

Molecular Virology of Hepatitis B Virus

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ABSTRACT

Hepatitis B virus (HBV) has evolved a unique life cycle that results in the production of enormous viral loads during active replication without actually killing the infected cell directly. Because HBV uses reverse transcription to copy its DNA genome, mutant viral genomes emerge frequently. Particular selection pressures, both endogenous (host immune clearance) and exogenous (vaccines and antiviral drugs), readily select out these escape mutants. Which particular viral mutations or combination of mutations directly affect the clinical outcome of infection are not known. Further studies are clearly needed to identify the pathogenic basis and clinical sequelae arising from the selection of these mutants.

KEYWORDS: Reverse transcription, covalently closed circular DNA, viral mutants, drug resistance, vaccine escape

HBV can be transmitted by sexual contact, through the skin, by inoculation with contaminated blood or blood products, by transplantation of organs from infected donors, and perinatally from infected mothers. Serum hepatitis B surface antigen (HBsAg) and HBV DNA are considered reliable indicators of active infection. The risk of transmission from infected individuals, however, is unpredictable because of the substantial variability in HBV replicative activity that occurs during the various phases of chronic HBV infection. Nonetheless, the presence of HBsAg is still regarded as the most important marker of infectivity, irrespective of viral load. This article addresses the unique method of HBV genomic replication, including the viral mutations that influence expression of hepatitis B e antigen (HBeAg) and the development of antiviral drug resistance.

MOLECULAR PATHOGENESIS

Liver damage in chronic hepatitis B results mainly from the direct interaction between the host's immune system

and HBV-infected hepatocytes. The antiviral cytokines, such as interferon alpha, beta, and gamma (IFN- α , IFN- β , IFN- γ) as well as tumor necrosis factor alpha (TNF- α), have been implicated as the major contributors to viral clearance, whereas destruction of infected hepatocytes by cytotoxic T lymphocytes contributes to both viral clearance and the development of liver disease.¹

In patients with self-limited, acute HBV infection, viral clearance occurs through a polyclonal and multispecific peripheral blood mononuclear cell response mounted against the epitopes of several HBV proteins, including the viral envelope, polymerase, and core proteins.²⁻⁵ This response involves human leukocyte antigen class II-restricted CD4⁺ helper T lymphocytes and CD8⁺ cytotoxic T lymphocytes. A predominantly type-1 helper T-lymphocyte response develops and leads to the secretion of cytokines such as interleukin (IL)-2 and IFN- γ . These cytokines contribute to liver cell injury as well as to recovery from disease.

In patients with chronic HBV infection, the peripheral cytotoxic T-lymphocyte response is usually

HBV Viral Kinetics and Clinical Management: Key Issues and Current Perspectives; Editor in Chief, Paul D. Berk, M.D.; Guest Editors, Emmet B. Keeffe, M.D., and Jules L. Dienstag, M.D. *Seminars in Liver Disease*, volume 24, supplement 1, 2004. Address for correspondence and reprint requests: Professor Stephen Locarnini, Victorian Infectious Diseases Reference Laboratory, 10 Wreckyn Street, North Melbourne, Victoria 3051, Australia. E-mail: stephenlocarnini@compuserve.com or stephen.locarnini@mh.org.au. ¹Head, Research and Molecular Development, Victorian Infectious Diseases Reference Laboratory, North Melbourne, Australia. Copyright © 2004 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New York, NY 10001, USA. Tel: +1(212) 584-4662. 0272-8087;p;2004,24,s1,003,010,ftx,en;sld00258x.

weak or undetectable and narrow in focus. An activated humoral response develops, characterized by the production of IL-4, IL-5, and IL-10 secreted by type-2 helper T lymphocytes; this response promotes antibody production rather than viral clearance. Low levels of intrahepatic HBV-specific cytotoxic T lymphocytes have been detected in such patients and are probably responsible for the hepatic flares that occur in patients with chronic disease.⁶⁻⁸ However, these activated cytotoxic T lymphocytes are unable to clear HBV.⁹

Both the adaptive and the innate immune responses play critical roles in viral clearance.^{1,10} Activation of innate immunity occurs early in HBV infection. Animal studies have highlighted the important role of IFN- γ and TNF- α in controlling the bulk of viral replication without necessarily inducing the perforin- or Fas-dependent apoptotic pathway of cell killing.^{1,10} This marked reduction in viral replication occurs typically before the peak infiltration of T cells and the onset of liver injury. Thus, activation of innate immunity during the early stages of infection appears to be essential for the control of HBV infection and accounts for the bulk of the antiviral activity of the host's immune response.

Viral Genomics

Human HBV is the prototype member of the family *Hepadnaviridae*, which includes a variety of avian and

mammalian viruses that share similar genomic organization, organ tropisms, and a unique strategy of genome replication.¹¹ Hepatitis B virus can be further classified into eight major genotypes (A to H) based on nucleotide (nt) diversity of $\geq 8\%$.^{12,13} These genotypes have a distinct global geographical distribution (Table 1).

The HBV genome comprises a partially double-stranded 3.2 kb DNA organized into four open-reading frames. The longest open-reading frame encodes the viral polymerase (Pol open-reading frame). The envelope open-reading frame is located within the Pol open-reading frame in a frame-shifted manner. Partially overlapping with the envelope open-reading frame are the core (C) and the X open-reading frames. The covalently closed circular DNA (ccc DNA) is the template that is transcribed to generate four major RNA species: the 3.5-, 2.4-, 2.1-, and 0.7-kb viral RNA transcripts.¹¹ Expression of these four transcripts is directed by the enhancer II/basal core, large surface antigen (L), major surface antigen (S), and enhancer I/X gene promoters, respectively.¹¹

Most studies of HBV have shown that the Pre-S1 domain is required for receptor binding and initiation of infection.¹¹ Once the virus is inside a susceptible cell, viral cores disassemble, and genomic DNA is transferred to the cell nucleus.¹⁴ Inside the nucleus, the partially double-stranded viral DNA is converted by the host cell to ccc DNA. The ccc DNA becomes a viral minichromosome, acting as the major transcriptional template for

Table 1 Overview of the 8 Major Genotypes of HBV

Genotype	Subtype	Genome Length (nt)	HBV Proteins				Frequency of Mutation		Global Distribution
			PreS1 ^a	Pol	Core	PC	BCP		
A	adw2, ayw1	3221	119	845	185	Uncommon (C1858)	Common	Western Europe, United States, Central Africa, India	
B	adw2, ayw1	3215	119	843	183	Common (T1858)	Common	Japan, Taiwan, Indonesia, China, United States	
Bj	adw2, aywl	3215	119	843	183	Common	Uncommon	Japan	
Ba	adw2, aywl	3215	119	843	183	Low	Uncommon	China, Taiwan, Indonesia, Vietnam	
C	adw2, adr, ayr	3215	119	843	183	Common T/C1858	Common	East Asia, Taiwan, Korea, China, United States, Japan, Polynesia	
D	ayw2, ayw3	3182	108	832	183	Common T1858	Common	Mediterranean region, India, United States	
E	ayw4	3212	118	842	183	ND	ND	West Africa	
F	adw, ayw	3215	119	843	183	Uncommon (C1858)	ND	Central and South America, Polynesia	
G	adw2	3248	108	842	195	Very common (insertion)	ND	United States, Europe	
H	adw	3215	119	843	183	ND	ND	Central and South America	

^aPre-S1 = 55 aa; S = 226 aa.

BCP, basal core promoter mutations such as A1762T, G1764A; PC, precore mutations such as G1896A; ND, not described. Common = up to 50% of isolates; uncommon = <10% of isolates; very common = most isolates.

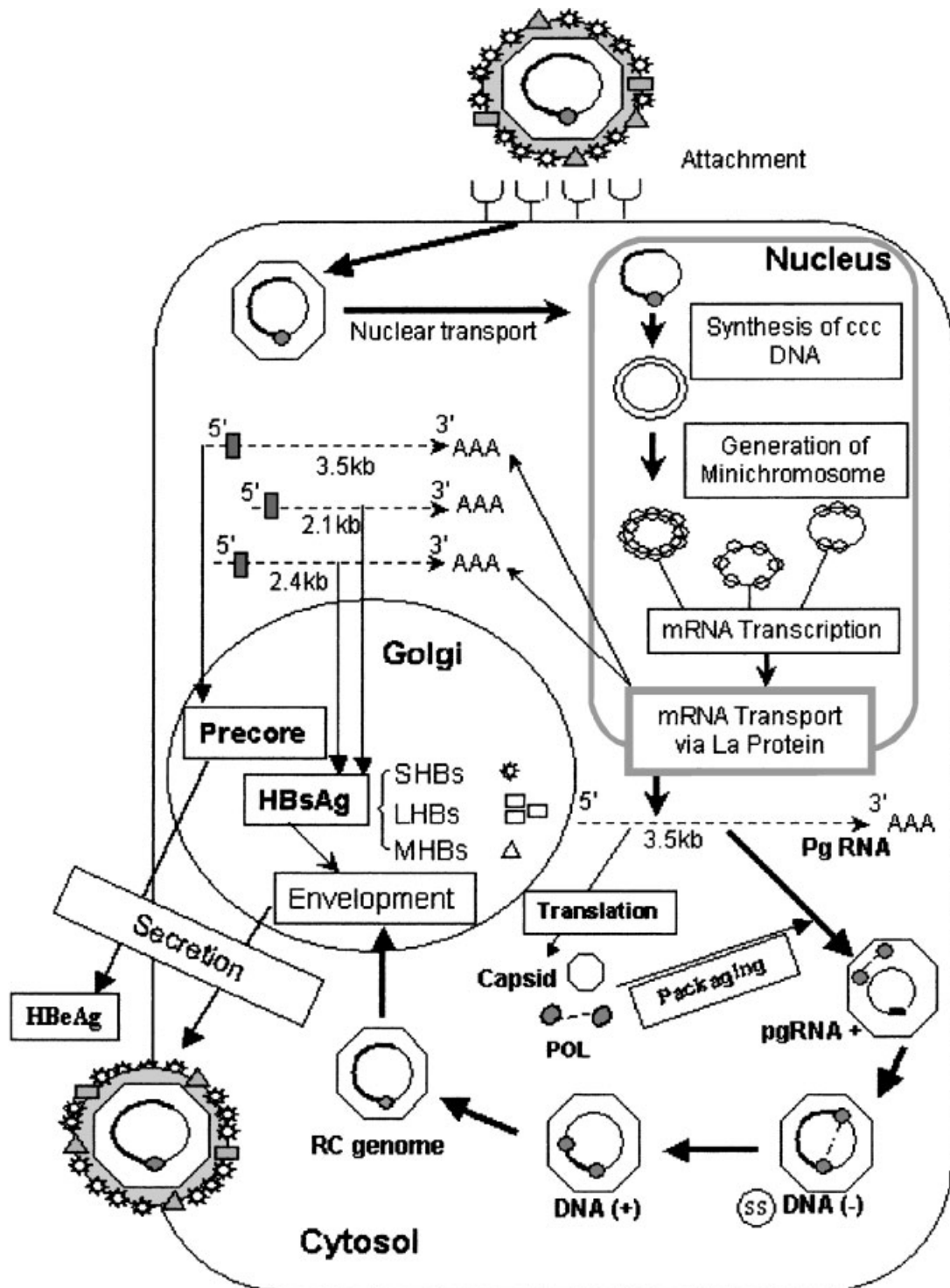


Figure 1 Life cycle of the HBV showing the major virus-specific events, including ccc DNA generation in the nucleus from genomic DNA and its subsequent processing into viral mRNAs. Reverse transcription occurs within the core structure in replication complexes, composed of nucleocapsids in the cytosol. The envelope proteins subsequently assemble around the nucleocapsids to form mature virions. (Reprinted with permission from Summers and Mason. ¹⁷)

the virus (Fig. 1). The four sets of mRNAs are transcribed from these viral minichromosomes. ^{15,16}

These RNAs are then translated to produce the viral proteins, namely, hepatitis B core antigen (or nucleocapsid protein, from the 3.5-kb RNA); the soluble

and secreted HBeAg (from the 3.5-kb RNA); the Pol protein (from the 3.5-kb RNA); the viral envelope proteins, which express HBsAg (from the 2.4- and 2.1-kb RNAs); and hepatitis B X protein ([HB_x] from the 0.7-kb RNA). In addition to serving as

messenger RNA (mRNA) for the nucleocapsid and Pol proteins, the 3.5-kb pregenomic RNA acts as the template for reverse transcription of the viral genome (Fig. 1).¹⁷

Viral Mutations

In addition to viral and host factors, exogenous selection pressures define the predominant HBV species in an infected individual. Exogenous pressures include treatment with nucleoside or nucleotide (nt) analogues as well as immune-based interventions such as hepatitis B immunoglobulin (HBIG) and vaccination. The presumably immune-based selection pressures that cause reduction in, or loss of, HBeAg and eventual elimination of HBsAg are probably responsible for the selection of particular mutants, such as those associated with HBeAg-negative chronic hepatitis B.

Viral reverse transcriptases (rt) lack a proofreading function and are thus inherently error prone. As a result, HBV populations exist in the host as heterogeneous mixtures known as quasi-species. The frequency of HBV mutation has been estimated to be approximately 1.4 to 3.2×10^{-5} nt substitutions per site per year,¹⁸ around 10-fold higher than that of other DNA viruses. The magnitude and rate of virus replication are also important in the process of mutation generation; the total viral load in serum frequently approaches 10^{11} virions/mL. Most estimates place the mean half-life of the serum HBV pool at approximately 1 to 2 days, translating to a rate of de novo HBV production approaching 10^{11} virions/day. The high viral loads and turnover rates coupled with poor replication fidelity influence mutation generation and the complexity of the HBV quasi-species pool. These mutation rates, however, are lower than those of other retroviruses, mainly because of the constraints imposed by the overlapping reading frames.

Mutations in the Basal Core Promoter, Precore, and Core Genes

Two major groups of mutations that result in reduced or blocked HBeAg expression have been identified. The first includes a translational stop-codon mutation in the precore gene.¹⁹ At nt 1896 (codon 28: TGG; tryptophan), located in the ϵ structure of the precore gene, a single base substitution (G to A) gives rise to a translational stop codon (TGG to TAG; TAG = stop codon) in the second to last codon (codon 28) of the precore region. The ϵ structure is a highly conserved stem-loop RNA structure critical in viral replication; the nt G1896 forms a base pair with nt 1858 at the base of the stem loop. In HBV genotypes B, D, E, and G and in some strains of genotype C, the 1858 nt is a thymidine (T) (see Table 1). Thus, the stop-codon mutation created by G1896A (T-A) stabilizes the ϵ structure. In

contrast, the precore stop-codon mutation is rarely detected in HBV genotypes A or F or in certain strains of HBV genotype C because the nt at position 1858 is a cytidine (C), maintaining the preferred Watson-Crick (G-C) base pairing.

The second group of mutations affects the basal core promoter, which results in a transcriptional reduction of precore and core mRNA.²⁰ These mutations occur at nt 1762 and nt 1764. Mutations such as A1762T plus G1764A in the basal core promoter may be found in isolation or in conjunction with precore mutations, depending on the genotype (see Table 1). Occurrence of the double mutation of A1762T plus G1764A results in a decrease in, but not a disappearance of, HBeAg production and an increase in viral load. In general, this pattern of mutation is often found in genotype A-infected individuals.²⁰ Mutations in the basal core promoter result in reduced binding of liver-specific transcription factors, transcribing fewer precore and core mRNA transcripts and, consequently, less precore protein. The basal core promoter mutations, however, do not affect the transcription of pregenomic RNA or the translation of the core or polymerase proteins. Thus, by removing the inhibitory effect of the precore protein on HBV replication, the basal core promoter mutations appear to enhance viral replication by suppressing precore and core mRNA relative to pregenomic RNA.²⁰

The core protein can be divided into two major domains, the N-terminal assembly domain (up to amino acid position 144) and the functionally important, arginine-rich C-terminal domain. The C-terminal domain is required for binding of the pregenomic RNA and subsequent genomic replication and has been shown to contain important B-cell and cytotoxic T-lymphocyte epitopes.^{8,9} During periods of cytotoxic T cell clearance of virus-infected hepatocytes in chronic HBV infection, escape mutations within those epitopes are readily selected.^{20,21} These "hot spots" have been linked to major cytotoxic T-lymphocyte regions (amino acids 18–30) and T-helper cell regions (amino acids 50–70) and to two B cell (HBc/e1 and HBc/e2) epitopes at residues 75–90 and 120–140, respectively.²¹ The frequency of core gene mutations is associated typically with the presence of precore stop-codon mutations, HBeAg-negative status, and active liver disease.²²

Mutations in the X Gene

Mutations in the X region can involve the regulatory elements that control replication, such as the basal core promoter and Enhancer II. Because the basal core promoter encompasses nt 1742 to 1802 and overlaps with the X gene in the concomitant reading frame, the A1762T plus G1764A core promoter mutations also

Genotype-Independent Numbering Scheme for HBV Polymerase/RT Domain

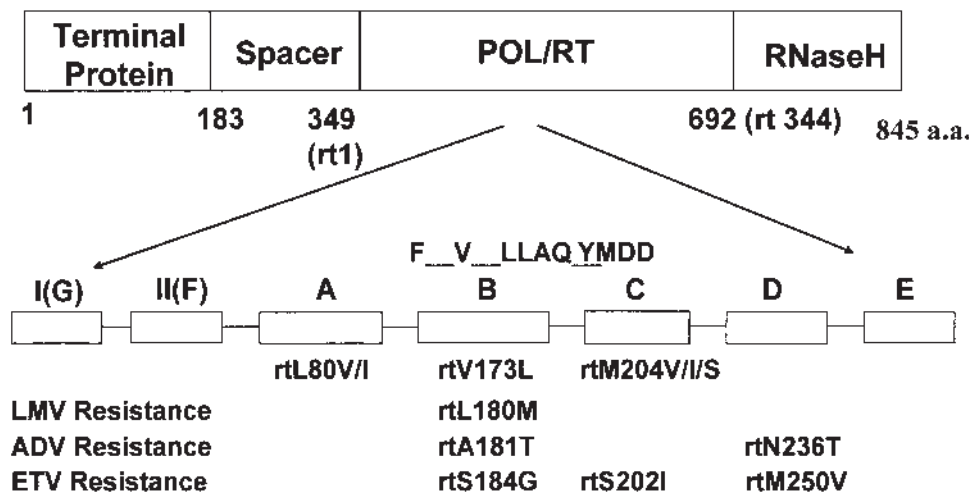


Figure 2 The location of the major antiviral drug-resistant mutations associated with lamivudine (LMV), adefovir (ADV), and entecavir (ETV) resistance. (Reprinted with permission from Angus et al.²⁶)

cause changes in the X gene at xK130M and xV131I. In addition, nearly all deletions or insertions in the basal core promoter shift the X gene frame and lead to the production of truncated X proteins. These shortened X proteins lack the domain in the C terminus (amino acids 130–140) that is required for the transactivation activity of HBx antigen.²¹

Mutations in the Envelope Gene

The Pre-S sequences exhibit the highest heterogeneity of the HBV genome.²¹ Point mutations, deletions, and genetic recombinations within the Pre-S genes have been identified in HBV DNA sequences obtained from the sera of inactive carriers. HBV genomes that cannot synthesize Pre-S2 proteins occur frequently and are the dominant virus populations in these persons.²¹ The Pre-S2 region overlaps the spacer region of the Pol protein, which is not essential for enzyme activity.

Most hepatitis B vaccines contain the major HBsAg protein and induce an immune response to the major hydrophilic region, located from residue 99 to 170. This anti-HBs response produces protective immunity. Mutations within this epitope have been selected during vaccination²³ and after treatment of liver allograft recipients with HBV.²⁴ Most isolates contain a mutation from glycine to arginine at residue 145 of HBsAg (sG145R) or from aspartate to alanine at residue 144 (sD144A). The sG145R mutation has been associated with vaccine failure.²³

Polymerase Mutations: Antiviral Drug Resistance

The advent of treatment with nucleoside and nucleotide analogues has resulted in the outgrowth of otherwise minor quasi-species containing mutations in the HBV Pol gene. Antiviral resistance to lamivudine has been mapped to the YMDD locus in the catalytic or C domain of HBV Pol,²⁵ whereas resistance to adefovir dipivoxil is associated with mutations in the D and B domains of the enzyme.^{25,26} According to the new nomenclature,²⁵ the mutations within the reverse transcriptase (rt) gene that were selected during lamivudine therapy are designated as rtM204I/V/S (domain C) ± rtL180M (domain B).²⁵ The major mutations associated with adefovir-resistant HBV are designated as rtN236T (domain D) and rtA181T (Fig. 2).²⁶

Lamivudine Resistance

Lamivudine resistance increases progressively during treatment at rates between 14 and 32% annually, approaching 70% after 48 months of treatment.²⁷ Factors that increase the risk of resistance include high pretherapy serum HBV DNA and alanine transferase levels and incomplete suppression of viral replication.^{27,28} Lamivudine resistance does not confer cross-resistance to adefovir dipivoxil.

Mutations that confer lamivudine resistance decrease in vitro sensitivity to the drug from ≥20-fold to >100-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 motifs. Numerous other secondary changes in the rt sequence have also been found to occur in conjunction with rtM204V/I/S,²⁹ and some of these are probably compensatory (see later).

Adefovir Dipivoxil Resistance

HBV resistance to adefovir occurs less frequently (around 2% after 2 years) than resistance to lamivudine does. Adefovir resistance is conferred by substitution of threonine for asparagine at codon 236, located in the D motif of HBV (rtN236T) and the B-domain mutation rtA181T.²⁶ These changes do not appear to affect sensitivity to lamivudine.

Entecavir Resistance

Resistance to entecavir was not reported in early clinical trials but has been observed in two patients who were also resistant to lamivudine (A. Bartholomeusz et al, unpublished data, 2003). Mutations in the viral polymerase associated with the emergence of entecavir resistance were mapped to domain B (rtS184G), domain C (rtS202I), and domain D (rtM250V) (see Fig. 2).

PATHOGENICITY OF DRUG-RESISTANT HBV: ROLE OF COMPENSATORY MUTATIONS

Ogata and colleagues³⁰ observed the co-occurrence of rtL80V/I (domain A) and rtL180M in conjunction with the M204V/I changes (see Fig. 2) that confer lamivudine resistance in Japanese patients (genotype C) treated with lamivudine. The presence of double and triple changes has been associated with higher viral loads, increased lamivudine resistance, and disease exacerbation. Longitudinal studies have shown that the mutations responsible for the sequence changes occurred almost simultaneously, just before viral breakthrough occurred, and also that the mutants were displaced by wild-type genotype-C HBV after completion of therapy.

Similar observations have been recorded after liver transplantation among patients in whom life-threatening recurrence of HBV infection developed.³¹ HBV isolates from these patients contained compensatory mutations that enhanced their *in vitro* replication efficiency in the presence of lamivudine. Even greater enhancement and drug dependency occurred when mutations resulting in sG145R or sP120T, key changes in the envelope protein, were also present.³¹

Mutations that abolish or decrease the expression of HBeAg are known to affect replication efficiency. HBeAg-negative chronic hepatitis B, which is most common in the Mediterranean region, is usually characterized by lower serum HBV DNA levels than

those found in HBeAg-positive chronic hepatitis B. Hadziyannis et al³² and Papatheodoridis et al³³ studied a group of patients with HBeAg-negative chronic hepatitis B who were infected with genotype D HBV that contained both basal core promoter and precore stop-codon mutations. Development of lamivudine resistance in these patients was associated with relatively rapid increases in viremia, culminating frequently in biochemical breakthrough, severe hepatitic flares, and disease progression.^{32,33} *In vitro* studies by Chen and colleagues³⁴ confirmed that the presence of the typical precore mutation (G1896A) could compensate for the replication deficiency in lamivudine-resistant HBV quasi-species. Other anecdotal reports confirm that drug-resistant HBV mutants are capable of causing severe, even fatal, disease.³⁵⁻³⁹ Such findings challenge the notion that drug-resistant HBV mutants and other minority quasi-species are invariably benign.

A recent publication documenting a case of transmission of lamivudine-resistant HBV between male homosexual partners⁴⁰ stresses the significance of drug resistance as an emerging public health issue and emphasizes the importance of constant surveillance for drug-resistant virus.

STRATEGIES TO OVERCOME RESISTANCE

Until new, more effective therapeutic agents for chronic HBV infection can be developed, issues such as long-term efficacy of, and drug resistance to, existing therapies will continue to pose challenges in patient management. Furthermore, whether combinations of antiviral drugs and immunomodulatory agents will necessarily improve clinical end points in chronic hepatitis B is unknown. Based on the paradigm in HIV infection, no single drug is likely to be able to permanently control or to eliminate chronic HBV infection. At present, the aim of combination chemotherapy is to arrest disease progression, improve patient quality of life, minimize the risk of disease progression, and decrease the rate at which resistance emerges.⁴¹ Because of the similarities between HBV and HIV infection, experience with HIV therapy provides a rational basis as well as valuable guidelines for developing combination therapy of HBV infection.⁴²

CONCLUSIONS

After nearly 30 years of clinical research, safe and efficacious antiviral drugs with which to treat HBV infection are finally becoming available. However, as their clinical use becomes more common and patient adherence decreases, the emergence of viral resistance to these drugs will undoubtedly increase. Control of these HBV mutants, which will require new drugs, vaccines, and treatment strategies, will become the next major

challenge on the path to eventual elimination of HBV infection. Nevertheless, prospects for control of chronic HBV infection have never been better, given the development of more sensitive and sophisticated diagnostic tests⁴³ and the renewed impetus provided by immunization programs.

The probability that viral resistance will develop is directly proportional to the grade of selection pressure and the diversity of quasi-species. Sufficiently potent inhibition of HBV replication should be able to prevent the development of drug resistance, mainly because mutagenesis is replication dependent. If viral replication can be suppressed for a sufficient length of time, viral load will decline to a level at which continued production of quasi-species with the potential to resist new drug treatments is no longer possible. As was the case with the refinement of highly active antiretroviral treatment for HIV infection, clinical application of this concept in HBV infection will require discovery of optimal combination therapies.^{41,42}

ABBREVIATIONS

HBV	hepatitis B virus
HBsAg	hepatitis B surface antigen
IFN	interferon
ccc DNA	covalently closed circular DNA
rt	reverse transcriptase
nt	nucleotide

DISCLOSURE OF COMMERCIAL FINANCIAL SUPPORT

Dr. Locarnini has received research support from Gilead Sciences, San Francisco; Bristol Myer Squibb, Connecticut; Bayer Diagnostics, San Francisco; Innogenetics, NV, Belgium.

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