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Antiviral Research 55 (2002) 381–396

Antiviral Research

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Review

Hepatitis B treatment: rational combination chemotherapy based on viral kinetic and animal model studies

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Received 12 March 2002; accepted 3 May 2002

Abstract

Hepatitis B virus (HBV) causes a generally non-cytopathic infection in the liver. Even though HBV is a DNA virus, it replicates via reverse transcription which is coordinated within the viral nucleocapsid by the virus-specific polymerase. The major transcriptional template is the viral mimichromosome from which the viral DNA exists as a covalently closed circular (ccc) molcule. The virus infects hepatocytes but can also be found in non-hepatocyte reservoirs such as bile-duct epithelium, mesangial cells of the kidney, pancreatic islet cells and lymphoid cells. When patients infected with HBV are treated with either interferon alpha or lamivudine, responses are variable and unpredictable. Sophisticated mathematical models analysing the dynamics of viral clearance during antiviral therapy have recently been applied to chronic hepatitis B. Typically complex profiles, rather than the usual biphasic responses seen with other diseases have been observed, indicating that antiviral efficacy requires substantila improvement. This may be achieved with combination chemotherapy. However, chronic hepatitis B is a complex and heterogeneous disease entity, and the challenge for the future is to define measurable end-points of treatment and address key virological issues such as the role of cccDNA and extra-hepatocyte replication in treatment failure. Clearly, new therapies and effective combination therapy protocols are urgently required in order to improve the present poor response rates in patients undergoing treatment.

Keywords: Hepatitis B virus; Combination chemotherapy; Animal model; Viral dynamics

1. Introduction

Persistent infection with the hepatitis B virus (HBV) is a major health problem worldwide as it can lead to cirrhosis and primary hepatocellular carcinoma (HCC; Szmuness, 1978). There are over 400 million people who are chronically infected with HBV (Mast et al., 1999; Mahoney, 1999) and overall, there is a 14–25% mortality associated with this chronic infection (Mast and Alter, 1993).

Analysis of the viral dynamics during antiviral therapy has provided critical insights into the pathogenesis of chronic infection with human

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0166-3542/02/$ - see front matter
PII: S0166-3542(02)00071-2

immunodeficiency virus (HIV; Ho et al., 1995; Perelson et al., 1997a) and hepatitis C (HCV Neumann et al., 1998). Most importantly, these insights have led to the development of a rational framework from which to design treatment strategies that would more effectively bring the infection (i.e. viral replication) under control. Similar studies of viral dynamics in acute or chronic HBV have also been performed (Nowak et al., 1996; Tsiang et al., 1999; Lau et al., 2000), but improved HBV DNA testing technologies and mathematical model development have resulted in several important advances in the field (Lewin et al., 2001; Whalley et al., 2001). This review will focus on the field of viral dynamics in HBV infection and attempt to provide a more rational basis for the use of combination chemotherapy for HBV.

2. HBV and its replicative strategy

2.1. Viral life cycle

The replication cycle of HBV has been reviewed extensively (Nassal and Schaller, 1996; Tavis and Ganem, 1996; Seeger and Mason, 2000) and is outlined in Fig. 1. The HBV is an enveloped virus containing a 3.2 kilobase (kb) partially double-stranded (ds) open circular DNA genome that is encapsidated in a nucleocapsid together with a virally encoded polymerase. The compact genome is organized into four overlapping open reading frames (ORFs) that encode the viral polymerase, the core (HBcAg) and precore proteins, three envelope proteins of HBsAg consisting of the large (LHBs), the medium (MHBs) and small (SHBs), and the X protein. The precore and X proteins are classified as accessory proteins; precore protein is processed and secreted via the Golgi pathway into the serum as the hepatitis B e antigen (HBeAg) whilst X protein is a multifunctional protein which includes transcriptional transactivating properties. The co-ordinated expression of HBV genes occurs by the regulation of HBV promoters located within two enhancer regions as well as other *cis*-acting promoter elements.

Following entry and uncoating of the virus in the cytoplasm, the viral nucleocapsid is translocated into the nucleus where the HBV genome is released, undergoes repair and the genomic DNA is then organized around nucleosomes to form the viral minichromosome (Bock et al., 1994; Newbold et al., 1995) in which the viral DNA exists as a covalently closed circular (ccc) DNA molecule. The viral minichromosome is the template for HBV transcription using cellular RNA polymerase II. At least four major transcripts can be found including the 3.5 kb terminally redundant pregenomic RNA and three subgenomic mRNAs (Schaller and Fischer, 1991). The pregenomic RNA is used both as a template for genomic replication by reverse transcription and as the mRNA for the synthesis of the core, HBeAg and polymerase proteins. The subgenomic RNAs encode the HBV envelope proteins and the X protein (Seeger and Mason, 2000).

Following transcription of the viral minichromosome in the cell nucleus, pregenomic RNA is transported to the cytosol where formation of the viral nucleocapsid occurs by the encapsidation of the pregenomic RNA along with the polymerase into core particles (Hirsch et al., 1991). During maturation of the core particles, the pregenomic RNA is reverse transcribed into a relaxed circular partially dsDNA (reviewed in Tavis and Ganem, 1996; Seeger and Mason, 2000). Viral assembly is initiated when the newly synthesized viral nucleocapsid buds into the endoplasmic reticulum where it acquires a lipid bilayer containing the viral envelope proteins (Huovila et al., 1992). The resulting virions are then released from the cell via the constitutive pathway of secretion (Simon et al., 1988).

2.2. Role of HBV cccDNA

Virus persistence in infected hepatocytes during chronic infection depends on the maintenance of the HBV cccDNA pool (Locarnini et al., 1996; Seeger and Mason, 2000). During antiviral therapy of chronic HBV infected patients, this cccDNA replicative form is almost certainly a major contributor to the relapse phenomenon commonly observed following cessation of therapy (Yokosuka et al., 1985).

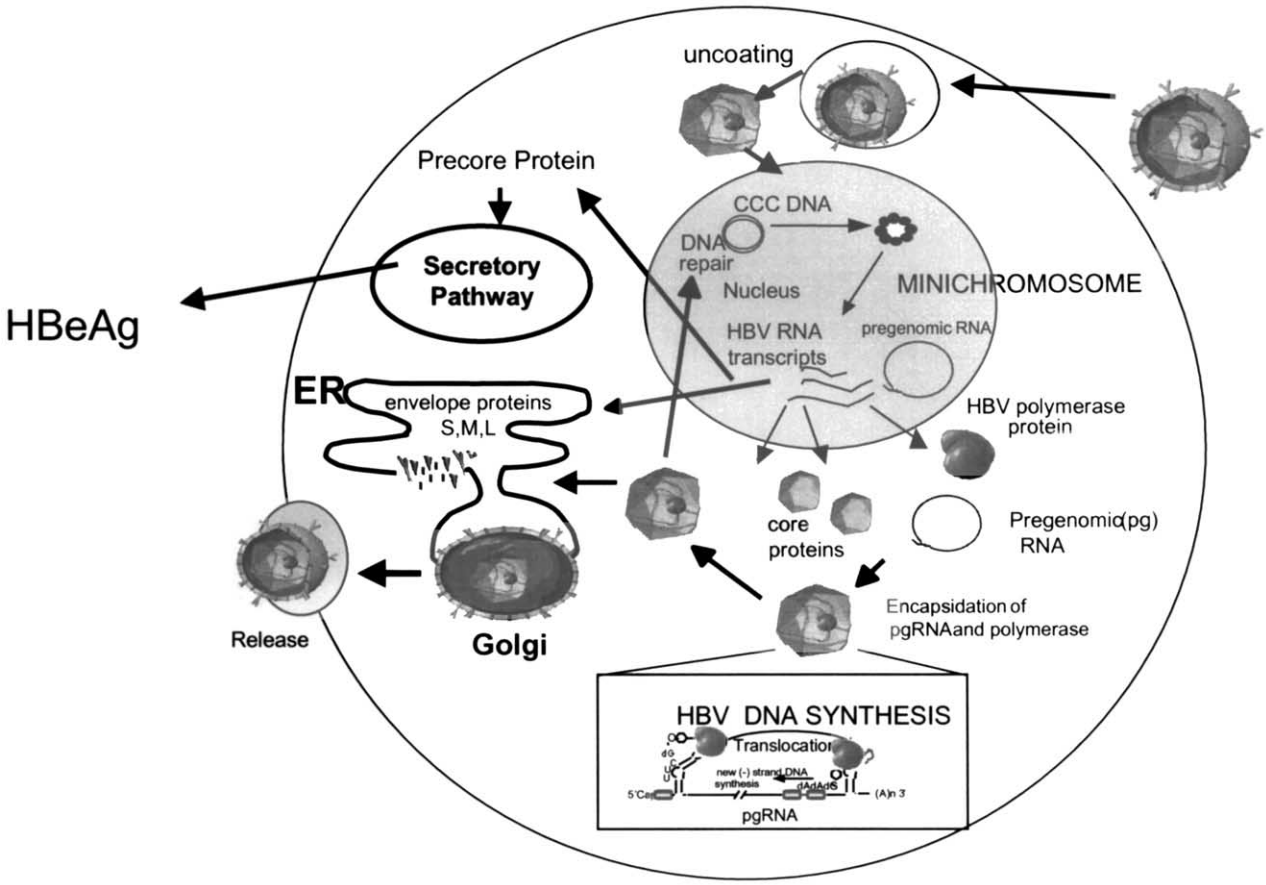

Fig. 1. Schematic view of the life cycle of HBV. In many details, this model is still speculative. It is assumed that the virus is endocytosed after attachment, the nucleocapsid is released to the cytosol and binds to the nuclear pore. The genome is transported into the nucleus, converted to cccDNA, and transcribed to generate three classes of mRNA. Translation of the pregenomic (pg) transcript in the cytosol allows for assembly of core particles that contain the pregenome. The transcripts for LHBs (Pre-S1 proteins) and M/SHBs (Pre-S2 and S proteins) are translated at the rough endoplasmic reticulum (ER) and HBs proteins are inserted in that membrane. The HBs particles bud to the lumen of the intermediate compartment, and at least a part of LHBs-rich ER-membrane areas envelopes core particles. The HBV and HBs particles are secreted thereafter by the constitutive pathway.

Viral nucleocapsids from infecting virions from mature intracellular nucleocapsids provide the source of cccDNA in the nucleus. During viral replication, the nucleocapsids can be directed for virion formation or transported into the nucleus, where the released viral genome is converted into cccDNA. This latter pathway is referred to as the intracellular conversion pathway and results in the amplification of the cccDNA pool (Tuttleman et al., 1986; Wu et al., 1990; see Fig. 1). This pathway is in part regulated by the viral envelope proteins via negative feedback (Summers et al., 1990; Lenhoff and Summers, 1994); early in infection when there are low amounts of viral envelope proteins, mature nucleocapsids are shunted into the nucleus whereby viral genomic DNA is converted into cccDNA. The number of cccDNA molecules increases as infection progresses and this leads to a rise in viral transcription. The viral envelope proteins are subsequently generated at high levels translationally and exert a negative feedback on cccDNA amplification by redirecting nucleocapsids into the pathway for virion formation. Failure of this regulatory pathway such that

high levels of cccDNA accumulate has been shown to result in the death of the hepatocyte (Summers et al., 1991; Lenhoff and Summers, 1994). Controlled cccDNA generation and processing is clearly an important element in maintaining viral persistence.

2.3. Extrahepatic sites of replication

Although the major source of virus production comes from HBV-infected hepatocytes, several studies in human and animal models have demonstrated the presence of HBV DNA and protein in other cell types. Within the liver, bile duct epithelial cells that form part of the biliary system have been shown to support viral replication (Luscombe et al., 1994, 1996a; Lin et al., 1996, 1998; Nicoll et al., 1996, 1997). Conclusive demonstration of de-novo replication of duck HBV in primary cultures of bile duct epithelium has recently been provided by Lee and colleagues (Lee et al., 2001) in which the complete life-cycle of duck HBV occurred including cccDNA generation, maintenance and processing. Extrahepatic sites such as the pancreas, kidney, spleen, peripheral blood mononuclear cells and lymphoid tissues (Fig. 2) have been found to harbour HBV DNA and proteins (Blum et al., 1983; Lie-Injo et al., 1983; Romet-Lemonne et al., 1983; Halpern et al., 1986; Tagawa et al., 1985; Walter et al., 1991). The presence of HBV genomes in these non-hepatic cells may have important implications in determining the outcome of antiviral therapy (Lee et al., 1998; see Fig. 2).

3. Pathogenesis of hepatitis B virus infection

3.1. Anti-HBV immune responses

The classic pathological picture during active liver disease caused by HBV infection is one of infected cell death in the presence of an inflammatory cellular infiltrate. The virus is believed not to be directly cytopathic. Rather, it is the immune response to infected hepatocytes that, even as it clears the infection, results in hepatocyte lysis (Bertoletti and Maini, 2000).

Clearance of HBV-infected hepatocytes is principally mediated by major histocompatibility complex (MHC) class-I restricted cytotoxic lymphocytes (CTL's; Chisari and Ferrari, 1995; Pignatelli et al., 1987). Analysis of HBV-specific CTL responses in acute HBV infection have demonstrated that effective viral control is associated with CTLs specific for a number of different epitopes within HBV core, polymerase and envelope proteins (Rehermann et al., 1995, 1996b; Penna et al., 1991; Maini and Bertoletti, 2000). Direct analysis of the frequency of CD8+ cells in acutely infected patients demonstrated that HBV core 18–27 specific CD8+ cells are the most numerous accounting for 1.3% of circulating CD8+ cells (Maini and Bertoletti, 2000). HBV-specific CD8+ T cell responses are present at greater frequencies in patients who successfully control HBV replication, at least 1 year after the recovery from infection, but are virtually absent from HBeAg-positive HBV chronic carriers except following treatment with lamivudine (LMV) or interferon-alpha (Bertoletti et al., 1994; Rehermann et al., 1996a,b; Boni et al., 1998). These data support the theory that a strong and multi-specific CTL response is linked to the ability to control HBV infection. Because of the circular minichromosome structure of cccDNA (Newbold et al., 1995) and its long term stability, clearance of cccDNA can probably only be achieved by the destruction or turnover of infected cells (Seeger and Mason, 2000). Data from chimpanzees, however, have suggested that hepatocellular cccDNA can also be controlled nonocytopathically, at least during the acute phase of acute self-limiting viral hepatitis (Guidotti et al., 1999).

The recognition of HBV-infected hepatocytes by antigen specific CTL's is believed to be the principle event determining the intensity of liver damage (Maini and Bertoletti, 2000) and the principal effectors to be the Fas/Fas-ligand and perforin/granzyme systems (Lowin et al., 1994). This hypothesis is supported by a transgenic mouse model of HBV infection (Moriyama et al., 1990), in which liver damage occurs after transfer of virus-specific CD8+ cells. In human HBV infection, it has been difficult to study intrahepatic pathogenesis as frequent liver biopsies of infected

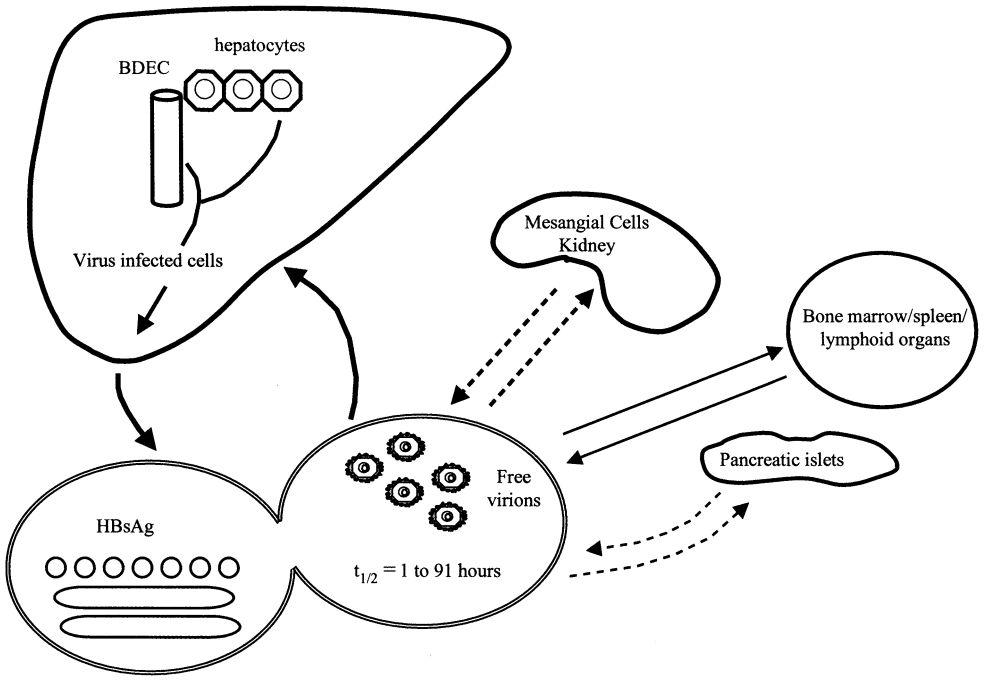

Fig. 2. Diagrammatic representation of the role both intrahepatic and extrahepatic sites play on viral dynamics during chronic HBV infection. While hepatocytes are the major source of HBV virions, bile duct epithelial cells (BDECs) and cells from extrahepatic sites such as the spleen, bone marrow, lymphoid organs, pancreas and kidney are reservoirs for the virus. The minimum virus production and clearance per day in an individual with chronic HBV has been calculated to be approximately 10^{11} virions per day (Zeuzem et al., 1997). Infectious virions produced from the various sites have an estimated half life ($t_{1/2}$) of 2–3 days. In contrast, the half-life of virus-producing infected cells has been calculated to be 10–100 days (Nowak et al., 1996; Zeuzem et al., 1997) (see Table 1).

individuals are not possible. For these reasons, knowledge of the HBV-specific CTL response has been mostly restricted to the circulating compartment, and confined to HLA-A2+ patients (Chisari, 1997). Overall these data have shown a better correlation between the virus-specific CD8+ response and protection, rather than liver damage. Recent studies using HLA-peptide tetrameric complexes have demonstrated HBV-specific CD8+ cells in the liver of HBV chronic carriers with and without liver disease. Contrary to expectations, a high frequency of intra-hepatic HBV-specific CD8+ cells was found in the absence of hepatic immunopathology. In contrast, virus-specific T cells were more diluted among liver infiltrates in viraemic patients, but their absolute number was similar because of the massive cellular infiltration (Maini and Bertoletti, 2000). Natural killer (NK) cells are also implicated in hepatocyte lysis (Chisari and Ferrari, 1995), and as discussed above, recent data point to a significant role for non-cytopathic mechanisms in the elimination of viral DNA including the cccDNA form, from the infected liver (Guidotti et al., 1999). The molecular mechanisms have not been identified but appear to precede and be independent of cytolytic processes, and have considerable effect on the generation, processing and turnover of cccDNA (Guidotti et al., 1999). In fact, viral cccDNA has been shown to be eliminated from the nucleus of infected cells in the absence of hepatocyte injury during transient infections (Guidotti et al., 1999).

An HBsAg-specific MHC class-2 restricted B-lymphocyte response is responsible for clearance of circulating virus (Ferrari et al., 1992; Beasley et al., 1983). Viral envelope protein epitopes are the principal targets. Particularly important is the conformational ‘a’ determinant and its flanking

subdeterminants as well as linear epitopes of the pre-S domains (Iwarson et al., 1985; Brown et al., 1984).

Critical to co-ordinating the integrated antiviral immune response are CD4+ T-helper (T_H) lymphocytes and a complex array of cytokines derived primarily from these cells (Chisari and Ferrari, 1995; Matloubian et al., 1994; Milich et al., 1986). T_H lymphocytes recognise linear peptides bound to MHC class-II molecules expressed by antigen presenting cells (APC's). Expression of peptides follows endocytosis of the viral antigens by APCs. The majority of active T_H cells in HBV infection respond to HBc- and HBe-derived epitopes bound to MHC class II molecules (Ferrari et al., 1990, 1992; Chisari and Ferrari, 1995). T_H cells, depending upon their microenvironment, differentiate into T_{H1} or T_{H2} functional T cell subsets. T_{H1} and T_{H2} cells differ in their cytokine profile (Mosmann et al., 1986; Thomas and Thurz, 1998); TH_1 cells produce interleukin-2 (IL-2), interferon-γ (IFN-γ) and lymphotoxin which potentiate cellular immune function, whilst TH_2 cells express CD40-ligand and secrete interleukins-4, 5 and 10, which support antibody production by B-lymphocytes (Chisari and Ferrari, 1995; Matloubian et al., 1994). The predominance of T_{H1}-type cytokine responses appears to play an important role in viral clearance of acute and chronic HBV infection (Milich, 1997). A single nucleocapsid-specific population seems able to co-ordinate both the anti-nucleocapsid cellular immune responses (Matloubian et al., 1994) and envelope specific humoral responses (Milich et al., 1987).

3.2. Dynamics of HBV Infection

Pathogenesis of chronic infection with HBV, HIV and HCV is characterized by a dynamic equilibrium between viral production and clearance, (Ho et al., 1995; Neumann et al., 1998; Nowak et al., 1996). The introduction of antiviral therapy can upset this equilibrium by inhibiting virus production and causing a decline in the viral load. Indeed, the rate of decline in the viral load is a measure of the rate of viral clearance and by inference, must be equivalent to the rate of virus production before therapy (Ho et al., 1995; Neumann et al., 1998; Nowak et al., 1996). Using a system of differential equations, mathematical functions describing the level of virus over time can be derived, which predict the fall in serum virus concentration following the introduction of effective antiviral therapy. Using regression analysis one can fit the experimentally derived viral load data and derive quantitative estimates for the various kinetic parameters associated with the viral infection. An essential prerequisite is the ability to accurately quantify the level of serum virus, and so along with the development of potent antiviral agents, the development of sensitive and specific DNA-based methods of viral quantification has been fundamental to the exploration of HIV, HCV and HBV dynamics.

In chronic HCV infection, the most important determinant of a sustained virological response is the clearance of infected cells during the period of viral inhibition (Neumann et al., 1998). All studies to date have identified a biphasic decay in HCV viral load following the administration of interferon-alpha; where the first phase decay has a half-life of 1.5–4.6 h and the second phase has a half-life of 1.7–70 days (Bekkering et al., 1998; Neumann et al., 1998). The first component of the decay curve represents clearance of free HCV virions. The second, slower phase of decay represents clearance of infected cells that have a longer half-life. Prolonged decay of this compartment may result from low-level production of interferon-resistant virus or alternatively, virus replicating in an interferon 'non-responding' cell population. The conclusion of most investigators is that decay of the 'second phase' will only occur by death of the infected cell population or by introduction of a second antiviral agent that 'accesses' the resistant cells (Thomas et al., 1999). Thus the inclusion of ribavirin in the therapeutic regimen has substantially improved the sustained response rates in infection.

The various models proposed to describe the fall in serum HBV differ principally in their underlying assumptions regarding the nature and efficacy of the inhibition of viral replication that is imposed upon the virus-host system by nucleoside analogue therapy, the behavior of the infected cell population after the commencement of therapy and

ultimately the residual effect of this population on the level of viraemia.

The experimental data can be analyzed with a model used by us and others (Neumann et al., 1998; Nowak et al., 1996; Zeuzem et al., 1997; Tsiang et al., 1999; Lewin et al., 2001). In the model, illustrated in Fig. 3, the dynamics of uninfected target cells (T, presumably hepatocytes), infected cells (I) and free virions (V) are considered. The equations for these populations are:

$$\frac{dT}{dt} = s - dT - (1-\eta)\beta VT + \rho I$$

$$\frac{dI}{dt} = (1-\eta)\beta VT - \delta' I - \rho I$$

$$\frac{dV}{dt} = (1-\varepsilon)pI - cV \qquad (1)$$

One assumes that target cells are created at rate s and die at rate d per target cell. These cells are infected at rate β per uninfected cell per virion. Infected cells die at rate δ' per cell, which may be different from the uninfected cell death rate due to viral or immune effects. In addition, infected cells may also revert to the uninfected state by loss of all cccDNA from their nucleus (Guidotti et al., 1999), at a rate ρ per infected cell. Thus the total rate of disappearance of infected cells is $\delta = \delta' + \rho$. Infected cells produce virions at an average rate p per infected cell, and viral particles are cleared from the circulation at rate c per virion. The virion production rate is assumed to be constant. In more complex models that also keep track of the number of copies of cccDNA per cell, the production rate could be made proportional to the cccDNA content of a cell. HBV-specific reverse transcriptase inhibitors will reduce the production rate of new virions i.e. p is decreased. Drug efficacy, ε, is defined such that the virion production rate under therapy is $(1-\varepsilon)p$. A drug that is 100% ($\varepsilon = 1$) efficient results in complete suppression of new virion production. To incorporate the possibility of reverse transcriptase inhibitors affecting infection, a parameter can be introduced that accounts for the efficacy of the drug in blocking new infection, η, so that the infection rate in the presence of drug is $(1-\eta)\beta$.

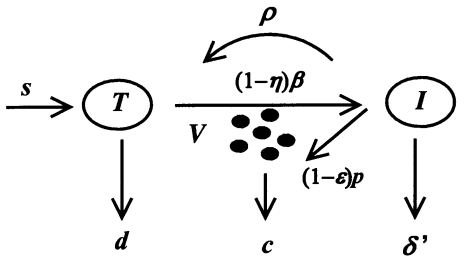

Fig. 3. Diagrammatic representation of the mathematical model for HBV treatment. Model depicts target cells, T; infected cells, I; and HBV virions, V. Model parameters are d = death rate of target cells, δ' = death rate of infected cells; ρ = rate of 'cure', i.e. non-cytolytic loss of infected cells; ($\delta' + ? = \delta$ = net loss rate of infected cells); c = clearance rate of free virions; ε = efficiency of drug therapy in inhibiting viral production; η = efficiency of drug therapy in preventing new infections; p = rate of production of virions per infected cell; β = rate of infection of new target cells; s = rate of production of new target cells.

To solve the model equations, one needs to assume that the number of target cells (Gao et al., 1993) remains approximately constant and that the virus and infected cells are initially at steady state. In the solution one can also account for the possibility of an initial delay existing between the start of treatment and its effect in reducing viral load, due to pharmacokinetics or the intrinsic viral life cycle time. For example, the initial delay in decline in viral load may differ between LMV, a nucleoside analogue that requires intracellular phosphorylation to an active triphosphate metabolite (Gao et al., 1993), and tenofovir, an acyclic phosphonate which can be regarded as a 'nuclease monophosphate' (nucleotide) analogue that does not require intracellular phosphorylation (Balzarini et al., 1991). This delay is accounted for in the model by allowing a temporal delay (τ) between the start of treatment and the initial decline of virus load (Neumann et al., 1998). The solution is then given by:

$$V(t) = \frac{1}{2} V_0 \left[\left(1 - \frac{c + \delta - 2\varepsilon c}{\theta} \right) e^{-\lambda_1 (t-\tau)} + \left(1 + \frac{c + \delta - 2\varepsilon c}{\theta} \right) e^{-\lambda_2 (t-\tau)} \right] \qquad (2)$$

where λ_1, λ_2 (the eigen values) are given by $1/2(c+\delta+\theta)$ and $1/2(c+\delta-\theta)$, respectively, and $\theta=\sqrt{(c-\delta)^2+(1-\varepsilon)(1-\eta)c\delta}$. This solution is valid for $t>t$. For $t<t$, the solution is $V(t)=V_0$, where V_0 is the initial viral load. The model can then be fitted to the data using non-linear least squares regression to find the parameters that best describe the data. From the fits one can obtain the parameters δ and c. From these one can calculate the infected cell and virion half-lives ($t_{1/2}$), given by $\ln(2)/\delta$ and $\ln(2)/c$, respectively, where the natural logarithm of 2, $\ln(2)\approx 0.693$.

Tsiang et al. (1999) followed the decline in serum virus caused by adefovir dipivoxil monotherapy, and quantified the level of serum HBV using the Roche COBAS AMPLICOR HBV MONITOR assay. They reported a clear and consistent biphasic decay. Regression analysis allowed the calculation of a mean viral half-life of 1.1 ± 0.3 days, a mean infected cell half-life of 18 ± 7 days, and a mean antiviral efficacy of 0.993 ± 0.008 (see Table 1).

Nowak et al. (1996), evaluated the result of lamivudine monotherapy through the Abbott Genostics solution hybridisation assay. They reported a similar virus half-life, dose-dependent anti-viral efficacy, and a broad range of infected cell half-lives from 10 to 100 days (Nowak et al., 1996; see Table 1).

Lau et al. (2000), used the Digene Hybrid Capture II assay (Murex Diagnostics Ltd., Dartford, UK) to monitor and compare the effect of lamivudine monotherapy and LMV and famciclovir (FCV) combination therapy on the pre-treatment dynamic equilibrium. A mean virus half-life of 2 days was generated, along with a mean infected cell half-life of roughly 40 days. The antiviral efficacy of the combination therapy was found to be significantly greater than that of lamivudine monotherapy (Lau et al., 2000).

Chronic HBV infection is a very dynamic process. However, studies of HIV and HCV dynamics suggest that these infections may be even more dynamic, with both infected cells and circulating virions showing a significantly shorter half-life for HIV and HCV, in comparison with HBV (see Table 1).

As will be discussed below, the characterization of HIV dynamics has provided a rational explana-

Table 1
Comparative dynamics among the three viral infections caused by HBV, HIV and HCV (modified from Tsiang et al., 1999)

	HBV ADV[a]	HBV LMV[b]	HIV ritonavir[c]	HCV IFN-a[d,e]	HBV[f] LMV or LMV+FCV
Plasma virus					
Half-life (h)	26.4	24	5.8	2.7–7.2	18.9
Mean lifespan (h)	36.9	34.6	8.4	3.9–10.4	270
Mean viral generation time (days)	24.8	24.7	2.7	3.8–7.3	20.5
Daily turnover (%)	48	50	95	94–99.8	56
Daily production (plasma)	2.1×10^{12}	10^{11}	10^{10}	$(1.1–12.7)\times10^{11}$	9.8×10^{11}
Total load	1.9×10^{12}	2×10^{11}	1.2×10^{9}	$(3.8–5.6)\times10^{10}$	1.4×10^{12}
Infected cells					
Half life (days)	11–30	10–100	1.6	2.4–4.9	2.4–>120
Mean lifespan (days)	23.3	23.3	2.3	3.5–7.1	19.4
Daily turnover (%)	2.3–6.2	1–7	38	13–25	4.7

ADV, adefovir; LMV, lamivudine; FCV, famclovir.
[a] The HBV ADV data are from Tsiang et al. (1999).
[b] The HBV LMV data are from Nowak et al. (1996).
[c] The HIV data is from Perelson et al. (1997a).
[d] The HCV data for the plasma virus are from Lam et al. (1997), Zeuzem et al. (1998), Neumann et al. (1998).
[e] The HCV infected cell data are Zeuzem et al. (1998) and Neumann et al. (1998).
[f] The HBV LMV/LMV+FCV data are from Lewin et al. (2001).

tion for the observed rapid development of phenotypic resistance to antiviral agents used as monotherapy, and has indicated the need for sustained, effective antiviral therapy if the infection is to be eradicated (Bonhoeffer et al., 1997; Perelson et al., 1997b). The smaller number of studies of HBV dynamics, the variation between them in the therapeutic agents used, the use of relatively insensitive assays for quantification of serum HBV DNA in two publications, and the subsequent variation in both the mean virus half-life and, in particular, the mean infected cell-half-life, has made such conclusions less easy to draw for chronic HBV infection.

4. Newer mathematical models and newer techniques

4.1. Kinetics of acute versus chronic HBV infection

Whalley et al. (2001), using data from a single source outbreak of HBV infection and the Roche Monitor PCR Assay for HBV DNA, characterized the kinetics of acute HBV infection in humans. After a peak viral load in serum of nearly 10^{10} viral copies ml^{-1}, clearance of HBV DNA followed a two or three phase decay pattern with an initial rapid decline characterized by a mean half-life ($t_{1/2}$) of 3.7 ± 1.2 days. Interestingly, this figure is similar to the half-life observed in the non-cytolytic clearance of cccDNA for other hepadnaviruses (Guidotti et al., 1999; Civitico and Locarnini, 1994). The final phase of decline in HBV viral load occurred at a variable rate ($t_{1/2}$ of 4.8–284 days) which the authors attributed to the rate of loss of infected hepatocytes. Free virus was found to have a mean $t_{1/2}$ of 1.2 ± 0.6 days and peak HBV production was estimated to be at least 10^{13} virions per day with a maximum production rate of an infected hepatocyte of 200–1000 virions per day on average. This last figure correlated well to estimates for duck HBV of the total DNA load in the liver of approximately 400–500 copies per cell (Lin et al., 1996).

The authors also noted several interesting clinical correlates with their viral dynamic analysis. For example in some patients, a faster viral doubling time appeared to correlate with the severity of acute infection. The non-specific symptoms experienced by patients as the virus titre started to fall were similar to those experienced by patients treated with interferon alpha, suggesting a role for cytokine mediated non-cytolytic down-regulation of the infection. This study also represented clinical confirmation of the observations reported by Guidotti et al. (Guidotti et al., 1999) of acute HBV infection in chimpanzees where 90% of virus-infected hepatocytes were cleared of viral infection by a cytokine driven (presumably interferon-gamma and IL-2) non-cytolytic process. Finally, Whalley and colleagues (Whalley et al., 2001) claimed that the first phase elimination (P-1) was due to the non-cytopathic removal or inhibition of HBV cccDNA replenishment.

4.2. New assays for quantification of HBV

More recently, Lewin et al. (2001) developed a real-time PCR assay using molecular beacon hybridization probes to analyze HBV viral load decline during antiviral therapy in patients with chronic hepatitis B on LMV versus combination (LMV plus FCV) therapy. The PCR reaction is carried out in a spectrofluorometric thermal cycler (ABI PRISM 7700, Applied Biosystems Inc.) that monitors changes in the fluorescence spectrum of each reaction tube during the annealing phase, while simultaneously carrying out programmed temperature cycles. The cycle number during PCR that yields a fluorescence intensity significantly above the background is designated as the threshold cycle (C_T). As reported previously, the C_T is directly proportional to the log of the copy number of the target sequence in the input DNA (Heid et al., 1996; Lewin et al., 1999). This assay is efficient (<4 h), specific, sensitive (detects HBV DNA in vivo at 100 copies ml^{-1}), dynamic (8 log range), and accurate (10% coefficient of variance). The assay has a high correlation with the Digene assay for viral loads $>10^6$ copies ml^{-1} but overcomes the limitations of the Digene assay, which has a low level of sensitivity (equivalent of 10^6 copies ml^{-1}) (Lewin et al., 2001). Although the Roche Amplicor Monitor assay is sensitive, sam-

ples with high viral loads must be diluted which increases the chance of error.

Lewin et al. (2001) demonstrated that the decline in viraemia of chronic HBV infection during potent antiviral therapy could not be entirely described by biphasic curves. Once therapy was initiated, there was a mean delay of 1.6 (1–5) days, before an antiviral effect could be detected, and in some patients, an increase in viral load was seen, after which a biphasic or multiphasic decay of viraemia occurred. In most patients, two patterns for the first phase elimination (P1: decay of viraemia) were observed with either a rapid ($t_{1/2}$ of 1.0 h) or a slow ($t_{1/2}$ of 92 h) slope being detected. This was not associated with pre-treatment viral load, serum ALT level or type of antiviral therapy (lamivudine monotherapy versus LMV plus FCV combination therapy). For these patients, the second phase elimination (P2: elimination of infected cells) was then either flat or slow with a $t_{1/2} = 7.2 \pm 1.2$ days. In some patients, a complex or 'stair-case pattern' was observed with further phases of viral decline and phases with little change in viral load.

These complex decay profiles could possibly represent both cytolytic and non-cytolytic mechanisms of infected cell loss and highlight the complexity of the chronic hepatitis B carrier state, reflected in the outcome of the virus–cell–host immune response interaction. However, these profiles need to be better understood in terms of developing alternative therapeutic approaches to improve the management of HBV infected individuals.

5. Combination chemotherapy for hepatitis B virus infection

5.1. Current therapies and the empirical approach

There are presently two licensed therapies for chronic hepatitis B: interferon alpha and LMV. The currently recommended regimen for interferon alpha is 5 million units (mu) given daily or 10 mu given 3 times a week by subcutaneous (sc) injection for 4–6 months. This regimen results in long term beneficial responses in around 35% of patients with HBeAg-positive chronic hepatitis B (Hoofnagle and Lau, 1997; Wong et al., 1995). The current recommended regimen for LMV is 100 mg given daily orally, for 1 year. This regimen results in beneficial responses in 16–20% of patients with typical HBeAg-positive chronic hepatitis B (Lai et al., 1998; Dienstag et al., 1995).

It is, therefore, logical to progress to the next stage and ask whether combination therapy of interferon alpha and LMV can increase the therapeutic benefit, especially since the two drugs appear to complement each other in terms of antiviral and immunomodulatory activities. Several studies have now combined interferon alpha and LMV with initially encouraging results, but to date no published conclusive evidence of significant benefit has really emerged.

The first pilot studies showed no adverse pharmacological interaction (Johnson et al., 2000), good tolerability with similar adverse event profiles to interferon alpha monotherapy, but unfortunately no significant increase in efficacy in those patients who were originally non-responders to interferon alpha (Mutimer et al., 1998). In a large randomized controlled trial of 226 previously untreated patients (Schalm et al., 2000), the HBeAg seroconversion rate for combination therapy was 29% and for the two monotherapies 19 and 20%, respectively. The difference between the response with combination and those with monotherapy was not significant on the intention-to-treat analysis, but in the 180 patients who adhered to the study protocol, the rate of response with combination therapy (36%) was significantly higher than the response with LMV (19%) or interferon alpha (22%).

More recently, Barbaro et al., 2001 compared 24 weeks of interferon alpha combination therapy with 52 weeks of lamivudine monotherapy in 151 patients with HBeAg-positive chronic hepatitis B. The sustained HBeAg seroconversion rate of 33% for combination therapy was, on the intention-to-treat basis, significantly different from 15% for lamivudine monotherapy. These results do support the concept of combining LMV and interferon alpha for the treatment of chronic hepatitis B.

5.2. Future directions based on viral dynamics and animal model studies

The variability of the profiles for the first and second phase elimination of viraemia in patients with chronic hepatitis B undergoing antiviral therapy discussed above highlights the challenges confronting treating physicians.

HBV infection is a complex and heterogeneous disease entity (Hadziyannis and Vassilopoulos, 2001). Furthermore, the end-points of therapy are still not entirely clear. For HBeAg-positive chronic hepatitis B, HBeAg seroconversion with normalization of serum ALT has been regarded as the goal of therapy. However, the positive impact of this end-point on the HCC risk, if any, is still not known. For HBe-Ag-negative chronic hepatitis B, total viral suppression to PCR-negativity has been associated with an improved biochemical and histological outcome. However, the relapse rate is high once therapy is stopped or drug resistance emerges (Hadziyannis et al., 2000), and again the HCC risk rate in responding patients is unclear.

In animal model studies (Luscombe et al., 1994; Luscombe and Locarnini, 1996b; Lin et al., 1996, 1998), the major effect of nucleoside analogue therapy on intrahepatic expression of viral antigens was on the level of cytoplasmic hepatitis B core antigen (HBcAg). Typically, by 4 weeks of therapy in the duck model of HBV, most nucleoside analogues had reduced intrahepatic HBcAg expression by an equivalent amount to the level of inhibition achieved for the liver viral DNA (Luscombe et al., 1994; Luscombe and Locarnini, 1996b; Lin et al., 1996, 1998). If similar virological events are occurring with human HBV infection during antiviral therapy, then it could be argued that when combinations of nucleoside analogues (such as LMV) and cytokines (such as interferon-alpha) are used clinically, that timing of the therapy may be an issue and that for the best antiviral effect, interferon-alpha therapy should be commenced first. The reason for this is because the major antiviral epitopes for immune-elimination driven by interferon-alpha against HBV are located on the viral nucleocapsid protein (HBcAg) (Chisari and Ferrari, 1995; Rehermann et al., 1996b). Thus, the intuitive better timing in the combination would be for the introduction of nucleoside analogues at the time when the antiviral effect of interferon-alpha is occurring which is around 8–10 weeks after the commencement of therapy (Gunther et al., 1992). This approach could ensure that patients would develop a rapid first-phase elimination profile with viral load dropping to below 10^4 copies ml^{-1}, a level that is significantly associated with HBeAg seroconversion (Gauthier et al., 1999).

However, in those interferon-alpha treated patients in which no antiviral response occurs (currently $> 50\%$), what should the approach be? Should the pegylated form of interferon-alpha, which ensures better pharmacokinetics and pharmacodynamics of the drug and antiviral state be evaluated? There has certainly been a significant improvement in the efficacy for treating chronic HCV infection with the introduction of pegylated interferon (Lindsay et al., 2001; Manns et al., 2001). A number of European investigators are presently comparing 52 weeks of combination pegylated interferon-alpha and LMV with monotherapy arms in more than 200 patients with HBeAg-positive chronic hepatitis B (Schalm et al., 2000).

Based on the interferon-alpha and lamivudine monotherapy and combination therapy trials, some important clinical and virological observations can be made. Firstly, during interferon-alpha therapy, there is typically a delayed effect on viraemia of up to 8–10 weeks after which the viral load drops very rapidly to less than 10^4 genome equivalents ml^{-1} in responder patients (Gunther et al., 1992). This is typically associated with HBeAg seroconversion and a hepatic flare. Secondly, with LMV treatment, the antiviral responses are much more rapid, occurring over the first 4–6 weeks of treatment (Dienstag et al., 1999). If the viral loads do not drop below 10^4 genome equivalents ml^{-1}, then HBeAg-seroconversion will not occur (Gauthier et al., 1999). As with interferon-alpha, increases in serum ALT are observed whilst on treatment over the 8–12 weeks period (Dienstag et al., 1999).

In the viral dynamic study of Lewin et al. (2001) comparing nucleoside analogue monotherapy with combination, all patients demonstrated a first

phase. The slope varied and this was not associated with pre-therapy viral load, serum ALT level or type of therapy. However, the antiviral efficacy was 0.951 for monotherapy and 0.985 for the combination with the HBV DNA drop in terms of logs of 2.9 and 5.2, respectively. The antiviral efficacy (ε) may be related to 'activation' of the nucleoside analogue to the antiviral state. Salvage enzymes in the liver are responsible for this activation (Shaw and Locarnini, 1995, 1999, 2000), and polymorphic variations are not uncommon (Tanaka, 1999; Poolsup et al., 2000). Thus, in order to achieve a greater efficacy, combinations of nucleoside/nucleotide analogues should be found which are able to reduce the viral load quickly as demonstrated by the clinical study of Lau et al. (2000) and Lewin et al. (2001). Rapid drop of HBV viraemia to below 10^4 copies ml^{-1}, as what occurs during acute hepatitis B infection with recovery (Guidotti et al., 1999; Whalley et al., 2001), and for HBeAg seroconversion during successful antiviral therapy (Gauthier et al., 1999) is an important efficacy endpoint for which clinical treatments need to achieve. A subset of patients in the Lewin et al. (2001) analysis demonstrated a flat or slow second phase, or complex 'stair-case' pattern. Since this phase reflects elimination of virus-infected cells, clearly some form of cytolytic T cell response (CTL) will be required to ensure this clearance. Is the hepatic flare associated with interferon-alpha secondary to activation of HBV specific CTL?

In animal model studies of acute hepadnavirus infection using woodchuck HBV, Guo et al. (2000) found that viral clearance occurred subsequent to the appearances of $CD4^+$ and $CD8^+$ T-cells as well as the production of interferon gamma and tumour necrosis factor alpha within the infected liver. These events were accompanied by a significant increase in apoptosis and regeneration of hepatocytes. Importantly, accumulation of virus-free hepatocytes was delayed for several weeks following this initial influx of lymphocytes indicating alternative mechanism(s) of immune sterilization to that described by Guidotti et al. (1999). Quite surprisingly, animals that did not clear virus infection and went on to become chronically infected, exhibited similar levels of T-cell accumulation, cytokine expression and apoptosis comparable with those observed during the initial phase of transient infections (Guo et al., 2000), implicating previously unrecognized pathway(s) to viral clearance.

Other animal model studies have firmly established that hepatocyte turnover is required in order to achieve rapid clearance of virus during antiviral therapy (Fourel et al., 1994a,b). These authors and others have concluded that in the absence of an immune clearance mechanism for the accelerated elimination of infected hepatocytes, inhibitors of virus replication would have to be administered for very long periods in order to substantially reduce the burden of infected hepatocytes in the liver. Obviously over this time period, breakthrough resistance becomes an issue (Richman, 2000).

Thus, previously unrecognized virological factors may be responsible for the inconsistent outcomes for treating chronic HBV infections. Further insights from animal model experiments have been recently described by Zhu et al. (2001) who have measured the kinetics of virus loss from the liver during antiviral therapy for up to 8 months in chronically infected woodchucks. Viraemia and intrahepatic replicative forms disappeared within 6 weeks of therapy but the cccDNA form declined more slowly, confirming previous studies of the recalcitrant nature of HBV cccDNA to susceptibility with antiviral agents (reviewed in Locarnini and Birch, 1999). Zhu et al. (2001), further demonstrated that the loss of cccDNA was comparable to that expected from the estimated death rate of hepatocytes in their woodchucks, suggesting that death of infected cells was the major route for elimination of cccDNA. However, the decline in the actual number of infected hepatocytes lagged behind the decline in cccDNA implicating the possibility that some fraction of cccDNA was actually distributed to daughter cells in those infected hepatocytes that passed through mitosis. These authors did not follow virological events in extrahepatic sites which could very well serve as another potential source of 're-infecting' virus.

In conclusion, as well as carefully defining intrahepatic events during antiviral therapy, the

role of sequestered virus in unique reservoirs within the liver such as the bile duct cells (Lee et al., 2001) and outside the liver including the lymphoid compartment, kidney and pancreas (Walter et al., 1991) needs to be re-appraised in light of the viral dynamic data and clinical responses seen following mono- and combination chemotherapy for chronic HBV infection. Clearly, new antivirals and immune modulators are needed to deal with these viral reservoirs either by accelerating their lysis (immunostimulation) or by inhibiting viral replication at the level of cccDNA. Furthermore, as well as new antiviral and immune modulating agents, a considered review and reappraisal of the existing therapeutic strategies is required in order to significantly improve our presently low successful treatment rates for chronic hepatitis B.

Acknowledgements

The authors would like to thank Dr. R. Ribeiro for critical review of the manuscript.

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