Complex dynamics of hepatitis B virus resistance to adefovir.

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Resistance to Adefovir

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Abbreviations: HBV: hepatitis B virus; HBe: hepatitis Be; Ag: antigen; IU: international unit; HIV: human immunodeficiency virus; PCR: polymerase chain reaction; ALT: alanine aminotransferase; PHYLIP: phylogeny inference package; IC50: 50% inhibitory concentration.

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ABSTRACT

In patients with hepatitis B e antigen-negative chronic hepatitis B, adefovir dipivoxil administration selects variants bearing reverse transcriptase rtN236T and/or rtA181V/T substitutions in 29% of cases after 5 years. The aim of this work was to characterize the dynamics of adefovir-resistant variant populations during adefovir monotherapy in order to better understand the molecular mechanisms underlying hepatitis B virus resistance to this class of nucleotide analogues. Patients included in a 240-week clinical trial of adefovir monotherapy who developed adefovir resistance-associated substitutions were studied. The dynamics of hepatitis B virus populations were analyzed over time, after generating nearly 4000 full-length reverse transcriptase sequences, and compared with the replication kinetics of the virus during therapy. Whatever the viral kinetics pattern, adefovir resistance was characterized by exclusive detection of a dominant wild-type, adefovir-sensitive variant population at baseline and late and gradual selection by adefovir of several coexisting resistant viral populations, defined by the presence of amino acid substitutions at position rt236, position rt181, or both. The gain in fitness of one or other of these resistant populations during adefovir administration was never associated with the selection of additional amino acid substitutions in the reverse transcriptase. Conclusions: Our results suggest that adefovir administration selects poorly fit pre-existing or emerging viral populations with low-level adefovir resistance, which subsequently compete to fill the replication space. Viral kinetics depends on the initial virological response to adefovir. Lamivudine add-on restores some antiviral efficacy but adefovir-resistant variants remain predominant. Whether these adefovir resistance-associated substitutions may confer cross-resistance to tenofovir in vivo...
will need to be determined.
INTRODUCTION

Treatment of chronic hepatitis B virus (HBV) infection is aimed at driving viral replication to the lowest possible level, in order to halt the progression of chronic hepatitis B and to prevent complications such as cirrhosis, decompensation of cirrhosis, and hepatocellular carcinoma. Two categories of drugs can be used to treat chronic hepatitis B, namely pegylated interferon alpha and nucleoside/nucleotide analogues that directly inhibit the reverse transcriptase function of HBV DNA polymerase. Five HBV inhibitors (lamivudine, adefovir dipivoxil, entecavir, telbivudine and tenofovir) are currently approved. Lamivudine and adefovir dipivoxil are most widely used worldwide.

Chronic administration of nucleoside/nucleotide analogues in monotherapy frequently leads to the selection of HBV variants with amino acid substitutions in the reverse transcriptase domain of HBV DNA polymerase, conferring reduced susceptibility to the inhibitory action of these drugs (1-4). HBV has a quasispecies distribution in each infected individual, meaning that the virus circulates as a complex mixture of genetically distinct but closely related variants that are in equilibrium at a given time in a given replicative environment (5). The quasispecies distribution of HBV implies that any newly generated mutation conferring a selective advantage in a given replicative environment will allow the corresponding viral population to overtake the other variants, following a classical Darwinian evolutionary process. As a result, HBV resistance is generally acquired gradually, through the selection of preexisting resistant variants and gradual accumulation of new amino acid substitutions. The latter may confer stepwise increases in the level of drug resistance, but they mostly improve the replication capacity of resistant variants in the presence of the relevant
drug. HBV resistance to antiviral therapy is a major issue in clinical practice because it can lead to HBV treatment failure and progression of liver disease.

Long-term administration of adefovir dipivoxil has been shown to select variants bearing reverse transcriptase rtN236T and/or rtA181V/T substitutions, both of which confer low-level resistance to adefovir in vitro. In patients with hepatitis B e (HBe) antigen (Ag)-negative chronic hepatitis B, these variants are selected in 29% of cases (cumulative incidence) after 5 years of monotherapy, essentially in patients who do not achieve HBV DNA levels below 1000 international units (IU)/mL (6). Their selection is associated with different patterns of HBV DNA kinetics in different patients: (i) typical escape, with a return to baseline replication levels in patients who had initially responded to the drug by a more than 3 log reduction of HBV DNA level; (ii) a gradual re-increase in viral replication preceding the emergence of the mutant virus by several months, generally in patients with a suboptimal response to adefovir (less than 3 log reduction of HBV DNA level); and (iii) no change in adefovir antiviral efficacy in patients who initially responded well to the drug (7-10). These different patterns point to differences in the replicative fitness of selected resistant variants in vivo.

To understand the mechanisms underlying viral resistance to a specific inhibitor, it is first necessary to determine the quasispecies dynamics during the emergence and amplification of resistance, together with the impact of the relevant amino acid substitutions on viral replication in vivo (11-13). We have recently shown that, in vivo, the fluctuating environment in which lamivudine-resistant variants replicate during lamivudine administration to patients potentially favors one variant population over another, regardless of the intrinsic replication capacities of the viral populations in vitro (11). The aim of the present work was to characterize the
dynamics of adefovir-resistant HBV variant populations during adefovir monotherapy in order to better understand the molecular mechanisms underlying HBV resistance to adefovir.

MATERIALS AND METHODS

Patients

This was a substudy of a recently published 240-week clinical trial of adefovir monotherapy in 185 nucleoside/nucleotide-naïve HBeAg-negative patients, with a double-blinded phase of 96 weeks followed by an open-label, long-term safety and efficacy study of 144 weeks (6). Serial HBV DNA assays were systematically performed during therapy, as well as direct sequence analysis of the entire HBV reverse transcriptase gene at baseline and, in patients with detectable serum HBV DNA, at weeks 48, 96, 144, 192, and 240. Twenty-nine patients developed adefovir resistance-associated substitutions during the 240-week study. Their viral dynamics were characterized by means of frequent HBV DNA assays, classifying the patients into the following three groups: (i) responders (HBV DNA level reduction of more than 3 log IU/mL) who experienced secondary treatment failure (reincrease in the HBV DNA level of more than 1 log IU/mL above the nadir); (ii) suboptimal responders (HBV DNA level reduction of less than 3 log IU/mL) who developed the amino acid substitution(s) in a context of a slow, gradual re-increase in viral replication; and (iii) responders who developed the amino acid substitutions without any virological breakthroughs. We selected the first seven patients displaying these three dynamic patterns in order to study HBV population dynamics during adefovir exposure.
The seven patients were five men and two women aged from 26 to 59 years at the start of the study. Their baseline characteristics are shown in Table 1. They were all Caucasian and none was coinfected by hepatitis delta virus, hepatitis C virus or human immunodeficiency virus (HIV). All were nucleoside/nucleotide treatment-naïve at inclusion. They were treated with 10 mg/day adefovir for the full study period. In four cases (patients A, B, E and G), lamivudine was added during adefovir therapy because of virological failure. Quasispecies analysis was performed with serum samples taken at baseline and frequently during therapy (15 to 24 samples). At every time point and in each patient, HBV DNA was extracted and the full-length HBV reverse transcriptase domain was polymerase chain reaction (PCR)-amplified. The PCR products were directly sequenced and cloned. For quasispecies analysis, 25 to 41 clones per time point were sequenced. The dynamics of HBV populations over time were studied.

Methods

Alanine aminotransferase and HBV DNA assays. Alanine aminotransferase (ALT) levels were determined at baseline and during treatment by using a Roche Modular Chemistry Analyzer (Roche Diagnostics, Basel, Switzerland). HBV DNA was measured at each time point by means of a “classical” reverse transcriptase PCR assay (Cobas Amplicor HBV Monitor® v2.0, Roche Molecular Systems, Pleasanton, California) or a real-time PCR assay (Cobas TaqMan® 48 HBV test, Roche Molecular Systems). The results were expressed in IU/mL.

PCR amplification of the HBV reverse transcriptase region. DNA was
extracted from 200-500 mL of serum with the QIAamp DNA blood kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer’s instructions. A 630-bp fragment encompassing domains A to E of the HBV reverse transcriptase, between amino acid positions 66 and 275, was amplified by PCR using primer pair pol1 (5’-CCCTGCTCGTGTACTGCGGG-3’, nucleotide position 186-206) and pol2 (5’-GTTGCGTCAGCAACACCTTGGCA-3’, nucleotide position 1196-1174) (14). Forty cycles of amplification were performed, with a denaturation step at 94°C for 30 s, annealing at 48°C for 30 s and extension at 72°C for 1 min. When the HBV DNA level was below 1000 IU/mL (3.0 log IU/mL), a nested PCR technique was used, with internal primers pol3 (5’-GACTCGTGTTGACTTCTCCT-3’, nucleotide position 251-272) and pol4 (5’-GGCATAAAGCAGGATACCACATT-3’, nucleotide position 1058-1033). PCR amplification consisted of 30 cycles at 94°C for 30 s. 55°C for 30 s and 72°C for 1 min. PCR products were analyzed by electrophoresis through 2% agarose gel and ethidium bromide staining. They were then purified with Montage PCR centrifugal filter devices (Millipore Corporation, Bedford, Massachusetts), using the manufacturer’s protocol.

**Direct sequence analysis of PCR products and determination of the HBV genotype.** Purified PCR products were directly sequenced with the primers used for PCR amplification. The HBV genotype was determined by phylogenetic analysis of the 630-bp fragment amplified at baseline, with respect to reference sequences of the principal HBV genotypes retrieved from GenBank, using the Phylogeny Inference Package (PHYLIP) program version 3.65.

**Quasispecies analysis of HBV RT.** Purified PCR products were cloned into
the pCR4-TOPO® vector (TOPO® TA cloning® kit, Invitrogen Corporation, Carlsbad, California) according to the manufacturer’s instructions. Recombinant plasmid DNA was transformed into One Shot® TOP10 chemically competent Escherichia coli, as recommended by the manufacturer, and transformants were grown on ampicillin plates. Cloned DNA was re-amplified by PCR and purified for sequencing. Twenty-five to 41 clones per sample were sequenced and their nucleotide and amino acid sequences were aligned with CLUSTAL X, version 1.83. Since all but one sample had an HBV DNA level above 1000 IU/mL, resampling resulting in expansion of multiple copies of the same viral species was unlikely to occur.

**Nucleotide sequence accession numbers.** The sequence data from this study have been deposited in GenBank under accession numbers EU026558 to EU028300.

**RESULTS**

Twenty-nine patients developed adefovir resistance-associated substitutions during the 240-week study. Based on their viral dynamics, the 29 patients were classified into response pattern 1 (initial virological response with secondary treatment failure: n = 9, 31%), response pattern 2 (suboptimal virological response followed by a gradual re-increase in viral replication: n = 4, 14%), response pattern 3 (sustained antiviral response throughout follow-up: n = 15, 52%), and mixed response pattern (n = 1, 3%). Given the number of samples studied per patient and the number of clones generated per sample, not all patients could be extensively studied. We selected the first seven patients, including three patients with response
pattern 1, two patients with response pattern 2, one patient with response pattern 3 and one patient with a mixed response pattern.

A total of 3843 nearly full-length HBV reverse transcriptase quasispecies sequences were generated and analyzed. At baseline, the HBV reverse transcriptase always had a typical quasispecies distribution, characterized by the coexistence of distinct but closely related viral populations, including one to three major variants each representing more than 10% of the quasispecies variants, and a variable number of minor variants bearing amino acid substitutions at sporadic positions relative to the most frequent variant. Figure 1 shows the HBV DNA dynamics over time in each patient and the proportions of resistant versus wild-type virus at baseline and at each time point during therapy. In all the patients, only a “wild-type”, adefovir-sensitive virus population (i.e. viruses without amino acid substitutions at positions rt181 and rt236) was detected prior to therapy (Figure 1). Figure 2 shows the dynamics of the different adefovir-resistant viral populations over time.

Dynamics of resistance associated with response pattern 1 (initial virological response with secondary treatment failure). Three patients (patients A, B and C) initially responded to adefovir, with a significant fall in the HBV DNA level (-4.6, -3.8 and -5.1 log IU/mL after 12, 17 and 8 months of adefovir administration, respectively; Figures 1A to 1C). The antiviral response only occurred after 14 months in patient B, after an ALT flare; this patient had initially responded suboptimally. The virological breakthroughs occurred at months 28, 31 (17 months after the antiviral response) and 37, respectively, and were always followed by a biochemical breakthrough (ALT elevation) when the HBV DNA level returned to baseline. Lamivudine add-on in patients A and B partially restored an antiviral response
No viral populations bearing amino acid substitutions at position rt181 or rt236 were detected before therapy. The wild-type viral population was always gradually replaced by the resistant viruses during therapy. The size of the resistant virus population grew gradually for several months before the virological breakthrough, and became predominant at the time of the biochemical breakthrough (Figures 1A to 1C). The adefovir-resistant viral populations remained predominant when lamivudine was added to adefovir in patients A and B (Figures 1A and 1B). Five resistant viral populations were found during adefovir administration in the three patients with viral dynamic pattern 1 (Figures 2A, 2B and 2C), including viruses with an rtA181V substitution alone (patients A, B and C), viruses with an rtA181T substitution alone (patient C), viruses with an rtN236T substitution alone (patients A, B and C), viruses harboring both rtA181V and rtN236T substitutions (patients A, B and C), and viruses harboring both rtA181T and rtN236T substitutions (patient B).

In the three patients, several different viral populations were present at several time points. However, the dynamics of HBV viral populations varied from one patient to the next. In patient A (Figure 2A), the rtA181V variant population predominated over the other adefovir-resistant populations throughout therapy. Despite the partial restoration of an antiviral response when lamivudine was added, the rtA181V population remained predominant during adefovir-lamivudine combination therapy (Figure 2A). In patient B (Figure 2B), the wild-type sensitive virus was initially replaced by a mixture, in similar proportions, of three variants harboring the rtA181V substitution, the rtN236T substitution and both substitutions together, respectively. However, after a few months the rtN236T-only variants took over and remained predominant until the end of adefovir monotherapy, even when lamivudine was
added, despite partial restoration of the antiviral response (Figure 2B). In patient C (Figure 2C), quasispecies analysis showed the simultaneous emergence of four resistant variants, but variants bearing both rtA181V and rtN236T substitutions rapidly became predominant. No reverse transcriptase positions other than rt181 or rt236 showed specific amino acid changes during therapy that might have led to improved fitness and thus explained how one specific resistant population overtook the others during adefovir exposure.

**Dynamics of resistance associated with response pattern 2 (suboptimal virological response followed by a gradual re-increase in viral replication).** Two patients (D and E) initially responded suboptimally to adefovir. In patient D, the HBV DNA level fell by -2.5 log IU/mL after 4 months of treatment and then gradually increased. A biochemical breakthrough occurred at month 22 in this patient, when the HBV DNA level had returned to baseline (Figure 1D). Patient E also responded suboptimally to adefovir (-1.9 log IU/mL). Adefovir withdrawal at month 10 was followed by HBV DNA and ALT flares. Treatment was restarted at month 13 and the patient again responded suboptimally (-2.4 log IU/mL in 2 months). The HBV DNA level then started to increase gradually, returning to baseline (the value before the second course of treatment) at month 30 (Figure 1E). No biochemical breakthrough occurred in this patient. Lamivudine was added at month 40 and resulted in a 2-log fall in HBV DNA (Figure 1E).

In these two patients the wild-type sensitive virus was gradually replaced by resistant viruses, which became predominant when the HBV DNA level had returned to baseline (Figures 1D, 1E). In patient D, five resistant populations (rtA181V alone, rtA181T alone, rtN236T alone, rtA181V plus rtN236T, and rtA181T plus rtN236T)
were detected during follow-up. Their dynamics were characterized by three successive waves, with rtA181V/T initially dominating, then being replaced by a dominant rtN236T population and, finally, by a dominant population bearing both rtA181T and rtN236T substitutions (Figure 2D). The biochemical breakthrough coincided with the shift from a dominant sensitive viral population to a resistant one at the time of the second wave (dominant rtN236T population) (Figure 2D).

In patient E, four resistant populations were detected during follow-up (rtA181V alone, rtN236T alone, rtA181V plus rtN236T, and rtA181T plus rtN236T), but the viral population bearing the rtA181V substitution was dominant throughout follow-up (Figure 2E). The same pattern (predominance of rtA181V variants) was observed when lamivudine was added (Figure 2E). No positions other than rt181 or rt236 showed specific amino acid changes during therapy in patients D and E.

Dynamics of resistance associated with response pattern 3 (sustained antiviral response throughout follow-up). Patient F was treated with adefovir for 57 months. He initially responded with a -4.3 log IU/mL decrease in HBV DNA after 7 months, and this level of response persisted throughout follow-up, despite gradual replacement of the wild-type, sensitive virus by the resistant virus population after month 40 (Figure 1F). Despite the predominance of resistant viruses, no virological or biochemical breakthrough was observed between month 40 and month 47, when follow-up ended for this patient (Figure 1F). As in the other patients, regardless of the viral dynamics on therapy, several resistant populations were detected but the population with only the rtN236T substitution predominated from the onset of viral resistance until the end of follow-up. No positions other than rt181 or rt236 showed specific amino acid changes during therapy.
Mixed response. Patient G had a mixed virological response (Figure 1G). This patient initially responded suboptimally to adefovir (-2.0 log IU/mL in 9 months) and the HBV DNA level remained stable thereafter, despite gradual replacement of the wild-type, sensitive viral population by a resistant population that predominated at month 22 when treatment was stopped (Figure 1G). Patient G had an HBV DNA flare one month after treatment withdrawal and, although the resistant viral population was still detectable, most of the variants present at the time of the flare were sensitive. The patient was then retreated at month 24 and responded (-4.0 log IU/mL in 2 months). However, the resistant population took over rapidly and virological and biochemical breakthroughs occurred a few months later. Lamivudine was added and this restored an effective antiviral response, even though an adefovir-resistant population predominated (Figure 2G). As shown in Figure 2G, the rtN236T population was nearly the only resistant population present during the first course of therapy, at the time of the flare after treatment withdrawal, during the second course of therapy, at the time of the virological and biochemical breakthrough, and when lamivudine was added (Figure 2G). No positions other than rt181 or rt236 showed specific amino acid changes during therapy.

DISCUSSION

We generated nearly 4000 full-length HBV reverse transcriptase sequences in order to unravel the dynamics of HBV variants during adefovir dipivoxil administration and thereby to understand how HBV resistance to this drug develops. The dynamics of HBV viral populations were compared with the replication kinetics of the virus
during several years of adefovir administration.

Whatever the viral kinetics, adefovir resistance was always characterized by: (i) exclusive detection of a dominant wild-type, adefovir-sensitive variant population at baseline; (ii) late (several years) and gradual selection by adefovir of coexisting resistant viral populations, defined by the presence of amino acid substitutions at position rt236, position rt181, or both; and (iii) during adefovir administration, an apparent gain in fitness of one or other of these resistant populations, which became predominant after several months of resistance. This gain in fitness was never associated with the selection of additional amino acid substitutions in the reverse transcriptase. In contrast, substitutions that improve viral replication without substantially enhancing resistance have been observed during lamivudine resistance (substitution at position rt180 or rt173) (15-18) and during entecavir resistance (substitution at position rt180, and positions rt184, rt202 and/or rt250). (19-22).

Our results are in keeping with, although they do not demonstrate, the hypothesis that the adefovir-resistant viral populations preexist in a subset of HBV-infected patients, who are those exposed to develop adefovir resistance. Indeed, amino acid substitutions occur randomly as a result of viral replication. They are not induced by the administration of an antiviral drug that can only select viral variants that bear them when they are present. Therefore, although it cannot be excluded that some substitutions occurred during the few months of therapy in patients with reduced HBV replication levels, the probability is much greater that these substitutions randomly occurred during the many years that preceded therapy during which replication levels were higher. HBV variants bearing rtN236T and/or rtA181V/T polymorphisms however remain undetectable at baseline, owing to their relatively poor in vivo fitness. Although these variants were not observed at baseline in our
clonal analysis, the sporadic detection of one or both mutations early in therapy, prior
to clear-cut outgrowth of resistant virus populations, was consistent with the
hypothesis of their preexistence. In addition, the substitutions at positions rt236 and
rt181 confer only low-level HBV resistance to adefovir (up to a 3-fold increase in the
50% inhibitory concentration (IC50) \textit{in vitro}), whereas the rtM204V/I substitution
confers a $>1000$ fold-increase in the lamivudine IC50 (23-25). Thus, their low-level
resistance and poor \textit{in vivo} fitness together explain why adefovir-resistant variants
emerge late and very slowly during adefovir administration.

The HBV replication kinetics provide further information on the relative \textit{in vivo}
fitness of adefovir-resistant variants in the presence of adefovir. In the patients with
pattern 1 replication kinetics (virological response followed by virological
breakthrough), the profound inhibition of wild-type, sensitive virus replication opens
the replication space to resistant variants. In this situation the gain in fitness can
allow HBV DNA to return to the baseline level when the resistant variant becomes
predominant. As during lamivudine resistance, selection of adefovir-resistant variants
preceded virological breakthrough by several months in our study. The interval
between the emergence of resistant variants and virological breakthrough was
longer, however, than during lamivudine administration (11), owing to the poorer
fitness of adefovir-resistant variants \textit{in vivo} relative to lamivudine-resistant variants.
The biochemical breakthrough generally occurred when the resistant population
accounted for 100% of circulating variants.

In viral kinetic pattern 3, no virological relapse was observed despite an initial
virological response (as in pattern 1) and gradual replacement of the wild-type,
sensitive population by adefovir-resistant variants. Two mutually compatible
hypotheses can be raised to explain this observation: (i) the selected variants may
have had such poor *in vivo* fitness that they could not be produced at levels similar to those of the wild-type, sensitive virus present before therapy; in that case, a viral breakthrough will be observed only if the variants acquire additional mutations susceptible to improve their *in vivo* fitness; (ii) owing to the poor replication capacity of the variants, more time may have been required for HBV DNA to reach high levels; this means that relapse could occur later in these patients. This observation could be reassuring in that detection of adefovir resistance-associated substitutions during therapy does not mean that a clinically relevant viral breakthrough will occur. However, such detection indicates that a breakthrough may subsequently occur and further research should be conducted to determine whether treatment should be altered in order to prevent it.

Viral kinetic pattern 2 was more difficult to interpret. Indeed, these patients had a suboptimal response to adefovir, owing to the use of a low dose in clinical practice (10 mg/day) (26). A suboptimal response to adefovir has been reported to occur in approximately 20% of patients with HBeAg-negative chronic hepatitis B. In these patients, the only moderate decrease in wild-type, sensitive virus replication did not open the replication space sufficiently for adefovir-resistant viruses to break through rapidly. This probably explains why the increase in HBV DNA levels was gradual and matched the progressive selection of resistant viruses, which became predominant roughly when HBV DNA levels had nearly returned to baseline.

Interestingly, we found no substitutions at positions other than rt236 and rt181 in the HBV reverse transcriptase that could explain a gain or loss of fitness by any of the adefovir-resistant populations in our patients. We cannot exclude the possibility, however, that substitutions in other genomic regions than the reverse transcriptase, which were not analyzed here, could have played a role, for instance by modifying
the intrinsic replication capacity of the corresponding variants or by inducing a shift in epitope immunodominance. It was indeed striking that, in two patients, the response to adefovir was directly associated with an ALT flare, with no visible sequence changes in the reverse transcriptase region. Indeed, patient B initially responded suboptimally to adefovir, but then responded fully to the same dose after an ALT flare occurred during therapy. Patient G also initially responded suboptimally, experiencing an ALT flare just after treatment was interrupted and subsequently responding fully to the same dose of adefovir. In both cases the response to adefovir was followed by virological breakthrough as a result of adefovir-resistant variant selection. 

Pharmacodynamic differences in adefovir metabolism between patients may also influence viral fitness and, as a result, the kinetics of viral populations and HBV DNA during therapy by influencing the replicative environment. For instance, differences in the uptake of antivirals, including adefovir, by organic anion transporters have been reported (27).

In patients on adefovir monotherapy who developed resistance to adefovir, lamivudine add-on partially restored the antiviral response. However, the circulating variants remained adefovir-resistant. This was not surprising, as adefovir-resistant variants theoretically have similar lamivudine sensitivity to wild-type, sensitive variants but remain fitter than the latter in the presence of adefovir. If adefovir had been replaced by lamivudine, an adefovir-sensitive viral population would likely have replaced the adefovir-resistant virus, allowing lamivudine resistance to occur rapidly, as in a treatment-naive population receiving lamivudine monotherapy. The fact that adefovir-resistant variants bearing substitutions at positions rt236 and/or rt181 would need to accumulate additional substitutions at position rt204 (and possibly positions rt180 and rt173) in order to become resistant to lamivudine and to become fit in vivo
is reassuring with respect to the time necessary for resistance to develop in patients receiving both drugs. Conversely, it has been demonstrated that adefovir add-on is a better strategy than a switch from lamivudine to adefovir in patients with lamivudine resistance (28). More potent antiviral drugs with no cross-resistance with adefovir, such as telbivudine and entecavir, should be added in adefovir-resistant patients. It is important to note that substitutions at position rt181 have recently been reported to confer cross-resistance to lamivudine and adefovir \textit{in vivo} and could also confer resistance to entecavir and telbivudine (29, 30).

Together our results suggest that adefovir administration selects poorly fit pre-existing or emerging viral populations with low-level adefovir resistance, which subsequently compete to fill the replication space. In patients who initially respond to adefovir, resistance may be associated with a virological breakthrough followed by a biochemical breakthrough. In patients who initially respond suboptimally to adefovir, resistance emerges slowly and is associated with a gradual re-increase in viral replication, in keeping with the small difference in \textit{in vivo} fitness between wild-type, sensitive and adefovir-resistant viral variants. Lamivudine add-on restores some antiviral efficacy but adefovir-resistant variants remain predominant.

Tenofovir disoproxil fumarate has recently been reported to reduce HBV replication more potently than adefovir in both HBeAg-positive and -negative patients with chronic hepatitis B (31, 32). Tenofovir has very recently received approval for use in the treatment of chronic hepatitis B in Europe and the United States. Conflicting results have been reported as to whether adefovir resistance-associated substitutions confer cross-resistance to tenofovir. However, several recent reports suggest that substitutions at positions rt236 may confer reduced HBV susceptibility to tenofovir \textit{in vitro} (33, 34). It remains to be seen whether adefovir resistance
mutations also confer reduced susceptibility to tenofovir in vivo, as suggested by the recent observation that adefovir resistance substitutions persist when adefovir is switched to tenofovir in non- or suboptimal responders to adefovir (35). If so, the dynamics of resistant viral populations will need to be carefully assessed. This will largely determine whether tenofovir eventually becomes the natural partner of nucleoside analogues in fixed-dose combinations for first- and second-line treatment of HBV infection.

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to adefovir in the absence of adefovir-resistant mutations. J Hepatol
FIGURE LEGENDS

Figure 1. Dynamics of serum HBV DNA levels (gray area), serum aminotransferase activities (gray line), adefovir-sensitive (black bars) and adefovir-resistant (white bars) HBV viral populations during adefovir (ADV) and eventually adefovir plus lamivudine (ADV + LAM) therapy in the patients with response pattern 1 (initial virological response with secondary treatment failure: patients A [Figure 1A], B [Figure 1B] and C [Figure 1C]), response pattern 2 (suboptimal virological response followed by a gradual re-increase in viral replication: patients D [Figure 1D] and E [Figure 1E]), response pattern 3 (sustained antiviral response throughout follow-up: patient F [Figure 1F]) and a mixed response pattern (patient G [Figure 1G]).

Figure 2. Dynamics of HBV variant populations during adefovir (ADV) and eventually adefovir plus lamivudine (ADV + LAM) therapy in the patients with response pattern 1 (initial virological response with secondary treatment failure: patients A [Figure 2A], B [Figure 2B] and C [Figure 2C]), response pattern 2 (suboptimal virological response followed by a gradual re-increase in viral replication: patients D [Figure 2D] and E [Figure 2E]), response pattern 3 (sustained antiviral response throughout follow-up: patient F [Figure 2F]) and a mixed response pattern (patient G [Figure 2G]). The proportion of wild-type, adefovir-sensitive HBV variants at the different time points is represented by white bars. The proportions of HBV variants bearing the rtA181V substitution, the rtA181T substitution, the rtN236T substitution, both rtA181V and rtN236T substitutions, and both rtA181T and rt N236T substitutions, respectively, are represented by red, orange, green, light blue and dark blue, respectively. HBV DNA levels are represented by the gray area and ALT levels by the gray line.
Table 1 – Clinical and virological characteristics of the 7 patients included in this study. M: male; F: female; Ag: antigen; Ab: antibodies.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex/Age</th>
<th>Race</th>
<th>Route of transmission</th>
<th>HBV genotype</th>
<th>HBeAg/ Anti-HBe Ab</th>
<th>HBV DNA (log IU/mL)</th>
<th>Baseline ALT</th>
<th>Ishak score*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>M/46</td>
<td>Caucasian</td>
<td>Unknown</td>
<td>D</td>
<td>-/+</td>
<td>7.8</td>
<td>170</td>
<td>4 3 2 2</td>
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<tr>
<td>B</td>
<td>M/59</td>
<td>Caucasian</td>
<td>Unknown</td>
<td>D</td>
<td>-/+</td>
<td>6.8</td>
<td>166</td>
<td>2 0 1 2 3</td>
</tr>
<tr>
<td>C</td>
<td>F/51</td>
<td>Caucasian</td>
<td>Unknown</td>
<td>D</td>
<td>-/+</td>
<td>7.6</td>
<td>52</td>
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<tr>
<td>D</td>
<td>M/55</td>
<td>Caucasian</td>
<td>Childhood acquisition</td>
<td>D</td>
<td>-/+</td>
<td>6.6</td>
<td>239</td>
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<tr>
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<td>M/43</td>
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<td>Childhood acquisition</td>
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<td>F/56</td>
<td>Caucasian</td>
<td>Blood transfusion</td>
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<td>G</td>
<td>M/26</td>
<td>Caucasian</td>
<td>Unknown</td>
<td>D</td>
<td>-/+</td>
<td>5.5</td>
<td>99</td>
<td>4 0 2 4 6</td>
</tr>
</tbody>
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*Ishak score: a: perportal injury; b: confluent necrosis; c: parenchymal injury; d: portal inflammation; e: fibrosis*