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**Stepwise process for the development of entecavir resistance in a chronic
Hepatitis B virus infected patient**

**Stéphanie Villet^{1,4}, Aurélie Ollivet^{1,4}, Christian Pichoud^{1,4}, Luc Barraud², Jean-
Pierre Villeneuve³, Christian Trépo^{1,4,5}, and Fabien Zoulim^{1,4,5}**

**¹ INSERM, U271, Laboratoire des virus hépatiques et pathologies associées,
Lyon, F-69003, France.**

**² Bioalliance Pharma, 59, boulevard Général Martial Valin, Paris, F-75015,
France**

**³ Division of hepatology, Hôpital Saint Luc du Centre Hospitalier Universitaire
de Montréal, Montréal, Québec, Canada.**

⁴ Université Lyon 1, Faculté de Médecine Laennec, Lyon, F-69008, France

**⁵ Hospices civils de Lyon, Hôtel Dieu, Service d'Hépatologie, Lyon, F-69002,
France**

Short title: Mechanism of entecavir resistance

Contact information: Mailing address: INSERM U271, 151 cours Albert Thomas,
69424 Lyon cedex 03, France. Phone: (+33)-472681970, Fax : (+33)-472681971. E-
mail address: zoulim@lyon.inserm.fr

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Abstract

Background/Aims: Complex mutants may be selected under sequential anti-VHB pressures. We analyzed the genotypic and phenotypic evolution of the viral quasi-species of a patient who developed resistance to entecavir following lamivudine breakthrough.

Methods: The polymerase gene was amplified, cloned and sequenced at different time points. Hepatoma cell lines were transfected to compare the replication capacity of HBV mutants and their drug susceptibility.

Results: A mixture of lamivudine-resistant HBV strains coexisted following viral breakthrough to lamivudine, all harboring the rtM204V mutation. The rtV173L+L180M+M204V dominant mutant displayed strong lamivudine-resistance and the highest replication capacity. Following the switch to entecavir, the viral load dropped but the lamivudine-resistant strains continued to be selected. Three years later, the viral load rose again, and a complex mixture of entecavir-resistant strains, all harboring the lamivudine-resistance signature rtL180M+M204V and the rtS202G mutation were observed. Although the rtL180M+S202G+M204V variant, that prevailed at the end of entecavir therapy, did not show the highest viral genome replication capacity, it conferred one of the strongest resistance level to entecavir.

Conclusion: We report the selection of complex HBV mutants that escaped lamivudine and entecavir antiviral pressures. Genotypic and phenotypic analysis provided additional information to understand the process of HBV variant selection.

Keywords : entecavir; antiviral-resistance; HBV variants; selection process; genome evolution

word count: 198 words

Introduction

Hepatitis B infection affects two billion people worldwide and nearly 400 million people are chronically infected [1], resulting in severe liver disease with eventual progression to cirrhosis and hepatocellular carcinoma [2]. Alpha interferon administration has been the only treatment option for many years, and is associated with a sustained response in approximately 30% of patients. However, dose-dependent side effects are also frequent [3,4]. The development of polymerase inhibitors for the treatment of Human Immunodeficiency Virus (HIV) infection has provided a new hope for the treatment of Chronic Hepatitis B. There are currently three approved nucleos(t)ide analogs for chronic HBV infection: Lamivudine (L-deoxycytidine analog), adefovir (adenosine monophosphate analog), and entecavir (deoxyguanosine analog). They all target the HBV reverse transcriptase (RT) activity, thus inhibiting viral replication and resulting in improvement in virologic, biochemical and histologic status in most of the patients [5-7]. However, lamivudine treatment results in the frequent emergence of drug-resistance HBV, approximately 20% after 1 year of treatment with a progressive increase to 70% at year 4. The main mutations associated with resistance are located in the YMDD catalytic motif of the HBV RT C domain (rtM204V/I) [8]. Compensatory mutations associated with lamivudine-resistance (rtV173L, rtL180M) are found in the B domain [9,10]. The rtM204V mutation induces a 1000-fold decrease in susceptibility to lamivudine *in vitro* in comparison to wt HBV [11]. Adefovir-resistance occurs at a lower rate, with resistance described in 3% of patients after 2 years of treatment; this increases up to 29% by year 5 of therapy [12,13]. Resistance to adefovir is associated with the selection of the rtN236T mutation within the D domain of the viral enzyme [14-16] or with a rtA181V amino acid change in the B domain [14,17,18]. The rtN236T mutation

results in a 3- to 6-fold reduction in susceptibility to adefovir *in vitro* [14-16,19]. Concerning entecavir, virologic breakthrough confirmed by genotypic analysis has been observed during phase II and III clinical trials in 5.8% of patients treated by entecavir for lamivudine failure for one year and 10% for 2 years [20-22]. The two patients reported with resistance to entecavir had two signature lamivudine-resistance mutations resulting in rtL180M and rtM204V with additional mutations of rtM250V or rtS202I. To date, phenotypic analysis revealed no *de novo* entecavir-resistance in the absence of preexisting lamivudine-resistance [21].

In this report, we describe a new case of a patient who developed resistance to entecavir following lamivudine breakthrough. A longitudinal genotypic analysis of HBV strains isolated from the patient revealed the presence of new resistance mutations to entecavir and the persistence of lamivudine-resistance signature after three years of entecavir therapy. *In vitro* assays were performed to analyze the replication level and drug susceptibility of the major HBV strains. Results showed that the lamivudine-resistance signature mutations, in addition to rtS202G substitution, are necessary to induce HBV resistance to entecavir *in vitro*, but remain sensitive to adefovir. These results led us to gain new insight in the mechanism involved in the process of selection of entecavir-resistant mutants.

Material and Methods

Patient

A 69 year-old Canadian man with a chronic hepatitis B and cirrhosis, was started on an Interferon therapy in May 1997. The patient was HBsAg positive and HBeAg positive. After 7 months of interferon treatment, serum HBV DNA had decreased by half. He then received lamivudine (100 mg daily). After 5 months of lamivudine-treatment, serum HBV DNA was consistently undetectable by Digene-I quantification method ($<1.4 \times 10^5$ copies/ml), but increased to its initial level 11 months later. He was rapidly switched to entecavir (1 mg daily). After one year of entecavir therapy, HBV-DNA had decreased by three logs but viral breakthrough (more than 1-log increase) was observed two years later. The patient is currently being treated with lamivudine and adefovir combination therapy, and HBV DNA has decreased progressively by 3 logs in 2 years. The evolution of viral load over time, quantified by Digene assay (Digene, Gaithersburg, MD) during the first 84 months and by bDNA assay (Versant HBV DNA 3.0 assay; Bayer Corporation, Tarrytown, NY) the following months, is shown in figure 1. The “rebound” observed under lamivudine+adefovir bitherapy corresponds to the change in HBV DNA detection method.

Clonal analysis of the HBV polymerase gene

HBV DNA was extracted from 200 μ l of serum sample by using QIAamp Ultrasens virus kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The HBV polymerase gene domain between amino acid position 323 to 770 [23], encompassing the whole RT domain, was amplified by PCR using primers and thermocycling conditions described by Villet et al. [14,24]. PCR products were cloned

into the pGEMt easy vector system (Promega, Madison, WI) according to the manufacturer's protocol. Following transformation into DH5 α cells (Promega), 20 colonies per serum sample were picked and the plasmid DNA inserts were sequenced.

HBV constructs for phenotypic analysis

A replication competent 1.1 HBV genome unit length vector containing HBV genome isolated from the baseline clinical sample was constructed as previously described [14,24]. The sequence and the replication capacity of these vectors containing HBV genome were verified.

Subsequently, the HBV polymerase mutants identified by the clonal analysis were inserted in the baseline viral genome by replacing the 1092bp KpnI-NcoI fragment of the vector containing HBV from baseline by the corresponding KpnI-NcoI fragment from selected pGEMt-HBV polymerase plasmids. The newly obtained plasmids were verified by DNA sequencing prior to cell transfection.

Construction of the rtS202G single mutant by site-directed mutagenesis

Plasmid pGEMt containing the 1320bp wt HBV RT sequence isolated from the baseline clinical sample was engineered with the Quick Change kit (Stratagene, La Jolla, CA) to install the rtS202G mutation. The construct was sequenced to confirm this modification. The 1092bp KpnI-NcoI fragment containing the main RT domain was then transferred to the vector containing HBV genome from the baseline clinical sample.

Analysis of HBV genome replication and drug susceptibility

Huh-7 cells were cultured in DMEM (Eurobio, Courtaboeuf, France) supplemented with 10% fetal bovine serum. Lamivudine, tenofovir and adefovir were obtained from Gilead Sciences and entecavir from Bristol-Myers.

HBV genome replication capacity was measured by co-transfecting transiently recombinant pTriex-HBV clones with a luciferase reporter plasmid (pGL3, Promega) as previously described [14]. Five days after transfection, purification of HBV DNA from intracellular core particles was performed following the protocol described by Summers et al. [25]. Intracellular HBV DNA was submitted to *Southern blot* analysis, quantified using PhosphorImager analysis, and adjusted for the efficiency of transfection according to the results of the luciferase assay.

The analysis of drug susceptibility was performed after transient transfection of HepG2 cells with recombinant wt and mutant pTriex-HBV clones. Sixty hours after transfection, the medium was changed and reincubated with drug-free medium or medium containing 0.01, 0.1, 10, 100, 500 μ M of lamivudine, or 10, 20, 50, 100, 200 nM (for lamivudine-resistant clones) or 10, 50, 200, 1000, 5000 nM of entecavir (for entecavir-resistant clones), or 6.25, 12.5, 25, 50, 100 μ M of adefovir or tenofovir. Treatments were renewed every day for 5 days. Then, intracellular HBV DNA was purified and was subjected to *Southern blot* analysis as described above. For each drug, the concentration inhibiting by 50% the amount of intracellular viral DNA (IC₅₀) detected in treated cells at the end of the treatment, compared with untreated cells, was determined by phosphorImager analysis.

Results

Evolution of the viral RT gene in the viral quasi-species

Viral genome analysis was performed for serum samples #1 to #6 throughout the clinical course of the patient (Figure 1). The DNA sequences from 20 clones per serum sample were analyzed. The sequence of the viral RT gene at baseline of lamivudine monotherapy was wild-type (wt). Five months after the beginning of lamivudine therapy, the HBV DNA titer was undetectable by Digene I assay but increased to its initial level 11 months later. At this time, surprisingly, the wt population was dominant, representing 43% of the viral population. In addition, 3 different mutants were also found to coexist: rtM204V, rtL180M+M204V and rtV173L+L180M+M204V representing 19, 9 and 29% of the viral population respectively (Figure 2). The patient was switched to entecavir and one year later, HBV DNA decreased by approximately 3 logs. This decrease of viral load was associated with the selection of the rtV173L+L180M+M204V variant which became the major variant species representing 88% of the viral population. When viral load increased significantly 3 years after the start of entecavir therapy, the sequence analysis revealed a complex mixture of variants. All the clones maintained the lamivudine-resistance signature mutations rtL180M+M204V despite the cessation of lamivudine treatment, but also harbored the rtS202G change, not already described in the literature. The rtL180M+S202G+M204V triple mutant emerged alone or in combination with the rtI169L, rtV173L+P177S or rtA181G mutations. The triple mutant represented the major variant species, with 41% and 50% of the HBV population at months 34 and 38 of entecavir therapy respectively. More complex mutants also coexisted during this period like the rtL180M+A181G+S202G+M204V and rtV173L+P177S+L180M+S202G+M204V variants that represented

approximately 30% and 15% of the HBV population respectively. Entecavir was switched to lamivudine+adefovir bitherapy, and viral load decreased very rapidly (1 log in 1 month) and then progressively (3 logs in 2 years). Ten months after the start of lamivudine+adefovir bitherapy, we observed a more pronounced dominance of the triple mutant, and an increased proportion of the rtV173L+P177S+L180M+S202G+M204V mutant over the rtL180M+A181G+S202G+M204V variant.

Viral genome replication capacity of the major HBV mutants

To gain insight into the selection process of the different mutants, the genome replication capacity of the major HBV variants identified at each time point, was analyzed in a Huh-7 transient transfection cell culture assay as described in Material and methods (Figure 3).

We selected the 3 main mutants that emerged at the end of lamivudine monotherapy, and compared their replication capacity with that of a mixture of 10 clones, obtained from the pre-therapeutic serum, and considered as wt. The results were consistent with the data from literature, showing a higher replication capacity of the rtV173L+L180M+M204V triple mutant, compared to rtM204V and rtL180M+M204V variants (Figure 3A). Indeed, the addition of the rtL180M mutation to the rtM204V mutant induced a 1.8-fold increase of viral replication level, and the addition of rtV173L to the rtL180M+M204V a 1.7-fold increase.

The replication capacity of the four main mutants that emerged under entecavir therapy was also analyzed (Figure 3B). The last mutant selected (rtL180M+S202G+M204V) showed a slightly decreased replication capacity (71%) compared to wt HBV. The addition of the rtA181G change to this triple mutant

resulted in a 1.5-fold replication impairment, whereas the addition of rtI169L and rtV173L+P177S increased by 1.3- and 1.8-fold respectively the replication capacity of the same triple mutant. The rtV173L+P177S+L180M+S202G+M204V mutant even showed an increased replication efficiency (by 1.3-fold) compared to wt HBV.

Drug susceptibility of the major HBV mutants

The three main mutants previously described during lamivudine monotherapy were analyzed for their susceptibility to lamivudine and entecavir in the HepG2 cell line. Results showed that the rtL180M+M204V and rtV173L+L180M+M204V mutants were strongly resistant to lamivudine with IC₅₀s >10,000-fold than that of wt HBV, and also induced a slight decrease of susceptibility to entecavir with IC₅₀s 19-fold (rtL180M+M204V) and 30-fold (rtV173L+L180M+M204V) higher than that of wt HBV (Table 1). The rtM204V single mutant, that coexisted at low levels under lamivudine therapy and disappeared during entecavir treatment, induced only a 30- and 2.4-fold decrease in susceptibility to lamivudine and entecavir respectively (Table 1).

We also analyzed the drug susceptibility of the four main mutants that emerged during entecavir therapy. As shown in table 2, all the tested mutants were strongly resistant to lamivudine with IC₅₀s >500 μ M. The triple mutant (rtL180M+S202G+M204V), which became the dominant strain at the end of entecavir and at the beginning of lamivudine+adefovir therapies, displayed with the rtI169L+L180M+S202G+M204V the highest resistance to entecavir (~1,300- and ~1200-fold compared to wt HBV). We also analyzed a mixture of the different HBV clones coexisting just before the switch from entecavir to adefovir (serum sample #5), with the same proportion of each clone observed in serum sample #5 by genotypic analysis. We tested the phenotype of this polyclonal mixture to adefovir and tenofovir,

and showed the susceptibility of the mixture as well as the triple mutant to both drugs (Table 3).

Implication of the rtS202G mutation in entecavir-resistance

To determine if the rtS202G mutation alone could induce resistance to entecavir, we constructed HBV mutant by site-directed mutagenesis harboring only this mutation, and analyzed its phenotype in tissue culture. Results showed that the rtS202G mutant had a similar replication capacity and does not induce any change in entecavir susceptibility when compared to wt HBV (Table 4). Therefore, in the absence of rtL180M+M204V lamivudine-resistant substitutions, the rtS202G change is not sufficient to induce entecavir-resistance. However, the addition of rtS202G change to the rtL180M+M204V mutant resulted in a ~1,300-fold increase in entecavir-resistance.

Discussion

This report describes virologic breakthrough after prolonged entecavir therapy in a patient who failed lamivudine therapy. A detailed clonal HBV genome analysis was performed on serum samples harvested during sequential therapies, which allowed the evolution of the viral quasi-species under the successive antiviral pressures to be shown.

Sequence analysis of HBV clones isolated from the patient's sera revealed the coexistence of 3 main populations during lamivudine therapy, all harboring the rtM204V mutation. Twenty seven months after the start of lamivudine therapy, the rtV173L+L180M+M204V triple mutant was the dominant HBV strain (30% of HBV population). Phenotypic analysis demonstrated that this triple mutant showed the highest replicative capacity (higher than wt HBV) compared to the other lamivudine-resistant variants. These results are consistent with previously published studies [10,11,26]. Interestingly, the triple mutant also induced a slight increase in entecavir-resistance compared to the other HBV variants (30-fold versus 2.4- and 19-fold for rtM204V and rtL180M+M204V respectively). This result, in addition to a high replicative capacity, may have contributed to its selection (>80% of the viral quasi-species) when lamivudine was replaced by entecavir therapy. This suggests that entecavir exhibits a positive selective pressure on lamivudine-resistant mutants *in vivo*.

Approximatively 3 years after the beginning of entecavir therapy, we observed the coexistence of 3 or 4 mutants, all harboring the rtL180M+S202G+M204V mutations. The rtS202G change, located in the C domain of the RT, has recently been observed in a patient who developed a resistance to entecavir following lamivudine breakthrough [27], in addition to lamivudine-resistance substitutions. Like the rtS202I

mutation described by Tenney et al. [21], the rtS202G mutation has never been found alone in patients who failed entecavir therapy. This observation is consistent with our phenotypic analysis showing no decrease of entecavir susceptibility for the single rtS202G mutant, compared to wt HBV. However, the addition of rtS202G mutation to the rtL180M+M204V variant induced a ~1,300-fold decrease in susceptibility to entecavir. This result suggests that the association of the lamivudine-resistance substitutions with rtS202G mutation was essential for the development of entecavir-resistance in this patient. The rtS202G change, located near the RT catalytic site, may induce a conformational perturbation of the dNTP binding pocket, preventing the binding of entecavir dGTP-TP analog [28]. This conformational change may not restore the fixation of lamivudine-TP analog, since the rtL180M+S202G+M204V mutant remained resistant to lamivudine. These data suggest that entecavir-resistance may require a two-step process with the first selection of lamivudine-resistance mutations, followed by the addition of new mutations conferring a higher replication capacity in the presence of entecavir. It is important to note that this triple mutant remains sensitive to adefovir and tenofovir *in vitro* (Table 3). This susceptibility to adefovir was confirmed *in vivo* with the decrease of viral load following a switch to adefovir.

New mutations were also found in some HBV clones from this patient in association with rtL180M+S202G+M204V mutations. The rtA181G substitution, was present in 30 to 40% of the HBV population at the end of entecavir therapy, but the proportion of this mutation decreased when entecavir was replaced by lamivudine+adefovir. This alanine in 181 position seems to be critical in the development of resistance to nucleos(t)ide analogs, since i) it is located in α -helix adjacent to the nucleotide binding site [29], and ii) rtA181V and rtA181T were already described to confer

resistance to lamivudine and adefovir [28]. Interestingly, phenotypic assays demonstrated that the rtL180M+A181G+S202G+M204V mutant does not reduce susceptibility to adefovir (data not shown), and has a slightly lower replication capacity when compared to the triple mutant, which may explain the decrease of proportion of this mutant under lamivudine+adefovir treatment.

The rtP177S mutation was always found in association with rtV173L in addition to rtL180M+S202G+M204V changes in the patient, except for one clone (data not shown). This mutant harboring five closed mutations in RT protein represented approximately 15% of the population at the end of entecavir therapy, and its proportion slightly increased after the switch from entecavir to lamivudine+adefovir. A mutation at this position in leucine, but not serine, has already been described for resistance to famciclovir [30]. This proline at the 177 position is conserved in HIV RT (P157), and seems to directly interact with M184 in HIV RT or M204 in HBV RT [28]. This substitution, like the mutation at the 181 position, could alter the positioning of the nucleic acid template strand relative to the catalytic region. In our phenotypic analysis, we found that rtP177S, in association with rtV173L, did not increase resistance of the triple mutant to entecavir, but induced a 1.8-fold increase in replication capacity of the same mutant. Since rtV173L was already described to support replication of the rtL180M+M204V lamivudine-resistant mutant, it would be interesting to study the implication of each mutation rtV173L or rtP177S in this phenomenon.

A third new mutation, rtI169L, was also found in a few HBV clones isolated from the patient, but only at one time point (34 months after the start of entecavir therapy). The mutation of this position was already described in the two patients described by Tenney *et al.*, who developed entecavir resistance, but threonine and not leucine

substituted the isoleucine [21]. This rtI169L seems to induce a modest increase in the triple mutant replication level (1.3-fold), but did not increase entecavir-resistance level.

When we compared the phenotype of these mutants coexisting during entecavir therapy in our tissue culture assay, we observed that the rtL180M+S202G+M204V triple mutant that was dominant during entecavir therapy, conferred one of the highest resistance to entecavir *in vitro* (~1,300-fold). This mutant did not show the highest replication level, but its replication capacity was only slightly impaired compared to wt HBV (by approximately 30%). Therefore, we can hypothesize that entecavir pressure selected the viral mutant that was the best fit in the presence of entecavir.

In conclusion, we report a case of resistance to entecavir following lamivudine breakthrough. Viral breakthrough was associated with the emergence of the entecavir-resistance substitution rtS202G, in addition to lamivudine-resistant mutations. There is currently no observation of entecavir-resistance in the absence of previous lamivudine treatment failure. The longitudinal genotypic analysis of HBV clones, combined with the *in vitro* phenotypic analysis of the major HBV variants, allows insight into the understanding of the selection process of the entecavir-resistant mutants, which followed a two-step model in our patient. Our findings highlight the importance of *in vitro* cross resistance testing, since adefovir was shown to inhibit *in vitro* the replication of these entecavir-resistant and reduced the viral load after the development of entecavir-resistance.

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Figure legends

Figure 1. Evolution of the viral load during the course of antiviral therapies. The duration of therapies is indicated by bars above the graph and the serum samples from the patient by the numbers below the graph. Lamivudine, entecavir, and adefovir dipivoxil were administered to the patient. The evolution of viral load over time was quantified by Digene assay (Digene, Gaithersburg, MD) during the first 84 months and by bDNA assay (Versant HBV DNA 3.0 assay; Bayer Corporation, Tarrytown, NY) the following months. The rebound observed under lamivudine+adefovir bitherapy corresponds to the change in HBV DNA detection method.

Figure 2. Genotypic analysis of HBV mutants evolution during sequential therapies. The different bars correspond to the percentage of each HBV RT mutant in the viral population. On the left are indicated the serum sample numbers.

Figure 3. Replicative capacity in Huh-7 cells of wt and HBV RT mutants isolated from the patient during lamivudine (A) and entecavir therapy (B). Viral DNA synthesis was analyzed as described in Material and Methods. After phosphorimager analysis, values obtained for each mutant were divided by wt HBV value that was assumed to represent 100% of viral replication. Values are reported as the mean and standard deviation from six independent experiments.