Hepatitis B virus: old, new and future approaches to antiviral treatment

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Patients chronically infected with hepatitis B virus (HBV) run the risk of developing cirrhosis and hepatocellular carcinoma in later life. Antiviral treatment offers the only means of preventing such an undesirable outcome. To date, interferon-α (IFN-α), an immunomodulator, and two synthetic nucleoside analogues, lamivudine and adefovir dipivoxil, are the only licensed antiviral agents for the treatment of chronic HBV infection. However, the standard treatment endpoints of loss of HBeAg with or without seroconversion to anti-HBe, normalization of serum transaminase levels, loss of HBV-DNA and improvement in liver histology following monotherapy with either types of agent are only achievable in ~20–30% of those treated. Long-term treatment with lamivudine is effective in suppressing viral replication, but drug-resistant mutants arise with increased length of treatment. Nevertheless, such mutants appear to be susceptible to adefovir and other nucleoside analogues that are undergoing Phase II/III clinical trials at the moment. Therapeutic vaccination and other molecular approaches such as antisense oligonucleotides, ribozymes, DNA vaccines, dominant-negative proteins and aptamers are possible future antiviral therapies, which will supplement our armamentarium against chronic HBV infection. It seems certain that combination therapies involving two or more nucleoside analogues, immunomodulators or gene therapies will be the future treatment regimens for chronic HBV infection.

Keywords: HBV, antivirals

Introduction

Conservative estimates place the number of persons chronically infected with hepatitis B virus (HBV) at >350 million. As these patients are at increased risk of developing cirrhosis, hepatic decompensation and hepatocellular carcinoma (HCC), therapeutic intervention offers the only means of interrupting this progression. The ultimate goals of treatment are to achieve sustained suppression of HBV replication and remission of liver disease. The agents currently available for the treatment of chronic HBV infection are divided into two main groups. The immunomodulators, which include interferon-α (IFN-α), thymosin α1, thymosin α2 and potential therapeutic vaccines, and nucleoside analogues, among which lamivudine (3TC), adefovir dipivoxil, entecavir, emtricitabine, β-L-2-deoxythymidine and famciclovir are the most well-known. At present however, only IFN-α and lamivudine are approved for chronic HBV treatment, and these have recently been joined by adefovir. The immunomodulators act by promoting cytotoxic T cell activity for lysis of infected hepatocytes and by stimulating cytokine production for control of viral replication. Nucleoside analogues on the other hand act by suppressing HBV replication at the level of DNA synthesis, and in addition there is evidence that they may enhance immune clearance of infected hepatocytes.

The hepatitis B virus

Classification

Hepatitis B is the prototype virus of the hepatadnaviridae, a name that signifies the hepatotropism and DNA nature of the
genome of its members. The family includes two genera. The \textit{Orthohepadnavirus} genus contains members that infect mammals, and other than HBV, includes hepadnaviruses that infect woodchucks (woodchuck hepatitis virus, WHV), squirrels, and primates such as chimpanzees, gibbons, gorillas, orang-utans and woolly monkeys.\textsuperscript{5} The \textit{Avihepadnavirus} genus contains members that infect birds such as ducks (duck hepatitis B virus, DHBV), herons, storks and geese.\textsuperscript{6,7} The WHV and DHBV animal models have proved invaluable in the assessment of the efficacy of potential antiviral agents before human trials, as discussed later on.

\textbf{Structure}

The infectious virion or Dane particle has an outer envelope, which consists of the hepatitis B surface antigen (HBsAg) in a lipid bilayer. This in turn encloses the nucleocapsid core of the virus, within which lies the viral genome. The latter is a relaxed circular, partially double-stranded DNA molecule of 3.2 kb in length, and contains four partially overlapping open reading frames (ORFs) (Figure 1).\textsuperscript{8} The Pre-S/S ORF encodes the three envelope glycoproteins that are known as the large (L), middle (M) and small (S) HBsAgs. The precore/core one yields two translation products, the precore polypeptide being the precursor of the soluble hepatitis B e antigen (HBeAg), and the nucleocapsid or core protein. One of the other two ORFs encodes for the X protein and the remaining one for the polymerase, which acts as a reverse transcriptase (rt) and also has DNA polymerase activity.\textsuperscript{8,9}

\textbf{Replication}

The life cycle of the virus begins with its attachment to the appropriate hepatocyte receptor, which still remains unknown. In contrast, the region between amino acids 21 and 47 of the Pre-S1 has long been known to be involved in virus binding to the hepatocyte membrane.\textsuperscript{10,11} Recently, it has been suggested that a domain within the small S protein may also be involved in attachment to the hepatocyte also, bringing the virus particle into close contact with the cell membrane, and thus facilitating the specific interaction of the Pre-S1 domain with its receptor.\textsuperscript{12} The virion is internalized and uncoated in the cytosol, whence the genome translocates to the nucleus, where it is converted into a double-stranded covalently closed circular DNA (cccDNA) molecule, following completion of the shorter positive (+)-strand and repair of the nick in the negative (−)-DNA strand.\textsuperscript{8,9,13} In this form, cccDNA serves as the template for viral transcript synthesis by host RNA polymerase II. Most antiviral agents so far have been unable to prevent the replenishment of the cccDNA pool from genomic HBV-DNA recycled from the cytoplasm, or to effect efficient clearance of cccDNA-containing hepatocytes.\textsuperscript{14} This explains the rather rapid rebound in serum HBV-DNA after cessation of antiviral treatment.

Viral kinetic studies have indicated that whereas virion half-life is about a day, the half-life of infected cells is much longer and variable, ranging from 10 to 100 days.\textsuperscript{15,16} This biphasic response pattern, however, in the case of HBV, and unlike hepatitis C virus (HCV) or human immunodeficiency virus (HIV), may not be universal. Recent findings suggest that viral decay patterns may be more complex or multiphasic (reviewed by Lewin et al.\textsuperscript{17}), possibly representing both cytolytic and non-cytolytic mechanism involvement in loss of infected hepatocytes. The implications of these findings have a bearing on the development of alternative therapeutic approaches in order to improve the management of HBV-infected individuals. The dosage, duration, timing, combination of antiviral agents and treatment regimens (concurrent, staggered or consecutive) will need to be further optimized if complete eradication of the cccDNA pool is to be achieved. However, this may not be possible in view of calculations that indicate that treatment will be necessary for very long periods of time, making such attempts expensive, impracticable and with an increased risk of breakthrough resistance.

One of the viral RNA transcripts, known as the pregenomic RNA (pgRNA), is longer than genome length (3.5 kb) and forms the template for (−)-DNA strand synthesis, but also constitutes the message for core and polymerase protein translation (Figures 1 and 2).\textsuperscript{18} The latter protein has three
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Figure 2. Schematic of the replication mechanism of hepatitis B virus. The viral polymerase binds to ε and directs the synthesis of a short primer using as template the nucleotide sequence of the bulge as shown (a). The polymerase–primer complex translocates to the 3′ end of the pgRNA and base-pairs with DR1, with which is homologous (b). As (−)-DNA strand synthesis proceeds (c), the pgRNA template is degraded by the RNase H domain of the polymerase, apart from its terminal 18 or so bases. These bases constitute the RNA primer which initiates (+)-DNA strand synthesis (d). The primer translocates to the 5′ end of the newly synthesized (−)-DNA strand and anneals with the homologous DR2 region. (+)-DNA strand synthesis then proceeds in the direction shown by the arrow, which necessitates yet another translocation event to the 3′ end of the (−)-DNA strand. Both of these events are most likely to be facilitated by the effective circularization of the (−)-DNA strand. This becomes possible as a result of the covalent attachment of the 5′ end of the strand to the polymerase, which is maintained during continued synthesis of the (−)-DNA strand. Thus, the two ends of the strand are brought into close proximity with each other (e).

The interaction between the polymerase and ε sets in motion the events that take place during viral replication. As a consequence of the terminal redundancy of the pgRNA, the epsilon sequence and flanking region containing direct repeat 1 (DR1) are duplicated (Figure 2). The bulge of the ε structure serves as a template for the synthesis of a 3–4 nucleotide-long DNA primer, which is covalently attached to the polymerase though a tyrosine residue of the terminal protein (position 96). This event involves the ε structure at the 5′ end of the pgRNA, and is then followed by the translocation of the polymerase–primer complex to the 3′, where it hybridizes with the DR1 region with which it shares homology. As the complex proceeds towards the 5′ end of the pgRNA, the (−)-DNA strand is synthesized by reverse transcription and the RNA template is concurrently degraded by the RNase H activity of the polymerase, except for the final 18 or so ribonucleotides. A second translocation event then occurs during which the ribonucleotide primer hybridizes with the DR1 region at the 5′ end of the newly synthesized (−)-DNA strand and anneals with the homologous DR2 region. (+)-DNA strand synthesis then proceeds along the 5′ end of the complete (−)-DNA strand. A template exchange occurs that allows the (+)-DNA strand synthesis to proceed along the 5′ end of the complete (−)-DNA strand, effectively circularizing the genome. Once the maturing nucleocapsid is enveloped by budding through the endoplasmic reticulum membrane, the nucleotide pool within the capsid cannot be replenished, hence the incomplete nature of the (+)-DNA strand.

Mutations

The HBV genome is not as invariant as originally thought. Natural variants of the virus exist, which give rise to well-recognized serological subtypes and its genotypes. However, since HBV replicates through an RNA intermediate that is reverse transcribed, this step in the replication cycle of the
virus is prone to errors. These may occur during pgRNA synthesis by the cellular RNA polymerase II, as RNA polymerases show inherently low copying fidelity, but also during reverse transcription due to the lack of proof-reading capacity by the viral polymerase.26 Fluctuations in the composition of the intracellular nucleotide pools is another possible contributing factor. Thus HBV has a higher mutation rate than other DNA viruses (2 × 10^{-4} base substitutions per site per year).27 Although a lot of these mutations would be deleterious to the virus, as a result of constraints imposed by the overlapping ORFs, some would be advantageous, either offering a replication advantage, or facilitating immune escape. Such are the HBsAg variants28 and the precore and core-promoter variants.29–31 The last two variants predominate in anti-HBe-positive patients with detectable levels of HBV-DNA and have important implications in the treatment of such patients with antiviral agents.32

The most common precore mutation is the G1896A substitution, which creates a premature termination codon that abrogates HBeAg production.33 This variant is commonly found in association with HBV genotype D, which prevails in the Mediterranean basin, genotypes B and C, which are prevalent in countries of the far East, and genotype E in Africa. In contrast, this mutation is rarely detected in genotype A strains found in Northern Europe and North America. The selection therefore of the G1896A mutation occurs in patients carrying HBV genotypes with a T at position 1858 in the precore region, which allows for stable base-pairing with the A change at position 1896.34 The double mutation affecting the core promoter region (A1762T, G1764A) results in decreased transcription of the precore mRNA, with a knock-on effect on HBeAg production, whilst pgRNA production is up-regulated.35 Patients with HBeAg-negative chronic liver disease tend to have lower HBV-DNA levels than HBeAg-positive patients,36 and may experience frequent exacerbations with fluctuating transaminase levels.32,37,38

**Patient groups**

HBV is transmitted following perinatal, percutaneous and sexual exposure, but also by contact with open cuts and sores, as may occur between children in hyperendemic areas.39 Following acute infection, the risk of becoming a chronic carrier of HBV is age dependent. This exceeds 90% in newborns of HBeAg-positive mothers, ranges between 25% and 30% in infants and very young children, but in adults this risk is only between 5% and 10%.40–42 The patient groups with chronic HBV infection, defined as the persistent presence of HBsAg in the serum of an individual for 6 months or longer,43 who could benefit from antiviral therapy include:

(i) HBeAg-positive patients who have transaminase levels greater than twice the normal, are positive for HBV-DNA and have necroinflammatory changes in liver biopsy material.

(ii) HBeAg-negative patients, who have active liver disease as shown by transaminase elevations (twice the normal), HBV-DNA positivity (>10^5 copies/mL) and moderate to severe hepatitis on biopsy.

(iii) Patients with compensated cirrhosis, and even patients with decompensated cirrhosis with treatments other than IFN.

(iv) Immunosuppressed patients, as a consequence of organ transplantation. Patients immunosuppressed as a result of HIV infection are normally excluded from treatment protocols.

**Interferon**

Interferons have immunomodulatory, but also antiproliferative and antiviral effects. Lymphoblastoid and recombinant IFN-α, have been used in turn since the early 1980s in attempts to achieve sustained suppression of HBV replication, and remission of HBV-related chronic liver disease. The drug is administered by subcutaneous injection and the recommended dosage for adults is 5 MU (million units) daily or 10 MU thrice weekly for a period of 16 weeks in HBeAg-positive patients, or 12 months for those who are HBeAg-negative. The recommended dose for children is 6 MU/m^2 thrice weekly with a maximum of 10 MU.

**Efficacy**

A meta-analysis by Wong et al.44 of 15 randomized placebo-controlled studies of IFN-α treatment in HBeAg-positive patients showed loss of HBV-DNA, HBeAg and HBsAg in 37%, 33% and 7.8% of patients, whereas in controls the respective figures were 17%, 12% and 1.8% (Table 1). In Asian patients (primarily Chinese), the treatment is generally less effective, particularly in patients with normal alanine transaminase (ALT) levels.45 In contrast, those with raised ALT respond similarly to Caucasian patients.46 This difference in response between Chinese and Caucasian patients is thought to be related to the duration of the chronic state. Whereas in Asian patients, exposure to the virus occurs early in life, either at birth or postnatally, in Caucasian patients infection is acquired during adolescence or adulthood, primarily through sex or intravenous drug abuse. In the former case, infection is followed by a lengthy period of immune tolerance (normal ALT),47,48 whereas in the latter patients, there is a more active host immune response directed towards clearance of the infection (active liver disease, raised ALT levels).49 In children, the response rate is similar to that in adults, being ~30% in those with raised ALT, as opposed to 10% in those with normal levels.47,50,51 The durability of HBeAg loss in the above patient groups is as high as 90%, after many years of follow-up, whereas HBsAg clearance during this time is more varied, ranging from 12% to 65%.52–58

Criteria for determining IFN-α treatment efficacy in HBeAg-negative patients include serum HBV-DNA nega-
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Table 1. Antiviral responses following therapy with either IFN-α or lamivudine in patients with HBeAg-positive and HBeAg-negative chronic hepatitis B. The figures given are those at the end of the follow-up period\textsuperscript{49,59,112}

<table>
<thead>
<tr>
<th></th>
<th>Interferon patients</th>
<th>controls</th>
<th>Lamivudine patients</th>
<th>controls</th>
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</thead>
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<tr>
<td>HBeAg-positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>loss of HBV-DNA</td>
<td>37%</td>
<td>17%</td>
<td>17–33%</td>
<td>11%</td>
</tr>
<tr>
<td>loss of HBeAg</td>
<td>33%</td>
<td>12%</td>
<td>16–18%</td>
<td>5%</td>
</tr>
<tr>
<td>HBeAg seroconversion</td>
<td>18%\textsuperscript{a}</td>
<td>1.2%</td>
<td>&lt;1%</td>
<td>0</td>
</tr>
<tr>
<td>loss of HBsAg</td>
<td>7.8%</td>
<td>1.2%</td>
<td>&lt;1%</td>
<td>0</td>
</tr>
<tr>
<td>HBeAg-negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>loss of HBV-DNA</td>
<td>28%</td>
<td>10%</td>
<td>25–30%</td>
<td>–</td>
</tr>
<tr>
<td>HBV-DNA –ve/ALT normal</td>
<td>18–25%</td>
<td>0</td>
<td>11–20%</td>
<td>–</td>
</tr>
<tr>
<td>loss of HBsAg</td>
<td>2.5%</td>
<td>0</td>
<td>0</td>
<td>–</td>
</tr>
</tbody>
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\textsuperscript{a}Difference in proportions.

\textsuperscript{–} Data not available.

activity and normalization of ALT. Response rates have been variable as a result of heterogeneity in disease patterns (continuous activity, fluctuating or intermittent), virus variation (genotypes, precore variants, basic core promoter variants), treatment regimens and their duration. Most of the data available originate from Greece and Italy,\textsuperscript{59,67} countries where HBV genotype D is prevalent and the precore stop codon mutation is common. Nevertheless, end of treatment response rates range between 38% and 90%, which, however, are not sustainable, as virological relapses are quite common, ranging from 54% to 87% (sustained response 18–25%). Similar rates of response (18–23%) have been reported in relapsed patients who have been retreated with IFN.\textsuperscript{68,69} About a third of the sustained responders may seroconvert to anti-HBs also.\textsuperscript{59,65,67,68}

More importantly, IFN-α treatment has been shown to slow down disease progression in comparison with untreated controls, improve survival and reduce HCC occurrence.\textsuperscript{64,65}

In decompensated HBV-related cirrhosis, IFN-α administration is not recommended even though it could be of benefit, particularly in Child’s A cirrhotic patients, since it is frequently associated with major complications such as variceal bleeding, aggravation of ascites or encephalopathy, development of pneumonia, bacterial infections and gastric ulcer bleeding.\textsuperscript{70,71} However, such severe side-effects were shown to be relatively uncommon following prolonged treatment (up to 48 months) with low doses of IFN-α (3 MU three times a week) and careful monitoring of the patients. Sustained loss of serum HBV-DNA and HBeAg, with normalization of ALT and clinical improvement with good survival rate, were observed in 65% of patients.\textsuperscript{72}

Patients with decompensated cirrhosis are potential candidates for orthotopic liver transplantation. Such patients with active viral replication have a high rate of HBV recurrence and reduced survival post-transplant due to accelerated HBV-related allograft disease, and this, in spite of passive immunoprophylaxis with anti-HBs (hepatitis B immune globulin; HB Ig) in the immediate post-transplant period. Interferon can therefore be administered pre-transplant to reduce viral load, which together with HB Ig post-transplant would prevent reinfection of the new liver graft. This has been shown to be the case in two studies using 1–3 MU three times a week of IFN-α.\textsuperscript{73,74} In both studies, attainment of HBV-DNA negativity before transplant was essential in preventing re-infection.

The efficacy of other more recently developed interferons, such as consensus and pegylated interferon, which have been used in the treatment of chronic HCV infection, is presently being assessed in clinical trials in HBV carriers.

Predictive factors

Factors that have been associated with a favourable outcome following IFN-α treatment include high pre-treatment ALT and low serum HBV-DNA levels,\textsuperscript{75–77} parameters that indicate that the patient is already in the immune clearance phase. In contrast, factors that have been associated with poor response include male sex, length of chronic state, Asian origin, precore mutations, homosexuality and HIV co-infection. HBV genotype in relation to IFN-α response rates has not been investigated in any of the clinical trials so far. In a recent retrospective study from Taiwan, however, it was established that patients with genotype B were more likely to respond to IFN treatment than those with genotype C (41% versus 15%).\textsuperscript{78} The latter patients had higher ALT levels and a higher frequency of core promoter mutations.
Prednisolone priming

Early observations suggested that steroid withdrawal is associated with an immunological rebound resulting in HBV-DNA decline. This might be beneficial to the patient if it is timed to coincide with the start of IFN-α therapy. A meta-analysis however of several clinical trials of IFN-α use after priming with prednisolone showed that such an approach was of marginal benefit and in only some of the patients. This contrasts with the findings of a European study reporting that prednisolone priming resulted in a higher rate of HBeAg seroconversion than IFN alone. A major drawback in this approach is the danger of hepatic decompensation following steroid withdrawal, an undesirable event that has led to the abandonment of this type of treatment.

Adverse effects

IFN-α therapy is associated with a number of adverse effects. Among these, flu-like symptoms that can be relieved with paracetamol, fatigue, leucopenia and depression are the most frequently reported. Hair loss, anorexia, mood swings and irritability have also been reported. Finally treatment with IFN may unmask or aggravate underlying autoimmune disorders, such as thyroiditis.

Other immunomodulators

It is generally accepted that patients with chronic HBV infection have weak and restricted T cell responses, whereas such responses are robust and multispecific during recovery from acute infection. Therefore, immunomodulators that can stimulate T cell responses may be effective in the treatment of chronic HBV patients. Thymosin-α1 is a synthetic peptide of 28 amino acids, which promotes T cell maturation and induces cytokine production, including IFN-γ and interleukin 2 (IL-2). Early experiments with thymosin in chronically infected woodchucks showed a 1000-fold reduction in serum WHV-DNA levels in four of the six animals that were treated. In human trials, thymosin was well tolerated, but data on efficacy gave somewhat mixed results. Two trials, one in Chinese patients and the other in patients from the USA, reported loss of HBeAg and HBV-DNA in 41% and 27% of patients treated for 6 and 12 months, respectively, versus 9% of controls in the former trial, and 14% versus 4% after 6 months of therapy in the second. Evaluation was at 18 and 12 months, respectively, after start of treatment. A recent meta-analysis of five trials indicated that thymosin is effective in suppressing viral replication in chronic HBV infection, but this effect is delayed and becomes apparent 12 months after the end of treatment.

Cytokines other than the IFN-α and -β have also been used in the treatment of chronic HBV infection. Interleukin-12 (IL-12), which favours the differentiation of T helper cells to Th1 cells, was evaluated in a pilot study during which 46 HBeAg-positive patients were treated for 12 weeks with different doses of a recombinant human IL-12 preparation. After a further 12 weeks of follow-up, seroconversions were seen only in the higher dose groups, which amounted to a modest 16%. More studies are therefore needed to establish whether IL-12 has any role in the treatment of chronic HBV. The efficacy of other cytokines such as IL-2 and IFN-γ, and immunostimulants like levamisole, in the treatment of chronic HBV patients has been disappointingly poor.

Nucleoside analogues

How they work

Nucleoside analogues are chemically synthesized drugs that are able to mimic natural nucleosides. As such, they are incorporated into newly synthesized HBV-DNA causing chain termination, and thus inhibiting viral replication. In addition, some of them competitively inhibit the DNA-dependent and reverse transcriptase activity of the viral polymerase. For this to occur, the analogues need to be phosphorylated within cells to their triphosphate counterparts. Nucleoside analogues can be produced in their natural D- or unnatural L-configuration, and these are often referred to as enantiomers. Template-dependent DNA polymerases add both L- and D-enantiomers of dNTP analogues to DNA with equal efficiency, when there is no 3’ substituent present. Interestingly, however, the HBV polymerase has a preference for the L- over the D-configuration enantiomers. Enantiomer reconfiguration enantiomers appear to have antiviral activity comparable to and sometimes greater than that of their D-counterparts, are less toxic and have greater metabolic stability. Steps in the life cycle of HBV that may be inhibited by nucleoside analogues include the synthesis of the (−) strand by reverse transcription, the synthesis of the (+) strand, the amplification and replenishment of the cccDNA pool in the hepatocyte nuclei from non-enveloped core particles, and the cccDNA formation in newly infected cells. Experiments in woodchucks suggest that nucleoside analogue treatment does not have an appreciable effect on the cccDNA pool in hepatocytes. Similar studies in a recombinant baculovirus—HepG2 cell system showed inhibition of cccDNA accumulation, only if lamivudine was present before infection. More recently, it was observed that cccDNA declined exponentially in DHBV congenitally infected ducks on combination therapy with lamivudine and a dideoxyguanosine prodrug. This decline was seen in animals whose liver biopsies had significantly greater numbers of nuclei staining positive for the cell division marker PCNA, than in animals in which cccDNA levels had reached a plateau. The effectiveness of nucleoside ana-
logues in reducing the cccDNA pool may thus be dependent on the cell cycle phase.

**Lamivudine**

Lamivudine, also known as 3TC (Epivir) is the L-enantiomer of the deoxycytidine analogue 2’,3’-dideoxy-3’-thiacytidine. This is metabolized within hepatocytes to the active triphosphate, by stepwise addition of phosphate groups (Figure 3). The drug contains a sulphur atom instead of carbon at the 3’ position of the sugar ring, which does not allow chain elongation by phosphodiester bond formation, in the absence of the normal 3’ hydroxyl group. Since lamivudine acts by terminating viral DNA synthesis and competitively inhibiting the viral polymerase/rt, it is equally effective in patients of any race, but also against both the wild-type virus and precore/core promoter variants. In addition, there is evidence to suggest that lamivudine treatment may overcome cytotoxic T cell hyporesponsiveness seen in chronically infected patients.

**Efficacy**

Several randomized clinical trials in HBeAg-positive patients showed that a 1 year course of lamivudine monotherapy induced HBeAg seroconversion in 16–18% of them, compared with 4–6% in controls. Histological improvement by at least two points in the histological activity score was observed in 49–56% of those treated and in ~25% of controls. Subsequent studies showed that HBeAg seroconversion rates increased with length of therapy rising from 17% at 1 year to 27%, 36% and 47% at years 2, 3 and 4, respectively. Despite the emergence of lamivudine-resistant variants, which are described further on, continued treatment results in further HBeAg seroconversions, and median HBV-DNA and ALT levels are maintained at lower than baseline values.

The most important predictor of a favourable response following lamivudine treatment is the pre-treatment ALT level. It has been shown that the higher the ALT, the higher the rate of HBeAg seroconversion, rising from 2% in those with normal ALT to 47% in those with levels five times the upper limit of normal.

The efficacy and tolerability of lamivudine as a treatment for chronic HBV infection in children have also been investigated. In a recent trial, the rate of HBeAg loss and HBV-DNA negativity was higher among 191 children who received lamivudine than among the 97 who received placebo, at week 52 of treatment (23% versus 13%, P = 0.04).

Treatment of HBeAg-negative patients with lamivudine results in response rates that are equivalent to those in HBeAg-positive patients.

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**Figure 3.** Chemical structure of some of the nucleoside analogues that are presently used, or have been used in the past, for the treatment of chronic HBV infection. POM, pivaloyloxymethyl.
positive ones. The results from several studies have recently been reviewed by Rizzetto\textsuperscript{112} and indicate that end points of loss of HBV-DNA and ALT normalization were achieved in 65–96% of patients at the end of treatment.\textsuperscript{97,98,113–118} but patient relapse rates after 1 year of follow-up amounted to 45–74% (sustained response of 11–20%). Histological improvement as demonstrated by a reduction in the Knodell necroinflammatory score was seen in 60% of patients.\textsuperscript{97} In the same study, the fibrosis score improved in 11%, and remained the same in 86% of patients. Two other studies reported histological improvements of 22%\textsuperscript{118} and 95%,\textsuperscript{119} and in addition, 35% of patients had improvement in fibrosis in the latter study. It appears therefore that lamivudine treatment results in histological improvement, which is comparable between HBeAg-positive and -negative patients. Moreover, the arrest or reduction in the immune-mediated inflammatory response in the liver leads to reduced scarring, with a consequent beneficial effect on fibrosis.\textsuperscript{120} Finally, lamivudine treatment has been used in patients with severe acute exacerbations complicating chronic HBV infection, and shown to be effective, even in patients with hepatic failure, 8/10 of whom had uneventful recoveries.\textsuperscript{121}

The efficacy of lamivudine in HBeAg-negative patients has also been evaluated for periods >12 months. Response rates as defined above generally dropped by 24 months of treatment, by between 14% and 36% of those at 12 months.\textsuperscript{98,114,115,122} For example, virological response rates of 68% at 12 months decreased to 52% and 41.6% at 18 and 24 months, respectively.\textsuperscript{114} Such breakthroughs are invariably due to HBV variants with mutations in the polymerase gene and these are discussed in detail later on. Other than the duration of treatment, core promoter mutations have been associated with the selection of lamivudine-resistant mutants.\textsuperscript{98}

Lamivudine treatment of patients with decompensated cirrhosis has shown that the drug is well tolerated and leads to improvement in the clinical picture of many patients, to the extent that some of them can be removed from liver transplantation lists.\textsuperscript{123–126} In the liver transplantation setting, treatment with lamivudine inhibits HBV replication and improves liver function in patients awaiting a liver transplant.\textsuperscript{127–131} HBV-DNA levels become undetectable after just 12 weeks of treatment,\textsuperscript{128,130} whereas histological improvement in the necroinflammatory and fibrosis scores was seen in 50% and 26% of patients, respectively.\textsuperscript{129,131} In a recent trial where lamivudine was used before and after transplantation, 60% (25/42) of patients were HBsAg-negative at their last visit (12 weeks or more after transplantation). At week 156, 59% (13/22) still remained HBsAg-negative. All of the re-infected patients (nine in all) were HBV-DNA-positive before transplantation.\textsuperscript{132} Prevention of recurrence of infection after liver transplantation has also been attempted with lamivudine in combination with HBIG.\textsuperscript{133} In this study, lamivudine treatment was initiated before or at the time of transplantation, and continued thereafter. At 1 year, actuarial patient and graft survival was 93%, and at a median 346 days after transplantation, all surviving patients (13/14) had undetectable HBV-DNA. In another study, low risk liver transplant patients (HBsAg-positive/HBV-DNA-negative) on HBIG treatment for 6 months after transplantation were randomized to receive either lamivudine or continue on HBIG: 11/12 patients on HBIG and 10/12 on lamivudine remained HBsAg-negative, but HBV-DNA was detectable by PCR in 6/8 patients on HBIG and 5/7 on lamivudine over a period of 6–22 months. All patients remained HBsAg-negative with normal graft function.\textsuperscript{134} Development of anti-HBs with prolonged lamivudine treatment has also been reported.\textsuperscript{135}

Finally, prednisolone withdrawal followed by lamivudine treatment gave virological responses in 60% of patients with ALT over five times the upper limit of normal, and only in 10% of those with no significant ALT rebound.\textsuperscript{136}

Long-term outcome

The durability of HBeAg seroconversion in lamivudine-treated patients is variable, ranging in some studies between 38% and 73%,\textsuperscript{107,108,137} whereas in others it was maintained.\textsuperscript{103,138} In one study, 9% of patients lost HBsAg also, after a median follow-up of 21 months.\textsuperscript{139} Post-treatment responses have also been evaluated in HBeAg-negative patients for up to a period of 2 years. A sustained virological and biochemical response was maintained in only 11–20% of patients.\textsuperscript{97,113,118} Relapse rates were 48% after 6 months of follow-up and rose to 74% by 12 months.\textsuperscript{113}

Drug resistance

Of major concern is the emergence of drug-resistant variants of HBV following lamivudine treatment. Breakthrough infections, indicated by virological and biochemical relapses, have been recorded in 14–32% of HBeAg-positive patients treated with lamivudine for a year.\textsuperscript{103–108} With longer periods of treatment, resistance was shown to increase from 14% at 1 year to 38%, 49% and 66% for years 2, 3 and 4, respectively.\textsuperscript{106–108} Lamivudine-resistant variants also arise in HBeAg-negative patients, but rates are more variable, ranging from 0% to 27% at 1 year to between 10% and 56% at 2 years.\textsuperscript{97,98,113,115} Emergence of the lamivudine-resistant variants may be accompanied by acute exacerbation of liver disease,\textsuperscript{140,141} and although rare, there have been isolated cases of hepatic decompensation. Continuation of treatment in patients with lamivudine-resistant variants sustains serum HBV-DNA and ALT at levels lower than those at the start of therapy. Moreover, HBeAg seroconversion has been reported to occur in about a quarter of the patients with breakthroughs who continue treatment.\textsuperscript{106,142} It has been observed that if after 24 weeks of lamivudine treatment the patient is still HBeAg/ HBV-DNA-positive and the ALT is higher than 1.3× the
upper limit of normal, then there is a 99% likelihood that polymerase mutants have arisen.142

As mentioned previously, the polymerase is divided into four domains, one of which functions as the rt of the virus. This region contains at least five subdomains (A–E), which are spatially separated but closely associated with the normal function of the protein. Similar to other rts,143,144 the HBV polymerase is thought to assume a three-dimensional, right-handed conformation, consisting of thumb, palm and finger domains. The latter contains subdomains A, C and D, which are most likely to be involved with dNTP binding and catalysis, whereas subdomains B and E interact with the pgRNA template and primer, and correspond to the thumb and palm of the structure, respectively.145,146 The HBV polymerase, as other RNA-dependent polymerases, contains the characteristic YMDD (tyrosine-methionine-aspartate-aspartate) motif of the catalytic site, located within subdomain C.147 The most common amino acid substitutions that have been described and are associated with lamivudine resistance occur in both the B and C subdomains, and have been clearly shown to confer drug resistance.141,148,149 These arise as a result of point mutations in the nucleotide sequence, affecting the relevant codons. It should be noted that not all point mutations result in amino acid changes, this being due to the degeneracy of the genetic code. Amino acid substitutions that confer drug resistance predominantly affect the YMDD motif, so that the methionine (M) at position 552 is changed either to valine (YVDD) or isoleucine (YIDD).104,141,148,150–154 The former mutation is almost always associated with a second one in subdomain B, a substitution of leucine with methionine at position 528 (L528M).148,151,153,154 A new numbering system by which changes in the amino acid sequence of the polymerase protein are identified has recently been proposed.155 In this system, the amino acids of each polymerase domain are numbered separately, so that the aforementioned rt domain mutations become rtM204V/I and rtL180M, respectively. In one study, about one-third of patients had the rtM204I mutation and the rest the rtL180M/M204V change.146 Other mutations that have been described include the rtV173L (V521L) and rtF166L (F514L), both in domain B (Table 2).141,156,157 One other mutant, rtA181T (A529T), has been shown to be resistant to lamivudine following prolonged treatment.158 The same study reported replacement of the original YMDD mutants with distinct ones during prolonged treatment. More recently, an rtM204S (M552S) mutant has been described, with the accompanying rtL180M (L528M) change.159

Crystallographic data have shed further light on the mechanism of lamivudine resistance. The YMDD mutations seem to affect the ability of the dNTP-binding pocket to accommodate the drug. This in turn leads to a reduction in the affinity of lamivudine for the rt domain, and possibly those of the natural nucleotides also.145,146,148,160,161 Moreover, the amino acid changes may alter their precise spatial arrangement necessary for optimum function during catalysis. Thus, the low affinity occupancy of the site is further compounded by suboptimal catalytic efficiency.145,146 The rtL180M mutation, which is spatially very close to the rtM204 position, may represent an attempt by the virus to partly redress this problem. This may also explain the observation that lamivudine-resistant variants are less replication fit than the wild-type

| Table 2. Amino acid mutations in the rt domain of the viral polymerase commonly associated with resistance to lamivudine and famciclovir. These are presented in both the old and new amino acid numbering systems155,156,157,186–189 |
|---|---|---|---|
| Numbering system | Subdomain B | Subdomain C |
| Amino acid position | old | new | old | new |
| Lamivudine | 511–537<sup>a</sup> | 163–189<sup>b</sup> | 545–558 | 220–210 |
| L528M | rtL180M | M552V/I | rtM204V/I |
| V521L | rtV173L | M552S | rtM204S |
| F514L | rtF166L | V555I | rtV207I |
| A529T | rtA181T | | |
| T532S | rtT184S | | |
| Famiclovir | L528M | rtL180M | V555I/E | rtV207I/E |
| V521L | rtV173L | | |
| P525L | rtP177L | | |
| T532S | rtT184S | | |
| R501Q | rtR153Q | | |
| A529V | rtA181V | | |

<sup>a</sup>Based on genotype A.
<sup>b</sup>Standardized numbering for all genotypes.155
P. Karayiannis

virus, whereas the rtL180M mutation has no impact on HBV replication on its own. Ono et al. established that the rtL180M mutation in combination with the rtM204V change partly restored the replication competency of the C domain mutants, and in addition increased resistance to nucleoside analogues. The reduced replication capability of the resistant variants may explain the rapid re-emergence and take-over by the wild-type virus once treatment is stopped. This in addition, confirms the failure of the drug to eliminate cccDNA-containing hepatocytes. To avoid the re-emergence of the wild-type virus and a possible rebound in ALT levels, it is recommended that patients with breakthrough infection are maintained on lamivudine treatment long-term. A recent study, however, suggested that in the liver transplantation setting, lamivudine treatment may result in the selection of polymerase mutants with increased levels of replication, confirmed in vitro in the presence of the drug. These were associated with mutations in the YMDD motif, and in addition in the ‘a’ determinant of the overlapping surface gene, which may represent compensatory changes to restore replication competency.

In vitro studies using transient transfection cell culture systems have confirmed that the above YMDD mutations confer lamivudine resistance. Moreover, such systems have been very useful in establishing sensitivity, or cross-resistance, to other nucleoside analogues. Lamivudine-resistant full-length HBV-DNAs containing the rtM204I, M204V or L180M/M204V mutations have been used to transfect human hepatoma cells. These experiments showed that lamivudine had no effect on the replication of the mutant viruses, whereas adefovir dipivoxil and lobucavir {9-[1β-2α-3β]-2,3-Bis(hydroxymethyl)cyclobutyl}guanine were active against all mutants, as well as DAPD [(−)-β-D-2,6-diaminopurine dioxolane] and DXG, its active metabolite following deamination. The rtM204V mutant appeared to be sensitive to L-FMAU and tenofovir. However, in a recent study, lamivudine-resistant mutants were shown to be resistant to L-FMAU also. Resistance was also maintained against drugs such as penciclovir, emtricitabine and others.

Other nucleoside analogues

A number of other nucleoside analogues have been tested or are being evaluated presently against HBV in Phase II and III clinical studies.

Famciclovir

This is the oral prodrug of penciclovir {9-[4-hydroxy-3-(hydroxymethyl)butyl] guanine}, an acyclic deoxyguanosine analogue (Figure 3). Famciclovir is deacetylated and oxidized to penciclovir, which is in turn phosphorylated in hepatocytes to the triphosphate by cellular enzymes. This then competes with dGTP as a substrate for the HBV polymerase. The drug is incorporated into nascent HBV-DNA strands causing premature termination, but it can also interfere with the priming of reverse transcription, by binding to the tyrosine residue of the terminal protein, which is involved in primer synthesis.

Penciclovir has been shown to inhibit viral replication in Pekin ducks infected with DHBV. However, after drug withdrawal there was a rebound of viral markers to pretreatment levels. This again is thought to be due to the persistence of cccDNA in hepatocytes. In clinical studies famciclovir has been found to be well tolerated and to inhibit HBV replication. A 500 mg dose thrice daily has been shown to give the best results, but the length of treatment in various studies has been variable. A pilot study showed >90% reduction in HBV-DNA levels in ~55% of patients treated for 10 days. A larger multicentre placebo-controlled trial showed that the 500 mg three times a day for 16 weeks, apart from inhibiting virus replication, was associated with sustained normalization of ALT and gave anti-HBe seroconversion in 14% of patients, by the end of the 8 month follow-up period. In another study, HBeAg seroconversion following famciclovir treatment was more modest at 9% compared with 3% in the control group.

Famciclovir has also been used in patients with decompensated cirrhosis and shown to improve liver function; 50% of patients given 500 mg three times a day became HBV-DNA-negative after 44 days of treatment. In the liver transplantation setting, patients treated with famciclovir had greatly reduced HBV-DNA levels, accompanied by histological improvement. In larger clinical trials, where patients with recurrent HBV after orthotopic liver transplantation were treated with famciclovir long-term, median HBV-DNA level reductions of 91%, 95% and 99% were seen at 6 months, and at 1 and 2 years, respectively. The median reduction in ALT levels was 58% and 90% at the yearly time points. Data on the efficacy of famciclovir in HBeAg-negative patients are rather lacking.

Prolonged treatment with famciclovir results, as in the case of lamivudine, in drug resistance. A number of mutations have been described affecting almost exclusively amino acids in the B domain of the polymerase (rtL180M, V173L, P177L (P525L), T184S (T532S), R153Q (R501Q), in domain B, and rtV207I (V555I) in domain C) (Table 2), although mutations at other positions have also been described, including intersubdomain regions and the terminal protein. The rtL180M mutation is however the dominant one seen in patients with breakthrough infections whilst on famciclovir treatment, and this is a risk factor in patients treated with lamivudine subsequently. Resistance to lamivudine occurs earlier in such patients who are sequentially treated with famciclovir followed by lamivudine.
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In *in vitro* studies, the rtV207I mutation exhibited the highest resistance to penciclovir, whereas the rtV173L and L180M mutations showed moderate resistance.\(^{190}\) All the fuciclovir mutations in cross-resistance studies were shown to be sensitive to adefovir, whereas rtV173L, L180M and T184S were also sensitive to lamivudine. The remaining ones displayed moderately decreased sensitivity to lamivudine.

In view of its low efficacy, high dosage and potential for cross-resistance with lamivudine, fuciclovir has not become one of the established treatments for chronic HBV. However, it has potential if used in combination therapies and this is discussed later.

**Adefovir dipivoxil**

Adefovir ( Hepsera) or bis-pivaloyloxymethyl-9-(2-phosphonylmethoxyethyl) adenine (PMEA) is a phosphonate of an acyclic nucleotide analogue (Figure 3). The drug, unlike other nucleoside analogues, contains a phosphate group already and requires an additional phosphorylation step (diphosphate), before it becomes active. This is preceded by the removal of the bis-pivaloyloxymethyl moiety. Adefovir, other than acting as a DNA chain terminator, is also thought to stimulate natural killer cell activity and to induce endogenous interferon production.\(^{191}\) Adefovir is a potent inhibitor of HBV replication,\(^{192,193}\) and has been assessed for efficacy in the clinical setting.\(^{167,193-196}\) Treatment with adefovir results in a rapid decline in HBV-DNA levels within 2 weeks.\(^{167,197}\) The drug is active against lamivudine-resistant mutants,\(^{166,167,198-200}\) and no resistance to it has been seen in patients treated with the drug for 48–60 weeks.\(^{198,201}\) At doses of 60–120 mg daily, as used in HIV-infected patients, the drug was shown to have mild to severe nephrotoxic effects.\(^{202}\) In the HBV trials a 10 mg daily dose was used, and no such side-effects have been recorded. Experiments in the woodchuck and duck hepadnaviral animal models have shown that adefovir, similarly to other nucleoside analogues, does not eradicate the hepatocyte cccDNA pool.\(^{203,204}\)

Adefovir appears to have potential as an effective drug against HBV, and may prove a strong candidate in combination therapies. The drug under the trade name Hepsera received approval by the FDA for use in the treatment of chronic HBV infection, in September of 2002.

**Emtricitabine (Coviracil)**

This is a 5-fluorinated derivative of lamivudine, [(-)-β-2’,3’-dideoxy-5-fluoro-3’-thiacytidine] or [(-)FTC], which is converted to the triphosphate by cellular enzymes and competes with dCTP as a substrate for HBV polymerase (Figure 3).\(^{213}\) (-)FTC was found to be a potent inhibitor of HBV replication in the human hepatoblastoma cell line 2.2.15,\(^{213}\) in primary human hepatocytes,\(^{215}\) and *in vivo* in nude mice.\(^{216}\) In the woodchuck animal model (--)FTC reduced WHV-DNA significantly in a dose-dependent manner, showing antiviral activity levels similar to those obtained with lamivudine.\(^{217,218}\) In a dose range study in chronic HBV carriers, daily doses of 100 mg or greater suppressed HBV-DNA levels by between 1.7 and 3.3 log\(_{10}\) over the 2 month dosing period.\(^{219}\) However, cross-resistance between lamivudine and FTC has been reported, thus precluding its use in the treatment of patients with lamivudine-resistant variants.\(^{220,221}\)

**Ganciclovir**

Ganciclovir or 2-amino-1,9-[(2-hydroxy-1(hydroxymethyl)-ethoxy)methyl]-6H-purin-6-one is a deoxyguanosine analogue (Figure 3), and has potent antiviral activity against DHBV. Ganciclovir has been shown to suppress viral replication and cause histological improvement, following recurrent HBV infection in liver transplant patients.\(^{222,223}\) In patients with chronic HBV infection, ganciclovir suppressed HBV-DNA levels by 99%, and this marker became negative in 26%, whereas ALT normalized or declined in most patients. However, there was a rebound in HBV-DNA levels on stopping therapy during the 8 week follow-up period in...
60% of patients. Oral administration of the drug was very well tolerated. However, the drug has frequent and potentially serious adverse effects, which are likely to limit its long-term use in the treatment of chronic HBV infection.

In the duck animal model, ganciclovir has shown potent antiviral activity. However, treatment of ducks with ganciclovir for 24 weeks did not have substantial impact on the cccDNA pool or viral RNA levels, and gave an increase in hepatic expression of envelope proteins.

This nucleoside analogue \([-{(2'\text{-deoxy}-2'\text{-fluoro}-1'\text{-\beta-D-arabinofuranosyl}-5'\text{-iodo})\text{-uracil}}]\) or fialuridine in short, was used in a Phase II clinical trial, which was stopped prematurely due to serious clinical side effects. In in vitro studies, FIAU was shown to be effective in suppressing HBV replication. However, FIAU was found to have a high affinity for polymerase \(\gamma\), which incorporates the drug into mitochondrial DNA. As a result, when it was used in vivo, several patients developed severe liver and kidney dysfunction associated with lactic acidosis, which led to some fatalities. This highlights the importance of having full in vitro toxicity data and complete evaluation of the drug affinities for cellular polymerases, well before use in human trials.

Other nucleoside analogues that have been developed and tested both in vitro and some cases in vivo include: lobucavir, which has been suspended due to possible drug-related increase in tumour incidence in rodents; L-FddC \([{(2',3',3'\text{-didehydro-5-fluorocytidine})}]\); L-FMAU or clevudine \([ {(2'-\text{fluoro-5-methyl-\beta-L-arabinofuranosyl})\text{-uracil}}]\); DAPD and DXG mentioned earlier; \([{(\beta-L-2',3'-\text{dideoxy-2',3'-didehydro-5-fluorocytidine})}]\) and MCC-478. Three additional simple and related nucleosides, \([{(\beta-L-2'\text{-deoxyxytidine})}]\), \([{(\beta-L-\text{thymidine})}]\) and \([{(\beta-L-2'\text{-deoxyadenosine})}]\), have been discovered to be potent, specific and selective inhibitors of HBV, WHV and DHBV replication. Finally, tenofovir disoproxil fumarate, an acyclic nucleoside analogue closely related to adefovir, which is directly incorporated into DNA, has recently been shown to cause significant (4 log10) reduction in serum HBV-DNA and seroconversion to anti-HBe in 25% of patients treated for 1 year. Moreover, it was active against lamivudine-resistant strains.

Combination therapy

In view of the shortcomings of antiviral monotherapy (restricted efficacy, drug resistance), combination therapy with a number of nucleoside analogues, with or without IFN-\(\alpha\), may result in greater sustained response rates.

**Combination of IFN-\(\alpha\) and lamivudine**

Combination therapy with IFN-\(\alpha\) and lamivudine was shown in one study to result in higher HBeAg seroconversion rates (29%) than either drug alone (18%), which did not reach significance. In this study, combination therapy was initiated after 8 weeks of lamivudine treatment, and continued for 16 weeks. Similar results were obtained following combination treatment for 24 weeks. HBeAg seroconversion and HBV-DNA negativity was seen in 33% of combination-treated patients as opposed to 15% of those treated with lamivudine alone. In addition, histological improvement was seen in 46% and 27% of patients, respectively. Lamivudine monotherapy in both studies was for 52 weeks. Treatment of chronically infected woodchucks with a staggered regimen involving lamivudine alone for 12 weeks, combination of lamivudine and recombinant human IFN-\(\alpha\) for a further 12 weeks, and then 12 weeks of IFN alone, was shown to be better at suppressing WHV replication than either monotherapy alone. Viraemia suppression was greater than expected by additive interactions, thus suggesting synergic antiviral effects.

Relapse rates after combined IFN-\(\alpha\) and lamivudine treatment are also high. In one study, patient relapse occurred within 1–3 months of follow-up, reaching 75%. Combination therapy of IFN-\(\alpha\) and lamivudine is therefore only marginally better than either drug alone. It appears therefore that such treatment has no added benefit, in contrast to the findings in the woodchuck animal model.

**Combination therapy of IFN-\(\alpha\) and famciclovir**

This has also been attempted in a pilot study. Of five patients treated for an overlapping period of 20 weeks with combination therapy, one lost HBV-DNA during treatment and the other during the follow-up period.

**Combination therapies with two or more nucleoside analogues**

This has also been tried. For example, 12 weeks of lamivudine plus famciclovir were shown to be more effective in reducing HBV-DNA than lamivudine alone. During a 16 week follow-up period, relapses in the lamivudine monotherapy group were 67%, but none was observed in the combination group. Synergic effects between the two drugs have in addition been reported in the animal models. A combination of penciclovir, lamivudine and adefovir in primary duck hepatocyte cultures had similar effects. In contrast, lamivudine and famciclovir in combination with IFN-\(\alpha\) were no
better than the combination of the two nucleoside analogues alone.252

Future treatments

Immunotherapy

Therapeutic vaccination. This is another approach that has been employed in an attempt to break tolerance and stimulate T-cell immune responses in chronic HBV carriers, using the licensed or newly developed vaccines, different adjuvants and by altering the route of administration.9 Immunization with recombinant HBsAg particles from transgenic mice expressing either HBsAg alone or replicating the virus resulted in marked reduction in serum HBsAg levels, loss of HBeAg or even development of anti-HBs.253,254 Pilot studies in chronic HBV patients suggested that standard HBV vaccination could lead to clearance or reduction of HBV replication in ~50% of such patients.255,256 In a larger controlled study of 118 patients receiving either GenHevac B (Pre-S2/S), Recombivax (S) or no vaccine, HBeAg to anti-HBe seroconversion was seen in 13.3% of vaccinees versus 3.6% of controls after 6 months of follow-up. After 12 months of follow-up, the seroconversion rate gap between the two groups narrowed to 18.9% versus 12.5%. None of the patients lost HBsAg.257

Alum-based vaccines, as the current HBV vaccine, promote production of antibodies and a Th2 biased immune response. For effective therapeutic vaccination, however, both humoral and cytotoxic T-cell responses may be necessary to eradicate infected cells. The use of alternative adjuvants such as MF59 already tested in healthy adults may improve vaccine efficacy.258 Preliminary results that appeared in abstract form suggest that 11 of 13 patients with chronic HBV developed an anti-HBs response to such a vaccine.259 Another adjuvant of potential benefit is CpG DNA, a synthetic oligonucleotide that preferentially stimulates Th1 responses, with production of IL-12 and IFN-γ.260 Immunization of transgenic animals with an HBsAg vaccine supplemented with CpG DNA led to clearance of serum HBsAg and development of anti-HBs, with concurrent down-regulation of HBV mRNA production in the liver. Adoptive transfer experiments of T-cells from such animals showed that they were able to partially control transgene expression in the liver and to clear the HBsAg from the sera of recipient transgenic mice, without an antibody requirement.261 A CpG-containing HBsAg vaccine was shown to overcome hyporesponsiveness normally seen in immunized orang-utans.262 It remains to be seen whether similar responses are observed in human trials.

Peptide-based T-cell vaccines have also been tested in patients chronically infected with HBV. A lipopeptide (CY-1899) containing a T-helper epitope from tetanus toxoid and a CTL epitope from HBV core (amino acids 18–27) was tested in 90 chronic HBV carriers by Heathcote et al.263 The vaccine induced CTL activity, which was not sufficient to clear the infection. Similar experiments in woodchucks co-immunized with WHVsAg together with a peptide from sperm whale myoglobin led to production of anti-WHVs in all immunized animals. However, two of the animals with the highest antibody levels developed severe liver damage, and one of them died.264 Care must therefore be exercised in the choice of T-helper epitopes.

DNA-based vaccines. Intramuscular injection of plasmids encoding HBV antigens is another novel approach to vaccination, which enables the expression of encoded proteins in vivo, in their native conformation and with the appropriate post-translational modifications. Moreover, such proteins are processed intracellularly and the correct epitopes are thus presented to the immune system. Plasmid DNA immunization results in the generation of humoral immune responses, but potent CD8+ CTL responses are also induced, as shown initially in mice using HBsAg- or HBcAg-expressing constructs.265,266 Similar experiments in HBsAg transgenic mice induced persistent clearance of circulating HBsAg.267,268 Moreover, adoptive transfer of HBsAg-primed spleen cells from DNA-immunized mice achieved control of transgene expression, in the absence of anti-HBs production.269 Augmented immune responses have been obtained in mice by including in DNA constructs sequences encoding T-helper epitopes such as PADRE (pan-DR epitope),270 and cytokines such as IL-2.271 DNA immunization has also been employed for prophylaxis in experiments carried out in animal models of hepatadnaviral infection with encouraging results.272,273 In addition, DNA vaccines have been evaluated for safety and induction of immune responses in naive primates such as chimpanzees,272,274 and rhesus macaques,273 and shown to produce high anti-HBs titres. Similar experiments in Aotus monkeys were not so promising.275 Nevertheless, a DNA vaccine in newborn chimpanzees was shown to protect from subsequent challenge in spite of poor anti-HBs responses.276 Immunization with HBsAg-encoding plasmid DNA, followed by recombinant HBsAg-expressing canarypox as booster in a chimpanzee HBV carrier, resulted in a 400-fold reduction in serum HBV-DNA levels, over a long period of time. In contrast, HBsAg levels in serum remained constant.277 In another study, an HbcAg-expressing retroviral vector was used to immunize three HBV carrier chimpanzees. One of the animals seroconverted from HBeAg to anti-HBe following an ALT flare, whereas the other two animals remained positive for HBeAg and viral load was unaffected, even though one of the animals had detectable HbcAg-specific CTL responses.278 A DNA vaccine against HBV has also been evaluated in healthy human volunteers using the PowderJect system to
deliver gold particles coated with plasmid DNA directly to skin cells. The vaccine proved safe, was well tolerated and produced preferentially Th1 helper cell responses. Humoral anti-HBs responses were however weak.279 There are no published reports as yet on the use of such vaccines in chronic HBV carriers.

**Molecular approaches**

**Antisense oligonucleotides**

Antisense oligodeoxynucleotides (ODN) are synthetic DNA molecules that can inhibit gene expression within cells by binding to complementary mRNA sequences, thus preventing translation.280 Phosphorothioate ODNs are nuclease resistant, so that they are still biologically active when they reach their intended site of action.281 Early experiments in cells transiently or stably transfected with plasmids encoding HBV proteins or the whole genome indicated that ODNs were effective in inhibiting viral protein expression and viral replication.282–286 Similar experiments in DHBV-infected ducks or avian cells in culture yielded promising results.287–289 However, in vivo efficacy particularly in man will be dependent on efficient delivery of the ODNs to the liver and at sufficient concentration. Such an approach will be quite expensive in view of the possible lengthy period of treatment that will be required, as the cccDNA pool of infected hepatocytes will persist. This approach is therefore unlikely to be used as monotherapy.

**Ribozymes**

Ribozymes (ribonucleic acid enzymes) are naturally occurring RNA molecules that catalyse RNA sequence-specific cleavage and splicing.291 The smallest of them, known as ‘hammerhead’ from their characteristic secondary structure shape, recognize a minimal target sequence for cleavage. RNA cleavage specificity is mediated by the ribozyme sequence, which is complementary to that of the target RNA, and flanking the catalytic sequence. A number of studies so far have demonstrated efficient cleavage of HBV mRNAs in *in vitro* experiments using transfected cells or cell-free systems.292–296 Ribozyme activity has been demonstrated by targeting regions that included the encapsidation signal in pgRNA,293 HBx RNA295–298 and the poly(A) signal region of HBV.214 Ribozymes are presently in Phase I/II clinical studies.

Other molecular approaches include the use of dominant-negative HBV core mutant proteins as inhibitors of nucleocapsid formation within hepatocytes,299 and peptide aptamers also targeting the core protein.300 These have been tested in the duck animal model299 and in transfected cells *in vitro*,299,300 and have been shown capable of inhibiting capsid formation and consequently HBV replication.

**Conclusion**

IFN-α or nucleoside analogue monotherapy treatments are effective in suppressing HBV replication, leading to HBeAg seroconversion, normalization of ALT levels, improvement in histology and in some cases even loss of HBsAg. However, such favourable outcomes are attainable in only about a third of those treated, at the best of times. In spite of this, certain groups of patients have benefited tremendously from the use of nucleoside analogues, such as those with decompensated cirrhosis and chronic HBV patients undergoing liver transplantation, when there was little hope for them before.

Patients who do not respond to monotherapy treatment protocols may benefit from combination therapies, as has been the case in HIV treatment. Drugs acting through different antiviral mechanisms may supplement each other, by additive or synergic effects. Such future therapies may include immunotherapies and molecular approaches, as discussed above. If successful, these may reduce the duration and cost of treatment, lessen the impact of side-effects, and more importantly prevent the emergence of drug-resistant variants of the virus. Combination therapies in Phase II and III clinical studies at the moment will hopefully prove successful and lead to the selection of the optimum cocktail of drugs and duration of treatment.

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