

Selection and Counterselection of the rtI233V Adefovir Resistance Mutation during Antiviral Therapy[∇]

Oliver Schildgen,^{1†} Cynthia Olotu,^{3†} Anneke Funk,^{3‡} Bernhard Zöllner,² Martin Helm,⁴
Jürgen Kurt Rockstroh,⁵ and Hüseyin Sirma^{3*}

Institut für Pathologie, Kliniken der Stadt Köln, Cologne, Germany¹; Institute for Medical Microbiology, University of Hamburg, Hamburg, Germany²; Heinrich-Pette-Institute, Hamburg, Germany³; Practice Abelein/Helm, Nuremberg, Germany⁴; and Department of Medicine I, University of Bonn, Bonn, Germany⁵

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Recently, we reported on three patients with chronic hepatitis B virus (HBV) infection for whom adefovir (ADF) therapy virologically failed, most likely due to a preexisting rtI233V HBV polymerase mutation. Here, we describe two further patients with chronic HBV infection who were found to develop the rtI233V mutation after initiation of ADF therapy. These patients represent the first cases known so far in which the rtI233V ADF resistance mutation evolved under persistent HBV replication during HBV therapy with ADF. Interestingly, one of the previously described patients, who was initially successfully switched from ADF to tenofovir (TDF) and became virologically suppressed subsequently, experienced a moderate but remarkable rebound of HBV viremia after switching from TDF to entecavir, due to the emergence of renal toxicity. Thus, we provide evidence for the selection and counterselection of the rtI233V ADF resistance mutation during antiviral therapy.

Despite safe and effective vaccines, chronic hepatitis B virus (HBV) infection remains a major medical challenge, with an estimated >350 million chronic carriers worldwide (19). These patients have an increased risk for subsequent liver damage that may lead to fibrosis, cirrhosis, and eventually hepatocellular carcinoma (HCC) with a poor survival prognosis (7, 9). The incidence of HCC correlates extremely well with the levels of HBV viremia; indeed, the higher the HBV viral load is, the higher the risk for HCC development becomes. Thus, a major therapeutic goal in HBV therapy is reduction of viremia for prevention of the emergence of fatal liver disease-related events (2).

An increasing number of nucleoside and nucleotide analogues that inhibit the reverse transcriptase (RT) activity of the viral polymerase have been licensed for HBV therapy (5, 6). That which became licensed was the nucleoside analogue lamivudine (LAM), which has been extensively used ever since, due to its impressive antiviral activity and clean safety profile. Unfortunately, the emergence of resistant mutant viruses under LAM therapy substantially diminishes the long-term success of this therapy. In 2003, the first nucleotide analogue, adefovir (ADF), was licensed for HBV treatment (1, 11). ADF displayed a resistance profile superior to that of LAM while maintaining an excellent antiviral activity. However, since its approval, an increasing number of cases showing initial non-response and clinical therapy failure have been reported, such as those associated with the emergence of the rtN236T mutation (13, 17, 18). One possible reason for an initial nonresponse to

ADF is infection with HBV variants harboring an rtI233V mutation, which also confers resistance to ADF *in vitro* (15). This variant has been detected in about 2% of all cases of naïve chronic HBV carriers (2, 3).

In this report, we used previously published sequencing and subspecies analyses (15) to further investigate, confirm, and extend the significance of the rtI233V mutation in a clinical setting. Our data further emphasize the necessity of HBV genotyping prior to antiviral therapy for chronic hepatitis B.

We investigated two new, epidemiologically unrelated cases showing resistance to ADF therapy due to the rtI233V mutation. This is in contrast to the previously described cases in which the rtI233V polymorphism was preexisting when ADF therapy was initiated and failed thereafter (15). The first patient, referred to as patient 1, a Caucasian male, now 21 years old, was first treated with LAM but subsequently was switched to ADF after developing virological failure. Initially, HBV viremia dropped significantly, from 10⁸ genome equivalents (GE)/ml to 2 × 10⁴ GE/ml. However, since the viremia remained at this relatively high level and the serum HBV surface antigen (HBsAg) level increased significantly during therapy, the case was considered to represent a therapy failure. To determine the reason for this failure, quasispecies analysis of the dominant HBV strain before and 24 months after initiation of ADF therapy was performed. Quasispecies analysis was performed by cloning products after PCR amplification of the RT region of the viral polymerase and sequencing of up to 20 clones (4 representative clones are presented in Fig. 1). We clearly identified the selection of resistance-associated mutations in the dominant HBV population. The genotypic analysis revealed genotype D and serotype ayw2, which converted to ayw4 after 24 months of ADF treatment. The patient was unsuccessfully pretreated with LAM and did not respond, most probably as there was a minor population bearing the rtY203C mutation, a known LAM resistance mutation (20). It was concluded that this patient may have been a LAM nonresponder.

* Corresponding author. Mailing address: Heinrich-Pette-Institute, Hamburg, Germany. Phone: 0048-040-48051226. Fax: 0049-040-48051103. E-mail: sirhus@rocketmail.com.

† The first two authors contributed equally to this work.

‡ Present address: Centre for Infectious Disease Research, School of Chemistry and Molecular Biosciences, University of Queensland, St. Lucia, Australia.

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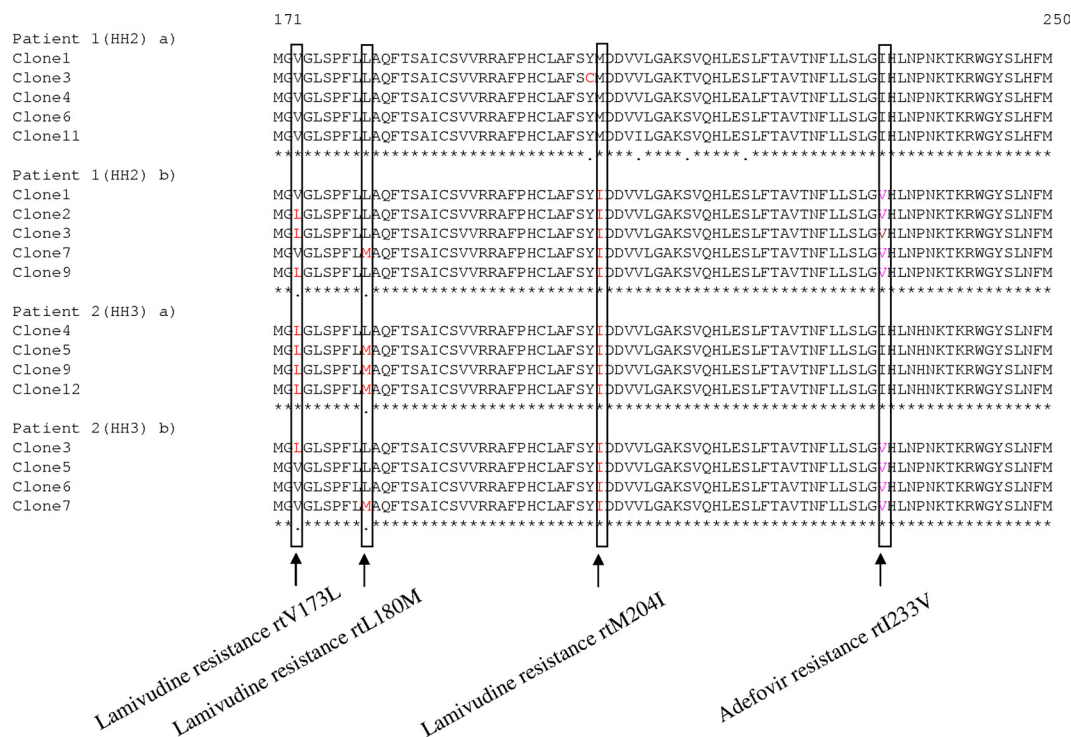


FIG. 1. Quasispecies analyses and alignments of four representative clones, each obtained from RT polymerase gene PCR amplicates before and after therapy for patients 1 and 2. In total, up to 10 clones per patient were sequenced, and of those, 4 representative clones are shown in the figure. Panel “a” indicates the sequences obtained before ADF therapy, whereas panel “b” indicates the sequences obtained after the increase of viral load, i.e., during or immediately after ADF therapy.

Surprisingly, after 24 months of ADF treatment, sequencing of the viral RT region revealed that the rtM204I mutation, a well-known LAM resistance mutation (20), was present in virtually all HBV subpopulations, whereas this mutation was not detectable before ADF therapy. Furthermore, minor populations with the rtV173L and rtL180M mutations, both of which are also associated with LAM resistance, were found after ADF therapy (20). These findings were confirmed by direct sequencing analysis of PCR products performed directly after the patient was introduced to the outpatient clinic. The observed type and frequency of these mutations are in agreement with the results for the previous LAM therapy. However, a delayed occurrence of the LAM-associated mutations after replacement of LAM therapy with ADF therapy is unusual and, to the very best of our knowledge, has not been described so far. We can only speculate on the reasons for this phenomenon, which may be caused by a reduced viral fitness that may be associated with the rtY203C mutation, but this would require further investigation and phenotypical characterization of this variant. Most importantly, the rtI233V mutation was present in all HBV subpopulations investigated after ADF therapy but not in those investigated before ADF therapy. Our findings thus clearly indicate that this mutation can develop during or is selected by ADF therapy.

The second patient, referred to as patient 2, a 39-year-old Caucasian male, was infected with HBV genotype D and serotype ayw3, which changed to ayw4 after ADF therapy. He was treated with ADF after failure of therapy with LAM. During ADF therapy, the viral load initially decreased below

the limit of detection but within 3 months rose to 2×10^5 GE/ml, probably due to resistance development (Fig. 2). The increase of HBV DNA was accompanied by an elevation of HBsAg in the serum. Sequencing of the RT region of the HBV genome revealed that the only mutation not detected prior to ADF therapy and present in all clones was the rtI233V ADF resistance mutation. This finding suggests efficient selection of this resistance mutation under ADF therapy and corroborates the findings for patient 1.

In conclusion, for both novel patients described in this report, who developed ADF resistance during therapy, quasispecies analyses revealed that the rtI233V mutation, a known ADF resistance mutation, was present, whereas before therapy, exclusively wild-type sequences were found in this region. This result strongly supports the previous conclusions, drawn from phenotypic analyses, that rtI233V mediates ADF resistance *in vivo*.

Follow-up sera from two previously reported patients who had been infected with HBV harboring rtI233V, which was thus resistant to ADF, leading to treatment with tenofovir (TDF), were investigated as described earlier (15). Clinical and serological markers, like levels of HBsAg, viral load, and liver enzymes, were monitored regularly during TDF treatment for 18 months. The female patient responded well to TDF treatment, with a sustained virological response in which virus genome levels rapidly decreased from $>5 \times 10^8$ genome equivalents (GE)/ml to 350 GE/ml and good overall tolerance of the treatment. This patient is still being successfully treated with TDF, and the HBV viral load is below the limit of detection

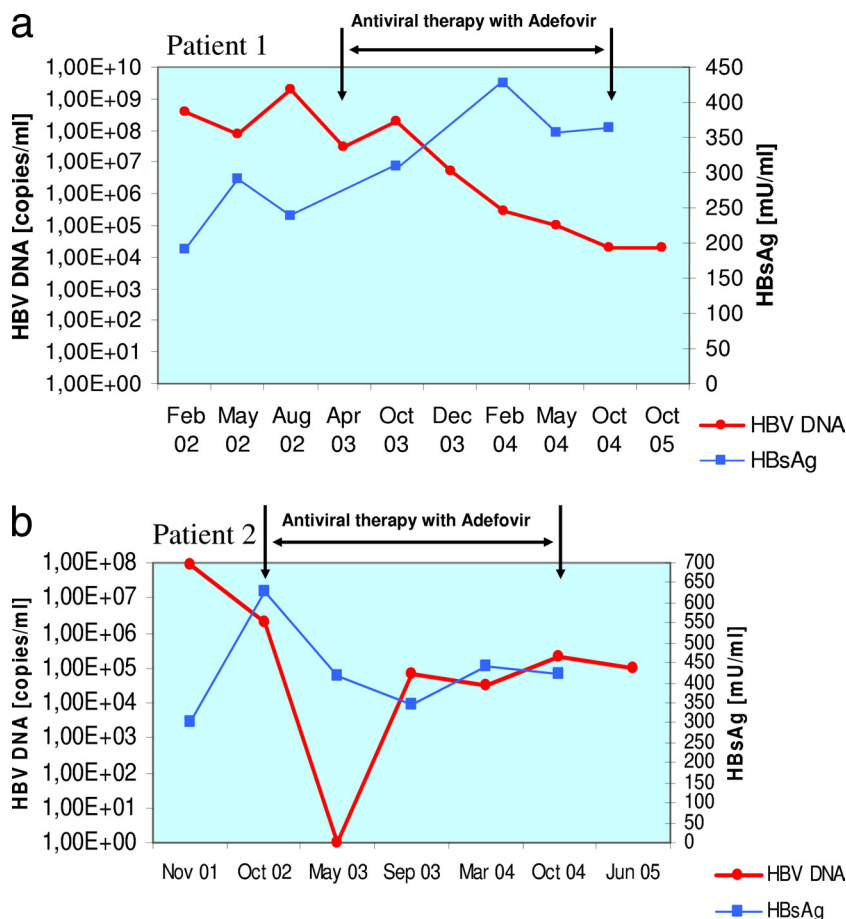


FIG. 2. Time course of viremia and HBsAg levels in two novel patients (patients 1 [a] and 2 [b]) for whom ADF therapy failed in association with the rtI233V polymerase mutation.

(<10 IU/ml, or 50 GE/ml). This shows that patients with ADF-resistant HBV can be successfully treated with TDF.

In the male patient, viremia also initially dropped from 4.5×10^7 GE/ml to 800 GE/ml and was undetectable 56 weeks after start of TDF therapy. However, TDF therapy of the male patient had to be stopped due to the emergence of drug-induced renal toxicity. Consequently, HBV therapy was switched to treatment with entecavir (Baraclude; Bristol Meyer Squibb), which was recently licensed for HBV therapy. To our surprise, the switch from TDF to entecavir resulted in a moderate but remarkable increase in viremia, from below the limit of detection to 10^4 GE/ml, while the rtI233V polymorphism partially reverted to the wild type (rtI233V \rightarrow rtI233I), as shown by direct sequencing of PCR products. As the rtI233V polymorphism was present before ADF therapy, this change to rtI233I might represent a novel form of counterselection in which the naturally occurring resistance-associated rtI233V polymorphism is presumably selected and changed by TDF therapy to wild-type rtI233I. This observation may reflect the response of the rtI233V variant HBV strains to TDF upon inclusion of TDF in a therapy regimen that replaces ADF.

In the present report, we provide two independent lines of evidence which suggest that the rtI233V HBV polymerase mutation is indeed associated with ADF resistance as recently

described (2, 15). Moreover, we show for the first time that the rtI233V mutation can be selected during ADF therapy in two patients. In addition, the follow-up of previously reported patients infected with rtI233V HBV mutants revealed that the cessation of ADF treatment can result in a loss of rtI233V and its conversion to the wild type (rtI233I). Interestingly, one of the novel patients, patient 1, showed an initial response to ADF that resulted in a decrease of about $4 \log_{10}$ in viral load, starting at 10^8 GE/ml and decreasing to 10^4 GE/ml, but the viral load remained constantly high, at 10^4 GE/ml, for more than 12 months (Fig. 2). Although a decrease of more than $1 \log_{10}$ in viral load is considered a therapy success and the reduced viremia may improve the further outcome of liver disease, the decrease was relatively weak and viral replication remained active at a relatively high level, which is corroborated by increasing levels of HBsAg in the serum. Fluctuation of the viremia should also be considered in this case, since it has been shown that viremia may fluctuate about $1 \log_{10}$ once chronic infection has been established, as observed in the woodchuck model (14). Since equivalent data from human patients has not yet been published, it remains unclear whether the fluctuation interval in humans may be more than $2 \log$ steps and whether a viral load decrease of $4 \log_{10}$ should be rated as a therapy success when viral load still remains high.

Moreover, these clinical cases give important insights into new treatment options that may be investigated in clinical studies in the future. First, it is worth noting that ADF can be efficiently replaced with TDF, provided that no side effects, such as nephrotoxicity, are observed. Thus, TDF is an efficient HBV-suppressing drug in ADF nonresponders or rebounders, although a cross-resistance between ADF and TDF was initially suspected. Second, the increase in viremia after replacement of TDF with entecavir in patient 1 may be a hint that the previous treatment primes for or even triggers entecavir resistance. Although, according to Chang and Lai (3, 8), the viremia in this patient may be considered low at a level of around 10^4 GE/ml, still, this level of viremia should be high enough to allow for the selection and emergence of resistant strains.

We conclude that the rtI233V mutation in the HBV polymerase gene mediates ADF resistance, consistent with previous reports (2, 15). Our data are divergent from those reported by Curtis et al. stating that the rtI233V mutation does not confer resistance to ADF (4). The *in vitro* resistance phenotype detection assay used by Curtis et al. is based on cytomegalovirus (CMV)-driven expression of the HBV genome and thus produces a large excess of viral pregenomes, a parameter used as a readout in their assay. Thus, CMV-driven *in vitro* systems seem less sensitive than those involving viral replicons driven by authentic promoters and thus seem inappropriate for measuring the relative extents of susceptibility of RT mutant genomes to nucleoside or nucleotide analogues (10, 12, 16). Nevertheless, the patients reported by Curtis and colleagues were susceptible to ADF therapy *in vivo* despite the rtI233V mutation. In the context of the data presented here, the hypotheses that arise are (i) that HBV resistance mechanisms are much more complicated than believed so far and (ii) that additional factors of the host or the virus contribute to clinical and phenotypical resistance or dose-dependent resistance against a distinct antiviral therapy.

Consequently, we recommend that in cases where the rtI233V mutation is detectable, ADF should be replaced with TDF or, with some limitations, with entecavir (3, 8).

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