

VIROLOGY: VIRAL REPLICATION AND DRUG RESISTANCE

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Links to: Chapter 7: Treatment of chronic hepatitis B virus infection

KEY POINTS

- The two key events in the viral life cycle of the hepatitis B virus (HBV) are the generation from genomic DNA of the covalently closed circular DNA transcriptional template and the reverse transcription of the viral pregenomic RNA to form the HBV DNA genome.
- Since the virus employs reverse transcription to copy its genome, mutations in the viral genomes are frequently found. Particular selection pressures, both endogenous (host immune clearance) and exogenous (vaccines and antivirals), readily select out escape mutants.
- The introduction of nucleoside/nucleotide analogue therapy has seen the emergence of drug resistance as the major factor limiting drug efficacy.
- The development of drug resistance is not unexpected if viral replication continues in the setting of ongoing treatment, especially monotherapy.
- Prevention of resistance requires the adoption of strategies that effectively control virus replication.

Introduction and pathogenesis

The hepatitis B virus (HBV) is a highly evolved pathogen and, under normal circumstances, viral infection and subsequent replication within hepatocytes is not cytopathic. Thus, the replication of the virus in hepatocytes in the liver does not directly cause cell death. The liver damage associated with acute or chronic hepatitis B (CHB) occurs mainly as a result of attempts by the host's immune response to clear HBV from infected hepatocytes.¹ In particular, it is the adaptive immune response arm, the CD4+ and CD8+ T-cells responding to HBV antigens on virus-infected cells, and not the virus directly, that causes most of the liver damage in CHB.

The HBV is a member of the family *Hepadnaviridae*. The hallmarks of viruses of the *Hepadnaviridae* family include a DNA genome, which is copied by a virus-specific reverse transcriptase (rt), and the production of excess viral coat/envelope material, the hepatitis B

surface antigen (HBsAg). The important point to note is that viral reverse transcriptases lack a proof-reading capacity and so, by using reverse transcription to copy its genome (RNA → DNA), substantial 'errors' (or diversity) are made in the progeny viruses from a single round of replication. This diversity ensures the survival of HBV because, as its environment changes due to the introduction of an antiviral agent, a resistant viral sub-population will already be present in the pool of newly replicating HBVs in that patient. Not surprisingly then, a number of immune or antiviral drug 'escape' (resistant) mutants of HBV are selected out as the new dominant populations, whenever a new selection pressure (in immune response or nucleoside analogue) is stimulated or introduced.

HBV replication: the virus and its life cycle

The HBV is a DNA virus. The viral DNA is found inside the viral core structure (or hepatitis

B core antigen [HBcAg]) with the viral reverse transcriptase/DNA polymerase. This core structure is then surrounded by its envelope, the hepatitis B surface antigen (HBsAg). The life cycle of HBV begins when its envelope protein attaches to a receptor on the hepatocyte cell surface. This allows the virus to enter the cell. The viral core structure is transported to the nucleus where the viral genomic DNA is converted into a covalently closed circular (ccc) DNA form, the major transcriptional template of the virus. Using host cell enzymatic machinery, viral RNA is made and transported out to the cytoplasm of the hepatocyte where the viral structural proteins (core [HBcAg] and surface [HBsAg]) are made, as well as the replication protein, HBV reverse transcriptase/DNA polymerase. The HBV reverse transcriptase (rt) then copies the HBV pregenomic (pg) RNA to DNA inside the core particle. The viral envelope proteins now coat those replicating core complexes, and mature virions are made and then released from the cell, completing the life cycle (Figure 2.1). The only viral enzyme

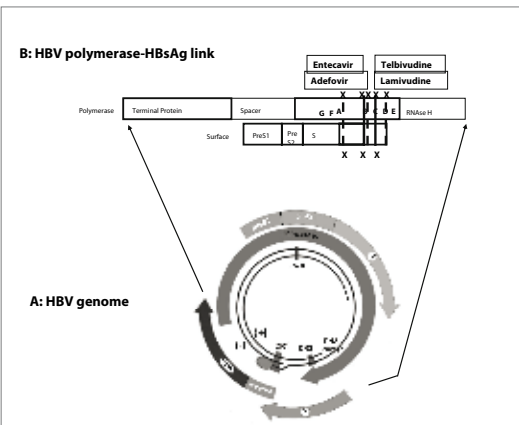


Figure 2.2 A: Hepatitis B virus (HBV) DNA genome showing the circular arrangement of the four overlapping but frame-shifted reading frames
 Figure 2.2 B: The hepatitis B virus polymerase – HBsAg link, demonstrating the changes observed in the HBV Pol due to the emergence of drug-resistance, affect the HBsAg directly for all four nucleos(t)ide analogues approved for chronic hepatitis B treatment

The life cycle of HBV revolves around two key processes (Figure 2.3):

- Generation of HBV ccc DNA from genomic DNA and its subsequent processing by host enzymes to produce viral RNA; and
- Reverse transcription of the pregenomic (pg) RNA within the viral nucleocapsid to form HBV DNA, completing the cycle (see Figure 2.1).

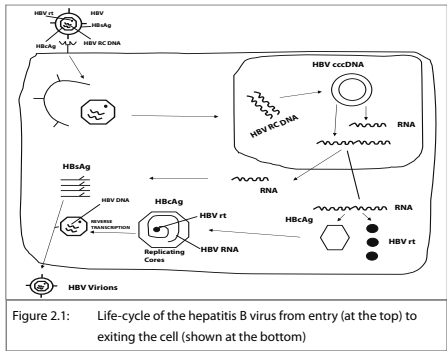


Figure 2.1: Life-cycle of the hepatitis B virus from entry (at the top) to exiting the cell (shown at the bottom)

identified to date is the viral rt and this is the target for nucleoside analogue (NA) therapy.

The HBV DNA genome is organised into four overlapping open reading frames (ORF), the longest of which encodes the viral reverse transcriptase/DNA polymerase (Pol ORF) (Figure 2.2 A). The envelope ORF is located within the Pol ORF, while the core (C) and the X ORFs partially overlap with it (Figure 2.2 B).

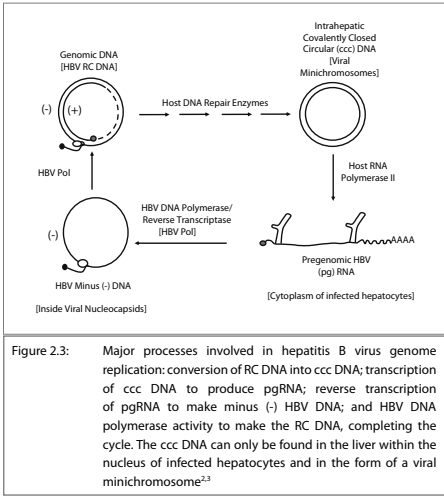


Figure 2.3: Major processes involved in hepatitis B virus genome replication: conversion of RC DNA into ccc DNA; transcription of ccc DNA to produce pgRNA; reverse transcription of pgRNA to make minus (-) HBV DNA; and HBV DNA polymerase activity to make the RC DNA, completing the cycle. The ccc DNA can only be found in the liver within the nucleus of infected hepatocytes and in the form of a viral minichromosome^{2,3}

As discussed previously, because HBV uses reverse transcription to copy its genome (RNA → DNA) and the viral *rt* lacks a proof-reading or editing function, many 'mistakes' are introduced into the newly replicated HBV DNA, resulting in substantial diversity in the viral genome. As discussed below, this gives the virus a great survival advantage as every single nucleotide in the viral genome of 3200 base-pairs is mutated or changed every day. Thus, single and double mutations associated with antiviral drug resistance exist even before therapy is introduced. However, three or four mutations in the HBV DNA that would be needed to escape NA treatment are very unlikely to be found pre-therapy. These observations are the basis for the use of combination chemotherapy for chronic viral diseases (such as HIV, AIDS, e.g.: Highly Active Antiretroviral Therapy [HAART]).

Common mutants of HBV

1. Mutations affecting HBeAg

As well as HBcAg and HBsAg, the HBV also encodes for an accessory protein, the hepatitis B e antigen (HBeAg). The HBeAg protein is a soluble form of the HBcAg and is thought to act as a tolerogen.⁴ The HBeAg is classified as an accessory protein of HBV, since the virus can replicate without an HBeAg. However, the production of HBeAg is an important strategy for the virus to help avoid immune elimination by the host's immunological response. When put under the immunological pressure of HBeAg seroconversion, which is part of the natural history of CHB, HBV has a number of ways of 'escaping' such pressure.

Two major groups of mutations have been identified which result in reduced or blocked HBeAg expression.

The first group refers to mutations that affect the basal core promoter (BCP) typically at nucleotide (nt)1762 and nt1764, resulting in a transcriptional reduction of the Pre-C/CmRNA.⁵ Mutations in the BCP, such as A1762T plus G1764A, may be found in isolation or in conjunction with precore mutations (see below). The double mutation of A1762T plus G1764A results in a significant decrease in

HBeAg levels and has been associated with an increase in viral load. Importantly, these BCP mutations do not affect the transcription of HBV pg RNA or the translation of the core or polymerase protein. Thus, by removing the inhibitory effect of the precore protein on HBV replication, the BCP mutations appear to enhance viral replication by suppressing Pre-C/C mRNA relative to pregenomic RNA.⁵

The second group of mutations includes HBV mutants with a translational stop codon mutation at nt position 1896 (codon 28: TGG; tryptophan) of the precore gene.⁶ The single base substitution (G-to-A) at nt1896 gives rise to a translational stop codon (TGG to TAG; TAG = stop codon) in the second last codon (codon 28) of the precore gene located within the ϵ structure of pgRNA. The ntG1896 forms a base pair with nt1858 at the base of the stem loop.⁶ Other mutations have been found within the precore transcript, which block HBeAg production, including the abolition of the initiation codon methionine residue.⁷

2. Envelope gene mutations

Viral genomes that cannot synthesise the envelope proteins have been found to occur frequently and are often the dominant virus populations in patients with chronic hepatitis B.⁸ The envelope region overlaps the Pol protein (Figures 2.2 A and 2.2 B).

The existing hepatitis B vaccine contains the major HBsAg. The subsequent anti-HBs response to the major hydrophilic region (MHR) of HBsAg located from residue 99 to 170 induces protective immunity. Mutations within this epitope have been selected during vaccination⁹ and following treatment of liver transplant recipients with hepatitis B immune globulin (HBIG) prophylaxis.¹⁰ Most vaccine-HBIG escape isolates have an amino acid change from glycine to arginine at residue 145 of HBsAg (sG145R) or aspartate to alanine at residue 144 (sD144A). The sG145R mutation has been associated with vaccine failure⁹ and has been shown to be transmitted and cause disease.

3. Polymerase mutations: antiviral drug resistance

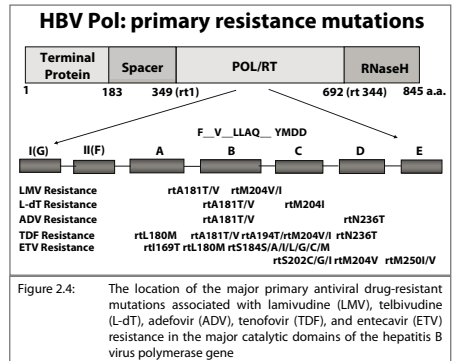
Antiviral drug resistance in clinical practice is discussed further in Chapter 7: Treatment of chronic hepatitis B virus infection.

As a result of the development of safe and efficacious orally available antiviral nucleoside and nucleotide analogues (NA), the treatment of CHB has advanced significantly during the past ten years. Lamivudine, a synthetic deoxycytidine analogue with an unnatural L-conformation, is the first of these NA and gained approval from the Food and Drug Administration (FDA) of the USA for treatment of CHB in 1996. Related L-nucleosides, including emtricitabine, telbivudine and clevudine, have since progressed to late stage clinical trials. Adefovir dipivoxil, a prodrug for the acyclic dAMP analogue, adefovir, gained approval in 2002 and clinical trials of structurally similar tenofovir disoproxil fumarate, which is currently used to treat HIV infection, are underway. The most potent anti-HBV drug discovered to date is the deoxyguanosine analogue, entecavir,¹¹ which has recently been approved by the FDA for first-line use against HBV. Telbivudine (L-dT) has also been recently approved for the treatment of CHB.

(a) Lamivudine and other L-nucleoside analogue resistance

Antiviral resistance to lamivudine (LMV) has been mapped to the YMDD locus in the catalytic or C domain of HBV Pol.¹² The mutations within the rt gene that have been selected during LMV therapy encode amino acid changes, which are designated rtM204I/V/S (Domain C) +/- rtL180M (Domain B)¹² with mutations in other regions of the HBV Pol being detected (Figure 2.4). For other L-nucleosides, such as telbivudine (L-dT), the B-domain (rtA181T/V) and C-domain (rtM204I), changes are most important for the development of resistance (Figure 2.4).

LMV resistance increases progressively during treatment at rates between 14% and 32% annually. At four years of therapy, rates of LMV resistance reach 70% in HBV mono-infection



and exceed 90% in HBV-HIV coinfection.^{13,14} Factors that increase the risk of development of resistance include high pre-therapy serum HBV DNA and alanine aminotransferase (ALT) levels, and incomplete suppression of viral replication.^{13,15} LMV resistance does not confer cross-resistance to adefovir (Table 2.1).

	LMV/FTC	ADV/TDF	L-dT	ETV
LMV-R	X	√	X	reduced
ADV-R	√	X	√	√
LdT-R	X	√	X	√
ETV-R	X	√	X	X

LMV = lamivudine L-dT = telbivudine
 FTC = emtricitabine ETV = entecavir
 ADV = adefovir R = resistant
 TDF = tenofovir
 X = resistant √ = sensitive

Mutations that confer LMV resistance decrease *in vitro* sensitivity to LMV from at least 100-fold to greater than 1000-fold. The rtM204I substitution has been detected in isolation, but rtM204V and rtM204S are found only in association with other changes in the B or A domains.¹⁶ The four major patterns of resistance can be identified as follows: 1) rtM204I; 2) rtL180M+rtM204V; 3) rtL180M+rtM204I; and 4) rtV173L+rtL180M+rtM204V.

(b) Adefovir dipivoxil resistance

Resistance to adefovir dipivoxil (ADV) was initially associated with changes in the B

(rtA181T/V) and D (N236T)-domains of the reverse transcriptase¹⁷ (Figure 2.4). HBV resistance to adefovir occurs less frequently (around 2% after two years, 4% after three years and 18% after four years) than resistance to LMV. These ADV-associated mutations in HBV Pol result in only a modest (three- to eight-fold) increase in IC50 and provide partial cross-resistance to tenofovir. The rtN236T change does not significantly affect sensitivity to LMV¹⁷ but the rtA181T/V change confers partial cross-resistance to LMV.

(c) Entecavir resistance

Resistance to entecavir (ETV) has been observed in patients who were also LMV-resistant.¹⁸ Mutations in the viral polymerase associated with the emergence of ETV-resistance were mapped to the B-domain (rtI169T or rtS184G and rtL180M), C-domain (rtS202I and rtM204V), and E-domain (rtM250V) of HBV Pol (Figure 2.4). In the absence of the LMV mutations, the rtM250V causes a nine-fold increase in IC50, while the rtT184G+rtS202I have no effect.¹⁸⁻²¹

(d) Multi-drug resistance

Recently, multidrug-resistant HBV has been reported in patients who received sequential treatment with NA monotherapies.^{18,22-26} The development of multidrug resistance will almost certainly have implications on the efficacy of rescue therapy, as in the case of multidrug-resistant HIV.^{27,28} Successive evolutions of different patterns of resistant mutations have been reported during long-term LMV monotherapy.^{29,30} The isolates of HBV with these initial mutations appear to be associated with decreased replication fitness compared with wild-type HBV; however, as treatment is continued, additional mutations that can restore replication fitness are frequently detected.^{31,32}

(e) Public health issues: Pol-env overlap

The polymerase gene overlaps the envelope gene (see Figure 2.2 B) and changes in the HBV Pol selected during antiviral resistance can cause concomitant changes to the overlapping reading frame of the envelope. Thus, the major resistance mutations associated with LMV, ADV, ETV, and L-dT failure also have the potential of

altering the C-terminal region of HBsAg. For example, changes associated with LMV and ETV resistance, such as the rtM204V, result in a change at sI195M in the surface antigen, while the rtM204I change that is associated with LMV and L-dT, is linked to three possible changes, sW196S, sW196L or a termination codon. To date, only one published study has examined the effect of the main LMV resistance mutations on the altered antigenicity of HBsAg.³³ One of the common HBV quasiespecies that is selected during LMV treatment is rtV173L + rtL180M + rtM204V, which results in change in the HBsAg at sE164D + sI195M. Approximately 20% of people with HIV-HBV co-infection¹⁴ and 10% of those with mono-infection encode this 'triple Pol mutant'.³¹ In binding assays, HBsAg expressing these LMV-resistant associated residues had reduced anti-HBs binding.³³ This reduction was similar to the classical vaccine escape mutant, sG145R.

The ADV resistance mutation rtN236T does not affect the envelope gene and overlaps with the stop codon at the end of the envelope gene. The rtA181T mutation selected by ADV and LMV results in a stop codon mutation at sW172stop. The ADV resistant mutation at rtA181V results in a change at sL173F. The HBV with mutations that result in a stop codon in the envelope gene, such as those for LMV and ADV, would be present in association with a low percentage of wild-type to enable viral assembly and release.

The ETV resistance-associated changes at rtI169T, rtS184G and rtS202I also affect HBsAg and result in changes at sF161L, sL/V176G, and sV194F. The rtM250V is located after the end of HBsAg. The sF161L is located within the region that was defined as the 'a' determinant or major hydrophilic region, which includes amino acids 90 to 170 of the HBsAg.³⁴ This region is a highly conformational epitope, characterised by multiple di-sulphide bonds formed from sets of cysteines at residues 107-138, 137-149, and 139-147.³⁴ Since distal substitutions such as sE164D significantly affect anti-HBs binding,³³ the influence of other changes to HBsAg driven by NA resistance, (such as sF161L), needs further investigation in order to determine the effect on the envelope structure and subsequent anti-HBs binding. The HBsAg change linked to rtM204V has already been covered.

Although evidence for the spread of transmission of antiviral-resistant HBV is limited, there has been a report of the transmission of LMV-resistant HBV to a HIV patient undergoing LMV as part of antiretroviral therapy.³⁵ In addition, HBV encoding LMV resistant mutations was also found in a cohort of dialysis patients with occult HBV.³⁶ Therefore, it is important to recognise that both primary and compensatory antiviral-resistant mutations may result in associated changes to the viral envelope that could have substantial public health relevance.

Conclusions

Resistance will remain an important issue in the management of patients with CHB because long-term, or probably life-long, therapy with NAs will be required in the majority of patients. One of the important lessons learned from the HIV paradigm is that resistance will occur if viral replication is present during treatment, as may occur with existing monotherapy regimens.³⁷ Combination therapy for CHB, either as an initial strategy or in selected groups of patients with inadequate response to monotherapy, is likely to be required in the future, and clinical trials are currently underway to investigate combination treatment strategies. Theoretically, combination therapy is likely to reduce not only the viral load and quasispecies pool, but also the risk of selecting resistance, especially if differing resistance mutation profiles of the various antiviral agents are used. Another strong argument for combination therapy is that, because of the overlap between the Pol and S genes, the selection of drug-resistant HBV may have important clinical, diagnostic, and public health implications. Envelope changes in HBV have been detected with LMV, ADV, ETV, and L-dT usage. The significance of these changes warrants further investigation to determine what affect they may have on the natural history of drug-resistant HBV and its possible transmissibility in the hepatitis B-vaccinated community at large.

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