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Acknowledgements: This work was part of the activities of the ViRgil network of excellence (ViRgil LSHM-CT-2004-503359).

Antiviral therapy of chronic hepatitis B remains a major clinical challenge (1). On the one hand, the development of new antivirals has been shown to be efficacious in controlling viral replication, decreasing the inflammatory activity within the liver and preventing the progression of chronic hepatitis towards its main complications including decompensation of liver cirrhosis and development of hepatocellular carcinoma (2). On the other hand, due to the unique mechanism of viral replication and persistence of HBV in infected individuals (3), long-term antiviral therapy is required in most individuals and thus places the patients at risk of selecting drug resistant mutants and of developing progressive liver disease (4). Therefore, new anti-HBV agents are needed in the armory for combating chronic hepatitis B and to design the best management strategies.

The recent approval of entecavir in the USA and in Europe is providing, as a newcomer, new hope for the treatment of HBV chronically infected patients. In clinical trials, entecavir administration for one year has shown a clear antiviral potency with more marked viral load suppression and a significant benefit in liver histology improvement as compared to lamivudine therapy. Its clinical efficacy was demonstrated in large numbers of patients enrolled in different phase III studies covering the most relevant clinical situations, including HBeAg –positive and –negative patients and lamivudine refractory patients (5-7). These results allowed the approval of entecavir for the treatment of chronic hepatitis B by the FDA and EMEA. However, clinically relevant recommendations can only come from long-term evaluation. Thus, results from studies beyond one year of therapy are urgently needed. Indeed, one of the major problems faced by anti-HBV therapy is the slow kinetics of cccDNA clearance from the infected liver using nucleoside analogs either as monotherapy (3) or in combination with pegylated interferon-alpha (8). One study performed in chronically infected woodchucks showed the effect of long-term entecavir treatment on cccDNA clearance without selection of drug resistant mutants (9).

As a result of viral persistence, the subsequent selection of drug resistant mutants from the viral quasi-species was considered inevitable with nucleoside analog monotherapy, as clearly shown by lamivudine and adefovir studies. The first cases of entecavir resistance were observed in patients receiving entecavir for lamivudine failure (10). In this population of patients, genotypic resistance to entecavir was observed in 10 out of 141 patients and virologic breakthrough in two patients after one year of therapy (5). The resistance mutations were characterized genetically and phenotypically, and were shown to occur on a lamivudine-resistance mutation background. From these studies, a two hit model was suggested. The initial requirement for lamivudine resistance mutations was suggested by studies showing that these mutants have an approximately 10 fold reduced sensitivity to entecavir *in vitro* (10, 11). Afterwards, one or two additional mutations are required on the same mutant genome to confer full resistance to entecavir (10). A few studies have shown that entecavir exhibits a selective pressure on lamivudine resistant mutants in lamivudine resistant patients and that mutants harboring additional polymerase gene mutations have a better replication capacity in the

presence of entecavir leading to their selection and to virologic rebound (12)**.** These mutants are then resistant to both lamivudine and entecavir (12, 13). Although *in vitro* data showed that these lamivudine and entecavir resistant strains are sensitive to adefovir and tenofovir, selection of these mutants raised the concern of entecavir resistance in nucleoside naive patients (12)**.**

In this issue of Hepatology, Colonno et al report the results of an extensive genotypic and phenotypic analysis of HBV isolates in 679 nucleoside naive patients who were enrolled in several phase III trials and received entecavir for up to two years *(Reference from this issue of Hepatology)*. Entecavir reduced HBV DNA to undetectable levels by quantitative PCR in 91% of HBeAg-positive and -negative patients by Week 96. In lamivudine treated patients, 13% patients experienced a virologic rebound (≥1 log increase from nadir by PCR) in the first year, with 74% of these having evidence of lamivudine-resistance mutations. In contrast, only 3% (n=22) of entecavir-treated patients exhibited virologic rebound by Week 96. Three entecavir rebounds were attributable to lamivudine resistant virus present at baseline, one of which also had a S202G entecavir resistance substitution emerging at Week 48. None of the other isolates from rebounding patients had emerging genotypic resistance or loss of entecavir susceptibility. The study was very comprehensive, as the authors performed a genotypic analysis of all additional entecavir patients with PCR-detectable HBV DNA at Weeks 48, 96 or end of dosing. This allowed the identification of 7 additional patients with lamivudine resistant substitutions, including one with emerging lamivudine + entecavir resistance mutations. Generally, in entecavir treated patients who developed lamivudine resistance mutants, these strains were detectable at baseline (8/10) and most patients subsequently achieved undetectable HBV DNA levels on entecavir therapy (7/10). Other substitutions in the viral polymerase were identified in the HBV genomes of entecavir-treated patients, but none were associated with decreased ETV susceptibility by *in vitro* phenotypic analysis.

The results of this important study raise several questions regarding the mechanism of selection of entecavir resistant strains and the management of chronic hepatitis B patients receiving antiviral therapy. It is interesting to note that several cases of virologic rebound were observed with only lamivudine resistance mutations, and fewer cases had both lamivudine and entecavir resistance substitutions. This may suggest that the decreased susceptibility of lamivudine resistant strains may not only be responsible for the selection of these strains during entecavir monotherapy, but in some cases, for virologic rebound. The authors also suggest that in nucleoside naïve patients who do not harbor lamivudine resistant strains at the initiation of treatment, no genotypic or phenotypic evidence of emerging entecavir resistance occurred while experiencing a virologic rebound on entecavir therapy. However, this can be challenged by the fact that drug resistant mutants pre-exist in the viral quasi-species in different amounts and the ability to detect them is dependent on the sensitivity of the method. The authors acknowledged that two patients may have had pre-existing lamivudine resistant mutants at undetectable levels

that were subsequently enriched during prolonged entecavir therapy. One of those patients developed a viral rebound without entecavir resistance mutations, and the other developed entecavir resistance mutations but did not present a virologic breakthrough.

It is important to recall that due to the high error rate in HBV replication, all possible mutations can be generated spontaneously, and pre-existing lamivudine resistant mutants can be present at low frequencies in the viral quasi-species of nucleoside-naïve patients, as already shown in previous studies with other nucleoside analogs (14). If the lamivudine resistant mutants are selected during entecavir treatment because their susceptibility is lower than wild type HBV, resistance to entecavir may occur if additional entecavir resistant mutations are selected during continued treatment. The frequency and rapidity of development of entecavir resistance in nucleoside naïve patients may depend on the actual proportion of primary resistance mutations (i.e. the lamivudine resistance strains) in the viral quasi-species when treatment starts. This highlights the importance of the sensitivity of the method when attempting to detect primary mutations. However, because of the potency of entecavir, HBV replication is rapidly suppressed and so the opportunity for the pre-existing lamivudine resistant mutants to be enriched and for additional entecavir resistant mutations to be selected is small in nucleoside naïve patients, provided that no specific enrichment of mutants occurs beyond two years of therapy. The scenario may not be the same in patients receiving entecavir for lamivudine failure, because the viral quasi-species is already enriched in primary resistance mutations (i.e. the lamivudine resistant strains). The results of previous clinical trials in this specific population have already shown a higher incidence of entecavir resistance after one year of therapy (5).

Several studies have characterized the dynamics of HBV quasi-species during lamivudine therapy (15-17). To better understand the pathway towards entecavir resistance, a longitudinal analysis of viral quasi-species is mandatory together with an *in vitro* phenotypic analysis of the identified HBV mutants. This would tell us if spontaneous generation of lamivudine resistance mutants and/or their selection from the pre-treatment quasi-species by entecavir could provide a pathway towards entecavir resistance, and if lamivudine resistant strains can also be considered as true entecavir resistance mutants. The longitudinal genetic studies performed in lamivudine resistant patients who subsequently failed entecavir therapy may favor a two hit model where the lamivudine resistance mutations are selected first (primary resistance mutations) and the entecavir resistance mutations are acquired in a second step to restore the fitness of the virus in the presence of entecavir (secondary resistance mutations) (10, 12)**.** As this issue has clear clinical implications for the decision of treatment strategies, detailed HBV mutant fitness studies are required. By contrast to HIV, these studies have been hampered by the lack of easy to use cell-culture systems and animal models to investigate the fitness of these mutants, including their infectivity and their capacity to archive the mutations in cccDNA. Several *in vitro* and *in vivo* models are in development and may help in the understanding of this

process of selection of drug resistant mutants (18, 19). Furthermore, based on results obtained in the woodchuck model regarding the kinetics of viral clearance and drug-resistant mutant selection, mathematical modeling allowed drafting of the hypothesis that treatment success is dependent on two main determinants: 1) the rate of hepatocyte lysis and cell turn-over involved in viral clearance; 2) the fitness of the drug resistant mutants, i.e. their capacity to spread in the liver in the presence of the antiviral drug, including their capacity to outgrow wild type virus and emerge during treatment (20)**.** These findings may have major implications regarding entecavir therapy, as this is a potent antiviral drug that exhibits a profound antiviral effect even on the lamivudine resistant strains that may represent the first step in the resistance process. In addition, the lamivudine + entecavir resistant strains may have an altered fitness which may hamper their spread in the liver, and therefore allow viral clearance despite their initial selection, depending on the rate of liver regeneration.

This interesting publication raises many questions on the management of patients receiving entecavir therapy. Long-term clinical and virological studies are needed outside the setting of clinical trials to determine the incidence and clinical impact of entecavir resistance in both nucleoside naïve and lamivudine resistant patients. Based on the available results, recommendations should be given to monitor viral load carefully in patients undergoing entecavir therapy, even in those who are nucleoside naïve. In cases of viral breakthrough, characterization of viral strains and early adaptation of antiviral therapy should be recommended. An increase in viral load associated with the detection of “primary lamivudine resistance” mutation during entecavir therapy should lead to a change in antiviral therapy to avoid the risk of selecting the additional “secondary” mutations that would confer multi-drug resistance to both lamivudine and entecavir. While entecavir is potent and has a low resistance rate, it is not yet known whether it will be the ideal first line therapy as a single agent (21, 22). Long-term follow-up of entecavir treated patients with both genetic and phenotypic analysis of viral strains is required to determine the optimal use of entecavir. This will also avoid, in the long-run, the selection of multiple drug resistant strains in the hepatitis B treated population, as already characterized in some patients who have been carefully followed (13, 23). Clearly, the major question regarding antiviral therapy of chronic hepatitis B in the future remains the evaluation of a *de novo* combination therapy to prevent drug resistance (24).

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