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Therapy of chronic hepatitis B virus infection: inhibition of the viral polymerase and other antiviral strategies

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Abstract

Chronic hepatitis B infection remains a major public health problem worlwide. The hepatitis B virus belongs to the family of hepadnaviruses that replicate their DNA genome via a reverse transcription pathway . The chronicity of infection in infected hepatocytes is maintained by the persistence of the viral covalently closed circular DNA. The main strategies to combat chronic HBV infection rely on the stimulation of the specific antiviral immune response and on the inhibition of viral replication. While the prolonged administration of reverse transcriptase inhibitors is most often associated with a control of viral replication rather than eradication, it may select for resistant mutants. The search for new viral targets is therefore mandatory to design combination strategies to prevent the emergence of resistant mutants and eventually clear viral infection.

Keywords: Chronic hepatitis B; Hepadnaviridae; Hepatitis B virus; Antiviral therapy; Nucleoside analogs; Viral resistance

1. Introduction

Despite the availability of efficient vaccines, hepatitis B virus (HBV) infection remains a major public health problem worldwide. Indeed more than 1 billion people have been infected and 300 million people are chronic carriers of the virus (Lee, 1997). Chronic carriers are exposed to a high risk of developing cirrhosis and hepatocellular carcinoma. This indicates that mass vaccination is required to decrease the prevalence and eventually eradicate HBV infection from the planet to prevent hepatocellular carcinoma (Chang et al., 1997). The development of new antiviral agents for the therapy of chronic hepatitis B remains a major goal since interferon alpha therapy is only moderately effective and often limited by dose-dependent side effects (Hoofnagle and Di Bisceglie, 1997). Moreover, the majority of patients receiving this therapy remain chronically infected and at risk of developing liver cirrhosis and its complication. Recently, the efficacy of new nucleoside analogs has been assessed for therapy of chronic hepatitis B. These compounds have shown very promising results in terms of antiviral effect and tolerance (Dienstag et al., 1995; Trépo et al., 1996). Lamivudine has been recently ap-

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proved in USA, Europe, China and other countries for the therapy of chronic hepatitis B. However, short-term monotherapy is not sufficient to clear viral infection and long-term monotherapy has been associated with the emergence of resistant mutants. Research efforts are ongoing to improve the current antiviral protocols with the development of new nucleoside analogs and new approaches to stimulate the host immune response against HBV (Fontana and Lok, 1997; Zoulim, 1997).

2. Hepatitis B virus replication

HBV belongs to the hepadnaviridae family which includes the woodchuck hepatitis virus (WHV) (Summers et al., 1978), the duck hepatitis virus (DHBV) (Mason et al., 1980), the ground squirrel hepatitis virus (GSHV) (Seeger et al., 1984), and the recently described viruses heron hepatitis virus (HHV) (Sprengel et al., 1988), snow goose hepatitis virus (SGHV) and woolly monkey hepatitis virus (WMHV) (Lanford et al., 1998). The members of the hepadnavirus family share homology in genome organization, viral particle structure and strategy of genome replication. The HBV genome is a short circular partially double stranded DNA molecule that comprises four open reading frames encoding for the capsid proteins, the envelope proteins, the polymerase and the X protein. Experiments performed in animal models of HBV infection have contributed to the molecular characterization of the replication cycle of HBV in infected hepatocytes (Fig. 1) (Summers, 1981). This led to the identification of viral targets for antiviral therapy of HBV infection. It was indeed demonstrated, that hepad-

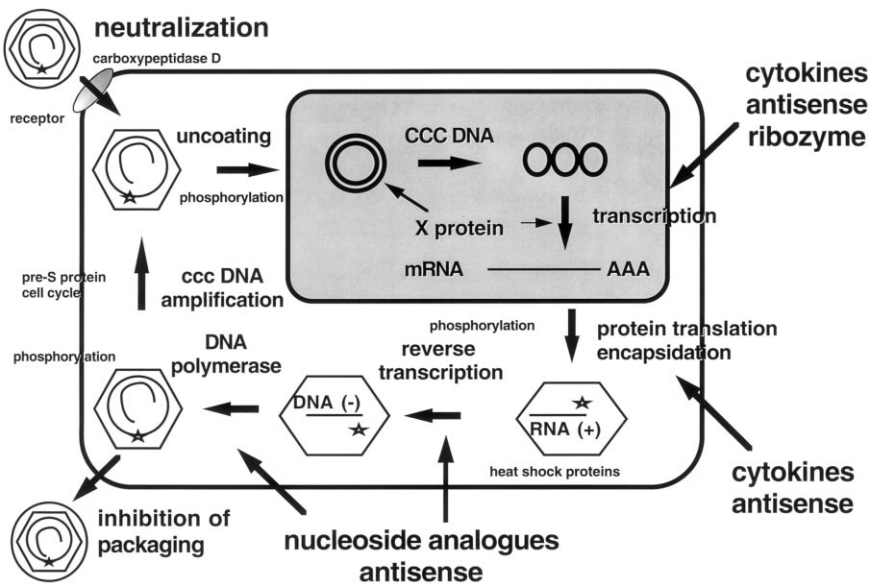

Fig. 1. The viral life cycle and targets for antiviral therapy. HBV particles interact with carboxypeptidase D at the cell surface, which is supposed to be one of the components of the cellular receptor for viral entry. After intracellular penetration and uncoating, the viral DNA genome is transported to the nucleus where it is transformed in CCC DNA. The degree of phosphorylation of capsid proteins may be critical for this step. Viral CCC DNA is the template for viral transcription. The transcripts are translated in the different viral proteins. The X protein is thought to activate at some extent the transcription of the viral genome. One of these transcripts, the pregenomic RNA is encapsidated together wtih the viral polymerase and heat shock proteins. It is then reverse transcribed in viral minus strand DNA and plus strand DNA synthesis occurs therafter. Capsid containing partially double stranded circular genome may follow two pathways: (i) formation of infectious particles after envelope assembly and secretion in serum, (ii) or recycling to the nucleus to amplify or maintain the number of CCC DNA copies in the nucleus of infected cells. The potential targets for antiviral therapy have been indicated.

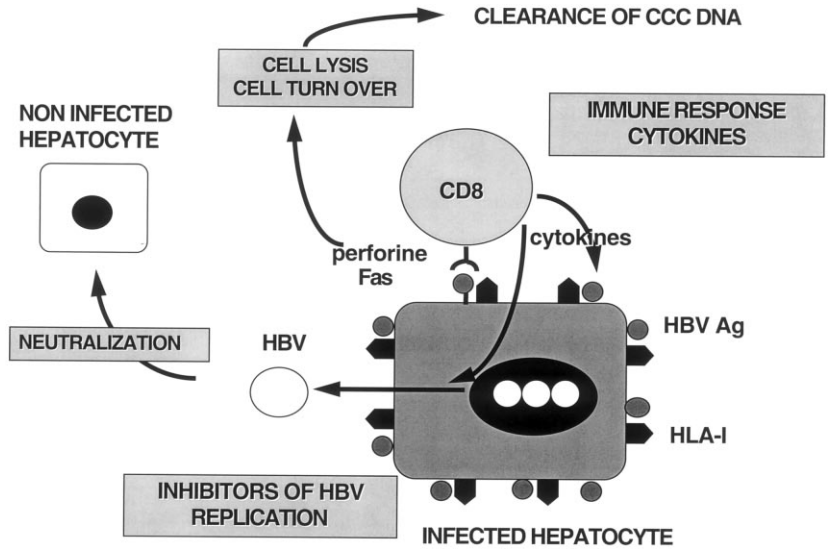

Fig. 2. Pathogenesis of chronic hepatitis B. HBV is not cytopathogenic. Viral replication leads to the production of viral particles that infect continuously other cells and to the expression of viral antigens at the cell membrane. These antigens are presented by MHC class I molecules to cytotoxic T cells that kill infected cells via the fas/perforin pathways. Cytotoxic T cells may also exhibit a direct antiviral effect by the secretion of antiviral cytokines (TNF alpha, IFN gamma, IL12) independently of cell lysis. It was also suggested that viral clearance and curing of infected hepatocytes may result from hepatocyte division. Chronic hepatitis B results from the persistence of viral replication and the continuing attack of infected hepatocytes which is not sufficient to clear all infected cells. The main anti-HBV approaches have been indicated: antivirals, immune modulation, neutralization, and cell turn-over.

naviruses replicate their DNA genome via a reverse transcription step (Summers and Mason, 1982). It was further shown that the template for reverse transcription is the pregenomic RNA. Later, the ends of viral minus and plus strand DNA were mapped and it was shown that viral minus strand DNA is covalently attached to a protein and that viral plus strand DNA synthesis is initiated by a short oligoribonucleotide sequence (Seeger et al., 1986). The key role of the viral covalently closed circular DNA (CCC DNA) as a template of viral transcription and for the maintenance of viral infection at the level of a single hepatocyte was well documented in the duck and the woodchuck models (Aldrich et al., 1989, Tuttleman et al., 1986). Viral genome determinants and cellular factors that are involved in the regulation of viral transcription or virion assembly have been studied in detail (Schaller and Fischer, 1991). There are still important issues that remain unsolved, such as the characterization of the cellular receptor required for viral entry as well as the determination of the exact role of the X protein during the viral cycle (Kay et al., 1985, Kuroki et al., 1994). Since HBV is not cytopathogenic, cell–virus interactions are thought to play a very important role in the regulation of viral replication and the chronicity of viral infection. Liver cell damage during chronic viral infection results from the immune attack of the infected cells by cytotoxic T lymphocytes that recognize viral antigens expressed at the cell surface together with HLA class I molecules (Chisari, 1995). Experiments in transgenic mice demonstrated that the activated cytotoxic T cells may exhibit a direct antiviral effect independent of hepatocyte lysis via the production of antiviral cytokines, i.e. IL12, TNF alpha, and IFN gamma (Chisari, 1995). Chronic hepatitis B is the result of a continuing attack of infected cells by the host immune system that is not vigourous enough to eradicate all infected hepatocytes (Fig. 2). Therefore, two main non-exclusive strategies can be envisionned to eradicate viral infection: inhibition of viral replication and/or enhancement of the specific immune response.

Another important aspect of chronic HBV infection is the integration of the viral genome in the host genome. By contrast to retroviruses, this phenomenon is not required for viral replication but is one of the major factor involved in virus induced hepatocarcinogenesis (Mason and Taylor, 1989). This represents another important target for which novel approaches are required to prevent the development of liver cancer.

3. Viral polymerase

3.1. Functions of the viral polymerase

The HBV polymerase has multiple functions including signals for viral pregenome encapsidation, priming of reverse transcription, RNA dependent-DNA synthesis, DNA dependent-DNA synthesis, and RNase-H activity. It is therefore an ideal target for antiviral compounds (Mason, 1993; Seeger and Mason, 1993). Alignment of viral genome sequences and comparison with the polymerase gene of retroviruses showed significant homology and predicted the presence of an RNAseH domain at the carboxy-terminus of the polymerase polypeptide, and a reverse transcriptase domain including the very conserved YMDD motif thought to be part of the catalytic site of the reverse transriptase (Toh et al., 1983; Xiong and Eickbush, 1990). Interestingly, the HBV polymerase also comprises an amino-terminal extension which is unique among all known reverse transcriptases and includes a spacer domain dispensable for enzymatic activity and the terminal protein domain harboring the primer for reverse transcription (Bartenschlager and Schaller, 1988; Zoulim and Seeger, 1994). The functional domains of the viral polymerase polypeptide were confirmed after extensive mutagenesis and careful functional analysis of the mutants (Fig. 3). Results of in vitro experiments, after transfection of viral genome mutants, showed that the hepadnavirus polymerase is expressed by internal initiation of translation and acts preferentially in *cis* on the pregenomic RNA that served as template for its own translation (Chang et al., 1989). It was also demonstrated that the viral polymerase ex-

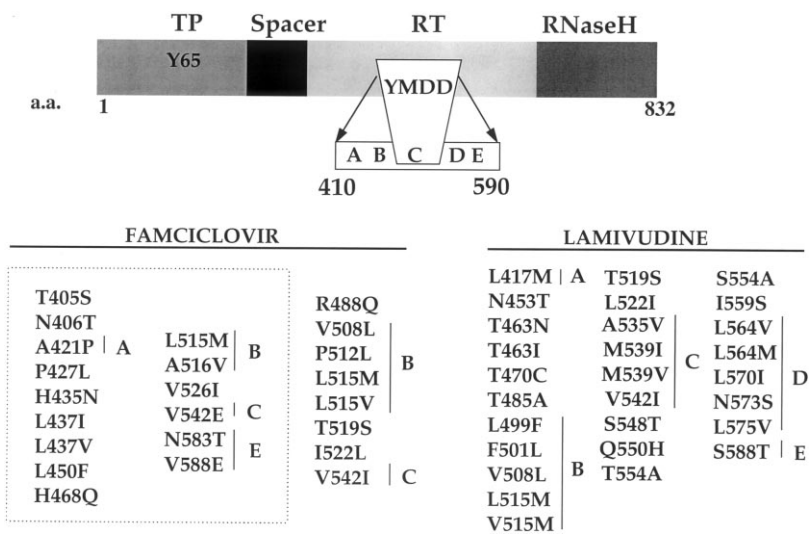

Fig. 3. Structural organization of the HBV polymerase and mutations associated with resistance to nucleoside analogs. The Figure depicts the structure of the viral polymerase gene and its conserved domains that have been determined by comparison with the HIV reverse transcriptase sequence and confirmed by genetic and functional studies (TP, terminal protein; RT, reverse transcriptase domain). A recent update of the mutations, which have been observed in case of resistance to famciclovir or lamivudine, and their position has been indicated (for references see Bartholomeusz et al., 1997; Allen et al., 1998; Pichoud et al., 1999a). Newly described mutants associated with famciclovir resistance are included in the dotted box (Seignères et al., 1999).

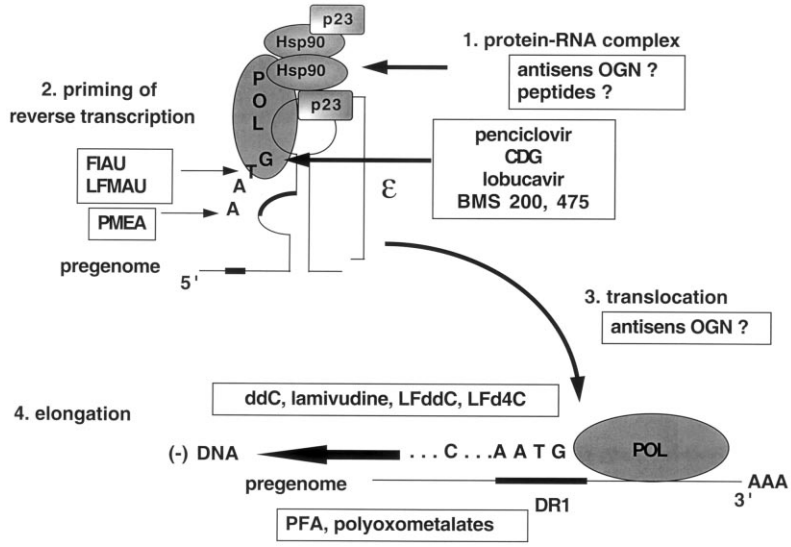

Fig. 4. Model for the initiation of reverse transcription and targets of nucleoside analogs. Viral reverse transcriptase interacts first with heat shock proteins, epsilon (the pregenomic encapsidation signal), and gains enzymatic activity. Reverse transcription is primed by the formation of a covalent bond between a conserved tyrosine of the TP domain and the first nucleotide of minus strand DNA, dGTP. This is followed by the addition of three more nucleotides, TAA in a template dependent manner. Following an arrest in viral DNA synthesis a strand transfer occur and the complex between the polymerase and the short DNA primer GTAA is translocated to the 3′ DR1 where elongation of viral minus strand DNA continues. In vitro studies have shown that the triphosphate forms of penciclovir, CDG, lobucavir, BMS 200, 475, L-FMAU and FIAU have an inhibitory activity on the priming reaction. By contrast, cytidine analogs such as lamivudine, ddC, LFddC, LFd4C, or other inhibitors, i.e. PFA and polyoxometalates, interfere with viral minus strand DNA elongation. Other agents such as antisens oligonucleotides or peptides may inhibit the interaction of the polymerase with epsilon or the translocation of the viral polymerase covalently attached to the short DNA primer. This may serve as a basis to design new strategies combining drugs with different mechanism of action.

hibits RNA dependent-DNA polymerase, DNA dependent-DNA polymerase, and RNase-H activities (Chang et al., 1990; Radziwill et al., 1990, Roychoudhury et al., 1991). Using an in vitro assay for the expression of an enzymatically active DHBV polymerase polypeptide in a reticulocyte lysate system, Seeger et al. could obtain new insight in the mechanism of initiation of reverse transcription in hepadnaviridae. It was shown that the hepadnavirus polymerase gains enzymatic activity after a complex interaction with cellular chaperones, i.e. heat shock proteins (hsp90, p23, hsp70), and the encapsidation signal on the pregenomic RNA (Wang and Seeger, 1992, 1993; Wang et al., 1994; Hu and Seeger, 1996; Hu et al., 1997). This was confirmed independently by other groups using the DHBV polymerase expressed in a yeast system (Tavis and Ganem, 1993; Tavis et al., 1994) and the HBV polymerase expressed in a baculovirus system (Lanford et al., 1995, 1997; Urban et al., 1998a; Lanford et al., 1999). By contrast to other reverse transcriptases, the presence of the viral polymerase was shown to be required for viral pregenome encapsidation (Bartenschlager et al., 1990; Hirsch et al., 1990). It was also demonstrated that, unlike all the other known reverse transcriptases, which utilize nucleic acid primers exclusively, the hepadnavirus polymerase uses a tyrosine residue near its amino terminus as a primer for reverse transcription. Viral DNA synthesis is initiated by the formation of a covalent bond between the polymerase and dGMP, followed by the addition of three or four in a template dependent manner (Wang et al., 1994; Zoulim and Seeger, 1994). After transfer of the polymerase-primer complex to the 3′DR1, the viral DNA chain is further extended by the addition of deoxynucleotides by the polymerase (Fig.

4) (Wang et al., 1993, 1994). Specific results were obtained in a baculovirus system for the expression of the human HBV polymerase (Lanford et al., 1995, 1997, 1999). These first steps of the reverse transcription process, being biochemically and genetically unique, may represent an ideal target for new antiviral strategies.

3.2. Inhibition of the HBV polymerase activity by lamivudine

3.2.1. Lamivudine

Lamivudine belongs to the L-nucleoside analogs family that includes compounds with a non natural configuration. Compounds belonging to this family have been shown to exhibit a very potent activity against HIV and HBV reverse transcriptases without significant toxicity, compared with their dextrorotary counterpart (Bridges and Cheng, 1995; Nair and Jahnke, 1995). The introduction of a sulphur atom in the 3′ portion of the sugar ring of dideoxycytidine led to the synthesis of a new nucleoside analog, 2′,3′dideoxythiacytidine (SddC) that was discovered in the racemic form containing the D(+) and the L(-) enantiomers (Doong et al., 1991). The L(−) enantiomer (lamivudine, or 3TC), was shown to be the stereoisomer responsible for the anti-HBV activity of the racemic SddC in hepatoma cells permamently replicating HBV (2.2.1.5.) (Bridges and Cheng, 1995; Chang et al., 1992). In this cell line L(−)SddC was found to be 50-fold more potent than the D(+) enantiomer and 280-fold more active than ddC. Furthermore, lamivudine was found to be less toxic than the D(+) enantiomer and ddC, with a very high slectivity index (Nair and Jahnke, 1995; Van Draanen et al., 1994). Antiviral activity of L(−) SddC (lamivudine) was attributed to its efficient conversion to the 5′ triphosphate metabolite and resistance to deamination. Biochemical studies showed that L(−)SddC triphosphate, the active metabolite, is a potent inhibitor of HBV DNA polymerase/reverse transcriptase activity (Zoulim et al., 1996), being more active as a chain terminator than as a competitive inhibitor of dCTP incorporation in viral DNA (Severini et al., 1995). It was also shown that the active metabolite of lamivudine is a poor substrate for nuclear and mitochondrial DNA polymerases (Parker and Cheng, 1994). Furthermore, double-stranded DNA with 3TC 5′-monophosphate incorporated at the 3′-terminus of the primer strand by DNA polymerase gamma was also shown to be a substrate for the 3′-5′ exonuclease activity of this enzyme. This may explain the low levels of mitochondrial toxicity observed with 3TC (Gray et al., 1995). Pharmacokinetic data obtained in the woodchuck model of HBV infection gave also some meaningful insight to design clinical trials (Rajagopalan et al., 1996).

Clinical trials have shown that short-term oral administration of lamivudine at 100mg/day is well tolerated and induces a dose dependent but transient inhibition of viral replication (Dienstag et al., 1995, Lai et al., 1997). Longer regimen were then evaluated in clinical trials (Nevens et al., 1997). Interestingly, HBV DNA as well as HBeAg and HBsAg concentrations in serum declined but sustained anti-HBe seroconversion was observed in only 4% of the patients. Relapse of viral replication was observed in the majority of the patients within 1 month, and was accompanied by a severe exacerbation of the liver disease in some patients (Nevens et al., 1997). A large multicenter placebo controlled trial of lamivudine therapy for 12 months was performed in Asia (Lai et al., 1998). This study confirmed that lamivudine was well tolerated. Serum HBV DNA dropped to undetectable levels in 100% of the patients. At 1 year, anti-HBe seroconversion was observed significantly more frequently in lamivudine treated patients (16%) compared with the patients receiving placebo (4%). In parallel, serum ALT levels decreased, and liver histology, at the end of the 12-month therapy, improved significantly in lamivudine treated patients, compared with placebo. A viral breakthrough was observed from the 6th month of therapy and became progressively more frequent until the end of 12 months therapy and reached 14% of the patients (Lai et al., 1998). The antiviral efficacy of lamivudine was also confirmed in patients with HBV recurrence on their liver graft (Perrillo et al., 1999).

3.2.2. Long-term treatment and drug resistance

These clinical data underline that in most of the patients with chronic hepatitis B, prolonged therapy with a single nucleoside analog is required for viral eradication. Using the results of clinical trials, Nowak et al. have elegantly determined the kinetics of viral clearance in chronic hepatitis B patients treated with lamivudine. When the viral load, half-life of infected hepatocytes, cell turnover, daily production of HBV by infected cells, half-life of HBV in serum and the inhibition of viral replication by lamivudine were taken into account, it was calculated that a prolonged therapy, for more than 12 months and more likely up to 5 years, is required to clear viral infection (Fig. 5) (Nowak et al., 1996). All these findings imply that in the absence of anti-HBe seroconversion or HBsAg clearance, antiviral therapy using this class of nucleoside analogs, should be continued to control viral infection and avoid the withdrawal flare. However, this strategy of prolonged antiviral therapy will place the patient at risk of developping drug toxicity (Lee, 1995) or viral resistance (Zoulim and Trépo, 1998).

The first cases of resistance to nucleoside analogs were described in patients treated with lamivudine for at least 6 months for HBV recurrence after liver transplantation or as prophylaxis to prevent HBV recurrence on the liver graft. Sequencing of the viral polymerase gene showed

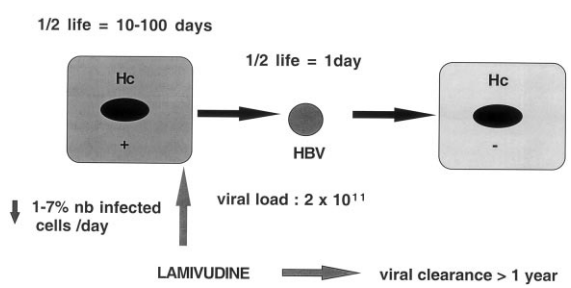

Fig. 5. Kinetics of viral clearance during lamivudine therapy. Kinetics of viral clearance were calculated from the data obtained with clinical trials of lamivudine. The model predicted that taken into account viral load, the kinetic of HBV in serum, the long life duration of infected hepatocytes and the inhibition of replication by lamivudine, long term treatment with lamivudine is necessary to eradicate viral infection (see Nowak et al., 1996; Tsiang et al., 1999).

the selection of viral mutants in the catalytic site with a M539V or a M539I change at the time of the viral breakthrough (Ling et al., 1996, Tipples et al., 1996). As these mutations were described already in HIV strains resistant to lamivudine the link between phenotypic and genotypic resistance was established (Arts and Wainberg, 1996, Richman, 1997). The rate of resistance after 1 year of therapy in patients with HBV recurrence on liver graft is 30%, and 17% in the case of prophylactic administration of lamivudine pre-/post-transplant (Perillo et al., 1997a,b). In immune competent patients with chronic hepatitis B treated with lamivudine for more than 6 months, viral breakthrough defined as the reappearance of viral DNA in serum while the patients is still under therapy was observed from week 36. In a large cohort of 335 patients included in a randomized trial for a duration of 1 year, the incidence of viral resistance documented by HBV DNA detection in serum and HBV polymerase gene sequence analysis was 14% (Lai et al., 1998). The determination of the pathogenicity of these mutants requires detailed clinical studies.

With the use of famciclovir, viral breakthrough has been observed first in liver transplant patients who received famciclovir on compassionate use for HBV recurrence (Aye et al., 1997; Bartholomeusz and Locarnini, 1997; Bartholomeusz et al., 1997). The rate of viral breakthrough in retrospective studies is estimated to be $\sim$19% after more than 1 year of therapy (Tillmann et al., 1997). Another particular feature of famciclovir therapy is the relatively high proportion of non response independently of mutations in the viral polymerase (Pichoud et al., 1999a; Tillmann et al., 1997), suggesting that this phenomenon may be due to host factors such as drug metabolism in the liver or resistance of a population of infected cells to penciclovir.

The occurrence of HBV polymerase mutants was expected due to the high viral turnover (Nowak et al., 1996) and the error rate of the viral polymerase (Norder et al., 1994). This is responsible for the heterogeneity of the viral genomes that led to the identification of six genotypes (A–F). It was also demonstrated recently that HBV circulates as a complex quasi-species that evolves dur-

ing the course of infection depending on the host antiviral pressure (Argentini et al., 1999). Resistant virus may therefore be normally present before therapy as a minor viral population and be selected when the antiviral pressure is applied. However, all mutants are not viable since they may disrupt an overlapping gene in the viral genome. For example, mutations in the HBV viral polymerase may affect the overlapping *S* gene and disrupt its expression. The precise kinetics of appearance of resistant mutants and its complexity in terms of the spectrum of the mutant populations has been studied in detail in the woodchuck model. It was shown that the drug resistant mutants are detected prior to the raise in viral DNA titers. Studies of the CCC DNA species showed that viral breakthrough occurs when the pool of CCC DNA is depleted in wild type species and progressively replaced by the drug resistant mutant species. This suggests that hepatocytes infected with wild type virus may not be susceptible to mutant virus superinfection (Mason et al., personal communication). Apart from the mutations described in the YMDD motif of the viral polymerase (Ling et al., 1996; Tipples et al., 1996), which is well conserved in all known reverse transcriptases and is the major component of the catalytic site within domain C of the enzyme (Toh et al., 1983; Chang et al., 1990; Radziwill et al., 1990; Xiong and Eickbush, 1990; Kohlstaedt et al., 1992; Wang and Seeger, 1992), other mutations located mainly in the B domain of the HBV polymerase were found in association with the major mutations residing in the catalytic site of the viral enzyme (Bartholomew et al., 1997; Niesters et al., 1998).

Lamivudine resistant mutants have been classified into two groups: group I with mutations in domains C and B (M539V + L515M) being the most frequently encountered drug resistant strain and group II with mutations in domain C only (M539I). Fig. 3 shows the main mutations found in association with lamivudine and also famciclovir resistance. By contrast with lamivudine, famciclovir resistance has been associated mainly with mutations in the B domain of the viral polymerase (Aye et al., 1997; Bartholomeusz and Locarnini, 1997; Bartholomeusz et al., 1997; Locarnini et al., 1997) and in only one case with a mutation in domain C of the viral polymerase at the border of the catalytic site (Pichoud et al., 1999a). Recent systematic and sequential studies of the viral polymerase sequence during famciclovir therapy demonstrated a spontaneous variability of the viral polymerase gene and a complex variability in relation to famciclovir therapy. Furthermore, sequential monotherapy with nucleoside analogs was shown to select more rapidly for drug resistant virus that may be cross-resistant (Seignères et al., 1999) (Fig. 6). Assays for the rapid determination of genotypic changes in the viral polymerase for the monitoring of antiviral therapy are currently in development. Specific hybridization assays which have been already developed for HIV (Stuyver et al., 1997) or restriction fragment length polymorphism analysis (Allen et al., 1998) may represent clinically relevant approaches for the rapid diagnosis of resistance to antiviral therapy. Using a specific line probe assay, it was shown that the selection of complex mutations in the pre-core promoter and the polymerase gene may allow HBV to escape both the anti-HBe immune response and the antiviral pressure of lamivudine (Pichoud et al., 1999b). Furthermore, it was shown in non selected patients that (i) group I mutants are more frequently observed, and (ii) polymerase gene mutant detection precedes by a mean of 14 weeks the occurrence of a viral breakthrough (Stuyver et al., 1999; Si Ahmed et al., 1999), confirming some of the data obtained in the woodchuck model (Mason et al., personal communication).

3.3. Inhibition of the priming of reverse transcription and viral minus strand DNA elongation

3.3.1. Famciclovir

Another nucleoside analog, originating from research on anti-herpes virus agents, showed a potent anti-HBV activity. The purine nucleoside analogue, penciclovir (9-(4-hydroxy-3 hydroxymethylbut-1-yl) guanine; BRL 39123), which is a highly selective antiviral agent, having activity against herpes viruses (Boyd et al., 1987; Vere Hodge and Perkins, 1989; Earnshaw et al., 1992),

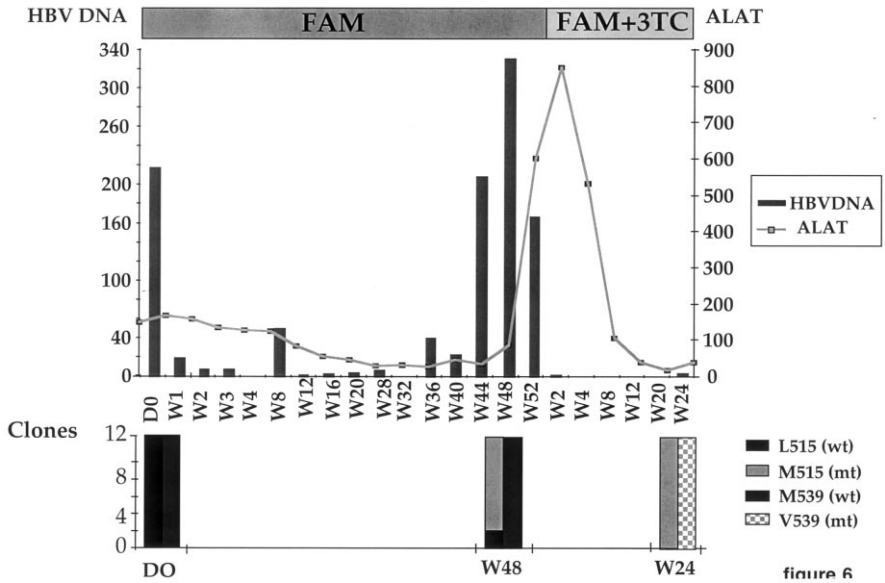

Fig. 6. Emergence of drug resistant mutants during sequential antiviral treatment. Long term treatment with nucleoside analogs may be associated with viral resistance. The figure shows the sequential selection of a L515M mutation in a patient treated first with famciclovir, followed by the selection of a double mutation L515M + M539V when lamivudine was added to famciclovir, leading to a the selection of a cross-resistant mutant.

was also shown to have a potent anti-hepadnavirus activity in the duck model of HBV infection (Shaw et al., 1994; Tsiquaye et al., 1994; Lin et al., 1996). Penciclovir inhibited duck hepatitis B virus (DHBV) replication in vitro in primary duck hepatocytes infected with DHBV (Shaw et al., 1994). It was also found to be a selective inhibitor of HBV replication in cultured human hepatoma cells (Korba and Boyd, 1996). Furthermore, results of in vivo experiments showed that the intraperitoneal administration of penciclovir induced a profound inhibition of viral DNA and protein synthesis in ducks congenitally infected with DHBV (Lin et al., 1996). Its oral form, famciclovir, was also shown to inhibit viral replication in congenitally DHBV infected ducklings (Tsiquaye et al., 1994, 1996). In these experiments, no significant toxicity was observed either in cell culture or in animal models of HBV infection. Small bile duct epithelial cells remained resistant to antiviral therapy and it was suggested that this reservoir may contribute, at least in part, to the rebound of viral replication after cessation of therapy (Lin et al., 1996). Further experiments showed that in the HBV infection model, cellular enzymes catalyze penciclovir phosphorylation to its active triphosphate metabolite, contrary to the herpes simplex infection model where both viral and cellular enzymes phosphorylate penciclovir (Shaw et al., 1996). Penciclovir triphosphate was also shown to be stable in hepatoma cell culture and to have a long half life which may explain in part its antiviral efficacy. Interestingly, (*R*)-PCV-TP was more efficient than (*S*)-PCV-TP in inhibiting DHBV reverse transcription (Dannaoui et al., 1997) as well as the HBV DNA polymerase activity, acting as a secondary chain terminator (Shaw et al., 1996).

3.3.2. Carbocyclic analog of 2′-deoxyguanosine (CDG)

This compound was found to inhibit dramatically viral DNA synthesis in DHBV infected primary duck hepatocyte cultures. However viral CCC DNA formation could not be blocked completely by this compound (Fourel et al., 1994b). In vivo, in DHBV infected ducks, CDG administration induced a rapid drop of viremia titers which

was sustained throughout the treatment period. In parallel, the number of infected cells decreased rapidly and was associated with an increased turnover of hepatocytes (Fourel et al., 1994a). However long-term therapy (> 1 year) was not sufficient to clear viral infection and was followed by a rebound of viral replication after drug withdrawal (Mason et al., 1994). Later, CDG-triphosphate was found to inhibit dramatically the incorporation of the first nucleotide of viral minus strand DNA, dGTP (Dannaoui et al., 1997).

3.3.3. Other guanosine analogs

Lobucavir and cyclopentyl guanosine (BMS 200, 475) were discovered recently as potent anti-HBV agents. The in vitro evaluation showed that these compounds exhibit a potent inhibitory against the hepadnavirus reverse transcriptase as well as an anti-priming activity (Seifer et al., 1998). The same group showed elegantly, using an endogeneous polymerase sequencing reaction, that BMS 200 475 is incorporated in viral minus strand DNA and terminates its synthesis. Both compounds exhibited a potent inhibitory activity on HBV DNA replication in the 2215 cell line (Innaimo et al., 1997). Cyclopentyl guanosine was found to be 40 times more active than lamivudine in this cell line and to have a very good cytotoxicity profile. This drug exhibits a less dramatic inhibitory effect on Herpes viridae replication and no significant anti-HIV and anti-influenza activity. It was demonstrated that both compounds strongly inhibit viral replication in WHV infected woodchucks without being associated with toxicity in this animal model. Furthermore, both compounds were found to have a more potent antiviral effect in WHV infected woodchucks than lamivudine (Genovesi et al., 1998). Lobucavir has been evaluated in phase II trials (Bloomer et al., 1997; Heathcote et al., 1998b) but its clinical development was recently stoped because of the occurrence of tumors in rodents during long-term dosing.

3.3.4. Thymidine analogs

The non natural derivative of FMAU, L-FMAU, was synthesized recently and was shown to inhibit specifically EBV and HBV replication but not that of HIV (Chu et al., 1995). In the 2.2.1.5. cell line, L-FMAU administration did not result in any decrease of mitochondrial content, did not affect mitochondrial function and was not incorporated in cellular DNA (Pai et al., 1996). L-FMAU was shown to be a substrate not only for cytosolic thymidine kinase and mitochondrial deoxypyrimidine kinase but also for cytidine kinase (Liu et al., 1998), which may explain why L-FMAU and L-FddC did not exhibit any synergistic antiviral effect in hepatocyte culture (Aguesse-Germon et al., 1998a). L-FMAU administration inhibits strongly hepadnavirus replication in the 2.2.1.5. cell line as well as in duck primary hepatocytes (Pai et al., 1996; Aguesse-Germon et al., 1998a). Furthermore, it was shown that L-FMAU-triphosphate inhibits the synthesis of the DHBV primer for reverse transcription as well as DHBV replication in vivo in infected ducklings (Aguesse-Germon et al., 1998a). L-FMAU gave promising results in the woodchuck model with a very potent antiviral effect together with viral clearance from infected hepatocytes after 4 months of administration. L-FMAU was not toxic in these animals by contrast with D-FMAU administration which was associated with severe mitochondrial toxicity (Tennant et al., 1997; Wright Witcher et al., 1997).

3.3.5. PMEA

Adefovir dipivoxil is the oral prodrug of an acyclic nucleotide monophosphate analog, (9-(2-phosphonylmethyl)-adenine (PMEA), that inhibits hepadnavirus replication in vitro in the 2215 cell line and decreases DHBV replication in vivo in DHBV infected ducks (Nicoll et al., 1998). This compound exhibits an inhibitory effect on both the HIV and HBV reverse transcriptases and was shown to inhibit the enzymatic activity of YMDD mutant of the HBV polymerase as well as wild type polymerase (Aguesse-Germon et al., 1999; Xiong et al., 1998). Its evaluation in phase II clinical trials showed a dose dependent antiviral effect associated with an increased rate of anti-HBe seroconversion compared to the placebo group (Heathcote et al., 1998a). Phase III clinical trials are ongoing to further demonstrate its efficacy in patients.

3.3.6. Cytidine analogs

Apart from lamivudine, (−) *cis*-5-fluoro-1-(2-(hydroxymethyl)-1,3-oxathiolan-5-yl) cytosine or (−) FTC which exhibits an inhibitory effect against HIV replication in tissue culture was shown to inhibit HBV replication in human primary hepatocyte cultures (Condreay et al., 1996). Furthermore, its antiviral effect was confirmed in WHV infected woodchucks after i.p. administration for 25 days and was not associated with significant toxicity (Cullen et al., 1997). All these results indicate that (−) FTC is a potent antiviral compound which needs to be further evaluated as an anti-HBV agent. 2′,3′-dideoxy-β-L-5-fluorocytidine or L-FddC was shown to inhibit HBV replication in the 2.2.1.5. cell line (Lin et al., 1994). Interestingly, its inhibitory effect on the viral reverse transcriptase was comparable to that of ddC-triphosphate, but was significantly more important in primary duck hepatocyte culture and in vivo in experimentally infected ducklings. These later results suggested that L-FddC was more efficiently metabolized in its active metabolite in infected hepatocytes than its parental compound, ddC (Zoulim et al., 1996). In vivo administration of ddC in experimentally infected ducklings was associated with liver toxicity, i.e. microvesicular steatosis, consistent with its toxicity on mitochondrial functions (Aguesse-Germon et al., 1998a). Recently, we have evaluated the anti-HBV activity of a novel L-nucleoside analog, LFd4C. It was shown to inhibit HIV replication in MT2 cells (Dutschman et al., 1998) and HBV replication in the 2.2.1.5. cell line (Zhu et al., 1998) with a good selectivity index. In the duck model, it was shown that the IC50 of L-Fd4C-triphosphate on the incorporation of dCTP in viral minus strand DNA by the viral polymerase was 30-fold less than 3TC-triphosphate. Furthermore, in vitro experiments showed that L-Fd4C administration in primary duck hepatocyte culture dramatically suppresses viral DNA synthesis and inhibits the initiation of viral infection. Prolonged administration of L-Fd4C early after experimental infection was able to suppress viral replication and viral spread in the liver but was not able to clear viral infection since viral replication could be detected 2 weeks after drug withdrawal. Altogether these results indicate that L-Fd4C is a potential anti-HBV agent that needs further evaluation in the woodchuck mammalian model of HBV infection (Le Guerhier et al., 1999).

3.3.7. Other inhibitors

The pyrophosphate analog, phosphonoformic acid, was shown to inhibit dramatically viral minus strand DNA elongation but not the priming of DHBV reverse transcription. Non nucleoside inhibitors belonging to the polyoxometalate family were also shown to inhibit preferentially DHBV reverse transcription without affecting the priming reaction in vitro (Aguesse et al., 1997).

3.4. Biology of the drug resistant mutants

The biology of these mutants has been studied in vitro. The first study was performed in the duck HBV model and showed no significant difference in the replication level of lamivudine resistant mutant by comparison with the wild type strain (Fischer and Tyrell, 1996). Melegari et al. have engineered by site directed mutagenesis the major mutations associated with resistance to lamivudine or famciclovir (F501L, L515M, M539V, M539I) (Melegari et al., 1998). The different clones were transfected in hepatoma cell lines and analysis of viral nucleic acid showed that these mutants are defective for viral replication. Furthermore, using different cell lines and treatment with hydroxyurea to deplete the pool of intracellular nucleotides, it was determined that this impaired viral replication capacity is due to defect in the fitness of the viral polymerase to synthesize viral DNA. Our group has engineered mutations in the YMDD motif of the viral polymerase of the duck hepatitis B virus (YIDD and YVDD) which are resistant to lamivudine. Our study showed that these mutants have a lower replication capacity after transfection of LMH cells, compared with the wild type virus. Moreover, using an in vitro assay for the expression of an enzymatically active DHBV polymerase, it was shown that these YMDD mutants have a decreased reverse transcriptase activity and that B domain mutations restore a higher enzymatic ac-

tivity (Aguesse-Germon et al., 1999). In one case of famciclovir resistance, a single viral polymerase mutant (V542I) was identified and was shown to have a 20-fold decreased viral replication capacity when the mutant clone was transfected in Huh7 cells (Pichoud et al., 1999a). All these data are consistent with the decreased activity and processivity of the M184V mutant of the YMDD motif of the HIV reverse transcriptase (Back et al., 1996; Wainberg et al., 1996; Back and Berkhout, 1997).

These observations may explain why wild type virus replication, originating from the very stable pool of viral CCC DNA in the nucleus of infected cells, reappears after drug withdrawal due to the replication advantage of the wild type strain in the absence of antiviral pressure. This was recently described in detail by Chayama et al. who demonstrated a takeover by wild type virus after lamivudine withdrawal (Chayama et al., 1998). One unexplained finding is the discrepancy of the low level replication observed in vitro with these mutants and the relatively high level of replication observed in vivo in infected patients. This will require further investigations to examine if immune suppression, hepatocyte turn over, cellular factors present in non cultured hepatocytes, the presence of other compensatory mutations, or viral interference with wild type virus may influence the replication capacity of these mutants.

The biology of clinical isolates, which were resistant and selected during lamivudine treatment was analyzed in vitro in human primary hepatocyte culture. The results showed that these HBV strains were at least 45 times less sensitive to lamivudine than those in the pre-treatment serum (Bartholomew et al., 1997). This was the first demonstration that the mutations of the viral polymerase which were selected during lamivudine therapy were indeed confering resistance to lamivudine in a cell culture model using non tumoral hepatocytes. The same group extended its observation with HBV clones bearing the different mutations in the viral polymerase, alone or in combination with each other (Allen et al., 1998). The sensitivity of the different mutants to lamivudine was analyzed after transfection of these clones in HepG2 cells. The results showed an increase of the IC50 of lamivudine by more than 10 000-fold for the L515M + M539V as well as for the M539I and L515M + M539I mutants, and milder effect of other mutants with a 153-fold increase in the lamivudine IC50 for the M539V strain, and 18 fold increase for the L515M mutant (Allen et al., 1998). The authors also made a prediction of the three-dimensional structure of the wild type and the mutant HBV reverse transcriptase domain based on the HIV reverse transcriptase crystal structure. Although the crystal structure of the HBV polymerase is not available, the authors suggested that the interaction of lamivudine with the catalytic domain of the HBV reverse transcriptase domain was modified in the case of the mutant, explaining the lower affinity to the drug (Allen et al., 1998). These results were confirmed in vitro in HepG2 cells transfected with wild type and mutant polymerase HBV and treated with lamivudine (Fu and Cheng, 1998; Ling and Harrison, 1999; Ono-Nita et al., 1999).

The sensitivity to penciclovir of clinical isolates resistant to famciclovir in vivo, was analyzed in vitro. Using the serum of one patient at the time of viral breakthrough, which contained the viral polymerase mutants L515M and V508L, it was shown that this viral population has a reduced sensitivity to penciclovir-triphosphate in an endogeneous polymerase reaction (Aye et al., 1997). We have also studied one HBV mutant with a sole V542I change in the viral polymerase which was associated with famciclovir resistance. Results of our in vitro studies could demonstrate, after transfection of hepatoma cells, that this mutated clone was indeed resistant to penciclovir, the active metabolite of famciclovir but remained sensitive to lamivudine (Pichoud et al., 1999a).

One of the major problems with these viral polymerase mutants is the possibility of cross resistance to other available nucleoside analogs. Indeed, Fig. 3 shows an update of the mutations described in drug resistant strains. The figure shows that mutations located in domain B of the viral polymerase may be associated with either famciclovir or lamivudine resistance. While some of the B domain mutations are sufficient to confer resistance to famciclovir, this may be also true for lamivudine with the human HBV. However, in

WHV infected woodchucks treated with lamivudine, viral resistance was found to be associated with mutations in the B domain of the polymerase without any mutation in the catalytic site (C domain) of the viral polymerase (Mason et al., 1998) which suggests that mutations located only in the B domain may potentially emerge in lamivudine treated patients. Our in vivo observation confirms that sequential re-treatment with the same nucleoside analog, or the sequential treatment with famciclovir followed by lamivudine, alone or a combination of lamivudine + famciclovir, select more rapidly for multiple resistant viral strains (Seignères et al., 1998). Recently, new nucleoside analogs with a potent anti-HBV activity have been developed. Results obtained in vitro with PMEApp, indicate that this compound may be equally active against wild type or lamivudine resistant mutants of the HBV or DHBV polymerase (Aguesse-Germon et al., 1999; Xiong et al., 1998).

3.5. Other approaches to inhibit viral polymerase activity

One approach could be the combination of different nucleoside analogs with different modes of inhibitory action of the viral polymerase to obtain a synergistic antiviral effect and therefore prevent or delay the emergence of resistant mutants (Fontana and Lok, 1997) (Fig. 4). Compounds such as penciclovir, CDG, PMEA, FIAU, L-FMAU, lobucavir and BMS 200, 475 were shown to inhibit at least in some extent the priming of reverse transcription (Staschke and Colacino, 1994; Dannaoui et al., 1997; Aguesse-Germon et al., 1999; Seifer et al., 1998). On the other hand, it was shown that other compounds such as lamivudine, L-FddC, L-Fd4C, phosphonoformic acid and polyoxometalates inhibit the elongation of viral minus strand DNA (Staschke and Colacino, 1994; Aguesse et al., 1997; Zoulim et al., 1996; Le Guerhier et al., 1999). The characterization of compounds which exhibit an inhibitory effect either on the priming reaction or on the elongation of viral minus strand DNA, may represent the basis for the development of combination therapy protocols using compounds exhibiting different modes of action, either to obtain a synergistic effect or to prevent the emergence of resistant mutants (Aguesse et al., 1997; Zoulim, 1997). Interestingly, a very elegant study performed in primary duck hepatocyte cultures showed that lamivudine and penciclovir inhibit DHBV replication to comparable extent when used alone and that the combination of both drugs is synergistic, especially in the inhibition of the viral CCC DNA (Colledge et al., 1997). These very interesting results, supporting the clinical use of a combination of lamivudine and famciclovir, may be explained by the different mode of action of these compounds on the viral polymerase (Severini et al., 1995; Aguesse et al., 1997; Shaw et al., 1996; Dannaoui et al., 1997). However, combination therapy should be started early to obtain a synergistic antiviral effect. Indeed, we have observed that once a L515M mutant has been selected during famciclovir therapy, the addition of lamivudine to famciclovir although initially active does not prevent the selection of a double mutant L515M + M539V resistant to both lamivudine and famciclovir, suggesting that the L515M was already resistant to lamivudine at least at some extent (Seignères et al., 1998). The development of other very potent nucleoside analogs with different modes of action is therefore required to increase the spectrum of the combination of nucleoside analogs. The number of potentially effective and non toxic drugs is increasing and will allow to design clinical trials of the combination of nucleoside analogs with different modes of action to obtain synergistic antiviral effect, prevent the selection of resistant mutants, and/or cure infected cells including non hepatocyte liver cells and extrahepatic cell reservoirs. Interestingly, it was also shown in tissue culture experiments that combination of L-nucleoside with D-nucleoside analogs may decrease the mitochondrial toxicity of the latter (Bridges et al., 1996). For all these reasons combinations carefully selected, in experimental systems, for their lack of toxicity and synergistic antiviral effect should be evaluated in clinical trials.

Other strategies not relying on the use of nucleoside analogs may help to circumvent the problem of HBV polymerase gene variability.

Recently, monoclonal antibodies directed against the terminal protein, the spacer region, the RNAseH domain of the human HBV polymerase were characterized. Monoclonal antibodies directed against the terminal protein domain but not those specific for the spacer or the RNAseH domain inhibited viral polymerase function in vitro. It was therefore suggested that recombinant antibody fragments expressed intracellularly may reduce the priming activity of the viral polymerase and the efficiency with which the enzyme is encapsidated within nucleocapsids (zu Putlitz et al., 1999a). Approaches using antisens oligonucleotide targeted against specific regions of the viral pregenomic RNA or peptides blocking viral RNA-polymerase interactions may also prove useful in the inhibition of viral polymerase activity.

4. Viral CCC DNA

Viral CCC DNA is a critical but difficult target for anti-HBV therapy. This form of viral DNA is the result of the transformation of the partially double stranded genomic DNA in covalently closed circular DNA in the nucleus (Summers and Mason, 1982; Tuttleman et al., 1986; Seeger and Mason, 1993). While more knowledge is available

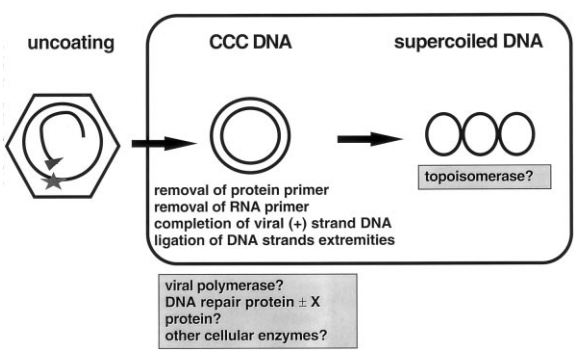

Fig. 7. Formation of viral covalently closed circular DNA. Viral CCC DNA formation results from the removal of the protein primer for reverse transcription that is covalently attached to viral minus strand DNA, the removal of the RNA primer for plus strand DNA synthesis, the completion of viral plus strand DNA, the ligation of DNA strand extremities, and supercoiling of the CCC DNA. It is still unclear what are the cellular and/or viral determinants involved in this reaction.

for the mechanism involved in the regulation of the recycling of the viral genome in the nucleus via the pre-S envelope protein, little is known on the actual enzymatic steps required in CCC DNA formation (Summers et al., 1990). CCC DNA formation results from the removal of the protein primer for the initiation of viral minus strand DNA synthesis, the removal of the RNA primer for the initiation of plus strand DNA synthesis, the completion of viral plus strand DNA, ligation of viral DNA extremities and supercoiling of this DNA molecule (Fig. 7). Preliminary results obtained in primary duck hepatocyte cultures suggest that cell cycle may influence CCC DNA accumulation in the nucleus of infected cells (Turin et al., 1996). The different cellular or viral enzymes involved in these key enzymatic steps still remain to be discovered.

Studies using the in vitro and in vivo model systems of hepadnavirus replication have shown that chronic infection of the hepatocyte is maintained by the presence in the nucleus of 30–40 copies of viral CCC DNA. This viral DNA species is the template for viral transcription and its production is regulated and amplified by an intracellular pathway in which newly synthesized genomic DNA is transported to the nucleus (Wu et al., 1990). Thus, new CCC DNA synthesis can be blocked by reverse transcriptase inhibitor. However, short-term therapy can not deplete the CCC DNA pool, which is the source of renewed viral production after cessation of therapy (Fourel et al., 1994b). With long-term therapy it may be possible to clear CCC DNA from the liver even if it has a long half-life if infected hepatocytes are cleared either by natural cell death or as the result of the immune response to viral antigens (Fourel et al., 1994a). This also imply that new hepatocytes arising from cell division are not susceptible to viral infection or protected by neutralizing antibodies (Mason, 1993). It was shown recently, using long-term woodchuck hepatocyte cultures infected with WHV and treated with different nucleoside analogs, including lamivudine, that viral CCC DNA half life is very long and that the decline of intracellular CCC DNA observed after 36 days of treatment in tissue culture may have been the result of dying hepatocytes rather than

the clearance of CCC DNA (Moraleda et al., 1997). These results were also confirmed in vivo in infected woodchucks that were treated with lamivudine. Although viral DNA titers in serum droped by 2–3 logs, the number of infected hepatocytes remained stable even after 18 months of therapy (Mason et al., 1998). Altogether these results suggest that not only the persistance of intracellular CCC DNA but also the longevity of infected hepatocytes are the key issues to the curing of viral infection. Strategies aimed at specifically inhibiting the formation of CCC DNA are attractive but the determination of cellular and/or viral enzymes involved in this process is still required.

Using another nucleoside analog, Ganciclovir, Luscombe et al. showed that a prolonged administration of this DNA polymerase inhibitor in DHBV infected ducklings could inhibit viral DNA synthesis but had little effect on the viral CCC DNA and RNA in the liver (Luscombe et al., 1996). An accumulation of the viral pre-S envelope protein was observed within the liver and the authors suggested that this may be a precursor for hepatotoxic liver disease and that such therapeutic approach should be used with caution in patients (Luscombe et al., 1996). Administration of penciclovir in DHBV infected ducklings for 12–24 weeks showed a very different antiviral effect. Indeed, a decrease of all viral replication intermediates was observed and remarkably of the viral CCC DNA and viral proteins in the liver. However, viral replication relapsed after drug withdrawal (Lin et al., 1998) again emphasizing that a prolonged therapy with a single nucleoside analog may not be sufficient to clear viral infection in an immune tolerant host.

Other studies of the clearance kinetics of HBV from patients during adefovir dipivoxil therapy showed a biphasic clearance of viral infection with a first decline corresponding to clearance of HBV particles from serum followed by a second slower phase corresponding to the loss of infected cells (Tsiang et al., 1999). It appears clear from these studies that clearance of intrahepatic CCC DNA should represent a major endpoint of antiviral therapy in experimental models as well as in clinical trials. PCR assays that are specific of viral CCC DNA detection have been set up (Kock and Schlicht, 1993; Kock et al., 1996) and may be used in the future to monitor antiviral therapy and adapt antiviral protocols to the persistence of this recalcitrant form of viral DNA.

New in vitro and in vivo models for the study of HBV infection have been studied recently and may help to gain insight on the mechanism of curing infected hepatocytes or of clearance of infected cells from the liver. A novel transient mechanism for studying HBV gene expression and replication using recombinant HBV baculovirus to deliver the HBV genome to HepG2 cells was generated by Delaney et al. In HBV baculovirus infected HepG2 cells, HBV replication could be readily detected as well as viral CCC DNA. This model represents a simple to use and flexible system for the study of the effect of new antivirals and cytokines (Delaney and Isom, 1998). Ilan et al. have developed a mouse Trimera model for human HBV infection. This system consists on the transplantation of ex vivo HBV-infected human liver fragments in lethally irradiated mice or rats, radioprotected with SCID mouse bone marrow cells. In this model viremia could be detected by PCR and followed for 2–3 weeks. Administration of LFddC or lamivudine resulted in the inhibition of viremia levels. The authors therefore concluded that this novel experimental model may be useful to evaluate new anti-HBV strategies (Ilan et al., 1999).

5. Viral assembly

A detailed knowledge of viral assembly may provide the basis for the inhibition of the encapsidation of the viral pregenome (Hirsch et al., 1990, 1991; Bartenschlager and Schaller, 1992; Nassal, 1992) (Fig. 8). One group has used with success HBV core mutants which exhibit a dominant negative effect in tissue culture by interfering with viral capsid formation and encapsidation of the viral pregenome (Scaglioni et al., 1996). Strategies to deliver such mutants with retrovirus or adenovirus vectors have been evaluated in vitro in tissue culture. In vivo transfer of such mutants should be evaluated in experimental models.

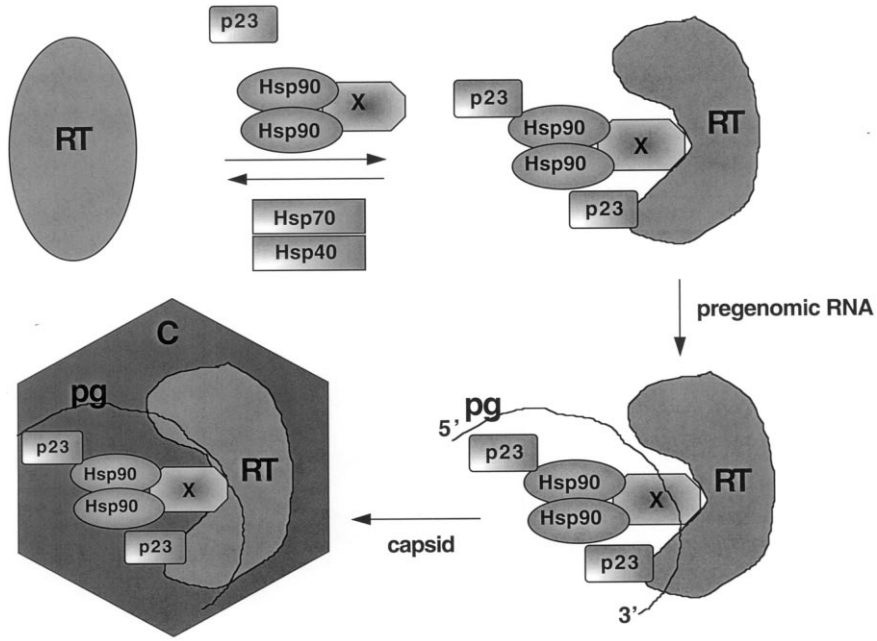

Fig. 8. Model for the packaging of pregenomic RNA. Packaging of viral pregnomic RNA (pg) results from a complex and sequential interaction of the viral polymerase (RT) with heat shock proteins (Hsp90, p23) and other cellular proteins (X), the encapsidation signal on pregenomic RNA and capsid proteins (C).

The specific inhibition of virion formation may be facilitated by a knowledge of envelope formation and the process of viral secretion from infected hepatocytes (Bruss and Ganem, 1991; Bruss and Thomssen, 1994; Bruss and Vieluf, 1995; Bruss, 1997). A strategy based on peptides to block hepatitis B virus assembly was shown to inhibit the interaction between the capsid and the large envelope protein after analysis by cryomicroscopy, mutagenesis and transfection (Böttcher et al., 1998). This gives a first evidence that peptides may be used as therapeutic agents for hepatitis B. Block et al. have studied the impact of the inhibition of cellular glucosidase to prevent the secretion of enveloped HBV particles, as a potential new antiviral strategy. They used the woodchuck model of HBV infection to evaluate the antiviral effect of an inhibitor of endoplasmic reticulum alpa glucosidases (*N*-nonyl-dexynojirimycin or *N*-nonyl-DNJ), in vivo and showed a dose dependent decrease in enveloped virus in serum together with an increase of hyperglucosylated glycan in serum. These results suggest that disruption of folding and transport of hepadnavirus glycosylated envelope proteins may represent a novel antiviral strategy. However, with the use of compounds that may interfere with intracellular trafficking, it will be mandatory to demonstrate the lack of toxicity during long-term treatment (Block et al., 1998). Another recent report showed that intracellular antibody fragment directed against the viral envelope protein and delivered by gene transfer was successful in inhibiting viral envelope protein secretion in tissue culture (zu Putlitz et al., 1999b). It remains to demonstrate its capacity of inhibiting viral replication in vitro and to find convenient ways for gene delivery, in vivo.

6. Viral gene expression

Viral gene transcription and translation represent other theoretical targets, but may be difficult to inhibit specifically since viral transcription uses the cellular machinery (Schaller and Fischer,

1991). The use of anti-sense oligonucleotides to block viral gene expression has been evaluated and may act on viral replication either by stimulating RNAseH activity and degradation of viral messenger RNAs, by inhibiting ribosome assembly, by modifying the secundary structure of messenger RNAs, or by inactivation of RNA sequences required for viral replication (von Weizsäcker et al., 1997).

Antisense oligonucleotides were used with success in the duck model and were shown to inhibit dramatically viral replication, in vitro in primary hepatocyte culture and in vivo in chronically infected animals (Offensperger et al., 1993). However, among a panel of oligonucleotides, only one directed against the 5′ end of the pre-S region, was found to be therapeutically active. The exact mechanism of action of this antisense oligonucleotide needs to be determined. Other approaches may be used in the future, such as ribozymes that catalyze sequence-specific cleavage of RNA to specifically cleave viral transcripts and inhibit viral replication (von Weizsäcker et al., 1992). More recently, zu Puplitz et al. have shown that inhibition of HBV replication may be obtained by the catalytic activity of the ribozymes in an in vitro cell based assay. They could identify catalytically active RNAs by combinatorial screening that mediate intracellular antiviral effects on HBV (zu Putlitz et al., 1999c). This new strategy therefore warrants further evaluation.

7. The X protein

The X protein may also represent a very specific target since it was shown that the X protein exhibits a promiscuous transactivating activity on the expression of a variety of viral and cellular genes in many cell lines (Schek et al., 1991). This pleiotropism and the lack of a DNA binding activity led to the conclusion that X exerts its transactivation function through one or several interactions with cellular protein targets and signal transduction pathway, including the tumor suppressor p53, transcription factors, CREB and ATF2, and components of the protein kinase C and ras signal transduction pathways (Colgrove et al., 1989; Seto et al., 1989; Maguire et al., 1991; Kekule et al., 1993; Seeger, 1997). Other in vitro experiments showed that X may also interacts with DNA repair proteins (UVDDB) (Sitterlin et al., 1997) and a subunit of the proteasome complex (XAPC7) (Huang et al., 1996). According to the many published works, in which the X protein was overexpressed in different artificial systems, the X protein may either interact with a cellular partner in the nucleus via the UVDDB protein or p53 (Sitterlin et al., 1997; Terradillos et al., 1998), or in the cytoplasm via the raf/ras pathways, NFkB or others (Benn et al., 1996; Huang et al., 1996; Su and Schneider, 1996) (Fig. 9). However, functional studies to attribute biological significance to these observations in terms of viral replication, infectivity or carcinogenesis, are lacking. This is all the more important since experiments performed in the woodchuck model showed that although X is not required for viral genome replication in transfected cells, it is essential to initiate viral infection in vivo (Chen et al., 1993; Zoulim et al., 1994). This may imply that X either acts as early protein or by inducing the expression of cellular genes required for the initiation of viral infection. In a recent study, Sitterlin et al. using WHV X mutants have shown a correlation between the transactivation activity, the interaction with DNA repair proteins and the in vivo infectivity of the mutants. The hypothesis drawn from these experiments is that the interaction between X and UVDDB may be required for the nuclear transport of X, allowing its function as a transcriptional activator. However, a direct demonstration of the X function in the viral life cycle is still lacking (Sitterlin et al., 1999). In vitro studies of the infectivity of these X mutants, using human or woodchuck primary hepatocyte cultures, may be required to clearly identify the function of this viral protein. The elucidation of its function at the molecular level may be very important to design new antiviral strategies to block viral infection.

8. Interaction with the cellular receptor

Identification of other viral targets may prove very useful to inhibit viral replication using differ-

ent strategies. Blocking the interaction of HBV and the cell membrane receptor as well as the transport of the viral genome to the nucleus awaits for the identification of the different cellular and viral determinants involved in these critical steps. Research performed with primary duck hepatocyte cultures allowed to identify a specific interaction between the viral pre-S envelope protein (Le Seyec et al., 1999) and a cellular glycoprotein, i.e. gp180 which was then identified as carboxypeptidase D (Kuroki et al., 1994; Breiner et al., 1998; Urban et al., 1998b). This interaction was confirmed by different groups using independant experimental approaches (Li and Tong, 1996; Sunyach et al., 1999). However, transfection of plasmid expressing this cellular protein in non susceptible cells could not restore infectivity, suggesting that this protein may represent a subunit of the cell receptor complex. Recently, it was also reported that annexin V is a candidate receptor (Gong et al., 1999b). More efforts are being pursued to identify the other components of the cellular receptor required for viral entry into the cell. This may lead in turn to the design of new strategies to neutralize infectivity by targeting the specific interaction between viral envelope proteins and the cellular receptor(s). The first successful attempts have been made in the duck model using neutralizing antibodies directed against the viral pre-S envelope proteins which were shown to protect animals from viral infection but also to select for escape mutants of the viral envelope protein (Lambert et al., 1990; Sunyach et al., 1997). Ryu et al. have assessed the capacity of anti-pre-S1 and anti-pre-S2 monoclonal antibodies to neutralize HBV infection in human primary hepatocyte cutltures. Their results showed that anti-HBs and anti-pre-S2 antibodies exhibited neutralizing activity against both the adr and ayw subtypes of the virus, but the anti-preS1 antibody scarcely neutralized the infection (Ryu et al., 1997). Passive administration of a mixture of human monoclonal antibodies against HBsAg was assessed, in vivo, in a chronically infected chimpanzee. The results showed that human monoclonal anti-HBs can effectively reduce the level of HBsAg in serum (Heijtink et al., 1999).

Other approaches have been evaluated with some interesting results with the use of com-

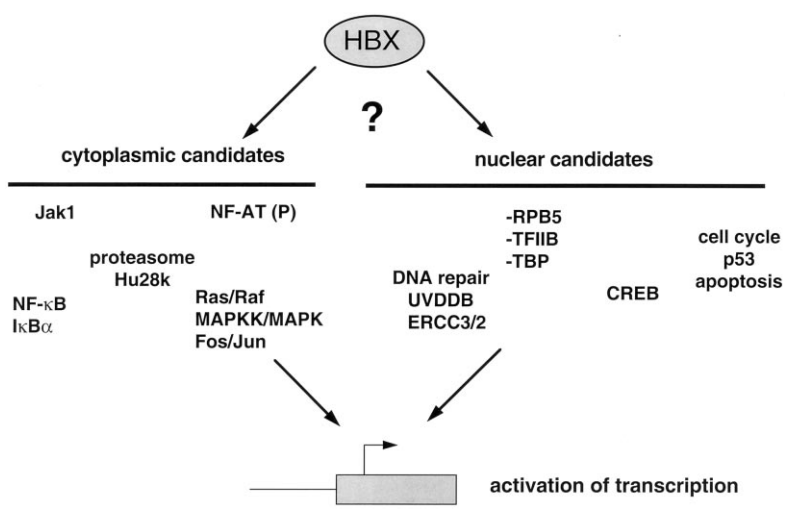

Fig. 9. Potential targets of HBV X protein. Many studies have shown that the HBV X protein exhibits a weak but promiscuous transcription transactivity. Since X does not bind to DNA, studies have been focused on the identification of interactions between X and cellular factors. Some follows a pathway in which the X protein interacts with a cytoplasmic partner to activate cellular gene transcription by the activation of several signal transduction pathways. In other studies the X protein was shown to interact with nuclear factors which may then activate the transcription of cellular genes. The X protein may also interfere with the tumor suppressor protein p53 and also with apoptosis and/or cell cycle.

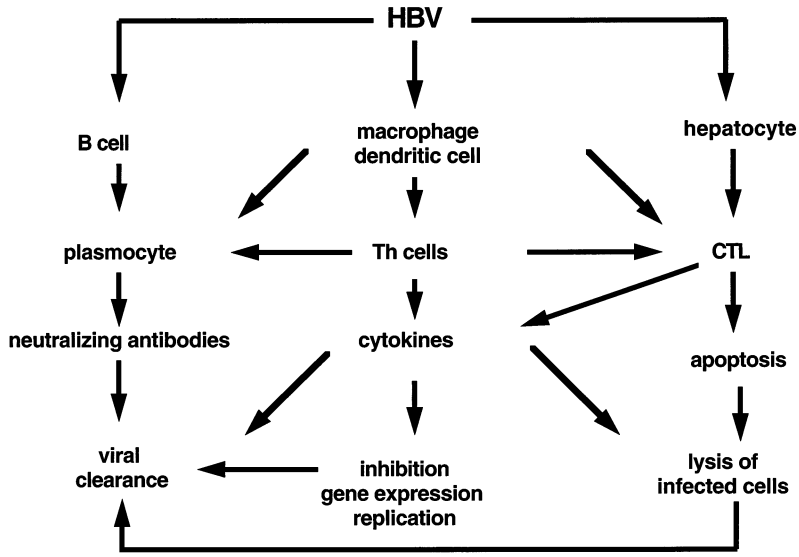

Fig. 10. Specific antiviral immune response required for viral clearance. Viral clearance requires the combined action of different components of the immune system with a potent B cell response to neutralize circulating virus, an efficient T cell response (CD8) that exhibits a cytotoxic activity against infected cells and produces antiviral cytokines, that are regulated by helper T cells (CD4) to optimize the immune response to eradicate viral infection.

pounds belonging to the tetraazamacrocycles family that were shown to reduce the ability of DHBV to initiate viral infection in primary hepatocyte cultures (Hantz et al., 1997).

9. Immune response

The role of the immune response in the clearance of HBV infection has been suspected long ago. More recently, several studies focused on allelic differences in the HLA complex and their influence in viral persistence or clearance. The importance of HLA class II mediated immune response in the resolution of viral infection was demonstrated clearly (Thursz et al., 1995; Thio et al., 1999).

Results of experiments performed in the transgenic mouse model of HBV infection have provided new insights into the immunopathology of HBV infection (Chisari, 1995) (Fig. 10). The role of different cytokines in the inhibition of viral replication was studied. It was shown that interleukin 2, TNF alpha and IFN alpha inhibit specifically the expression of viral transcripts within the liver (Gilles et al., 1992; Guilhot et al., 1993). Furthermore, it was shown that interleukin 12 inhibits viral replication by suppressing viral transcription at a post-transcriptional level and by inducing viral capsid degradation, via the induction of secretion of TNF alpha and interferon gamma in the liver (Cavanaugh et al., 1997). Evidence was also provided in this model that hepatocellular HBV RNA content is controlled by the stabilizing/destabilizing influences of RNA binding proteins whose activity is regulated by cytokine-induced signaling pathways (Heise et al., 1999). Furthermore, Chisari and colleagues elegantly showed that the cytotoxic T cell response may actually exhibit two independant antiviral effects: first by facilitating the clearance of infected cells via the stimulation of cell killing by CD8 positive cells through the fas and perforin pathways, and second by inhibiting independently viral replication via the production within the liver of cytokines (Chisari, 1995). An increased production of cytokines in the liver during the clearance of viral infection without massive cell death has been observed also by Mason and colleagues (Kajino et al., 1994). This may explain

why viral clearance may occur in massively infected animals without significant liver damage (Seeger, personal communication). These very interesting results may provide the basis for using a combination of nucleoside analogs and cytokines to hasten viral clearance. Therefore it will be crucial to evaluate different protocols of combination therapy based on our knowledge of the immunobiology of HBV infection. This was also recently and elegantly demonstrated by Guidotti et al. in the chimpanzee model for HBV infection. In this study they showed that HBV disappeared from the liver and serum of acutely infected chimpanzees long before the peak of T cell infiltration. These results may also indicate that noncytopathic antiviral mechanisms contribute to viral clearance during acute hepatitis by eliminating viral replicative DNA intermediates from cytoplasm and CCC DNA from the nucleus of infected hepatocytes (Guidotti et al., 1999).

Both interferon alpha and gamma from duck have been cloned and characterized recently by Schultz et al. (Schultz and Chisari, 1999; Schultz et al., 1999). It was shown in DHBV infected primary duck hepatocytes that recombinant duck interferon gamma inhibits DHBV replication in a dose-dependent fashion as well as the synthesis of progeny CCC DNA by amplification but does not affect the initial formation of CCC DNA. Another interesting finding was that treatment with duck interferon alpha resulted in a different antiviral effect with a depletion of pregenomic RNA containing nucleocapsids.

Since most chronic HBV carriers do not respond to interferon therapy, it has been hypothesized that the limited efficacy of interferon may rely at least in part to a low expression of IFN receptor in the liver. Since the asialoglycoprotein (ASGP) receptor is highly expressed in the liver, the ASGP receptor binding domain was generated within a *N*-glycosylated human interferon beta molecule. This modified IFN beta molecule exhibited a greater anti-HBV effect in vitro in tissue culture compared to its conventional counterparts, i.e. IFN alpha and beta. This was also supported by a stronger induction of the 2′,5′-oligo-adenylate synthetase. Moreover, this modified IFN beta was shown to significantly inhibit HBV replication in vivo, in a nude mouse model, in contrast to conventional IFN beta. These results indicate that directing IFN to ASGP receptor may facilitate its targeting to the infected liver and enhance the antiviral effect of IFN (Eto and Takahashi, 1999). Due to their many clinical implications, these new findings deserve rapid confirmation in clinical trials.

Another interesting approach to stimulate the anti-HBV immune response would be to use a therapeutic vaccine (Koziel and Liang, 1997). Preliminary studies have shown promising results in transgenic mice bearing an *S* transgene that received recombinant HBsAg particles and further developed an anti-HBs humoral response (Mancini et al., 1992). This has provided the basis for a clinical trial in patients with chronic hepatitis B who received the pre-S2/S recombinant vaccine and showed a trend towards a decrease of serum viral DNA (Pol et al., 1994). It was further shown that vaccine therapy using HBsAg containing vaccine in HBV-transgenic mice induces proliferation of T cells and production of anti-HBs antibody via the activation of dendritic cells. The results of this experiment showed the key role of dendritic cells in the antiviral response after administration of therapeutic vaccine in this HBV transgenic mouse model (Akbar et al., 1997, Akbar et al., 1999). Other approaches using a DNA based vaccine with the S or C genes have been evaluated in mice, woodchucks and ducks. It was demonstrated that this approach may be more effective in stimulating a HLA class I restricted cytotoxic T cell response against viral envelope proteins compared to HBs Ag particle injection (Schirmbeck et al., 1995). A broad cytotoxic T cell activation as well as a T helper cell response associated with a profile of T_{H1} cytokine production was observed in another study in mice and may represent a potential antiviral approach (Geissler et al., 1997). Experiments performed in the woodchuck and the duck models showed that DNA-based vaccination with viral genes can elicit protective immunity to hepadnavirus infection (Triyatni et al., 1998; Lu et al., 1999; Rollier et al., 1999). Furthermore, very interesting findings came from studies performed in ducks that were chronically infected with DHBV in whom DNA

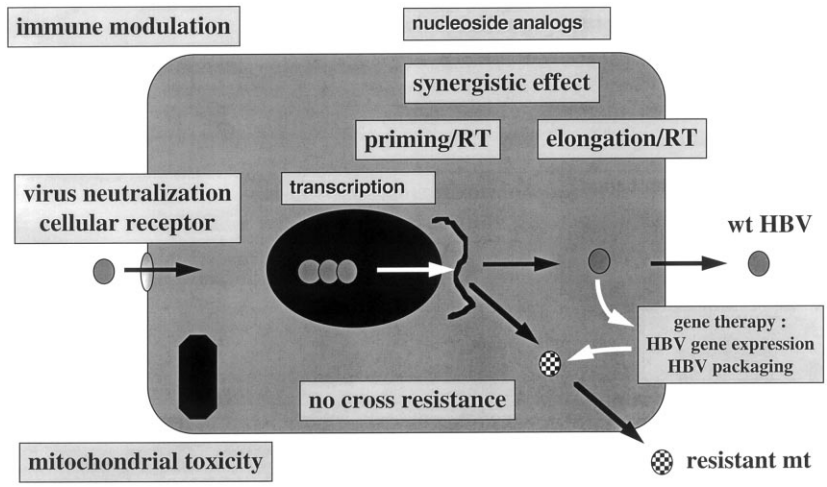

Fig. 11. Rationale for combination therapy in chronic HBV infection. The Figure indicates the viral targets of cytokines and nucleoside analogs which may help to design combination therapy using reverse transcriptase inhibitors having different mode of action and different resistance pattern to prevent the selection of resistant mutants. Mitochondrial toxicity should remain a concern when using this strategy. Other strategies of inhibition of HBV replication (packaging, viral assembly) and manipulation of the anti-HBV immune response (cytotoxic T cell response, neutralizing B-cell response) may also be used in combination to prevent the development of resistant mutants.

based vaccination with a pre-S envelope protein encoding plasmid induced a strong humoral response against the pre-S antigen and inhibited dramatically viral replication within the liver (Rollier et al., 1999). These important results deserve confirmation in animal models and in clinical trials before designing new protocols for the combination of nucleoside analogs and immune manipulation.

10. Integration

Viral genome integration is not required for viral replication. However, it is a major factor involved in liver carcinogenesis. Hepatocytes harboring integrated HBV genome may persist after the clinical resolution of viral infection and the clearance of cells replicationg the virus. Viral genome integration therefore represents an important target to prevent the development of liver cancer. During chronic hepatitis, viral DNA integration occurs randomly and at a low frequency in infected cells (Bréchot et al., 1980, 1981). Integration may be enhanced by the increased rate of hepatocyte cell division resulting from hepatocyte necrosis and cell turn over during chronic hepatitis. The presence of viral integrations in the cellular genome may stimulate the expression of cellular oncogenes as frequently observed and described in the woodchuck model with the *c-myc* and *N-myc* genes (Hsu et al., 1988; Fourel et al., 1990) and more rarely in human hepatomas (cyclin A gene, v-erb, etc.) (Dejean et al., 1986; Wang et al., 1990). This may also lead to chromosomal rearangements including deletions, translocations, transpositions or amplification of specific viral and cellular sequences (Hino et al., 1986, 1989; Nagai et al., 1997). Results of detailed molecular studies suggested that double stranded linear viral DNA molecules may represent precursors of viral DNA integration and that the r region of the genome may represent a preferred integration site for linear viral DNA molecules (Wang and Rogler, 1991; Gong et al., 1995, 1996, 1999a; Pourquier et al., 1999). Since HBV genome does not encode a viral integrase, in contrast to retroviruses, the precise mechanism involved in integration remains to be elucidated. Meanwhile therapeutic intervention aiming at inhibiting the consequences of viral genome integration should be explored (Wang et al., 1998). Recently, it was

shown that interferon alpha administration in C-myc-WHV transgenic mice is able to inhibit c-myc activation and delay the development of hepatocellular carcinoma in mice (Merle et al., 1997). This represents a good model to better understand the clinical findings that interferon treatment may prevent the development of liver cancer even in non responder patients. Alternatively, other approaches to inhibit the transctivation of cellular genes by the X protein may be also helpful in this aim.

11. Conclusion

Our knowledge of the HBV replication cycle, the viral dynamics during antiviral therapy, the selection of resistant mutants and the experience of antiviral therapy of HIV infection build a strong case for the evaluation of combination therapy for chronic hepatitis B (Fig. 11). The development of new inhibitors of the HBV reverse transcriptase activity and other key steps of the viral life cycle is clearly warranted. Efforts to optimize the protocol of stimulation of the specific antiviral immune response, possibly via naked DNA vaccine or other approaches, should open new perspective in clinical therapeutic research. The identification of the cellular receptor, the process involved in CCC DNA formation and the role of the X protein in viral infection remain a difficult challenge that clearly will open new avenues in terms of antiviral treatment of HBV infection. It will be interesting to see in the near future whether the gene based therapeutic approaches will be successful in animal models and will reach clinical trials.

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