reverse-transcriptase and S gene sequences. Remarkably, all three had the HBsAg subtype corresponding to an isoleucine at the polymorphic site 127 of the HBsAg protein, which is very rare (3 of 152 strains) among patients with the genotype D worldwide.[20] The frequency of the 3 HBV strains with the ayw4 subtype and the rtI233V mutation among the 80 sequenced HBV strains may not reflect the real frequency of such variants in Germany. A GenBank search revealed that only 3 of about 500 previously reported HBV strains had the rtI233V mutation. Two of these strains were from Southeast Asia and were genotype C,[21] and the third was a HBV strain found in gibbons.[22] A lamivudine-resistant genotype C strain found in Chinese patients had a threonine at that
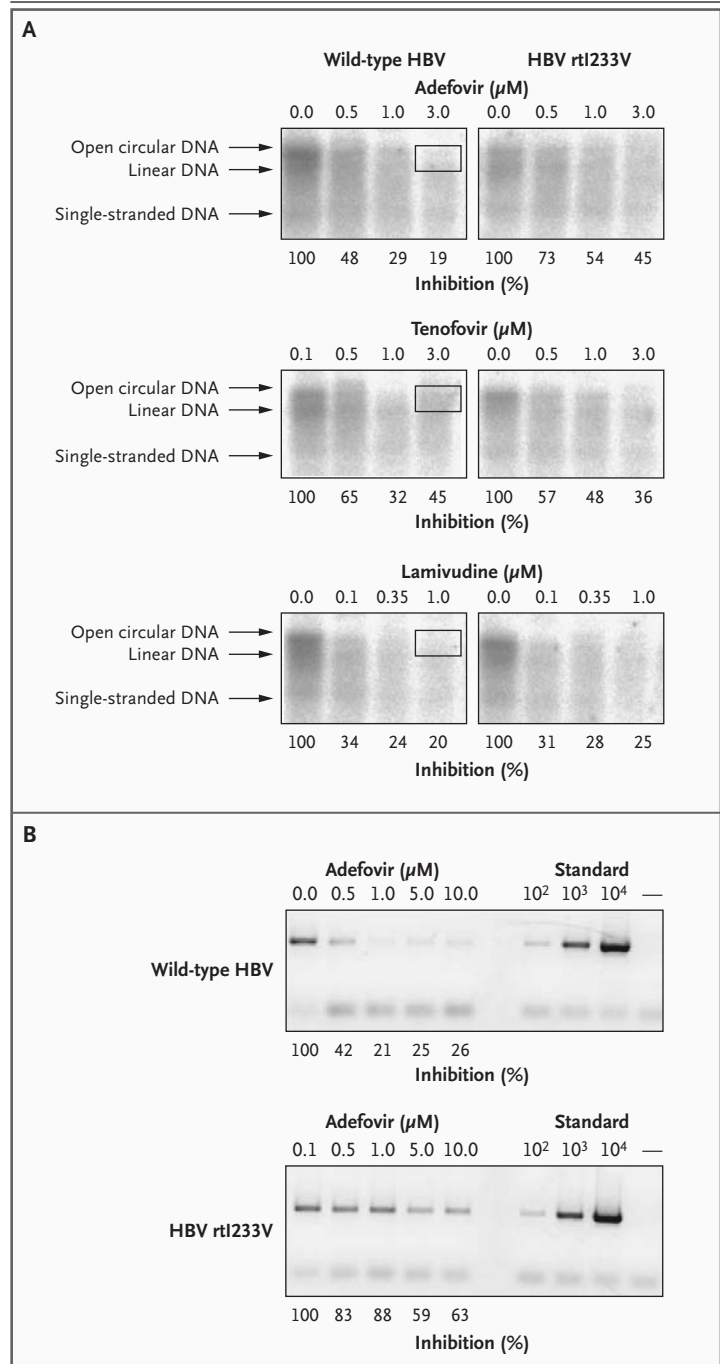
Figure 3. Inhibition of Intracellular HBV DNA Synthesis in Vitro by Increasing Concentrations of Adefovir, Tenofovir, and Lamivudine (Panel A) and the Effect of Adefovir on the Production of Secreted Virus Particles (Panel B).

Panel A shows the evaluation of the production of the wild-type or variant (rtI233V) HBV within transfected cells. Southern blots of extracts obtained six days after transfection were quantitatively evaluated by phosphoimaging (Fujifilm BAS 2500). The number of pixels in the boxed area corresponding to the open circular and linear forms of HBV DNA was quantitatively evaluated for all lanes and corresponded to a value of 100 percent for the untreated control. Panel B shows the effect of increasing concentrations of adefovir on the production of wild-type HBV DNA and the rtI233V mutant HBV DNA secreted to the transfected cells. The DNA was extracted and then amplified by PCR assay with the use of the subgenomic primers F1 5′CTCCAGTTCAGGAACAGTAAACCC3′ and the corresponding reverse primer R1 5′TTGTGAGCTCAGAAAGGCCTTGTAAGTTGGCG3′. Serial dilutions of a cloned HBV genome served as a control (Standard) for calibration of the PCR assay. Amplified products were analyzed on an ethidium bromide–stained agarose gel and quantified with the use of the Fluor-S MultiImager (Biorad) and Quantity One software.

A

Wild-type HBV / HBV rtI233V

Adefovir (μM)

0.0 0.5 1.0 3.0 0.0 0.5 1.0 3.0

Open circular DNA → Linear DNA → Single-stranded DNA →

100 48 29 19 100 73 54 45

Inhibition (%)

Tenofovir (μM)

0.1 0.5 1.0 3.0 0.0 0.5 1.0 3.0

100 65 32 45 100 57 48 36

Inhibition (%)

Lamivudine (μM)

0.0 0.1 0.35 1.0 0.0 0.1 0.35 1.0

100 34 24 20 100 31 28 25

Inhibition (%)

B

Adefovir (μM) Standard

0.0 0.5 1.0 5.0 10.0 10^2 10^3 10^4 —

Wild-type HBV

100 42 21 25 26

Inhibition (%)

Adefovir (μM) Standard

0.1 0.5 1.0 5.0 10.0 10^2 10^3 10^4 —

HBV rtI233V

100 83 88 59 63

Inhibition (%)

position, but this variant may not have been viable because it had a truncated S gene.[23] Thus, there is very little variation at rt233 and no obvious genotypic association. There is, however, at least one other example of primary adefovir resistance. Two of three cases of in vivo adefovir resistance that we discussed in a previous report[7] involved HBV strains that had an rtL217R mutation before adefovir therapy. This mutation is present in most HBV strains with genotype A2, which are prevalent in Europe and the United States, but not in strains with other genotypes, including A1, which are found in Africa and Asia.[20,24]

Our observations demonstrate that some naturally occurring HBV strains are primarily resistant to adefovir. The rapid and strong effect of tenofovir in all three patients along with the in vitro data suggest that the patients' nonadherence to the medication was not the reason for the failure of adefovir therapy. Reactivation of HBV replication, as occurred in Patient 1, has also been reported in three patients by Van Bömmel and Berg,[25] but sequencing data for HBV DNA have not been reported by these authors, and therefore it is not known whether these patients carried relevant HBV variants.

In agreement with a previous report,[26] we found that the in vitro efficacy of tenofovir against wild-type HBV was similar to that of adefovir. Van Bömmel et al. reported that tenofovir was generally superior to adefovir in vivo,[6] a finding that was most likely not caused by the presence of reverse-transcriptase variants. They ascribed this general superiority of tenofovir in vivo to the fact that the dose of the drug can be increased by

a factor of 10, whereas the dose of adefovir is fixed.[25] Since the dose of adefovir cannot be increased, the presence of naturally occurring HBV variants, as described here and in our earlier report,[7] may decrease its efficacy.

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