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Virologic Monitoring of Hepatitis B Virus Therapy in Clinical Trials and Practice: Recommendations for a Standardized Approach

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Abstract

Treatment of chronic hepatitis B virus (HBV) infection is aimed at suppressing viral replication to the lowest possible level, and thereby to halt the progression of liver disease and prevent the onset of complications. Two categories of drugs are used in HBV therapy: the interferons, including standard interferon alfa or pegylated interferon alfa, and specific nucleoside or nucleotide HBV inhibitors that target the reverse-transcriptase function of HBV-DNA polymerase. The reported results of clinical trials have used varying definitions of efficacy, failure, and resistance based on different measures of virologic responses. This article discusses HBV virologic markers and tests, and their optimal use both for planning and reporting clinical trials and in clinical practice.

Hepatitis B virus (HBV) infection is a major public health problem, with approximately 350 million individuals chronically infected worldwide.[1] Individuals with chronic hepatitis B are exposed to a risk of complications such as cirrhosis, hepatic decompensation, and hepatocellular carcinoma.[2] Treatment of chronic hepatitis B is aimed at suppressing viral replication to the lowest possible level, and thereby to halt the progression of liver disease and prevent the onset of complications. However, HBV infection cannot be eradicated fully because of the persistence of covalently closed circular DNA (cccDNA) in the nuclei of infected hepatocytes. Two categories of drugs are used in HBV therapy: (1) standard or pegylated interferon (IFN) alfa, and (2) specific nucleoside or nucleotide HBV inhibitors that target the reverse-transcriptase function of HBV-DNA polymerase. Four HBV inhibitors currently are approved for HBV therapy in the United States, Europe, and most Asian and Latin American countries: lamivudine, adefovir dipivoxil, entecavir, and telbivudine. Tenofovir disoproxil

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fumarate and the combination of tenofovir and emtricitabine have potent activity against HBV, but to date are approved only for use in the treatment of human immunodeficiency virus infection. Clinical trials are ongoing to assess their utility and the utility of other new anti-HBV drugs for the treatment of HBV infection.

The goal of treatment with specific HBV inhibitors is to produce an antiviral effect that is as profound and as sustained as possible to efficiently prevent the complications of chronic HBV infection in the long term. Chronic administration of HBV inhibitors frequently leads to viral resistance, particularly with incomplete suppression of HBV replication. The selection of HBV variants with amino acid substitutions in the reverse-transcriptase domain of HBV-DNA polymerase confers reduced susceptibility to the inhibitory action of the drug.[3,4] Resistance is a major issue in clinical practice because it leads to HBV treatment failures and progression of liver disease.[5]

Context and Objectives

Clinical trials of therapies of HBV infection have led to current drug approvals, including IFNs and nucleos(t)ide analogues. The reported results have used varying definitions of efficacy and failure based on different measures of virologic responses. In patients with hepatitis B e antigen (HBeAg)-positive chronic hepatitis B, trials generally report rates of HBeAg loss and HBeAg seroconversion, alanine aminotransferase (ALT) normalization, and suppression of serum HBV DNA. Trials of conventional IFN alfa[6,7] and the early lamivudine studies[8–10] reported suppression of serum HBV DNA as measured by hybridization-based methods, which had detection limits of around 10^5 copies/mL. With the advent of more sensitive assays for quantification of serum HBV-DNA level, recent trials with both pegylated IFNs and nucleos(t)ide analogues have used a variety of definitions of serum HBV-DNA response with levels of suppression ranging from less than 500,000 copies/mL to less than 300 copies/mL.[11–19] In patients with HBeAg-negative chronic hepatitis B, HBeAg seroconversion is not the end point: a combined end point of biochemical response (ALT normalization) and virologic (serum HBV-DNA suppression) response is used frequently. However, inconsistent levels of the target serum HBV-DNA level have been chosen. In addition, different results are reported in different units depending on the assay used. In clinical practice, different assays may be used, even for sequential assays for the same patient, making interpretation of results and identification of the emergence of resistance difficult.

Definitions and hence the reporting of “resistance” across clinical trials also vary. In some cases, the incidence of genotypic mutations (ie, nucleotide alterations that result in amino acid substitutions that are selected by antiviral drugs) may be reported with no reference to whether the mutations correlate with any virologic rebound (increase of levels of serum virus in a responder patient) or effect on clinical or biochemical parameters. Conversely, virologic and biochemical breakthrough may be reported with no description of associated viral mutation and pharmacologic data.

As more antiviral therapies become available for the treatment of chronic hepatitis B, the risk of emergence of resistance and cross-resistance will increase, and as more options for managing patients with antiviral drug resistance are developed, it will become important to define, understand, and be able to use and interpret the results of HBV virologic tools in the management of HBV therapy. The aim of this article is to discuss the virologic markers and tests and their optimal use both when planning and reporting clinical trials and in clinical practice. The authors met for 2 days. Four questions were discussed: what HBV markers should be used and what is their utility in clinical trials and practice? What are the definitions of treatment responses and failures and how should they be assessed virologically? How should HBV treatment be managed with virologic tools in clinical trials? How should HBV treatment

be managed with virologic tools in clinical practice? A consensus was reached on each point after extensive discussion. A draft summary of the group's conclusions was circulated and finalized with every author's comments and suggestions. In this article, evidence-based (EB) recommendations are identified and recommendations based on the experts' opinions (EO) are presented. The authors acknowledge that virologic testing is expensive and not readily available or affordable in many countries where hepatitis B is prevalent. Therefore, the recommendations for clinical practice should be considered best practice.

HBV Markers and Their Utility in HBV Clinical Trials and Practice

The accepted virologic and biochemical markers (HBeAg, anti-HBe antibodies, serum HBV DNA, and serum ALT) used in the diagnosis and monitoring of HBV disease clearly are useful for the evaluation of patients both in clinical trials and in clinical practice (EB). Other potentially useful markers include liver cccDNA and quantitative hepatitis B surface antigen (HBsAg), HBV genotype, and genotypic resistance markers (Table 1).

HBeAg/Anti-HBe Antibodies, Serum HBV DNA, and ALT

Among HBeAg/anti-HBe antibodies, serum HBV DNA, and ALT, the best viral marker for the management of HBV disease (including both HBeAg-positive and HBeAg-negative) is serum HBV DNA. Serum HBV-DNA level is an indicator of disease prognosis. Several studies have shown that increasing HBV viral level, starting at 10^4 copies/mL, is a predictor of risk for the development of cirrhosis and hepatocellular carcinoma, regardless of HBV genotype, HBeAg serostatus, and baseline serum ALT level.[20–22] It is unclear, however, if these data from a large Taiwanese cohort study of mostly HBeAg-negative patients can be generalized to other populations of patients chronically infected with HBV, such as those with adult-acquired infection, or to the individual patient. In addition, serum HBV DNA cannot be used for prognostication in individual patients and the clinical context is important for the decision regarding when to start treatment.

Importantly, the control of HBV replication and treatment outcome are correlated. Reduction of serum HBV-DNA level is associated with an increased rate of HBeAg seroconversion in HBeAg-positive patients.[5,6,9,14–19,23–25] However, with nucleoside or nucleotide analogues, more potent HBV-DNA suppression is associated with only a small increase in HBeAg seroconversion. Reduction of HBV-DNA levels also is associated with higher rates of histologic response and lower rates of complications of liver disease.[5,6,9,14–19,23–25] For instance, continuous reductions in the levels of HBV DNA with lamivudine delays clinical progression in patients with advanced fibrosis or cirrhosis and significantly reduces the incidence of hepatic decompensation.[5] Measurement of the HBV-DNA level is critical for the early detection of treatment failure that may be related to poor adherence to therapy or selection of a resistant virus.[5,18,26,27] In addition, viral kinetics while on therapy predict the emergence of resistance. The likelihood of resistance to nucleos(t)ide analogues is very low when HBV-DNA level is undetectable (<300 copies/mL) during therapy. It is significantly higher in patients with more than 10^3 copies/mL at week 24 or 48 of therapy and increases proportionally to the HBV-DNA level (note that the nonstandardized copy/mL unit has been used in the published trials).[18,27–29]

Liver HBV cccDNA and Quantitative HBsAg

The persistence of HBV in the liver, despite antiviral therapy, is owing to the maintenance of HBV cccDNA in the nuclei of infected cells. cccDNA levels have been assessed in liver biopsy specimens from patients in the different phases of chronic HBV infection, both during and after antiviral therapy, using selective polymerase chain reaction (PCR) assays for HBV cccDNA in liver biopsy specimens.[30–34] HBeAg-positive patients have a higher cccDNA copy number

per cell than do HBeAg-negative patients, and cccDNA levels correlate with the phase of HBV infection. Patients who achieve HBeAg seroconversion during antiviral therapy have lower baseline levels of cccDNA than do nonseroconverters, and cccDNA is reduced significantly in patients who received long-term nucleos(t)ide therapy compared with placebo.[30–36] In addition, a significant reduction in serum HBsAg titer has been observed with adefovir dipivoxil, which correlated with changes in cccDNA level, total intracellular HBV-DNA level, and serum HBV-DNA level.[30] Because cccDNA is the major template for transcription and translation of viral antigens, including HBsAg, this result suggests that change in serum HBsAg titer might be used as a surrogate for change in liver cccDNA, the latter requiring a liver biopsy.[37] However, this suggestion requires validation. Standardization of these assays is now needed.

Levels of hepatic cccDNA may provide a greater predictive value of response than alternative measures for a range of clinical therapies. In a trial of IFN–lamivudine combination therapy vs lamivudine monotherapy, liver HBV cccDNA levels at the end of therapy gave a higher predictive value for sustained virologic response than did serum HBV-DNA level or total intrahepatic HBV-DNA level.[31] Similarly, in patients with positive HBsAg and lymphoma, cccDNA levels before chemotherapy predicted reactivation of HBV after chemotherapy, with an optimal cut-off level of approximately 3 copies/cell predicting no reactivation.[38] Larger-scale trials are required to determine if quantification of cccDNA may provide an indicator of the efficacy of antiviral therapy and an independent predictor of outcome. For the immediate future, it is recommended that cccDNA assays and quantitative HBsAg assays be included as research tools to increase our understanding of clinical trial results (EO) (Table 1).

HBV Genotypes

HBV is classified into 8 HBV genotypes, A–H, based on an 8% or more DNA sequence difference over the whole genome.[39,40] Data suggest that HBV genotype may be related to disease outcome. In Asia, genotype C is associated with a higher risk of reactivation of hepatitis B and progression to cirrhosis than genotype B.[41,42] In Europe, genotype D is associated with more active disease than the other genotypes, but the fact that genotype D also is associated with long-standing infection (as a result of acquisition at a younger age) may constitute a bias.[39,43,44] Genotype F was implicated recently in an enhanced risk of hepatocellular carcinoma in Alaskan natives.[45] Genotype also may influence response to IFN-based therapy. After pegylated IFN therapy, the rate of HBeAg loss at the end of the follow-up period for genotypes A, B, C, and D, respectively, was 47%, 44%, 28%, and 25%.[46] A similar relationship was observed with the rate of HBsAg loss in the same study.[47] In another study, the rate of HBeAg loss at the end of the follow-up period for genotypes A and D was 52% and 22%, respectively. However, the role of HBV genotype in predicting clinical outcomes, including therapy, remains to be established. It is recommended that all clinical trials collect HBV genotype data, and consideration should be given to stratifying trials according to genotype (EO) (Table 1). In clinical practice, the positive and negative predictive values of the HBV genotype on disease progression and treatment outcome have not been determined at the individual patient level, but genotype determination may become more important in the future as the data matures.

Genotypic Resistance Markers

Sensitive assays are available that can detect resistant viral variants during therapy before an increase in HBV-DNA level.[48–56] Early detection of genotypic change allows one to switch to alternative therapies and to avoid virologic rebound and hepatitis flare. This is particularly useful in patients with cirrhosis. The value of early detection of resistance by genotyping is less clear for patients without significant hepatic fibrosis, in whom serum HBV-DNA monitoring may be adequate to diagnose the development of antiviral drug resistance. In the setting of virologic breakthroughs, detection of resistance by genotyping can be used to distinguish between medication noncompliance and the selection of resistant variants.

Systematic testing for resistance by genotyping is mandatory in clinical trials to understand fully the properties of new therapeutic agents (EB). Recommendations for the frequency of resistance testing in both clinical trials and clinical practice are discussed later.

Virologic Assessment of Treatment Responses and Resistance

Standardization of Quantification Units

Serum HBV-DNA levels are reported in many different units depending on the method used and the manufacturer of the assay (eg, copies/mL, genome equivalents [Eq]/mL, mega-equivalents [MEq]/mL, or international units [IU]/mL). The World Health Organization has defined an international standard for HBV DNA nucleic acid amplification techniques[57] that has been used to calibrate the IU/mL. Several HBV-DNA quantification assays are available that have been normalized to the World Health Organization international standard.[58] Serum HBV-DNA levels now should be expressed universally in IU/mL in all available assays to ensure comparability between the assays, between different trials in which different assays have been used, and to allow the creation of guidelines that can be applied to whatever assay was used (in general, an IU is equivalent to approximately 5– 6 copies, depending on the assay) (EB).

HBV-DNA Quantification Technology

With several HBV-DNA quantification assays available and with fund providers/insurers sometimes dictating which assay is to be used by a laboratory, it is important to recommend the required properties of the test rather than any specific technology (Table 2). An assay with a lower limit of detection of 10^3 IU/mL may be sufficient to monitor and manage the patient but, in some instances, for example, in patients with a low baseline HBV-DNA level or with profound inhibition or viral replication during therapy, a more sensitive assay with a lower limit of detection of the order of 10 IU/mL may be required, to ensure detection of the emergence of resistance as early as possible (EB). A dynamic range of quantification of at least 5 $\log_{10}$ is recommended and samples with an HBV-DNA level above the upper limit of detection of the assay should be diluted and retested to provide an end point. If no dilution is performed, the result should be reported as higher than the upper limit of detection, but this will not allow monitoring of primary response to therapy and primary treatment failure. For all of these reasons, real-time PCR quantification assays now strongly are recommended over other technologies, especially in clinical trials, because they are very sensitive and have a broad dynamic range of quantification (7– 8 $\log_{10}$ with the current assays) (EB).[59 – 65] In addition, the assay used should be proven to quantify equally and accurately all HBV genotypes. It is important to use the same assay for a given patient in clinical practice. In the case of an assay switch during a clinical trial or a cohort study of treated patients, the initial samples should be retested with the new assay. This also ideally should be performed in clinical practice if the initial sample(s) has (have) been stored. If not, HBV-DNA changes should be interpreted cautiously.

HBV Genotyping Technology

The reference method for HBV genotype determination is sequencing followed by phylogenetic analysis of generated sequences together with reference sequences.[59] This is the only method suitable for the analysis of new genotypes or recombination between genotypes but it is time consuming. Reverse hybridization techniques have proven very useful in clinical trials. They also can identify mixed genotype infections.[59] Real-time PCR or multiplex PCR are potential alternatives if appropriately validated against the gold standard (ie, sequencing).[66]

HBV Resistance Testing by Genotyping

Direct sequencing-based techniques are the gold standard because all mutations can be detected, which is particularly important with the increasing number of mutations reported. In contrast, hybridization assays can detect only known specific mutations and require new probes to detect novel mutations.[49–56,59] In clinical trials, direct sequence analysis must be used. Testing for HBV mutations also should evaluate the proportion of the mixed mutant and wild-type populations, using at least the data from direct sequencing (also called *population sequencing*), but more sensitive and quantitative results can be obtained from the sequencing of multiple clones.[54,56,67–69] Hybridization-based methods have the advantage of detecting resistant variants when they are present as minor populations (down to 10% of the total viral population).[55] More sensitive technologies, such as those based on mass spectrometry, are currently in development.[50] They will be able to detect smaller proportions of viral variants in complex viral mixtures, but the utility of such sensitivity remains to be determined. Real-time PCR-based techniques are potential alternatives,[66] but they may not be suited yet to clinical trials and clinical practice given the high number of substitutions of interest and variability among wild-type sequences.

Phenotypic Resistance Testing

Phenotypic analysis can determine in vitro inhibitory concentrations (IC) of specific HBV inhibitors (ie, it allows the testing of the susceptibility of a given HBV polymerase sequence to the antiviral action of a given drug). Methods based on transient transfection and continuous protein expression as well as transduction with recombinant HBV baculoviruses have been described.[70–75] Testing by phenotype permits the quantification of the magnitude of resistance to a drug and can interrogate for resistance without the need to know the responsible mutations. Phenotypic analyses can confirm and assess the drug susceptibility associated with a given amino acid substitution and cross-resistance to other drugs from the same or other families. However, one must be careful in interpreting the results of in vitro phenotypic analyses because the replication properties observed in vitro may not always translate in vivo where the virus is under the influence of a much more complex replicative environment.[54,56,68,69]

The IC_{50} is defined as the drug concentration that reduces replication in the in vitro model by 50%. A fold change in IC_{50} can be considered significant if it is greater than the natural variability of the in vitro assay. However, the IC_{50} has been shown to vary from 2- to 5-fold up to more than 50- to 100-fold with amino acid substitutions known to be associated with in vivo resistance to different drugs relative to the wild-type sequence.[70,76] Indeed, the in vivo pharmacodynamics of the drugs may have a significant impact on whether small variations in IC_{50} may translate into clinical resistance. It is not possible to predict with confidence what the impact of reduced susceptibility in vitro will have on the response to a given drug. A significant fold change in IC_{50} defines resistance clinically if it is associated with a diminished treatment efficacy in vivo. Thus, clinical trial data are necessary to determine a level of resistance that will impact response and thus guide treatment decisions. Phenotypic assays also are critical to provide information on cross-resistance.

Virologic Definitions

Definition of Baseline Viral Level

A baseline viral level is needed as a reference against which to assess treatment response or nonresponse. The baseline viral level ideally should be defined as the viral level taken within 24 hours before the patient starts treatment (EO). This is distinct from any previous viral level measurements performed to inform the decision to commence treatment. In the case of HBV-DNA fluctuations (more frequent in HBeAg-negative patients), this baseline HBV-DNA level is the viral level against which therapy will be assessed in the subsequent weeks and months.

In clinical practice, it may not be practical to obtain the baseline viral level and the viral level determination closest to the start of therapy should be used (EO).

Definition of Treatment Antiviral Effect and Efficacy and of End Points Achieved by Therapy

Three levels of virologic response can be defined: antiviral effect, antiviral efficacy, and end point.

Antiviral effect—When antiviral treatment is started, it is important to have an early indication that the patient is responding to therapy. An antiviral effect is defined as a 1 $\log_{10}$ IU/mL or greater reduction of serum HBV-DNA level from baseline within 3 months of starting therapy.

Antiviral efficacy—The quantitative $\log_{10}$ reduction relative to baseline is a measure of antiviral treatment efficacy and the aim is to reduce HBV DNA to as low a level as possible to avoid resistance, to increase the possibility of HBeAg loss within the first or second year of treatment in HBeAg-positive patients, and to ensure adequate virologic suppression that then will lead to histologic improvement in all patients. The ability to quantify treatment efficacy by measuring the $\log_{10}$ reduction relative to baseline is dependent on the baseline viral level and the lower limit of detection of the HBV-DNA assay used. Treatment efficacy also can be defined as the ability of a given therapy to achieve an undetectable HBV-DNA level in a given HBV-DNA assay. In clinical trials, treatment efficacy should be assessed by measuring both the mean or median $\log_{10}$ reduction of HBV-DNA level and the proportion of patients with undetectable HBV DNA (less than the threshold of the assay, see previously for technical requirements) at various time points in all treatment groups (EO).

End point—The goal of HBV therapies is to stop or slow the progression of liver disease to prevent cirrhosis, decompensation of cirrhosis, or hepatocellular carcinoma. Current therapies fall into 2 categories: IFN and pegylated IFN, and specific HBV nucleoside or nucleotide inhibitors. Patients also can be divided into whether or not they have a sustained response off treatment. The sustained response off treatment is defined as sustained HBe seroconversion, HBV-DNA level reduction, and ALT normalization in HBeAg-positive patients (EB). There is a recent controversy as to whether HBeAg seroconversion is an adequate end point for patients infected during infancy or childhood.[77,78] These patients probably need permanent suppression of HBV DNA to levels undetectable by sensitive PCR assays and reduction of ALT levels to less than 0.5 times the upper limit of normal.[77] In patients with HBeAg-negative chronic hepatitis B, the sustained response off treatment is defined as sustained HBV-DNA level reduction and ALT normalization. However, current evidence suggests that sustained virologic responses may be rare in HBeAg-negative patients (EB). This term may be more applicable to IFN treatment because of the finite duration of therapy. For nucleos-(t)ide analogues, treatment duration tends to be extended and the goal of therapy is to achieve profound and sustained inhibition of HBV replication in both HBeAg-positive and HBeAg-negative patients. Studies have suggested that nucleoside analogue therapy can be stopped 6 –12 months after HBe seroconversion in HBeAg-positive patients (EB). However, the guidelines for HBeAg-negative patients have not been defined. The ultimate goal of therapy for all patients is HBsAg seroconversion (EO).

Standardization of the end points achieved by therapy is needed in both clinical trials and in clinical practice. Three different end points can be defined to classify patient outcome. These do not signify that therapy can be stopped but serve to define end points as a goal that has been achieved by therapy, at any time. First, in HBeAg-positive patients, the goal is to achieve HBeAg seroconversion, ideally with short-term therapy. In addition, in both HBeAg-positive and HBeAg-negative patients the aim is to have sustained inhibition of viral replication to

improve liver disease and to avoid the development of resistance. The end points are as follows: (1) in HBeAg-positive patients: HBeAg seroconversion (HBeAg loss and gain of anti-HBe), sustained inhibition of viral replication (serum HBV-DNA level <2000 IU/mL or any lower threshold), and normalization of serum ALT level; (2) in HBeAg-positive patients with no HBeAg seroconversion: sustained inhibition of viral replication (serum HBV-DNA level <2000 IU/mL or lower) and normalization of serum ALT level; (3) in HBeAg-negative patients: sustained inhibition of viral replication (serum HBV DNA at most <200 IU/mL) and normalization of serum ALT level. In all instances, the HBV-DNA thresholds represent a minimal efficacy end point. With the more sensitive real-time PCR-based HBV-DNA assays and more potent anti-HBV drugs, HBV-DNA levels should be as low as possible and, ideally, undetectable (ie, below the lower limit of detection of the assays, which is of the order of 10 IU/mL) to ensure full prevention of liver disease progression and complications and the emergence of resistance.[77]

Definition of Treatment Failure

Because of the different mechanisms of action of nucleos(t)ide analogue-based and IFN-based therapies, separate definitions of treatment failure are needed (EO). Furthermore, a definition of treatment failure with nucleos(t)ide analogue-based therapy aims to identify the development of resistance, a problem that does not occur with IFN.

Nonresponse to antiviral treatment with nucleos(t)ide analogues or primary antiviral treatment failure is the failure to achieve more than 1 $\log_{10}$ decrease from base-line within 3 months of starting therapy. In some patients, a suboptimal response to therapy may be observed, characterized by a more than 1 $\log_{10}$ but less than 2–3 $\log_{10}$ IU/mL decrease at month 3 of therapy.[19] Secondary antiviral treatment failure is defined by a rebound of serum HBV-DNA levels of 1 $\log_{10}$ IU/mL or greater from nadir in patients with an initial antiviral treatment effect as confirmed by 2 consecutive determinations at a 1-month interval.[79] The main causes of primary and secondary antiviral treatment failure are poor adherence to therapy, lack of the drug's antiviral effect related to metabolic causes (problems with absorption, bioavailability, metabolism of prodrug to active metabolite, or phosphorylation of the antiviral agent to its triphosphate), and selection of drug-resistant HBV mutants.[80 – 82] Further research in pharmacogenetics and inherent metabolic alterations in bioavailability or differences in the rate of phosphorylation of nucleosides, similar to studies performed with antiretroviral or anticancer drugs,[83,84] may be important for understanding differences in primary response.

Although IFN therapy is directed toward HBeAg seroconversion, only about a third of selected patients achieve HBeAg seroconversion (ie, the majority do not seroconvert).[12,23] Treatment with IFN is normally for 6 –12 months and seroconversion may occur after treatment cessation. Hence, for IFN-based therapy, treatment failure is defined as failure to achieve a 1 $\log_{10}$ or greater reduction from baseline within 6 months of starting therapy or HBe seroconversion during treatment and follow-up evaluation (EO). This is based on experience with conventional IFN, is preliminary, and will require more data from studies with the pegylated IFNs.[12]

The cumulative incidence of primary and secondary treatment failures should be reported systematically in clinical trials according to the formula shown later (EB).

Definition of Resistance

Antiviral drug resistance reflects the reduced susceptibility of a virus to the inhibitory effect of a drug[85] and results from a process of adaptive mutations in the HBV polymerase gene. In clinical trials and practice, resistance is defined as the selection of variants bearing amino acid substitutions conferring reduced susceptibility to drug that result in primary or secondary treatment failure (see earlier definition). Although it is more likely that resistance is identified

owing to secondary treatment failure, resistance may become a cause of primary treatment failure either because of transmission of resistant HBV or because of cross-resistance resulting from previous therapies. In clinical practice, clinicians likely will be monitoring HBV-DNA levels and failure to respond to treatment will be the first indication of the emergence of resistance.

In clinical trials, the reporting of resistance has varied tremendously from one trial to another, thus considerably biasing the interpretation of the results and our knowledge of resistance incidence with a given anti-HBV drug.[5,10,14,18,26,27,86–90] The incidence of resistance, which now is assessed by sequencing, should be reported as a cumulative probability of occurrence.[18] In all clinical trials, the cumulative probability of the onset of (1) resistance, (2) resistance with virologic (HBV DNA) breakthrough, and (3) resistance with virologic (HBV DNA) and biochemical (ALT) breakthroughs, should be reported every year according to the duration of follow-up evaluation (EB). Calculation should be made by means of the following formula:

$$P=1-(1-n1/N1)(1-n2/N2)\ldots(1-nx/Nx)$$

where P is the cumulative probability that the event will occur, nx is the number of cases at year x, and Nx is the number of patients still followed up at year x. For example, this formula has been used to calculate the cumulative incidence of mutation selection over 5 years of adefovir administration in patients with HBeAg-negative chronic hepatitis B.[18] Clinical trials in which subsets of patients systematically stop treatment might create bias in results depending on what category of patient (responders or nonresponders) is taken off the trial. This therefore should be avoided in the design of clinical trials, especially with new drugs.

In addition to reporting resistance according to the earlier definition, it also would be useful to report the number of patients with virologic and biochemical breakthrough without a viral cause (EO). If a mutation is identified in a patient, follow-up evaluation is important because the virologic breakthrough can be delayed several weeks after the appearance of the viral mutant. In trials of new drugs it is important to evaluate the emergence and incidence of new mutations and then the time to loss of response, defined as an increase (1 $\log_{10}$) in HBV-DNA level. However, some mutations may show phenotypic resistance but not confer clinical failure within the time-frame of a clinical trial.

Management in Clinical Trials and Clinical Practice

How Do We Monitor Treatment Efficacy and Failure in Clinical Trials and Clinical Practice?

To aid standardization of reports and comparison of different clinical trials with IFN and/or nucleos(t)ide HBV inhibitors, assessments of HBV DNA, ALT, and HBeAg/anti-HBe should be performed at baseline and then every 1–3 months; although for a new drug, serum HBV DNA and ALT should be measured monthly to evaluate viral kinetics and incidence of ALT increases (EO) (Table 3). In clinical practice, 3- to 6-month assessments are adequate (EB). After the baseline assessment, the first assessment at month 3 will allow evaluation of the primary treatment response. In both clinical trials and clinical practice, if a serum HBV-DNA measurement indicates that the patient may have primary or secondary treatment failure, but there is no increase in serum ALT level, a second serum HBV-DNA sample should be assayed for confirmation 1 month later (EB).

How Do We Show Resistance in Clinical Trials and Clinical Practice?

In clinical trials with nucleos(t)ide HBV inhibitors, direct sequencing of the polymerase gene should be performed systematically at baseline, at the end of each year, at 3 months if there is

primary treatment failure, and at the time when secondary treatment failure is documented (EO) (Table 4).

In clinical practice, nucleos(t)ide analogue resistance testing based on direct sequencing or reverse hybridization on a baseline sample (if available) is required only in patients who have failed on their current treatment (EB). If primary or secondary treatment failure has been shown, non-HBV–related causes of failure such as noncompliance should be eliminated, for instance, by measuring circulating drug concentrations. Resistance testing should be performed to show resistance and to inform the treatment decision (EO). Similar to the clinical trial setting, it should be performed at 3 months in the case of primary treatment failure and at the time of secondary treatment failure (Table 4). However, consensus guidelines should be issued to guide treatment decisions according to the patient's genotypic resistance profile.

How Do We Explore the Mechanisms of HBV Resistance in Clinical Trials?

Phenotypic assays and viral quasispecies analysis are useful in clinical trials with nucleos(t) ide HBV inhibitors and as a research tool (EO). Phenotypic assays are used to establish the concentration of the drug that inhibits in vitro replication of HBV variants by 50% and 90% (IC_{50} and IC_{90}). These numbers quantify the level of resistance to a specific drug conferred by viral mutation(s). Phenotypic assays can use nearly full-length HBV polymerase sequences retrieved from patients before or during therapy, or prototype polymerase sequences into which resistance substitutions have been incorporated by mutagenesis.[70–75] The methods are complementary. The study of multiple clones isolated at different time points (quasispecies analysis) is useful to understand the dynamics of viral populations and the emergence of resistance during therapy.[54,56,68,69,91] It helps characterize the in vivo replication fitness of the variants in the presence of the drug(s). Improvement and standardization of these methods, however, is needed before their widespread application.

Conclusions

The virologic definition of HBV treatment effect, efficacy, and end points allows investigators and clinical practitioners to define treatment responses and failures. The currently available assays for HBV-DNA quantification (in particular those based on real-time PCR), serologic assays for HBeAg and anti-HBe antibodies, and molecular assays for the identification of genotypic resistance to HBV drugs can be used confidently to monitor treatment responses and diagnose primary and secondary treatment failures and their causes in HBV clinical trials and clinical practice. The utility of additional virologic parameters, such as the HBV genotype, quantitative HBsAg, or intrahepatic cccDNA remains to be established. These parameters should be assessed in clinical trials when possible (but not all clinical trials allow for sequential liver biopsy specimens for cccDNA assessment).

Diagnosis and monitoring of HBV treatment failure in clinical trials of new anti-HBV drugs involves 5 successive steps. First, primary or secondary treatment failure should be diagnosed by means of HBV-DNA measurement according to the earlier-described definitions. Second, nonviral causes of treatment failure should be sought. In this respect, drug dosages are particularly useful to show poor adherence to therapy, an unusual cause of treatment failure in clinical trials, but the principal one in clinical practice. Third, selection of resistant variants should be shown by means of sequencing assays. Fourth, to differentiate true resistance mutations from polymorphisms and to assess the level of resistance to the drug conferred by the identified mutations, the mutant HBV should be tested for estimation of the IC_{50} and IC_{90} against that particular (and other) agent in phenotypic assays, ideally both in and out of the context of the full-length patient's sequence. Fifth, the dynamics of sensitive and resistant viral populations during treatment should be characterized by means of quasispecies analyses at serial time points. In clinical practice, steps 1–3 generally are sufficient to diagnose viral

resistance when it is caused by known mutations. When treatment failure is diagnosed and mutations unknown to be associated with resistance to the specific drug are identified, steps 4 and 5 are necessary to confirm that the mutation is indeed responsible for treatment failure.

Standardization of HBV clinical trial reports is now needed based on these definitions and end points.

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Abbreviations used in this paper

cccDNA
: covalently closed circular DNA

IC
: inhibitory concentration

IFN
: interferon

PCR
: polymerase chain reaction

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Biography

Jean-Michel Pawlotsky acted as a consultant/advisor for Roche (Basel, Switzerland), Gilead (Foster City, CA), Bristol-Myers Squibb (Princeton, NJ), Idenix (Cambridge, MA), and Novartis (Basel, Switzerland), and received research support from Roche and Gilead; Geoffrey Dusheiko received consultancy fees from Glaxo Smith-Kline (London, UK), Roche, Novartis, Schering Plough (Kenilworth, NJ), Idenix, Gilead Sciences, and Bristol-Myers Squibb, and received research support from the same groups; Daryl Lau acted as a consultant/advisor and has received research grants from Roche, Gilead, Bristol-Myers Squibb, and Novartis; George Lau is a Consultant for Roche and Novartis; Stephen Locarnini has received royalties and is a patent holder for Melbourne Health (Parkville, Victoria, Australia), has received consulting fees from Evivar (Melbourne, Victoria, Australia), Gilead, Pharmasset, and Bristol-Myers Squibb, has received research support from Evivar and Gilead, and has ownership interests in Pharmasset (Princeton, NJ); Paul Martin is a consultant and speaker for Bristol-Myers Squibb, Gilead, Idenix, and Roche; Douglas D. Richman is a consultant for Bristol-Myers Squibb, Gilead, Idenix, and Roche; and Fabien Zoulim is a consultant and has received speaker fees from Gilead, Bristol-Myers Squibb, Idenix, and Novartis.

Table 1

Utility of Markers of Treatment Efficacy, Failure, and Resistance

Marker	Clinical trials	Clinical practice	Research tool
Serum ALT	Yes	Yes	No
HBeAg/anti-HBe	Yes	Yes	No
HBV DNA	Yes	Yes	No
Genotypic resistance	Yes	Yes[a]	Yes
HBV genotype	Yes	No	Yes
Quantitative HBsAg	Whenever possible	No	Yes
Quasispecies analysis	Whenever possible	No	Yes
Liver cccDNA	Whenever possible[b]	No	Yes

[a] Provided that consensus guidelines are available to guide treatment decisions.

[b] If liver biopsy specimens are available.

Table 2

Monitoring Treatment Efficacy and Failure in Clinical Trials and Clinical Practice

Marker	Clinical trials	Clinical practice
Serum HBV DNA	Baseline and every 1–3 months (every month for a new drug)[a]	Baseline and every 3 to 6 months[a]
Serum ALT	Baseline and every 1–3 months (every month for a new drug)	Baseline and every 3 to 6 months
HBeAg/anti-HBe	Baseline and every 3 to 6 months	Baseline and every 6 months

[a] If a serum HBV-DNA measurement indicates that the patient may have primary or secondary treatment failure, but there is no increase in serum ALT level, a second serum HBV-DNA sample should be assayed for confirmation.

Table 3

Demonstration of Resistance in Clinical Trials: Indication of Direct Sequence Analysis of the HBV Reverse Transcriptase (in Both Treatment-Naive and Previously Exposed Patients)

Baseline
At the end of 1 year, and every year irrespective of HBV-DNA level
At month 3 if primary treatment failure
Every case of secondary treatment failure

Table 4

Demonstration of Resistance in Clinical Practice: Indication of Direct Sequence Analysis of the HBV Reverse Transcriptase (in Both Treatment-Naive and Previously Exposed Patients)

	Treatment-naive	No information	Interrupted treatment	Failed on treatment
Baseline	No, store sample (pending treatment failure)	No, store sample (pending treatment failure)	No, store sample (pending treatment failure)	Yes
At month 3 if primary treatment failure	Yes	Yes	Yes	Notapplicable
Every case of secondary treatment failure	Yes	Yes	Yes	Notapplicable