Fitness and infectivity of drug-resistant and cross-resistant hepatitis B virus mutants: why and how is it studied?

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Summary

The emergence of HBV drug-resistant (and multidrug-resistant) strains during long-term therapy with nucleos(t)ides analogs is associated with treatment failure and represents therefore a clinical challenge. For clinicians, the close monitoring and management of resistance has become a key issue in clinical practice. For HBV virologists, the understanding of the mechanism of emergence of specific mutant strains in the viral quasispecies during treatment is also an important issue. If a particular viral strain can emerge in the quasispecies within a particular environment, it is likely because its fitness is superior to other strains. The present review focuses on viral fitness as well as viral infectivity, and in particular on technical means that are available to study this viral fitness *in vitro* and in animal models.

Chronic hepatitis B: clinical issues and current treatment

Infection by hepatitis B virus (HBV) can be resolved after an acute episode, or leads to persistence and chronic hepatitis B (CHB). Chronic hepatitis B virus infection is a serious clinical problem and a major cause of severe liver-related morbidity and premature mortality. Indeed, patients with CHB have an increased risk of developing decompensated liver disease, cirrhosis and hepatocellular carcinoma (HCC) [1]. The primary treatment goal is the suppression of HBV replication, ideally followed by a seroconversion (anti-HBe, and then anti-HBs), and the prevention of active disease in the long-term [2, 3]. In this respect, the treatment of CHB with either interferons or nucleos(t)ides analogs (NAs), including lamivudine (LMV), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (LdT) and tenofovir (TDF), has resulted in a significant reduction in patient morbidity and mortality. Yet the efficacy of treatments for CHB can be affected by a number of factors, including the development of adverse side effects, poor patient compliance, previous treatment with suboptimal regimes and/or inadequate drug exposure, individual genetic variation, or infection with drug-resistant virus. As therapy with interferons ("naked" or pegylated) alone remains quite inefficient, the clinical used of nucleos(t)ide analogs has played a major part in the substantial advances in CHB treatment that have occurred over the past decade. CHB requires long-term therapy, and resistance to therapy is a frequent consequence of treatment duration. The emergence of drug resistance during long-term therapy with NAs is almost inevitable, due to the high adaptability of viruses and the quasispecies nature of HBV, and represents a clinical challenge [2, 3]. For clinicians, the close monitoring and management of resistance has become a key issue in clinical practice. For HBV virologists, the understanding of the mechanism of emergence of specific mutant strains in the viral quasispecies during treatment is also an important issue. If a particular viral strain can emerge in the quasispecies within a particular environment, it is likely because its fitness has become superior to other strains. The present review will focus on viral fitness as well as infectivity, and in particular on technical means that are available to study this viral fitness *in vitro* or in animal models.

Treatment failure and HBV resistance

HBV is a DNA virus that replicates its genome via an RNA intermediate, the pregenomic RNA (pgRNA), that comes from the transcription of cccDNA, i.e. covalently-closed-circular-DNA, the nuclear form of HBV genome and main template for viral transcription. The pgRNA is reverse-transcribed by covalently-linked-HBV-polymerase after incorporation in the nucleocapsid [4]. This step of the viral life cycle is currently the target of NA-based therapy. Long-term therapies with NAs, which are theoretically necessary to get a chance to clear cccDNA from cells, are confronted with the emergence of drugs resistant strains in the viral quasispecies. HBV mutants are spontaneously produced by the low fidelity HBV polymerase, and a drug pressure may select for viral species that exhibit the best replication capacity in this new treatment environment. Mutations conferring resistance to NAs are located in the viral polymerase gene. The rapidity of selection of drug resistant mutants depends on their replication capacity and fitness, their level of resistance, and free liver space available for infection by these mutants [5, 6]. This may explain, at least in part, the

differences in the rate of resistance for the different drugs that are clinically available.

Different mechanisms are involved in drug-resistance under antiviral therapy [3]. First, a complex mixture of genetically distinct variants develops under selective pressure. A pre-existing or newly acquired mutation conferring a selective advantage to a variant will give rise to virions, which are fitter and can spread more rapidly in the liver. This mutant may accumulate and become the dominant (or at least a well represented) species in the infected liver, under the pressure of the antiviral drug. The kinetics of replacement of wild type virus in liver cells by a dominant mutant are generally slow. As resistant mutants mainly infect uninfected cells, the efficient spreading of the dominant mutant depends on its intrinsic fitness and the availability of free liver space for its propagation and replication [5, 6]. During antiviral therapy, several months may be needed for the immune system to clear hepatocytes infected with wild type virus and to generate new cells that are susceptible to infection by viral drug-resistant mutants. On the other hand, the specific infectivity of drug resistant mutants may have a major impact on the rapidity of selection of these strains during therapy. Indeed, some mutations in the viral polymerase gene may result in nucleotide changes in overlapping surface genes, which in turn may lead to reduced viral fitness, due to impaired assembly, secretion or infectivity [7, 8]. The level of resistance to a drug conferred by a given mutation may have profound implication on the fitness of the mutant. This may explain the difference in drug resistance rates observed with the different antivirals.

Definition of viral fitness and rationale for studying it

In a simple way, viral fitness can be defined as the sum of parameters that quantify the adaptation of a viral strain to a given environment. *In vitro*, it can be seen as the growth properties of a given strain as compared to a reference or wild type viral strain in a defined environment (i.e., in tissue culture or *in vivo* in the presence of drugs or an intact immune system). The fitness of a given viral strain is of great importance to understand its emergence or elimination in a particular microenvironment. Of course in the case of *in vivo* infection other features including the genetic makeup of host and immune system are also important to explain the emergence of a particular strain, but this will not be further developed here as this review focuses on virus related parameters. The therapeutic pressure represents also an important environmental parameter to explain the emergence of mutant strains in patients. In general, NA-resistant strains do not replicate as well as wild type counterparts, because mutations in the polymerase gene tend to affect the enzymatic activity of the protein. But this pattern of replication can be reversed in the presence of the drug(s) that selected the mutant in the first place [7, 9, 10].

The virologic parameters of HBV fitness are the capacity of a strain to synthesize its genome, to produce infectious particles and (re)-infect cells (i.e. enter and deliver the genome to nucleus). The synthesis of the genome itself includes the ability of the strain to produce pgRNA (i.e. transcription via precore promoter, some mutations may alter the level of transcription mediated by host RNA polymerase II) that is subsequently packaged into the nucleocapsid before being used for reverse transcription. The study of viral fitness is a complex matter as it relies on the models used to perform analysis. Cell culture systems or animal models capable of full replication and propagation

of the virus are necessary to properly measure the fitness of a viral strain. For HBV such models do no exist. Therefore to get insight into HBV fitness, several more or less artificial assays that will be hereafter named replication, assembly and infectivity assays have been developed.

Studying viral fitness *in vitro*: critical review of assays

Replication assays. Several *in vitro* assays have been developed to determine the replication capacity of clinically relevant HBV strains, including wild-type and drug-resistant strains. These assays are also critical to determine the role of a given mutation profile in drug resistance, as well as to determine the cross-resistance profile of those mutants. Several approaches and assays, also termed phenotypic assays as they describe the phenotype (i.e. replication capacity + drug resistance profile) of a given strain *in vitro*, have been described in literature. These assays are mainly based on the transfection of full-length HBV PCR-amplicons (i.e. vector-free) or plasmids containing 1.1 to 2 HBV genome units into hepatoma cell lines (e.g. Huh7 or HepG2).

The vector-free method relies on an original and efficient PCR amplification of full-length HBV genomes isolated from patients [11]. The linear amplicon is transfected into cells and serves as a ccc-like DNA template for the initiation of the intracellular HBV replication after re-circularization by host machinery. This step of circularization is rate limiting and explains the rather low absolute level of replication obtained with this approach; however the relative replication capacity of two different strains can be analyzed by this approach. Another advantage of this approach comes from the fact that the expression of pgRNA is driven by the HBV promoter itself, which is interesting if precore or core promoter mutants are analyzed.

Alternative approaches are based on vectors, that can be plasmid or alternatively recombinant adenovirus or baculovirus, carrying 1.1 to 3 HBV genome units [12-15]. These vectors contain the HBV genetic information necessary and sufficient to initiate an HBV replication cycle after transfection or transduction into cells. The synthesis of HBV pgRNA can be driven either by the HBV promoter (i.e. 1.3 to 2 genome unit) or a strong mammalian promoter (i.e. 1.1 genome unit). Until recently, the analysis of the replication capacity of naturally occurring or drug induced HBV mutants relied either on PCR-mediated transfer of HBV genome cassettes or on site directed mutagenesis of a well established replication-competent laboratory strain [16, 17]. Despite its obvious utility to quickly characterize new mutations *in vitro*, the methods based on the exchange of a cassette or site directed mutagenesis do not take into account the HBV genome variability existing in other parts of the genome.

Several methods based on a standardized cloning strategy of the entire HBV genome isolated from patient into plasmid vector have been recently described [10, 18] and have proven useful to study replication capacity and drug susceptibility, as well as cross-susceptibility of clinically relevant HBV strains [10, 18-23]. These cloning techniques enable the assembly of molecular clones, containing either 1.1 or 1.3 HBV genome unit, which allows the study of viral replication upon transfection of one clone or a mixture of clones into eukaryotic cells. By using a vector in which the expression of HBV pgRNA is driven by heterologous mammalian promoters (e.g. CMV or actin promoters), the replication level detected post-transfection, as measured by the neo-synthesis of

encapsidated relaxed-circular DNA (rcDNA), is artificially elevated. However the replication capacity of two given HBV strains can be determined in a relative fashion as it measures the intrinsic ability of HBV polymerase to transform pgRNA into rcDNA [10, 18].

Vector-free and vector-based replication assays are complementary approaches to determine the phenotype (replication capacity + drug susceptibility) of clinical HBV strains. The vector-free method is interesting as the whole viral population is represented in transfected amplicons, if we hypothesize that there is no bias during amplification. Vector based assays present the advantage of strong relative level of DNA synthesis, which render the approach more universal, but the disadvantage of a cloning step. Altogether, it may be interesting to use both approaches in order to generate complementary information. According to the approach used several methods can be utilized to detect HBV replication, including Southern blotting (if the replication level is high, i.e. when vector based assays are used) or qPCR. The first method is work intensive but gives qualitatively relevant results (i.e. visualization of all DNA forms of HBV genome), whereas the second is very sensitive but might be confronted to false positive (i.e. detection of infra-length DNA sequences). Moreover HBV replication may be monitored by quantifying intracellular or secreted DNA. If qPCR can be used in both cases, Southern blotting is more adapted for the detection of intracellular DNA due to the rather low sensibility of the methodology.

Beside the molecular approach used, the choice of the cell line for the transfection is also important as differences in term of replication capacity and drug susceptibility have been observed [9, 24]. Altogether an important effort to standardize assays intra and inter laboratory remains to be made in order to move from current data that are mainly valid in a relative context (i.e. comparison with the same approach in the same laboratory) to fully comparable data.

It is worth noting that another approach to characterize viral drug resistance in tissue culture is the use of cell lines permanently expressing the mutants, to allow a more reproducible measure of drug susceptibility [25-28]. These cell lines are extremely useful to assess the antiviral activity of approved drugs and those in development on the main resistant mutants. However, one of the problems of these permanent cell lines, is the need to design and produce new cell lines when new resistant mutants are identified.

Virion production, secretion capacity and infectivity - Assembly and infectivity assays. Beside the ability of a strain to replicate its genome in the absence or presence of drugs, the next important step to ensure a strain a better propagation is the ability to produce particles. Some mutations occurring in the polymerase gene have a consequence in overlapping envelope genes, in particular in the S gene. For instance, the rtM204V mutation is associated with the mutation sI195M in the surface antigen, whereas the rtM204I change is associated with three possible changes: sW196S, sW196L, or a termination codon. The mutation selected by adefovir, lamivudine or telbivudine at rtA181T typically results in a stop mutation in the envelope gene (sW172stop), and the ADV-resistance mutation at rtA181V results in a concomitant change sL173F. Mutations that result in a stop codon mutation in the envelope gene are present in association with a low percentage of wild type HBV in order to ensure rescue of viral packaging and release [8].

Beside their impact on viral assembly, mutations may also impact the antigenicity and therefore the

ability of a strain to be neutralised by circulating or administrated antibodies [29, 30]. This may also have an impact on viral propagation and therefore represent a component of viral fitness. Several studies have examined the altered antigenicity of common antiviral drug selected mutations that also alter HBsAg. In the study by Torresi *et al.*, the mutations rtV173L+rtL180M+rtM204V that resulted in the mutations sE164D+ sI195M in HBsAg were found to reduce antigen - antibody binding [31]. Although the reduction was not as great as for the classical vaccine-escape mutant at sG145R, it was greatly reduced compared with the wild-type virus. These results were recently confirmed and extended by Sloan and colleagues using cell derived HBVs [30].

To study the impact of a given mutation on HBV assembly there are two main methodologies. The first is based on hepatomas cells replicating HBV after stable or transient transfection. Stably transformed cell lines are useful to study the assembly and secretion of HBV strains, but are work-intensive to generate. Moreover, it can be quite difficult to compare two different cell lines as the sites and numbers of integration into the genome may vary from one cell line to another. By contrast, the transient transfection of cells with plasmids carrying 1.1 to 2 HBV genome units is more flexible, although, due to transfection efficiency, a lower number of cells produce HBV particles compared to stably transformed cell lines. The assembly and secretion of HBV particles is measured by quantification of secreted HBV DNA by qPCR (or Southern blot) and/or envelope proteins by Elisa or Western blot. Those measurements are not precise, as they do not distinguish between enveloped and non-envelopped nucleocapsids for qPCR analysis, and between subviral particles and Dane particles for Elisa or Western blot analysis. The amount of Dane particle produced can be more precisely quantified after immunoprecipitation with an anti-PreS1 antibody followed by qPCR [7]. A more laborious approach is to purify particles by ultracentrifugation in density gradients, and to analyse the production of each particles (Dane, subviral particles and non-enveloped nucleocapsids). Electron microscopy can be used to some extend to visualise and distinguish HBV particles, but is not a quantitative approach.

Other approaches to study the impact of mutation in envelope genes on viral assembly are based on the utilisation of naturally pseudotyped hepatitis delta virus (HDV) particles. Indeed, HDV utilizes the envelope proteins of HBV for propagation. When introduced into permissive cells, the HDV RNA genome replicates and associates with multiple copies of the HDV-encoded proteins to assemble a ribonucleoprotein (RNP) complex. The mechanism necessary to export the RNP from the cell is provided by the HBV envelope proteins, which have the capacity to assemble lipoprotein vesicles that bud into the lumen of a pre-Golgi compartment before being secreted [32]. Hence the co-tranfection of HDV genome (contained as trimers in a plasmid) and plasmid either expressing HBV pgRNA (i.e. like the ones used for replication assays; see above) or coding HBV envelope proteins leads to the production of either a mixture of HBV/HDV or pure HDV particles. These models have been successfully used to determine domains and residues of S protein important for HBV/HDV assembly [33, 34]. They can be used as surrogate model to study the impact of naturally or drug-selected mutations on HBV assembly. In this case assembly and secretion of HBV/HDV or HDV particles is measured by quantification of secreted HDV RNA by qRT-PCR (or Northern blot) and/or envelope proteins by Elisa or Western blot.

The infectivity of HBV particles represents another important component of viral fitness to be analyzed. To perform infectivity assays, HBV particles have to be produced. All the approaches to produced *in vitro* HBV particles have been already described above and include transiently-transfected or stably-transformed cells [7], as well as HDV based models [35, 36]. To perform comparative infectivity analysis, the critical issue is to standardize the inoculum to be used. Standardization based only on the dosing of HBV (or HDV) genome is not ideal as non-enveloped nucleocapsid can also be produced together with infectious Dane (or HDV) particles. Therefore a double standardization based on genome and protein dosing is necessary. An interesting method is to dose immunoprecipitated (with anti preS1 antibody) HBV (or HDV) genome. Infectivity assays are performed with cells that can be infected *in vitro*. Currently there are three main models available: primary human hepatocytes (PHH) [37], primary tupaia hepatocytes [38], or HepaRG cells [39], the last being the easiest to use because it is an established cell line. After inoculation with HBV or HDV particles, the intensity of infection is measured by either Northern blot or qRT-PCR to detect HBV or HDV RNAs. There are differences between HBV and HDV based models. With HDV, there is a disconnection between entry and replication, as the latter occurs irrespective of the HBV mutations that are assayed for their impact on entry. With HBV, the measurement of infectivity includes the ability to enter the cells and to initiate HBV replication, and both aspects are linked. One main problem with these infectivity models is that there is no propagation of infection in PHH and HepaRG cells. Thus no competition experiments have been reported with this experimental setting, although it is one of the main objectives when it comes to compare viral fitness of two strains.

Studying viral fitness in vivo: from human to animals models. Longitudinal studies in patients with CHB treated with antivirals are per se very interesting as they provide useful information on viral fitness. Hence, the emergence of a particular HBV mutant strain in patients treated with NAs indicates that this strain is fitter than others in this particular microenvironment. Villet *et al.* managed to obtain *in vitro* data on the viral fitness of a multi-resistant strain that emerged after treatment with lamivudine, adefovir, and anti-HBV immunoglobulins [7]. The *in vitro* data confirmed that the finally selected strain was the fittest one amongst the four strains that were yet present after few weeks of treatment in terms of replication capacity, assembly efficiency and specific infectivity. This was the first demonstration of a good correlation between clinical and laboratory findings. The main problem with longitudinal studies on patient cases is that they are retrospective. It would be interesting to have a small animal model to perform prospective studies.

One particularly important point when the viral fitness of a given drug resistant strain is concerned is to determine whether this strain can be transmitted. The first report of transmission of LMV resistant HBV was from Thibault *et al.* [40]. In this case report, the transmission of LMV-resistant HBV (rtL180M+rtM204V) was associated with an incubation period of 2-3 months and resulted in a typical acute hepatitis. Obtaining evidence for the transmission of HBV mutants in humans remains difficult and opportunistic. Animal models are necessary to get further information on the infectivity of HBV mutant strains. Using the chimpanzee infection model, Kamili *et al.* [41] tested the efficacy of the immunity induced by a commercial hepatitis B vaccine against challenge with a

tissue culture-derived, clonal HBV polymerase mutant that contained a combination of three polymerase mutations (rtV173L, rtL180M, rtM204V), two of which resulted in changes to the overlapping viral envelope of the HBsAg (sE164D, sI195M). Evidence of HBV replication was observed in the vaccinated chimpanzees after challenge with the mutant, as well as after rechallenge with serum-derived wild-type HBV, despite robust humoral and cellular anti-HBV immune responses after hepatitis B vaccination.

Although useful, the chimpanzee model remains difficult to handle. Amongst other animals that could be used for studying the infectivity of HBV mutant strains, liver-humanized mouse represents one of the most convenient models [42-44] SCID/Alb-uPA mice can be efficiently infected with *in vitro* produced HBV particles [45], and can be therefore used to study the infectivity of HBV mutants. Moreover HBV infected SCID/Alb-uPA mice can be treated with LMV and ADV, whereas infection with LMV-resistant strain was not sensitive to LMV treatment [46, 47]. Altogether this model could be potentially used to study viral fitness of HBV strains in mono or competitive challenges in the presence or absence of drugs. However, it is worth noting that quantitative and qualitative species-dependent differences in the enzymology of nucleos(t)ide metabolism in mouse tissues might limit the usefulness of this model since the hepatocyte's environment is influenced by other cells and tissues, that are of mouse origin in this model.

Concluding remarks

The study of HBV viral fitness *in vitro* is limited as there is currently no really relevant and unique cell culture model to perform such studies. In the absence of such a model, several assays have been developed to get insight into various parameters that define viral fitness, including the replication capacity, assembly efficiency, and specific infectivity of a given HBV strain. These assays have proven useful to get correlation between *in vitro* and *in vivo* data, and explain the emergence of a given strain *in vivo* by a better viral fitness of this one, as compared to other wild-type and mutants strains. Besides *in vitro* testing, animal models could be used to get further information about the fitness and the infectivity of HBV mutant strains. So far, using chimpanzees, it has been possible to demonstrate the transmissibility of HBV drug resistant strains, and using HBV vaccinated chimpanzees it has been possible to demonstrate the transmissibility of HBV vaccine escape mutants therefore pointing out the potential risk of spreading of such strains. Mice with humanized liver may also represent an interesting model to get prospective data on the fitness of clinically relevant and laboratory engineered HBV strains.

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