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REVIEW ARTICLE

Use of Current and New Endpoints in the Evaluation of Experimental Hepatitis B Therapeutics

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New hepatitis B virus (HBV) therapies are expected to have breakthrough benefit for patients. HBV functional cure is sustained hepatitis B surface antigen loss and anti-HBs gain, with normalization of serum aminotransferases off therapy. Virologic or complete cure additionally includes loss of HBV covalently closed circular DNA. Currently available endpoints of therapy are inadequate to evaluate the efficacy of many of the new therapeutics. Therefore, either new ways of using the existing virologic endpoints and laboratory values or entirely new biomarkers are needed. In this review, we discuss the currently used endpoints, potential new endpoints, as well as what new markers are needed to assess the ability of HBV therapeutics to achieve functional and virologic cure in various phases of HBV infection. In addition, we discuss how patient selection from differing phases of HBV impacts the choice of HBV drug(s) needed to achieve cure.

Keywords. virology; immunology; pathology; therapeutics; markers.

New drugs for chronic hepatitis B (CHB) should be superior to currently available medications [1], which do not achieve sustained, off-drug, virologic suppression in the majority of patients. HBV "cure" has been defined in 2 ways (Table 1). "Functional" cure is the sustained loss of circulating hepatitis B surface antigen (HBsAg) and gain of anti-HBs with normalization of liver enzymes off-therapy. Some patients do not develop anti-HBs but remain HBsAg negative for years. Whether achieving anti-HBs is essential for functional cure is under discussion. "Complete or virologic cure" additionally includes loss of covalently closed circular DNA (cccDNA) in hepatocytes [2]. As reduction in disease-associated morbidity and mortality can take decades to occur, surrogate markers are needed to evaluate the efficacy of medications. Tactically, markers will be needed to determine if a drug is superior and/or complementary to current medications; when therapy should be started, adjusted, or stopped; and, most importantly, which patients will benefit most from a particular therapy. In this review, we discuss how the current endpoints are used, as well as how they could be used. Also, we discuss new biomarkers that are in development and could be useful for evaluating the effectiveness of HBV therapeutics.

ROLE OF PATIENT SELECTION IN THERAPEUTIC STUDIES

Clinical trials and the endpoints used may need to differ for individuals in different stages of CHB that differ virologically, pathologically, clinically, and immunologically [3] (Table 2). In the immune-tolerant phase, liver biopsies reveal little inflammation, with HBsAg detectable in the cytoplasm of infected hepatocytes. This phase can last several decades in persons infected with HBV genotype C and is shorter in those infected with genotypes A or D [3]. The immune-tolerant phase may be followed by the immune-active phase, but most patients eventually evolve into the inactive phase [3]. However, 15%–25% of persons who clear HBeAg will develop HBeAg-negative (anti-HBe positive) immune active CHB. In addition, up to 15%–25% of inactive patients will reactivate with either HBeAg positive or, more likely, HBeAg-negative, immune-active disease with increased risk of fibrosis and hepatocellular carcinoma (HCC) [3]. Patients with inactive, chronic HBV for many years rarely lose HBsAg, <1% per year [3]. However, it appears that persons who spontaneously enter into the inactive phase and remain there have a lower risk of HCC and reactivation than those who achieve this phase on antiviral therapy [4].

Studies of new drugs must take into account both the various phases of CHB and the endpoints needed to determine successful therapy in each phase. Whereas new drugs will likely be evaluated first in patients who are already treated with HBV polymerase nucleos(t)ide inhibitors (nucs) [1], immune-tolerant

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Table 1. Assessment of Goals of Hepatitis B Virus Therapy After Drug Discontinuation

Cure Definition	Blood				Liver
	ALT	Hepatitis B virus DNA	Hepatitis B surface antigen	Anti-HBs	Covalently closed circular DNA[a]
Functional cure	Normal	Undetectable (quantitative PCR)	Undetectable	Detected	Present
Virological (complete) cure	Normal	Undetectable (qualitative PCR)	Undetectable	Detected	Undetectable

Abbreviation: ALT, alanine aminotransferase; Anti-HBs, antibody to Hepatitis B surface antigen; PCR, polymerase chain reaction.

[a]As detected in the liver. These assays are not currently commercially available or standardized.

patients may benefit the most and require different or combination therapies compared to those who are nuc suppressed.

ENDPOINTS

The virologic, biochemical, and serologic measurable endpoints of CHB management, which are used for the currently licensed drugs, are the reduction of HBV viremia, normalization of serum aminotransferases, loss of liver inflammation, reversal of liver fibrosis, seroconversion from HBeAg to anti-HBe, loss of HBsAg, and acquisition of anti-HBs [5] (Table 3). Creative use of existing markers or development of entirely new markers may be needed, as discussed below (Table 4).

Virologic Endpoints

Productive replication of HBV, a DNA virus that replicates its genomes via reverse transcription, is driven from its transcriptional template, known as the cccDNA [1]. cccDNA is found only in the nucleus of an infected hepatocyte and exists as a viral minichromosome [1, 2]. Transcription from cccDNA generates a 3.5-kb precore mRNA-producing HBeAg; pregenomic mRNA-producing core protein, hepatitis B core antigen (HBcAg), and polymerase/reverse transcriptase; and the subgenomic RNAs, which produce the viral envelope proteins (HBsAg) and HBx. Integration of HBV DNA, which occurs through a process of illegitimate recombination [6] that is assisted by host enzymes that act on double-stranded linear DNA [7], is not required for productive replication. These integrated sequences cannot provide an adequate template for productive replication; however, HBsAg can be produced from the usually intact open reading frame of the HBsAg S gene. Thus, the following 2 sources of HBsAg can be identified: cccDNA and integrated HBV DNA. This has relevance in terms of direct-acting antivirals and treatment goals including defining cure endpoints as functional or complete virologic, as described in Table 1 [2].

Circulating HBV DNA

A hallmark of effective nuc therapy is the reduction in HBV viremia in the blood. Viremia reflects, but is not directly proportional to, the number of infected cells and viral gene expression [1]. Current nucs can suppress viremia below detection, which is often not sustained after nuc withdrawal. Therefore, undetectable viremia on therapy does not reliably predict a drug's ability to achieve a functional cure.

HBsAg quantitation

In the United States, serum HBsAg levels are usually reported qualitatively. Most patients with CHB have concomitant anti-HBs, especially HBeAg-positive patients with high viral load in the immune-active phase [8]. The amount of HBsAg that is measured in serum as free HBsAg in the blood can be confounded by immune complexes with coexisting anti-HBs. Quantitative HBsAg assays are available outside the United States. During peginterferon (PegIFN) treatment, sustained responders tend to show greater HBsAg decline than the nonresponders. Levels after 12 weeks of PegIFN can predict nonresponders and be used for early termination [9]. The HBsAg levels can indirectly reflect the amount of viral transcriptional activity in the liver [10], but this is the case only during the HBeAg-positive phase of chronic HBV [11]. The exact role of HBsAg assays, such as ultimate cutoff points to predict response to therapy and clinical outcome, are still under investigation.

Table 2. Phases of Hepatitis B Virus Infection

Phase	HBeAg	Hepatitis B virus DNA	ALT	Liver Biopsy	Treatment Candidacy Based on Currently Approved Medications
Immune tolerant	Positive	Very high, >200 000 IU/mL	Normal	No/mild inflammation/fibrosis	Not currently considered candidates
Immune active	Positive or negative	Usually >20 000, >2000 IU/mL	Elevated	Inflammation and fibrosis; degree varies	Treatment candidates
Inactive	Negative	<2000 IU/mL	Normal	Normal or mild	Not currently considered candidates
Hepatitis B surface antigen clearance	Negative	Usually not detected/or <1000 IU/mL	Normal	No inflammation/improving fibrosis	Not currently indicated

Abbreviation: ALT, alanine aminotransferase; HBeAg, Hepatitis B e antigen.

Table 3. Currently Used Virologic and Host Markers and Targets

Assay	Specimen	Target	Reference
Virologic markers			
HBV DNA (q, non-q)	Blood	Viral replication	[5]
HBsAg (non-q)	blood	HBsAg gene expression	[5]
HBsAg (q)	Blood	HBsAg gene expression	[10]
HBeAg	Blood	HBeAg gene expression	[11]
Host markers			
Anti-HBs (q/non-q)	Blood	Immune restoration	[30, 32]
Anti-HBc (q/non-q) IgM, IgG	Blood	Exposure to HBV	[33]
Standard liver tests	Blood	Liver function and inflammation	[5]
Imaging	Liver	Detection of hepatocellular carcinoma	[5]

Abbreviations: anti-HBc, antibody to hepatitis B core antigen; anti-HBs, antibody to hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; Ig, immunoglobulin; q, quantitative test; non-q, nonquantitative test.

HBeAg quantitation

Serum HBeAg levels correlate with HBV viremia and HBV replication [3]. Loss of serum HBeAg, as reported qualitatively, has been a hallmark of successful therapy in HBeAg-positive individuals but can also reflect the emergence of basal core promoter variants, which are associated with poorer prognosis, lower HBeAg expression, and a lower chance of subsequent HBsAg loss [5, 12]. Quantitative HBeAg after 24 weeks of PegIFN predicted HBeAg loss after end of therapy [13]. This could be used with other markers such as HBV DNA and HBsAg.

Table 4. Examples of Experimental Virologic and Host Markers and Endpoints

Assay	Specimen	Measures	Reference
Virologic markers			
Hepatitis B core–related antigen (q)	Blood	Denatured HBeAg, HBcAg, precore protein p22cr	[14, 17]
cccDNA (q)	Liver	Number of infected hepatocytes	[20]
Integrated DNA (q)	Blood	Infected cell number	[2]
HBV RNA	Blood	cccDNA amount of transcription	[22]
Host markers			
PD1, Tim3, CTLA4 expression (q) on HBV-specific CD8 T cells by flow cytometry	PBMC	Exhaustion status of virus-specific T cells	[34]
CD127 on HBV-specific T cells by flow cytometry/ functional assays	Blood	Long-lived HBV-specific memory T cells	[27, 34]
Cytokines (q)	Blood	Inflammation	[27, 35]
HBsAg epitopes	PBMC	HBsAg clearance	[25]

Abbreviations: cccDNA, covalently closed circular DNA; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; PBMC, peripheral blood mononuclear cells; q, quantitative result; non-q, nonquantitative result.

Hepatitis B core–related antigens

All viremic CHB patients have core-associated antigens in their blood [14]. HBcAg forms the nucleocapsid that surrounds the genome, which is subsequently enveloped and secreted from infected hepatocytes as circulating virions. Because core proteins are difficult to detect in circulating virions, assays for hepatitis B core–related antigen (HBcrAg) have been developed, which simultaneously measure denatured HBeAg, HBcAg, and the precore protein p22cr (aa28 to aa150) with a range of 3–7 log U/mL [15]. In an Asian cohort, HBcrAg levels correlated with serum HBV DNA and intrahepatic cccDNA [16]. Low levels of HBcrAg in the serum reflect successful nuc discontinuation [17] and distinguish HBeAg-negative chronic HBV with active disease from inactive disease [15]. High levels of HBcrAg constituted an independent risk factor for HCC in both European [17] and Asian patients [18].

Time to virologic rebound as an endpoint

The most potent approved nucs effectively reduce HBV viremia to undetectable levels with minimal risk of developing antiviral resistance [5]. After stopping nucs, detectable levels of virus often appear in the blood ("rebound") and rise over time, even reaching pretreatment levels [3]. The kinetics of this rebound differs in HBeAg-positive and HBeAg-negative patients. Rebound occurs in <50% of HBeAg-positive patients and takes several months [19]. In contrast, 70%–80% of HBeAg-negative patients will rebound and HBV DNA elevation can be detected within weeks of drug withdrawal [19]. New therapeutics, provided as add-on therapy to those whose viremia is already controlled with nucs, may prevent or significantly delay rebound of viremia and would be considered very beneficial. The value of an add-on new therapy could be relatively quickly determined in HBeAg-negative patients since the time to rebound following cessation of nucs is short.

Virologic Markers in Development

Even after a year of nuc-mediated suppression of HBV viremia by 5–7 logs, the amount of intrahepatic/intracellular viral DNA (replicative and cccDNA forms) is reduced by only 1–3 logs [20]. HBV cccDNA persists, and considerable viral replication continues even after effective nuc therapy. Thus, lack of viremia does not accurately reflect intrahepatic viral DNA load, and new tests are needed to more closely reflect intracellular viral load.

Measurement of cccDNA in the liver and blood

Reducing the amount, or silencing the transcriptional activity, of HBV cccDNA is a critical and valuable goal of therapy. Intrahepatic cccDNA levels have been shown to change over multiple CHB phases [20], with a nearly 1.0-log drop in levels following 1 year of adefovir–dipivoxil therapy. However, direct assessment of levels of cccDNA, without reasonably large amounts of liver tissue, is problematic, and international

standards and consensus protocols for handling and processing liver samples for cccDNA testing have yet to be developed. Development of assays to detect and quantify HBV cccDNA in blood face many hurdles but, if validated to accurately reflect cccDNA in liver, would be extremely useful for determining the effect of therapeutic drugs on cccDNA levels without need for liver biopsy.

HBV RNA, encapsidated and in the circulation

HBV RNA is packaged within nucleocapsids, and both truncated and full-length HBV RNA forms can circulate in patients with CHB [21]. Rapidly falling levels of serum HBV RNA during nuc therapy are an early predictor of HBeAg seroconversion [22]. In the presence of nucs, it is possible that encapsidated RNA will accumulate since synthesis of the negative-strand DNA is inhibited. The detection of encapsidated viral RNA in the blood during nuc therapy may reflect cccDNA transcription and thus cccDNA levels. Conversely, undetectable HBV RNA has been associated with a durable off-treatment sustained virologic response [23]. Unfortunately, there are no commercial assays for HBV RNA in serum and no international reference standard.

HBsAg epitope changes

HBsAg conformation and epitope availability are influenced by therapeutic and host antiviral immune pressures that lead to a clearing (or blocking) anti-HBs response, which targets the "a" determinant region of the HBsAg [24]. A multiplex immunoassay maps the HBsAg antigenic profile across the major hydrophilic region (MHR) using a panel of anti-HBs monoclonal antibodies [25]. Epitope mapping of the HBsAg [26] differentiated patients who demonstrated an HBsAg clearance profile (reduced recognition/availability at both loops 1 and 2 regions of the MHR) and patients with a nonclearance profile (ie, no change in epitope recognition or reduced antibody binding at only 1 epitope). These changes in epitope recognition predicted HBsAg loss and anti-HBs seroconversion ($P < .02$, positive predictive value 83%) [26]. Additional assays to detect coexisting anti-HBs have shown that complexed anti-HBs development coincides with HBsAg decline and HBsAg clearance profile detection [25]. These assays could provide useful viral biomarkers to predict HBsAg loss and anti-HBs seroconversion.

Immunologic Endpoints

At least 10 of the experiential drugs being developed for management of HBV are intended as immune modulators [1], so the ability to detect immunologic activation as a function of therapy is becoming increasingly important. The best natural outcome of HBV infection, that is, recovery from an episode of acute self-limited HBV infection, represents functional cure, rather than complete or virologic cure, and life-long protective immunity. However, due to the persistence of cccDNA in some hepatocytes, trace amounts of HBV DNA below the detection level of quantitative commercial assays appear sporadically in the blood [27]. These trace amounts of HBV stimulate and are controlled by HBV-specific antibody and T-cell responses [27]. In the presence of immune suppression, high-level viremia may occur [27]. Likewise, HBsAg vaccination induces protective rather than sterilizing immunity [28].

Although we now understand a lot about the nature of protective immune responses induced either by resolved acute HBV infection or vaccination, less is known about immune responses in different phases of CHB. Immune responses in CHB are not as static as in other virus infections, as shown by spontaneous seroconversion to protective anti-HBs status in some patients [27]. However, the role of innate vs adaptive immune responses in the natural history of CHB is not completely understood. Neonates who are born to HBV-infected mothers display an activated innate response of monocytes and natural killer cells [29], and the progression of CHB from the immune-tolerant to the immune-active phase may not be driven by HBV-specific adaptive immune responses but rather by age-dependent changes in inflammatory bystander activation [29]. These changes need to be better understood in order to use immunologic biomarkers to assess the antiviral mechanisms, effectiveness, and potential clinical side effects of new antiviral regimens, particularly those agents with an immunomodulatory component. At the same time, immunological biomarkers could be useful in detecting subtle changes in host immune responses that may represent essential steps along the path to cure.

Anti-HBs quantitation

Using current methods of detection, CHB patients usually have little to no free anti-HBs but do produce anti-HBs that are complexed with circulating HBsAg [30]. The relative abundance of free and bound anti-HBs in combination with genotype-specific quantitative assays for HBsAg [31] may be useful biomarkers for assessing early effects of new treatment regimes. Quantitative assays for anti-HBs may be adapted for endpoint analysis [32].

Anti-HBc (immunoglobulin M and total)

In CHB the type (immunoglobulin [Ig]G vs IgM) and amount of anti-HBc in serum may vary as a function of disease status, making this a possible marker of outcome [33]. Total anti-HBc (IgM and/or IgG) declined significantly in patients with CHB as a function of virologic responsiveness to Peg-IFN and polymerase inhibitor therapy ($P < .001$) and in nuc-treated patients ($P < .001$) [33]. The lowest levels of IgM anti-core were seen in long-term responders who eventually lost HBsAg.

T-lymphocyte markers

HBV-specific T-cell responses are dysfunctional in CHB and exhibit decreased proliferation, cytotoxicity, and cytokine production in in vitro recall assays [27]. This is associated with a

molecular signature of increased expression of inhibitory molecules such as PD-1, Tim-4, and CTLA-4 [34]. The relative roles of viremia vs circulating HBsAg and HBeAg in driving this phenotype are currently not known. Interestingly, HBeAg-negative status is associated with a higher prevalence of T-cell responses to HBV core and HBV polymerase [34]. Furthermore, spontaneous HBeAg and HBsAg clearance after acute HBV infection has been associated with increased expression of the interleukin-7 receptor on HBV-specific T cells [35], indicating antigen-independent proliferation of T cells in response to low levels of homeostatic cytokines and development of long-lived memory cells.

Pathological Endpoints

Liver disease fibrosis stage

The natural history of CHB infection is characterized by a necroinflammatory disease, leading to liver fibrosis, liver cirrhosis, and HCC [3]. Using serum aminotransferases and by liver histology, an important endpoint and validation of all HBV therapies was reduction in levels of inflammatory hepatitis and liver fibrosis. Noninvasive methods to assess liver fibrosis include transient elastography [36], and serum markers such as aspartate aminotransferase to platelet ratio, Fibrotest, and Enhanced Liver Fibrosis Test [37] have been shown to predict severity of liver fibrosis in HBV-infected patients but do not reflect liver inflammation. However, long-term studies of current noninvasive markers and new markers and assays for assessment of liver fibrosis are still needed.

Quantitation of HBV-infected cells

The number of HBV-infected or HBV antigen–expressing hepatocytes is likely to be related to successful virus suppression, host control of infection, and the probability of rebound following cessation of therapy. Being able to determine predrug, on-drug, and off-drug infected cell numbers would be a very useful marker of efficacy. There are currently no reliable methods to quantify infected cell number. Recent studies in hepatitis C have used laser capture microdissection (focused laser with a fully automated light microscope) to study single hepatocytes [38] and highly sensitive in situ hybridization systems to simultaneously detect viral genomes and mRNA levels of antiviral host genes [39] in HCV-infected livers. These techniques could be used to study virologic and immunologic responses in the liver of HBV-infected patients.

Repurposing existing markers

It is possible to creatively use available markers that are not currently used for evaluation of HBV therapies. Perhaps this is best dramatized in fibrosis and cancer risk assessment, where the use of algorithms in which multiple markers are combined together, weighting each marker for its relative contribution to risk [40, 41]. Composites of currently used markers could be configured into algorithms that predict the desired endpoints of therapy, such as sustained, off-drug, long-term reduction in liver fibrosis and HCC.

CONCLUSIONS

CHB is dependent on persistence of intracellular genomic forms of the viral DNA (cccDNA) and immune incompetence of the host [2]. CHB is a dynamic disease characterized by phases that differ clinically, virologically, immunologically, and pathologically. Better definition of markers to assess each phase of disease is needed so that the efficacy of a given experimental compound, when used alone or in combination therapy with the currently used medications, can be determined. Currently there are markers that can be used, but more work is needed to identify how to use these and new markers as endpoints of successful HBV therapy and what markers and drug(s) will be of most benefit at which phase of CHB. Potent therapeutic immune stimulators may be needed to achieve the best response to therapy along with direct-acting antiviral agents. Thus, the immune status of the patient may play a critical role in the response to different new direct-acting antivirals and immune modulators.

Notes

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