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Hepatitis B virus: From diagnosis to treatment

Virus de l'hépatite B : du diagnostic au traitement

P. Dény [a,b], F. Zoulim [a,c,d,*]

[a] *Inserm, U871, 69003, Lyon, France*
[b] *Laboratoire associé au centre national de références des hépatites B, C et delta, hôpital Avicenne, université Paris-13, 93009 Bobigny cedex, France*
[c] *Service d'hépatologie, hospices civils de Lyon, Hôtel Dieu, université Lyon-1, 69002, Lyon, France*
[d] *Université de Lyon , IFR62 Lyon-Est, 69008 Lyon, France*

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ABSTRACT

During the next few decades, vaccination against hepatitis B virus (HBV) will dramatically change the epidemiological profile of this worldwide infection especially when Heath Policies encourage including HBV vaccination program for the newborns. However, it is still estimated that more than 2000 millions living people have met HBV. Symptomatic hepatitis with jaundice is less frequent than asymptomatic infection; however, as much as 350 millions of individuals remain chronically infected by HBV. In these cases, the need for efficient antiviral therapy remains clear when a viral replication is observed to control the risk of progression and the need for liver transplantation, which represents the only end-stage treatment. Indeed, patients having chronic hepatitis B (CHB) can now be successfully treated using nucleos(t)ide analogs (NA) or pegylated interferon (PEG-IFN). Therefore, beside vaccination, prevention of the progression of the disease to cirrhosis and liver decompensation, leading to end-stage liver disease and/or to hepatocellular carcinoma, by inhibiting viral replication seems to represent the best approach to improve survival. At last but not least, co-morbidities and other viral infections, leading also to chronic liver cirrhosis or liver inflammation such as the specific satellite delta virus (HDV), human immunodeficency virus (HIV) and/or hepatitis C (HCV) virus, are able to accelerate the progression and have to be taken in account. Interestingly, in treated infection, the dogma of the irreversibility of the liver fibrosis, when the cirrhosis is constituted, is tumbling down. In this review, we will focus on the clinical, virological and therapeutic aspects of hepatitis B infection in order to expose the proposals to follow-up and treat HBV-infected patients and the prevention of drug-resistant HBV mutants that frequently arise, leading to treatment failure and progression to liver disease.

Mots clés:
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Génotype
Traitement
Résistance

RÉSUMÉ

Au cours des prochaines décennies, la vaccination contre le virus de l'hépatite B (HBV) changera profondément l'épidémiologie de cette infection mondiale, en particulier lorsque les politiques de santé publique encouragent d'étendre systématiquement la vaccination aux nouveau-nés. À présent, on estime que plus de deux milliards d'individus ont rencontré l'HBV. L'hépatite symptomatique aiguë avec ictère est moins fréquente que l'infection asymptomatique, cependant plus de 350 millions d'individus sont chroniquement infectés par l'HBV. Dans ce cas, la nécessité d'une thérapeutique efficace est claire en cas de réplication virale pour contrôler le risque de progression et la nécessité ultime d'une transplantation hépatique qui demeure le seul traitement en cas d'insuffisance hépatocellulaire terminale. En effet, les patients atteints d'hépatite B chronique peuvent à présent bénéficier de traitements par des analogues de nucléos(t)ides (NA) ou par de l'interféron pégylé (PEG-IFN). Ainsi, à côté de la vaccination, la prévention de la progression de la maladie – vers la cirrhose et la décompensation hépatique, entraînant une insuffisance hépatique terminale et/ou un carcinome hépatocellulaire – en inhibant la réplication virale, semble être la meilleure façon de restreindre la mortalité. Parallèlement, les co-morbidités et les autres infections virales susceptibles également de conduire à des maladies inflammatoires chroniques du foie ou à la cirrhose (comme l'infection satellite spécifique par le virus de l'hépatite delta [HDV] et/ou l'infection par le virus de l'hépatite C), ou

* Corresponding author.
E-mail address: fabien.zoulim@inserm.fr (F. Zoulim).

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d'accélérer la progression (comme le virus de l'immunodéficience humaine), doivent être prises en compte. De façon intéressante, au cours des infections traitées, le dogme de l'irréversibilité des lésions de cirrhose constituée s'estompe. Dans cette revue, nous nous centrerons sur les aspects cliniques, virologiques et thérapeutiques de l'infection par l'HBV dans le but d'exposer les propositions de suivi et de traitement des patients infectés, ainsi que la prévention des mutants de résistance aux médicaments qui, survenant fréquemment, conduisent à l'échappement thérapeutique et à la reprise de l'évolution de l'atteinte hépatique chronique.

1. Hepatitis B virus and humans

At least five viruses (A-E) are susceptible to lead to inflammation of the liver (hepatitis), and three of them account for chronic infection: hepatitis B virus (HBV), hepatitis C virus (HCV) and the HBV-satellite hepatitis delta virus (HDV). HBV might be present in human being since a very long time. On the one hand, assessment from molecular analyses on nonoverlapping parts of the genome, for the origin of HBV in hominoids primates, was 6000 years ago [1]. On the other hand, the existence of primates, mammals and avian hepatitis B-related viruses argues for a long coexistence with human beings. Indeed, HBV has a peculiar spreading strategy by being transmitted from generation to generation in endemic areas favoured by contamination in childhood, which in turn allows the viral infection to persist. It is estimated that one third of the world's population has serological evidence of past or present infection with HBV and that 350 to 400 million people are still chronically infected, of whom 78% lived in Asia, 16% in Africa, 3% in South America and the remaining 3% in Europe, North America and Oceania.

HBV infection has a broad spectrum of clinical diseases, ranging from acute hepatitis (including fulminant hepatic failure) to a low viraemic asymptomatic ''inactive'' carrier state or to progressive chronic hepatitis, which may lead to cirrhosis with an annual rate of 2 to 5% in HBe-positive patients and hepatocellular carcinoma (HCC) with a cumulative 5-year incidence of 15 to 20% [2]. Both HBV-related end-stage liver disease and HCC are responsible for around 1 million deaths per year [3–6]. Therefore, patients with complicated cirrhosis require urgent antiviral treatment. Significant clinical improvement can be associated with control of viral replication, but patients with very advanced liver disease should also be considered for liver transplantation [7]. Host and viral factors, in addition to coinfection with other viruses, in particular HCV, human immunodeficiency virus (HIV) or the satellite HDV, together with other co-morbidities including alcohol use and exposure to aflatoxin B1, can affect the natural course of chronic HBV infection, carcinogenesis and efficacy of antiviral strategies.

2. Clinical data

2.1. Contamination and incubation time

Contamination occurs by parenteral routes for HBV, HCV and HDV. Beside blood contamination efficiently performed for these viruses, sexual or childhood transmissions are frequently observed for HBV and HDV and neonatal transmission is also a major risk factor in endemic countries for HBV.

After contamination, the incubation time occurs for 2 to 3 months (range 1–6 months) before the occurrence of liver hepatitis. Depending on the intensity of immune response against foreign viral antigens expressed in liver cells, hepatitis ranges from mild to severe. In the liver, features of hepatocellular necrosis are often associated tentatively explained by a viral direct cytopathic effect.

The incubation period is followed by a prodromal period of less than 2 weeks with mild fever, fatigue, anorexia, nausea, abdominal discomfort and body aches; rarely an eruption occurs.

2.2. Acute hepatitis

Indeed, in 60% to 80% cases, acute HBV infection is clinically asymptomatic and patients have a mild subclinical illness with a liver cytolysis syndrome, based on a rise in the level of transaminase enzymes: serum alanine aminotransferases (ALAT) and aspartic acid aminotransferases (ASAT) associated with high levels of HBsAg and HBV DNA. Mild symptoms also include fatigue and nausea. In less than one third of cases, jaundice associated with dark urine can occur together with a high level of glycuroconjugated bilirubin. After the acute phase which may last for 1 to 2 weeks, asthenia can persist for several months while HBsAg is cleared followed by the disappearance of serum detectable HBV DNA by PCR.

A severe liver failure may occur in less than 1% of symptomatic jaundice hepatitis. In such cases, the sudden appearance of fever, abdominal pain, vomiting and jaundice is followed by neurological symptoms characteristic of hepatic encephalopathy described as stage I: flapping tremor and disorientation, stage II: confusion and stage III: coma. Associated to the liver failure, HBsAg and HBV DNA levels fall rapidly and may become undetectable in some patients with hepatic coma. In such patients, access to a medical center with the availability of liver transplantation is required.

2.3. Chronic hepatitis

HBV are classically described as wild type (wt) viruses: virus able to express the HBeAg, (a soluble protein observed during wt viral replication) and PreC/C mutant virus (virus unable to express the HBe protein). Natural history of chronic hepatitis B without therapy has been recently described through five phases, which are displayed in Fig. 1. A classical definition of chronic HBV carriage includes the positivity of the surface HBsAg detection in the serum for more than 6 months.

2.3.1. The "immune tolerant" phase

This phase is characterized by high levels of HBV replication, normal levels of aminotransferases, mild or no liver necroinflammation and no or slow progression of fibrosis. During ''WT'' HBV infection, the rate of spontaneous HBeAg positivity loss is very low. This phase occurs for a prolonged time in subjects infected perinatally or when infection is acquired in early childhood.

2.3.2. The "immune reactive phase"

During this period, which accounts for several months to several years, a lower level of replication is usually recorded. By contrast, moderate to severe liver necroinflammation and rapid progression to fibrosis can occur. For WT viruses, the rate of

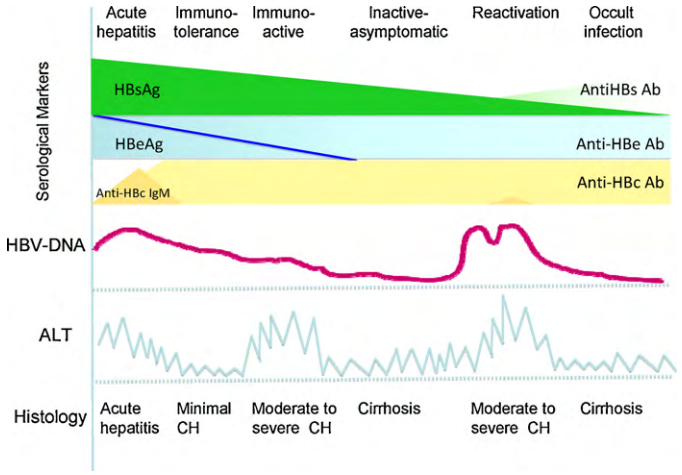

Fig. 1. Natural course of chronic hepatitis B infection. CH: chronic hepatitis.

spontaneous HBeAg loss is enhanced but may occur when fibrosis has already developed. This phase may follow a long period of immune tolerance and is more frequently reached in subjects infected during adulthood.

2.3.3. The "asymptomatic (inactive) hepatitis B virus carrier state"

It may follow seroconversion from HBeAg to anti-HBe antibody; it is characterized by low or even undetectable serum HBV DNA and normal aminotransferase levels. As a result of immunological control of the infection, this state is associated with a favourable long-term outcome with a very low risk of cirrhosis or HCC in the majority of patients. Furthermore, HBsAg loss and seroconversion to anti-HBs antibody may occur spontaneously after several years while HBV DNA is persistently undetectable.

2.3.4. "HBeAg-negative chronic hepatitis"

It may follow the seroconversion from HBeAg to anti-HBe antibody during the immune reactive process and represents a turn in the CHB history. It is characterized by periodic reactivation of the viral replication with a pattern of fluctuating levels of HBV DNA and aminotransferases and active hepatitis (Fig. 2). HBeAg is not detectable due to either nucleotide mutation inducing a stop codon in the pre-C sequence interrupting the synthesis of HBe protein, and/or substitutions in the basal core promoter region leading to the expression of low levels of HBeAg. HBeAg-negative CHB is associated with low rates of prolonged spontaneous disease remission. How to distinguish true asymptomatic inactive HBV carriers from patients with active HBeAg-negative CHB in whom phases of spontaneous remission may occur? The former patients have a good prognosis with a low risk of complications, while the latter patients have active liver disease with a high risk of progression to cirrhosis and subsequent complications. It is suggested that a follow-up of at least one year controlling serum ALAT and HBV DNA levels every 3 months usually will detect fluctuations of activity in patients with active HBeAg-negative CHB [8].

2.3.5. In the "HBsAg-negative phase"

After HBsAg loss, HBV DNA persists in the liver and low level HBV replication may occur [9,10]. Generally, anti-HBc antibodies are clearly detectable with or without anti-HBs and HBV DNA in the serum is very rarely detectable and sometimes needs "rolling circle amplification" [11]. HBsAg loss is associated to outcome improvement with reduced risk of cirrhosis, decompensation and HCC. The clinical relevance of occult HBV infection (detectable HBV DNA in the liver with low level (< 200 international units (IU)/ml), of HBV DNA in blood) is unclear [9]. Immunosuppression may lead to severe reactivation in these patients and in patients with resolved infection [12,13].

3. Diagnosis and complementary exams

Schematically, diagnosis and biological follow-up of HBV infection are based on complementary analyses based on virological and immunological markers (Figs. 2–4) and assessment of liver morphology and functions.

Direct virological markers

HBs Ag:
coded by *S* gene, 3 forms, S-HBs, M-HBs, L-HBs
present in acute and chronic HBV
HBc Ag:
coded by *C* gene, capsid
masked in the serum, present in the liver
HBe Ag:
coded by *Prec/C* gene
wt seric soluble protein, replication
HBV DNA:
partially double stranded circular DNA
Viral load, genotyping, resistance mutations

Humoral immunity

Anti-HBc:
Detectable early, underlines HBV infection
Anti-HBc IgM:
Acute HBV infection, Chronic HBV reactivation
Anti Hbe:
wt decrease of replication, Hbe-negative chronic hepatitis
Anti-HBs:
Past or occult infection, vaccination

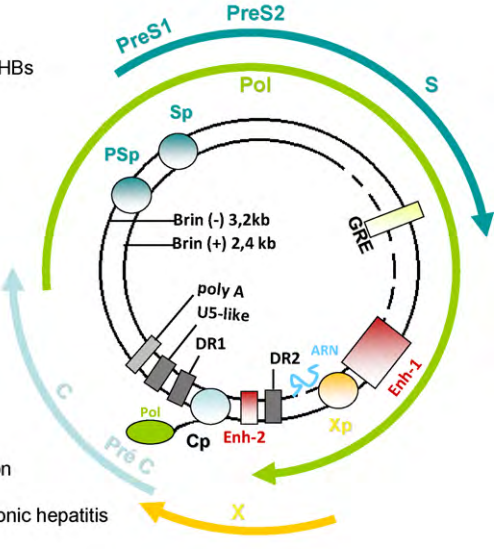

Fig. 2. Markers of viral infection include direct virological markers such as viral proteins coded by the viral genome and the hepatitis B virus DNA; indirect markers are based on humoral immunity. On the right, is a schematical diagram of the regulatory sequences and the open reading frames of the viral genome.

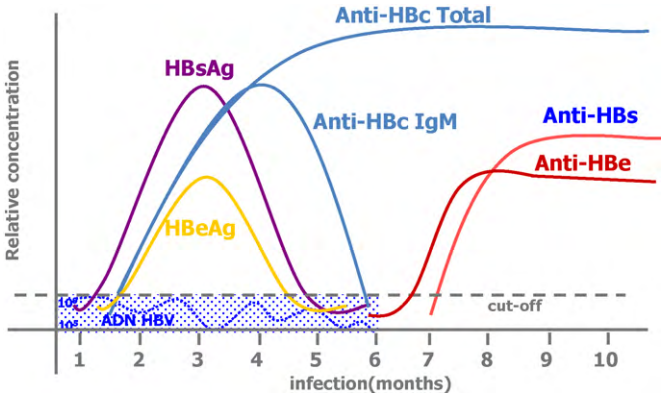

Fig. 3. Evolution of serological markers during resolved acute hepatitis B virus infection.

3.1. Virological approaches

3.1.1. Primary diagnosis

It relies on serological markers of HBV infection including the search for HBsAg in the serum and the antibodies reacting against the capsid antigen (HBcAg) bear by the capsid proteins, which are masked in the serum as they are embedded into the HBs-positive viral envelope. If both results are negative, there is no argument to sustain a WT HBV infection. If both are positive, acute or chronic infection can be differentiated by clinical history and anti-HBc IgM (Fig. 3); however, the detection of IgM anti-HBc is not synonymous of acute HBV infection and, especially at low level, might assess viral reactivation during chronic infection. If anti-HBc Ab are positive and HBsAg undetectable, antibodies against HBs can be useful: if HBsAg is negative and both anti-HBs and anti-HBc are positive, this would in theory reflect a "past infection", however an "occult infection" with a very low level of HBV DNA might also be suspected. A scheme of the viral markers detected is presented in Fig. 2. Modulation of this scheme can occur during occult and/or HBs-negative variants infections (Fig. 4).

3.1.2. Hepatitis B virus (HBV) DNA detection and HBV DNA level measurement

They are essential especially for treatment indication and monitoring, and to explore a viral reactivation. Follow-up using real-time polymerase chain reactions (RTq-PCR) quantification assays are now widely used because of their sensitivity, specificity, accuracy, broad dynamic range and positive predictive values. The World Health Organization (WHO) has defined international standards, of whom sample 97/746 was assigned a potency of 10E6 IU/ml [14]. However, because all commercially available or homemade RTq-PCR do not use the same HBV oligodeoxynucleotide primers, follow up of patients should still relies on the same technical approach in order to dynamically compare viral DNA load evolution.

3.1.3. Search for other viral infections

It is crucial and should include search for anti-HDV, anti-HCV and anti-HIV antibodies. If one or several co-infections are highly suspected, specific virological tests including confirmatory approaches (such as western blot for HIV) and viral loads should be assessed.

Schematically, the satellite HDV infection will be confirmed on HBs-positive sample by antidelta antibody and viral RNA detections in the serum or less frequently by the evidence of hepatitis delta (HD) antigens in the liver [15]. In HBsAg-positive carriers, it is important to check the status of HDV at least once in the early diagnosis time or in case of aggravation.

3.2. Hepatitis B virus genetic variability and clinical practice

3.2.1. Hepatitis B virus classification

Serotyping of viral particles, based on the antigenicity of the extracellular loop of the HBsAg had been useful for epidemiological studies, including studies of hospital-acquired or intrafamilial transmission. Briefly, serotyping classification comprises the major "a" determinant (residues 124–147) and relies on major subtype positions for the "d/y" (K122R) and "r/w" (K160R) determinants. However, there is no strict correlation between serotyping and genotyping classification, which also follows a geographical distribution [16]. HBV genetic variability results of several factors. During viral replication, the intracytoplasmic reverse transcription step of the viral polymerase introduces errors that are not corrected due to the lack of a 3' to 5' exonuclease activity leading to mutations. In a chronically infected patient, more than 10E11 viral particles are produced per day and viral polymerase introduces noncorrected errors every 10E4 to 10E5 nucleotides. However, all mutations do not lead to replication-competent clones. In fact, due to the genetic complexity of the overlapping open reading frames, encompassing regulatory and structural

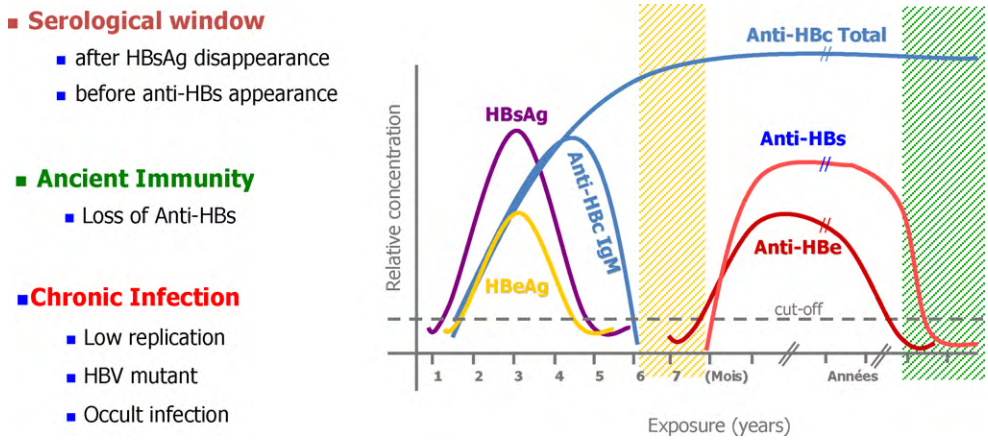

Fig. 4. Significance of anti-HBc antibodies detected alone. Anti-HBc can be detected during serological window after acute infection but can also reflect ancient immunity after a possible loss of anti-HBs antibodies. One the other hand, anti-HBc alone are sometimes found during chronic hepatitis B virus infection.

regions, the replication strategy of the HBV genome would block the majority of mutations to maintain [17]. On the other hand, even if the mutations arise from a replication competent clone, it can be maintained and propagated only if the mutated DNA is further included in the ccc DNA pool by infecting a new hepatocyte or replacing ancient ccc DNA molecules [18]. Therefore, in a chronically infected patient, several HBV distinct variants with a clear phylogenetic relationship coexist, joining the quasispecies concept of the viral RNA world.

3.2.2. Hepatitis B virus genotypes and subgenotypes

Eight different genotypes, labelled by alphabetical letters from A to H, have been previously described. Since the proposal of the four described genotypes (A–D) [19], four others (E–H) have been characterized during the two last decades [16]. Recently, a ninth "genotype" evidenced in North-West China [20], India [21], Lao [22] and Vietnam [22–24] and tentatively termed 'I' was suggested, although it is still subject to debate [1,25] as being a recombinant strain with a genotype C backbone. Finally, very recently, a tenth genotype provisionally assigned to genotype 'J' was proposed for a Japanese patient's HBV isolate [26].

Regarding indels, genotype-specific HBV genomes have sizes ranging from 3182 nt (HBV/D) to 3248 nt (HBV/G). This has consequences on the size of the viral proteins. In summary, the consensual "genotype" definition is based on the dissimilarity of the complete nucleotide sequence of more than 7.5% for strains from different genotypes; and of less than 7.5% for strains of the same genotype [27]. Methods to determine the viral genotype are based on hybridization and sequencing, and genotype affiliation relies on phylogenetic analyses.

Among various areas of the world, HBV genotype evolution had lead to different variants called "subgenotypes". Dogmatically, a subgenotype is suspected if intragenotypic dissimilarity is higher than 4%, for complete nucleotide sequence. Genotypes and subgenotypes might also be associated to specific geographic distributions reflecting ancient evolution [16]. However, as the patients might be chronically infected, this picture will evolve due to human migrations.

Furthermore, recombination events will complexify the HBV classification; indeed more than 42 full length complete sequences of HBV recombinants are now described in databases; and in some specific area, the recombinant strain represents the dominant variant such as the HBV/CD recombinant in Tibet [28]. In specific area, high endemy and coexistence of different genotypes in borders will favour a recombination process such as the recently described HBV/DE recombinant in Niger [29].

3.2.3. Hepatitis B virus genotyping and pathogenic power

Whether or not a specific viral genotype/subgenotype is linked to a specific pathogenic power or treatment sensitivity is a field of active research. Some emerging features have to take into account the genetic background and cofactors such as nutrition, alcohol and exposure to carcinogens (for example aflatoxin B). At last but not least, systematic search for coinfecting viruses such as HIV and HCV, and the satellite delta virus, has also to be taken in account when comparing the severity of the liver progression among cohorts. Finally, the recombination process will also be an important point to be followed in the near future because emerging strains might take benefit of the high possibility of recombinaison to resist to the only antiviral class of drugs or to escape from vaccine.

A lot of studies have explored a possible link between on the one hand, the HBV genotype and on the other, the severity of the liver disease and/or the profile of treatment response [16,30]. However, there are still conflicting results. Another important point relies on the fact that 78% of chronic carriers live in Asia. This high percentage might by itself contribute to a high number of severe cases in this part of the world where genotype B and C are predominant. From a French cohort of the Seine Saint-Denis district in the North-East of Paris (France), comparison among patients infected with genotypes HBV/A–E suggested that, genotypes HBV/A, /C and /D seemed to be more frequently associated to severe forms of the illness than genotypes B and E [31]. In the countries where genotypes A and D coexist, it has been suggested that patients infected by genotype A might evolve more rapidly during chronic infection than those infected with genotype D [32]. It is also true that there are more often severe forms of liver disease in Asia where genotype B and C are prevalent. Furthermore, different studies seem to sustain that patients infected with genotype C would progress to cirrhosis and liver cancer earlier than those infected by genotype B at the age of 30; however at the age of 45 years, the same proportion of patients have evolved to cirrhosis and liver cancer whatever the genotype was [33]. Other studies in the Amazonian basin indicated that the F genotype was frequently associated to severe acute hepatitis [34,35]. In such cases, most of hospitalized patients where co- or superinfected by the hepatitis delta virus genotype 3 (HDV3). Whether or not, the severity is attributed to the B or delta genotype is discussed. Indeed, it has been suggested that patients infected with genotype F might have a higher mortality rate than those infected with genotype A or D; however, in a retrospective study of the "Labrea black fever", HBV genotypes F, A and D were found in 50.0, 28.6 and 21.4% from liver samples from 14 patients who developed fulminant hepatitis and died during 1978–1989, respectively. Phylogenetic analyses of HDV sequences showed that they all clustered with previously characterized sequences of HDV genotype 3 (HDV 3) [36].

In contrast, several authors have found no link between HBV genotypes and the severity of hepatitis. For example, a study conducted in Uzbekistan didn't demonstrate any difference of the severity of the liver histology between infections by HBV genotype D and A [37]. Another recent study considering all case of liver damage (asymptomatic HBsAg carriers, acute or chronic hepatitis, cirrhosis and hepatocellular carcinoma) also found no concrete link between genotype A or D and the severity of the hepatitis or response to treatment [38].

In summary, there is not yet enough clear data to attribute to a specific genotype a clear severity predictive value. It is obvious that the intragenotypic heterogeneity needs also to extend investigations on the research for explanation of the observed differences in function of genotypes/subgenotypes. There is increasing evidence that in Asia, genotype C might be more frequently associated to HCC than genotype B [39]. It is also important to evaluate these genotypes in function of the therapeutic response as they could represent one of the predictive criteria. In a trial of PEG-IFN, patients infected with genotypes A and B had a higher rate of HBeAg loss (about 45%) as compared to patients infected with genotype C or D (about 26%) [40]. On the other hand, in a Japanese study, while genotype B and C carriers responded well to interferon treatment, genotype A carriers responded poorly [41].

3.3. Study of the liver morphology and function

The assessment of the severity of the liver disease should include: biochemical markers, including aspartate aminotransferase (AST) and ALT, gamma-glutamyl transpeptidase (GGT), alkaline phosphatase, prothrombin time and serum albumin; blood counts; and hepatic ultrasound. Usually, ALT levels are higher than those of AST. A progressive decline in serum albumin concentrations and

prolongation of the prothrombin time, often accompanied by a drop in platelet counts, are characteristically observed after cirrhosis has developed.

A liver biopsy is recommended to determine the degree of necroinflammation and fibrosis in patients with either increased ALT or HBV DNA levels greater than 2000 IU/ml (or both) since hepatic morphology can assist the decision to start treatment. Biopsy is also useful for evaluating other possible causes of liver disease such as steatosis or steato-hepatitis. Although liver biopsy is an invasive procedure, the risk of severe complications is very low (1/4,000–10,000) [42]. It is important that the size of the needle biopsy specimen be large enough to precisely analyse the degree of liver injury and fibrosis. A liver biopsy is usually not required in patients with clinical evidence of cirrhosis or in those in whom treatment is indicated irrespective of the grade of activity or the stage of fibrosis. There is growing interest in the use of noninvasive methods, including serum markers and transient elastography, to assess hepatic fibrosis to complement or avoid a liver biopsy [43].

4. Inducing a treatment for hepatitis B virus infection

4.1. Therapeutic goals

By contrast to acute HIV and HCV infection, a treatment for acute HBV infection has to be studied in terms of interest, efficacy and tolerance [44]. In adults, in contrast to acute HCV infection, less than 5% of acute HBV hepatitis infections will progress to chronic carriage. The distinction between acute HBV hepatitis and chronic hepatitis B with an acute flare has recently been reevaluated; of several diagnostic combinations, IgM anti-HBc jointing HBV-DNA is most effective and most practicable in distinguishing acute from chronic hepatitis B with flares [45].

Concerning chronic hepatitis, the major goals of anti-HBV therapy are to prevent the development of progressive disease, specifically cirrhosis and liver failure, as well as hepatocellular carcinoma development and subsequent death [46]. However, the currently available antiviral drugs are unable to eradicate HBV infection because of both the defective immune response against infected hepatocytes and the persistence of HBV covalently-closed circular DNA (ccc DNA) in the liver of infected patients [47]. Goals of treatment are to suppress viral replication to the lowest possible level, and thereby to decrease the progression of liver disease and to prevent the onset of complications. The optimal endpoint of therapy is sustained HBsAg loss with seroconversion to anti-HBs. This may indicate immune protection preventing viral relapse in the majority of patients. The registered drugs currently available for treatment are divided into two main groups: immunomodulators which include interferon (IFN) alpha and PEG-IFN, and NA such as lamivudine (LMV), adefovir dipivoxil (ADV), entecavir (ETV), tenofovir (TDF), and telbivudine (LdT). These approved therapies are associated with improvements in biomarkers, including HBV DNA, HBeAg loss or seroconversion, decreases in ALT levels, and improvement in liver histology. Furthermore, recently introduced antiviral agents consistently produce rapid and dramatic decreases in viraemia which allow the challenge of HBV DNA undetectability. Thus, the next step will be to achieve HBsAg clearance; this will contribute to prevent disease progression and antiviral drug resistance, and help to stop treatment. HBsAg clearance can be achieved in 3 to 7% of patients 6 months after PEG-IFN treatment and this rate increases during the post-treatment follow-up in responder patient. HBsAg loss can also be obtained in patients receiving NA; the most promising results have been obtained with TDF, since 3% of HBeAg-positive patients cleared HBsAg after 96 weeks of TDF [48].

Theoretically, HBsAg loss may result of the clearance of infected cells or the decrease of transcriptionally active ccc DNA in episomal-bearing hepatocytes. Data indicate that intrahepatic HBV total HBV DNA and ccc DNA levels at the end of therapy are better than serum HBV DNA to predict sustained virologic response [49]. Several studies also showed a correlation between intrahepatic ccc DNA and quantification of serum HBsAg. Since ccc DNA is likely to persist after HBsAg seroconversion, immunological control of infection might also become an alternative objective. Such restoration of specific CD4 and CD8 T cell responses might also be induced by targeted immune therapy. There are interesting data showing that innate and adaptative immune response may be critical to control viral replication and may perhaps be used as alternative endpoints.

4.2. Indication of an antiviral therapy

Patients should be considered for treatment when the serum ALT levels are raised above the upper limit of normal (ULN) for the laboratory and/or HBV DNA levels are above a critical value, and liver histology shows moderate to severe active necroinflammation and/or fibrosis. HBV DNA values are constantly being revised and should be set at a lower level for older patients who may have been infected for a longer period of time [50]. Indications for treatment must take into account age, health status, and availability of antiviral agents in individual countries.

Thus, patients with deteriorated cirrhosis require urgent antiviral treatment. Rapid and profound viral suppression and efficacious prevention of resistance are very essential. Significant clinical improvement may happen with control of viral replication, but patients with very advanced liver disease and with no benefit should be considered for liver transplantation. Patients with cirrhosis and detectable HBV DNA may be considered for treatment, even if ALT levels are normal. Patients with slightly elevated ALT (less than two times ULN) and mild histologic lesions (less than A2F2 with Metavir scoring) may not require therapy, however, follow-up is mandatory. Most patients, being in the immunotolerant phase, under 30 years of age with persistently normal ALT levels, a high HBV DNA level (usually above 10^7 IU/ml) and without any suspicion of liver disease, and without a family history of HCC or cirrhosis do not require immediate liver biopsy or therapy. Here again, follow-up is strategic [51].

4.2.1. Interferon alpha-based treatment

Interferon and its pegylated forms are licensed for chronic hepatitis B treatment. The main theoretical advantages of IFN alpha (conventional or pegylated) are the absence of resistance and the potential for immune mediated containment of HBV. Frequent side effects are the main disadvantage of IFN alpha treatment. IFN alpha is contraindicated in patients with deteriorated HBV related-cirrhosis, severe depression, or autoimmune disease. Pretreatment factors predictive of HBe seroconversion are low viral load (HBV DNA $< 10^7$ IU/ml), high serum ALT levels (greater than three times ULN), high activity scores on liver biopsy such as A2 in the Metavir scoring system [52]. It has also been suggested that HBV genotype A and B might be associated with a better response to IFN alpha than genotypes C and D [53]. It is a goal to reach a decrease in HBV DNA to less than 20,000 IU/ml at 12 weeks as there is a 50% chance of HBe seroconversion in HBeAg-positive patients and of sustained response in HBeAg-negative patients [54].

4.2.2. Antiviral agents such as analogues of nucleoside (lamivudine, entecavir, telbivudine) or nucleotide (adefovir, TDF) treatment

ETV and TDF are potent HBV replication inhibitors and have a high barrier to resistance [55,56]. Thus, each drug is now suggested

as first-line monotherapy. The choice of monotherapy with ETV or TDF could be modified if higher rates of resistance arise with longer treatment duration.

Factors predictive of HBe seroconversion are pretreatment low viral load (HBV DNA $< 10^7$ IU/ml), high serum ALT levels (greater than three times ULN), high activity scores on liver biopsy (at least A2).

During treatment with LMV, adefovir or LdT, a virological response at 12, 24 or 48 weeks (undetectable HBV DNA in a real-time PCR assay) is associated with a lower incidence of resistance and with HBe seroconversion in HBeAg-positive patients. Nowadays, there is no evidence that HBV genotype might influence the response to some NA [57].

The detrimental effect of HBV drug resistance on clinical outcome was established by a placebo-controlled trial of LMV in patients having advanced fibrosis [58]. Patients successfully treated with LMV who maintained WT HBV had a significantly lower risk of liver disease progression compared to those who received placebo. This effect was lost in patients that developed LMV-resistant mutant forms of the virus [58]. The kinetic of emergence of resistance to ADV, which are typically slower than those of LMV, follows the same sequence of events: polymerase variants with the specific resistance mutations can be detected initially, this is next followed by virologic breakthrough and then rising serum levels of ALT. In some cases, the emergence of ADV resistance was also associated with acute exacerbation of disease and liver failure [59].

4.3. Resistance to nucleoside and nucleotide analogs

4.3.1. Incidence and prevalence of resistance

Among the different NA available for CHB treatment, the lowest incidence of resistance corresponds to TDF and ETV; the latest in treatment-naïve patients. Recent trials of TDF reported that no resistance had developed by weeks 48 and 96 of treatment, although at week 72, the majority of viremic patients were given Truvada®, which is a combination of TDF and emtricitabine [55]. Very low rates of genotypic resistance to ETV have been reported in naïve patients after more than 6 years of therapy (Fig. 6). In contrast, in patients previously treated with LMV, having HBV-infecting strain bearing LMV-associated resistance, the cumulative genotypic resistance rates for ETV increase to almost 60% by year 6 [60].

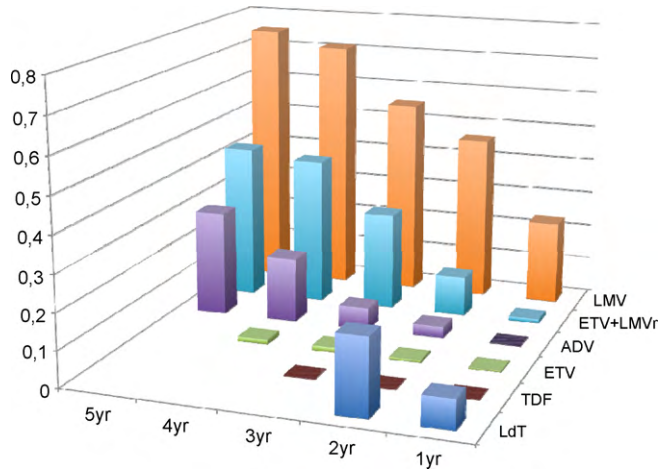

Fig. 5. Percentage of resistance isolates in clinical practice during several years of therapy. LMV: lamivudine, LMVres: LMV-resistant strain, ETV: entecavir, ADV: adefovir, TDF: tenofovir, LdT: telbivudine.

LMV resistance increases progressively over the course of treatment: 14% to 32% of patients become resistant to the drug each year after treatment was initiated and more than 80% are resistant after 48 months of treatment. The rate of emergence of LdT-resistant HBV is lower than that of LMV, but is still substantial. In a phase III trial that compared LdT with LMV, genotypic resistance occurred in 4.4% and 21% of HBeAg-positive patients after 1 and 2 years of treatment, respectively, and 2.7% and 8.6% in HBeAg-negative patients [61]. The rate of selection for ADV-resistant virus occurs in approximately 2% of patients with HBeAg-negative CHB after 2 years of therapy. However, following 4 to 5 years of ADV monotherapy, up to 30% of patients are found to be infected by resistant-viruses [59] (Fig. 5). When ADV has been used in patients that are resistant to LMV, primary ADV resistance detected by genotype analysis has been found in up to 20% of patients by 12 months after ADV therapy began.

4.3.2. Mutations in the polymerase gene that confers resistance-associated substitutions in the HBV-reverse transcriptase

The common mutations that confer resistance to LMV and LdT (eg: rtM204 V/I ± rtL180 M) confer cross-resistance to other L-nucleosides and reduce sensitivity to ETV but not to ADV or TDF. Conversely, mutants that are resistant to ADV (eg: rtN236T) and TDF generally remain sensitive to L-nucleosides and ETV. Both the L-nucleosides (LMV and LdT) and alkyl phosphonates (ADV and TDF) also select for the mutation rtA181T/V, thereby indicating a multidrug resistance-associated substitution. Multiple mutations (eg: rtA184A/A/I/L; rtS202G/L; rtM250I/V), in addition to those that confer resistance to LMV and LdT (rtM204 V/I ± rtL180 M) are required for high-level resistance to ETV. Cross-resistance across NA groups (eg: rtA181T) (Fig. 6) might eventually be overcome by development of drugs that block other stages of the viral life cycle. However, such drugs are unlikely to become available for clinical use in the near future. Thus, it is important to understand the molecular mechanisms of NA resistance, to optimize their use.

4.3.3. Resistance-associated substitutions pathways

At least eight codons in HBV polymerase are associated with primary drug resistance to NA: 169, 180, 181, 184, 202, 204, 236, 250. These eight codons have been shown to be involved in HBV antiviral drug resistance via different pathways [62,63]:

- the rtM204 V/I pathway for L-nucleosides;
- the rtN236T pathway for alkyl phosphonates;
- the rtA181T/V pathway which is shared between the L-nucleosides and alkyl phosphonates;
- the D-cyclopentante/entecavir pathway (rtL180M+rtM204V+I169T+T184S/G/C+S202C/G/I+M250I/V).

The first three pathways are associated with only one mutation whereas the fourth pathway requires at least three mutations for resistance. These resistance-associated substitutions pathways facilitate understanding HBV evolution during NA therapy, and can be used to predict patient outcomes and improve our understanding of cross-resistance patterns and profiles.

4.3.4. Multidrug resistance

Due to the overlapping of polymerase and envelope genes in Hepatitis B Virus (HBV) genome, nucleoside analog therapy can lead to the emergence of complex HBV variants that harbor mutations in both the reverse transcriptase and the envelope proteins. To understand the selection process of HBV variants during antiviral therapy, we analyzed the in vitro fitness (the

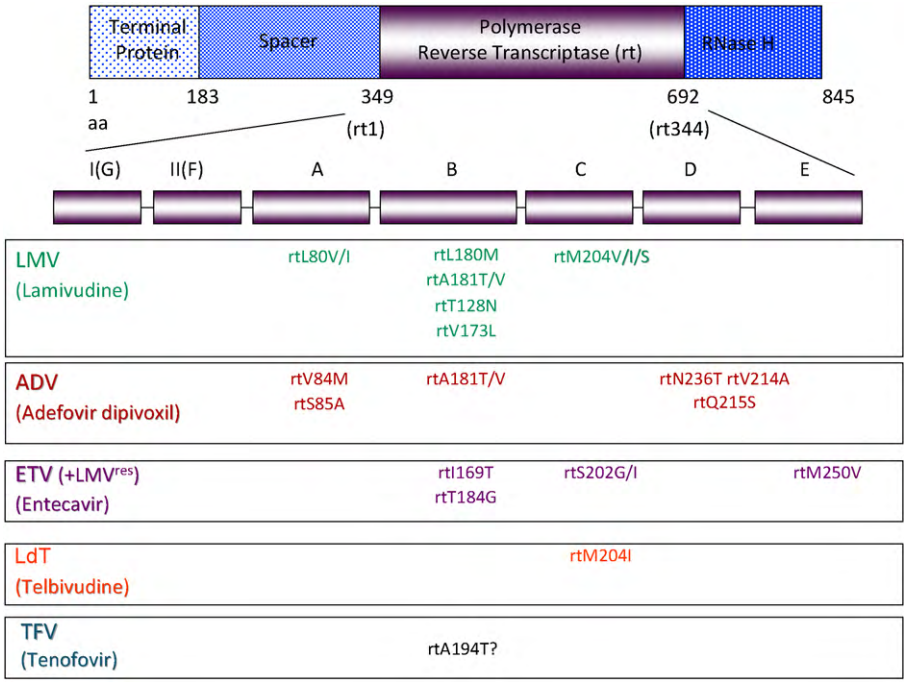

Fig. 6. Resistance-associated mutations in the reverse transcriptase of hepatitis B virus for the different nucleoside and nucleotide analogs (NA).

ability to produce infectious progeny) of four mutant viral genomes isolated from one patient who developed resistance to a triple therapy (LMV, ADV and anti-HBV immunoglobulins [63]. The dominant HBV variant that emerges in the long run was found to have the best replicative capacity in vitro in the presence of high concentrations of LMV and ADV. The expression of envelope proteins and secretion of subviral and Dane particles by this mutant were equal to that of WT HBV. HDV particles enveloped by surface proteins from the selected mutant had the highest rates of infection in HepaRG cells, compared with other mutants. These results illustrate the importance of viral fitness and infectivity as major determinants of antiviral therapy resistance in HBV-infected patients.

Indeed, sequential monotherapy can promote selection for multidrug resistant (MDR) strains of HBV, especially when patients are sequentially treated with drugs with congruent characteristics, such as with LMV followed by ETV or LMV followed by ADV. Clonal analyses indicated that MDR usually occurs via the sequential addition of resistance mutations to the same viral genome; mutants that arise from this selection process have full resistance to both drugs. Studies have shown that MDR strain arises if an 'add-on' therapeutic strategy does not result in rapid and complete viral suppression.

Some specific single mutations confer MDR. This was shown with the rtA181 V/T substitution which is responsible not only for decreased susceptibility to the L-nucleosides LMV and LdT, but also to the alkyl phosphonates ADV and TDF [64,65]. This emphasizes the need for genotypic testing in patients with treatment failure to determine the resistance mutation profile and adapt therapy to the major viral strain circulating in the patient [16].

Understanding HBV mutant selection phenomena will help to optimize new anti-HBV therapeutic strategies. Studies of the antiretroviral agents used to treat HIV have shown that drug resistance testing is useful to monitor response to therapy and help in the selection of new drug regimens for patients who have failed to respond to antiviral therapy. Since factors other than drug resistance (for example, poor patient compliance and/or pharmacogenomic factors) can affect viral load, it cannot be automatically assumed that a rising load is indicative of drug resistance. Therefore, a careful clinical, virological and pharmacological follow-up is mandatory to specify the best therapeutic approaches that will ultimately lead to the best possibility for a patient to prevent the life-treatening liver complications and to draw HBV infection to a sustain decent course.

Conflicts of interest statement

F.Z. has received consulting and speaker honoraria from Gilead Sc, Bristol Myers Squibb, Novartis, Roche, and received research support from Gilead Sc and Roche. P.D. has received speaker honoraria from Gilead Sc, Bristol Myers Squibb, Novartis, Abbott, and received research support from Diasorin and Altadis.

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