LAMIVUDINE TREATMENT

FOR CHRONIC HEPATITIS B

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LAMIVUDINE BEHANDELING VAN CHRONISCHE HEPATITIS B

PROEFSCHRIFT

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"Geef ons heden ons dagelijks brood."

Mattheus 6:11

Aan Wilma en Pieter jr.

Aan mijn moeder
Ter nagedachtenis aan mijn vader

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INTRODUCTION

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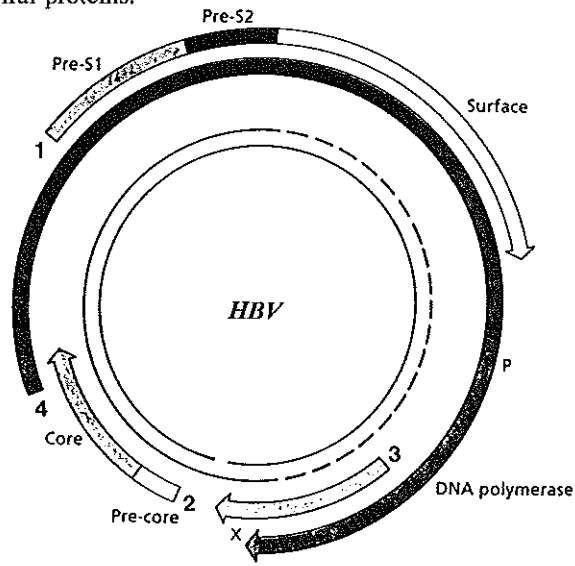
The hepatitis B virus

The hepatitis B virus (HBV) is one of the smallest human viruses known and belongs to the family of Hepadnaviridae; it was the first human hepatitis virus that could be characterized. Before the discovery of the virus two types of transmission of infectious hepatitis were distinguished on the basis of epidemiological observations: the classical hepatitis (type A) was transmitted by the faecal-oral route, while type B was transmitted parentally.[1]

In 1963, BS Blumberg discovered a previously unknown antigen in the blood of an Australian aboriginal (Australia antigen) and within a few years this was found to be related to the parentally transmitted type B hepatitis.[2] In the early seventies the virus was seen by electron microscopy[3] and the genome was found to be a small, circular DNA that was partially double-stranded (figure 1). The nucleotide sequence of the virus contains only 3200 nucleotides (3.2 kb) and revealed 4 overlapping genes for the production of seven viral proteins.



1- Surface antigen related peptides

2- Core antigen related peptides

3- HBxAg

4- HBV-DNA polymerase

Figure 1. *The genome of the hepatitis B virus consisting of 3200 nucleotides representing 4 open reading frames drawn as arrows. (Surface, Core, X and Polymerase)*

The first open reading frame encodes a family of hepatitis B surface antigen (HBsAg)-related polypeptides that make up the outer envelop of the virus. The major HBsAg polypeptide is encoded by the S gene. The pre-S2 sequences are cotranslated with S sequences to yield pre-S2/S or middle-sized polypeptides. Cotranslation of the upstream

pre-S1 sequences, yield pre-S1/pre-S2/S or large sized polypeptides. The second HBV open reading frame, the C gene, encodes the major hepatitis B core antigen (HBcAg) polypeptide and hepatitis B e antigen (HBeAg), these are the major components of the virus inner nucleocapsid. These particles are important targets for the immune response against the virus. The soluble HBeAg is secreted into serum and associated with virus replication and high infectivity. The third HBV open reading frame encodes the hepatitis B x antigen (HBxAg), which function is not clear. It is probably one of the co-factors in the pathogenesis of hepatocellular carcinoma.[4] The fourth and largest HBV open reading frame encodes the HBV-DNA polymerase, which is responsible for the endogenous DNA-polymerase activity in virus particles. HBV-DNA polymerase has been shown to be the reverse transcriptase (RT) polypeptide of the virus. In this context, any mutation that affects the polymerase activity will not only affect the amount of virus produced, but also the number of templates available to encode virus proteins, and elicit appropriate and timely immune responses.

Chronic hepatitis B

Inoculation with the hepatitis B virus causes hepatocellular necrosis and inflammation, which severity ranges from asymptomatic to acute liver failure. Acute infection will resolve spontaneously in 90% of the adults; the reason why in certain patients the acute infection will not resolve but progresses to chronic hepatitis B is not yet clear. Both viral factors and host factors can probably influence the outcome of the acute infection.
Chronic infection with the hepatitis B virus is a serious liver disorder which can result in chronic active hepatitis, cirrhosis and primary hepatocellular carcinoma. It is estimated that 5% of the world population is chronic HBsAg carrier,[5] the majority of these will even not show any disease progression. In our population about half of the HBsAg carriers did not show any signs of liver inflammation (normal serum transaminases) or viral replication (HBV-DNA negativity by PCR). Currently patients with detectable HBV-DNA by PCR in combination with the lack of any signs of liver inflammation do rarely show progression of their liver disease. Longitudinal studies on the natural history of chronic liver disease due to HBV have shown that ongoing replication (HBeAg *either* HBV-DNA positivity by hybridization technique) *or* liver inflammation are features which predict unfavourable evolution of the disease.[6,7,8] Permanent termination of HBV replication is found to be the major event that will influence outcome.[9,10,11] Spontaneous remission of disease activity may occur in approximately 10% of HBeAg positive carriers per year.

Antiviral therapy

The ultimate goal of antiviral therapy in chronic hepatitis B is to prevent progression of liver disease to cirrhosis, hepatocellular carcinoma or liver failure and subsequently

improve life expectancy and quality of life (table 1). This can be obtained by elimination of the virus from the body and the induction of protective antibodies against the virus. However, improvement of prognosis can be achieved by suppression of HBV replication or induction of HBeAg seroconversion despite the continued presence of HBsAg. This situation of viral latency is associated with normalization of serum transaminase levels and decrease in necro-inflammatory activity on liver biopsies. Therefore, in most clinical studies, the therapeutic outcome (response) is defined as clearance of HBeAg and HBV-DNA in serum, and subsequently improvement of liver disease.

Table 1. Goals of antiviral therapy for chronic hepatitis B

- Loss of markers of viral replication (HBV-DNA / HBeAg)
- Normalization of serum transaminase levels (ALT)
- Disappearance of liver cell inflammation
- Improvement of symptoms (quality of life)
- Decrease of progression of liver disease
- Decrease in incidence of hepatocellular carcinoma
- Improvement of survival
- Diminished infectivity
- Loss of HBsAg and development of anti-HBs

Until now the only agent known to have a lasting beneficial effect in chronic hepatitis B is alpha-interferon (α-IFN). It demonstrates both antiviral and immunomodulating effects of whom the last one is probably the most important in chronic hepatitis B. α-IFN will enhance the HLA class I antigen expression on the membranes of virus-infected hepatocytes, and thereby amplify viral antigen recognition and display. The immunomodulatory effects of α-IFN include enhancement of antigen-processing cells, natural killer cells and cytotoxic T-lymphocytes, and increased release of cytokines. Standard α-IFN treatment takes three to six months with weekly doses around 30 MU and will induces long-term remission in 25-40 percent of patients.[12,13]

However, polymerase chain reaction (PCR) techniques are able to detect products of viral replication in patients classified as 'healthy carriers'. In spite of HBeAg seroconversion the disease will probably continue to progress in these patients. Recently Fattovich et al. observed that ALT normalization is a crucial sign for inactivation of the progression of the disease.[14] This can be clarified by the fact that in a proportion of patients viral mutations arise which are able to escape the immunological control.

In conclusion α-IFN treatment can significantly improve outcome both after short-term and long-term follow-up analysis. However three major reasons forced us to develop new antiviral strategies:

1. Several patients will not fulfil virological and serological criteria for α-IFN therapy.
2. Since α-IFN has significant side effects there are contra-indications for interferon therapy, especially in patients with advanced liver disease.[15,16]

3. The majority (60-75%) of patients does not respond to α-IFN therapy.
Therefore, the majority of patients with chronic hepatitis B will not benefit from standard α-IFN therapy and new antivirals are needed.

Nucleoside analogues

Over the last decade, advances in the understanding of the molecular virology of the hepatitis B virus have resulted in a renewed interest in nucleoside analogues for the treatment of chronic hepatitis B. Since the discovery that the replicative cycle of HBV involves an obligate RNA intermediate and a cytoplasmic reverse-transcriptase step, the HBV polymerase has become the major focus in the search for novel therapeutics that could block replication. Because the reverse transcriptase is not involved in human cell replication, nucleoside analogues may have little toxicity. However, most of the first generation nucleoside analogues as vidarabine, acyclovir, didanosine, zidovudine and ribavirin were either ineffective, associated with unacceptable toxicity or both.[15,17,18,19,20] The advent of an in vitro cell line (human hepatoblastoma, Hep G2) which propagates the virus[21], allowed the development of a screening system[22] to test a promising new generation nucleoside analogues.[23]

Table 2. *Nomenclature of Nucleosides*

Purine	Adenine
	Guanine
Pyrimidine derivatives	Cytosine
	Uracil (RNA)
	Thymidine (DNA)

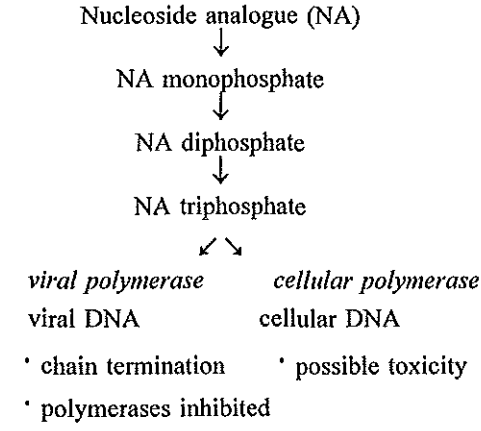


Figure 2. *Metabolism and activity of nucleoside analogues.*

A nucleoside analogue must first be phosphorylated to its triphosphate (TP) form, for antiviral activity. Antiviral drug selectivity is established by primary phosphorylation with a viral encoded enzyme followed by the conversion to the TP form by cellular nucleotide kinases (figure 2). Therefore virus infected cells accumulate the active triphosphate form of the drug more properly than the uninfected cells. Recently some new nucleoside showed very promising results *in vitro*, but first I would like to summarize the clinical studies on nucleoside analogues in the past (table 3).

Table 3. *Nucleoside analogues for chronic hepatitis B*

Generic name (analogue)	Abbreviation	Proprietary name	Results
Acyclovir	ACV	Zovirax	Little or no efficacy
Vidarabine	ARA-A	Vira-A	Inconvenient, toxic
Didanosine	ddI	Videx	No efficacy
Zalcitabine	ddC	Hivid	Inconvenient, toxic
Ribavirin	RTCA	Virazole	No efficacy
Zidovudine	AZT	Retrovir	Little or no efficacy
Ganciclovir	DHPG	Cymevene	Limited efficacy
Fialuridine	FIAU	-	Extremely toxic
new nucleoside analogues in clinical testing for chronic hepatitis B			
Lamivudine	3TC, SddC	Epivir	Effective, non-toxic
Famciclovir	FCV	Famvir	Effective, non-toxic
Lobucavir	BHCG	Cygalovir	Promising
Phosphonylmethoxyethyladenine	PMEA	Adefovir	Promising
Deoxythiafluorocytosine	FTC	-	More data required
Carbodeoxyguanosine	2-CDG	-	More data required

Acyclovir [9-(2-hydroxyethoxymethyl) guanine] is a non-cyclic guanosine analogue discovered in 1974 and is licensed for the treatment of herpes virus infections in many countries. In hepatitis B virus infections acyclovir inhibits viral DNA synthesis in an *in vitro* model. In human studies the observed poor antiviral effect and the possible renal and neurological toxicity of the drug suggested that it was not justifiable in chronic hepatitis B.[18]

Administration of *vidarabine* (adenine arabinoside, ARA-A) and its soluble monophosphate analogue ARA-AMP resulted in only a transient decrease in levels of HBV-DNA both after intravenous or intramuscular injection.[17,24] The inhibitory function is not virus specific and the triphosphate form has been recognized as a potent inhibitor of cellular polymerase-α. A combined approach with α-IFN also failed to show advantage to interferon therapy.[25] In addition after 4 weeks of therapy 47% of patients complained of myalgia, requiring withdrawal of treatment in 29% of cases; also neurotoxicity has been reported during therapy.[17]

2',3'-*Dideoxyinosine* (ddI) and 2',3'-*dideoxycytidine* (ddC) are nucleoside analogues that inhibit HIV reverse transcriptase and HBV-DNA polymerase *in vitro*.[26] Translating these results in clinical studies in HBV infected patients was disappointing.[19] The potential development of neuropathy as well as lactic acidosis make these drugs unsuitable for antiviral therapy in chronic hepatitis B.[27]

Ribavirin is a purine analogue with a structure similar to guanine and inosine. Although ribavirin has been shown to have a broad spectrum of antiviral activity against both RNA

and DNA viruses, it is not a direct inhibitor of viral polymerases but its main mode of action appears to be interference with viral mRNA synthesis. In a pilot study in 18 patients treated for 6 months with ribavirin at three different doses, HBV-DNA levels decreased by an average of 20 per cent. Also ALT levels decreased during therapy, however, all changes were transient and returned to pretreatment levels once ribavirin was discontinued.[20]

Zidovudine (3'-azido-3'-deoxythymidine, AZT) has proven to be a potent antiviral in HIV-1 infected patients.[28] These inhibitor of the reverse transcriptase activity also demonstrated marked decrease in HBV replication both *in vitro* and *in vivo*.[29] However a randomized placebo controlled trial in 24 patients could not find an additional effect when zidovudine was given in combination with α-IFN. In addition patients undergoing combination therapy exhibited more dose-limiting bone marrow toxicity than those on α-IFN monotherapy.[30]

Ganciclovir is a guanine nucleoside analogue that has been shown to be effective for the treatment of CMV. In spite of weak *in vitro* inhibition of HBV replication[31] some pilot studies showed a decrease in HBV-DNA levels after HBV recurrence after liver transplantation.[32] The poor oral bioavailability and the substantial myelosuppression limits the clinical use of the drug.

In two preliminary dose-finding studies on *fialuridine* marked suppression of HBV-DNA was observed during two to four weeks of treatment.[33] However prolongation of treatment in twelve patients resulted in the death of five and others survived after liver transplantation.[34]

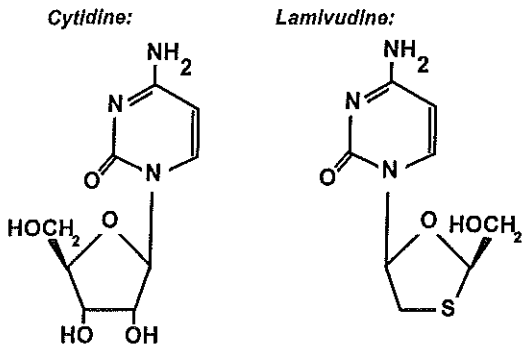


Figure 3. *Structure of the natural nucleotide cytidine and its analogue lamivudine.*

A number of new nucleoside analogues including *famciclovir* and *lamivudine* are in phase III clinical trials and others in phase I or II (lobucavir, adefovir). These agents all show promise by reducing the amount of complete viral particles in the serum and the expression of viral related proteins in the liver in the majority of patients. *In vitro* studies show that lamivudine is the most potent inhibitor of viral replication with cytotoxicity only at high dosages.[35] Therefore we decided to start a clinical evaluation of this drug which showed the largest therapeutic index (high efficacy with low toxicity).

Lamivudine

Lamivudine is the orally administered (-) enantiomer of the racemic mixture 2',3'-dideoxy-3'-thiacytidine (SddC, 3'TC, figure 3) and was discovered to have potent anti-HIV activity.[36] The (+)-form of the antiviral stereoisomer is susceptible to deamination by deoxycytidine deaminase and was found to be more toxic than the (-) enantiomer.[37,38] Anti-HBV activity takes place by direct inhibition of HBV-DNA polymerase and by termination of the pro-viral DNA chain during elongation. This in contrast to famciclovir that acts as inhibitor of initiation of the pro-viral DNA chain (figure 4). The potent and selective anti-HBV activity of lamivudine results from an efficient drug uptake, resistance to cytidine deaminase, a high affinity for deoxycytidine kinase, and high selectivity toward the polymerase, the target of nucleoside analogue toxicities.[39]

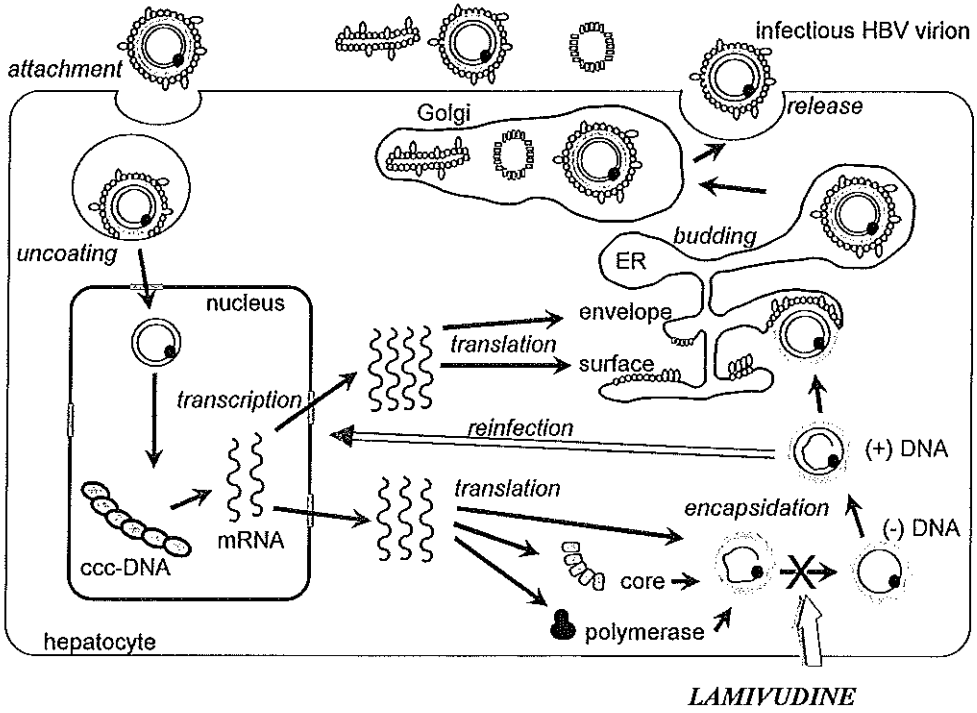


Figure 4. *Proposed life-cycle of HBV in hepatocytes. Lamivudine will terminate the prolongation of the proviral DNA chain, marked* X.

Objectives of the study are:

1. To assess the virological, biochemical and histological efficacy of lamivudine monotherapy in chronic hepatitis B patients.

2. To evaluate the pharmacokinetics, safety and efficacy of lamivudine and α-IFN combination therapy.

3. To evaluate the possible mitochondrial toxicity, as observed in fialuridine, during lamivudine therapy.

4. To describe the 'lamivudine withdrawal hepatitis' and the role of lamivudine influencing the outcome of the flare.

5. To evaluate the incidence, molecular background and clinical impact of lamivudine resistance.

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2

DYNAMICS OF HEPATITIS B VIRUS INFECTION *IN VIVO*

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Summary

Background/Aims: Information on the kinetics of the pretreatment steady-state of the hepatitis B virus (HBV) can be obtained from serial measurements of serum HBV-DNA concentrations following lamivudine ((-)enantiomer of 3'-thiacytidine)-induced perturbation of the balance between virus production and clearance.

Methods: In a placebo-controlled, dose-ranging trial lamivudine (5 to 600 mg per day) was administered for 4 weeks to 17 patients with chronic replicative hepatitis B. Serum HBV-DNA levels were quantified by standard liquid hybridization techniques. The time-dependent concentrations of serum HBV-DNA following lamivudine administration were subjected to iterative least-squares regression in order to obtain kinetic data on HBV life-time and viraemia.

Results: In patients with stable HBeAg positive chronic hepatitis B responding to lamivudine, HBV-DNA declined exponentially with a half-life of approximately 2-3 days. The minimum virus production and clearance per day in patients with chronic hepatitis B was calculated to be 6.09×10^{11} virions/day (range 0.26 to 21.06×10^{11} virions/day). Compared to the HBeAg levels before treatment, relative amounts of HBeAg were 1.00 ± 0.16 and 0.96 ± 0.20 at days 22 and 28 of treatment, respectively. Four weeks after termination of lamivudine treatment the relative amount of HBeAg was 1.04 ± 0.19.

Conclusion: The half-life of HBV in chronically infected patients is longer and *in vivo* turnover rates are higher compared to recently published data on the human immunodeficiency virus type 1 and the hepatitis C virus. The constant expression of HBeAg as observed in the present study during a 28-day lamivudine treatment period does not allow calculation of a definite decay rate for virus-producing cells. Our data, however, imply that the minimum half-life of infected cells may exceed 100 days.

Introduction

The hepatitis B virus (HBV) has a partially double stranded DNA genome and replicates via an RNA intermediate.[1,2] Measurement of serum HBV-DNA is an effective non-invasive method of assessing the replicative activity of HBV, since serum HBV-DNA represents virion DNA, and its presence correlates with replicating and infectious HBV.[3] Kinetic information on viral turnover can be obtained after the balance between virus production and clearance is disturbed.[4,5,6,7,8] Lamivudine, the (-)enantiomer of 3'-thiacytidine, is an oral 2',3'-dideoxy-nucleoside that interferes with the reverse-transcriptase activity of the hepatitis B virus polymerase and inhibits DNA synthesis by terminating the nascent proviral DNA chain. At concentrations sufficient to completely block HBV replication, the drug does not affect transcription of HBV-specific RNA.[9,10,11] In the present study, we administered lamivudine to patients who were chronically infected with hepatitis B virus. From serial measurements of viraemia in patients responding to lamivudine, we obtained kinetic information on the dynamics of HBV replication *in vivo*.

Patients and methods

In a placebo-controlled, dose-ranging trial, lamivudine was administered for 4 weeks to 17 patients with chronic replicative hepatitis B, in doses of 5 to 600 mg per day.[12] The diagnosis of chronic hepatitis B was based on elevated serum aminotransferase levels for at least 6 months, presence of HBeAg and histological examination. For inclusion into the study serum HBV-DNA had to exceed 10 pg/ml. All patients were anti-HCV, anti-HDV and anti-HIV-1/2 negative. Blood samples were obtained 4 weeks before and directly prior to initiation of treatment, and subsequently on days 3, 7, 14, 21, and 28. Further serum samples were taken 1, 2, 4, 6, and 8 weeks after termination of antiviral treatment. Serum was prepared under a laminar flow bench and frozen at -80°C. All patients consented to participate in the study, which was approved by the Ethics Committee for Medical Research in Rotterdam in accordance with the 1975 Declaration of Helsinki. Lamivudine doses of less than 20 mg had no effect on HBV-DNA levels, while a substantial decline in the amount of HBV-DNA was observed in 11 patients receiving 20 to 600 mg. For studies on the dynamics of HBV turnover, only the data of these 11 responders were taken into account. The mean age of these 11 patients was 31.9 years (range 18-60 years). Histological examination revealed chronic persistent hepatitis in 3/11 and chronic active hepatitis in 8/11 patients.

Serum HBV-DNA levels were quantified by standard liquid hybridization techniques (Genostics assay, Abbott Laboratories, North Chicago, Ill., USA). The lower detection limit of this assay is 2 pg/ml, i.e. 5.8×10^5 HBV genomes per ml serum.[13,14] Relative amounts of HBeAg were assessed by the micro particle ELISA IMx system (Abbott Laboratories, North Chicago, Ill., USA).

The time-dependent concentrations of serum HBV-DNA following lamivudine administration were subjected to iterative least-squares regression in order to obtain kinetic data on HBV life-time and viraemia. As rationalized below, the underlying three-compartment kinetic model[8] including HBV-releasing infected hepatocytes ([*A*]), serum HBV-DNA ([*B*]), as well as a fictitious degradation compartment ([*C*]) was reduced to a first-order problem. In the present case, it was found sufficient to determine the two parameters HBV-DNA concentration prior to lamivudine administration and the rate constant for HBV-DNA decline, B_0 and k_2, respectively. The fit protocol included a repeatedly initialized downhill simplex optimization.[15] Typically, less than 500 function evaluations were needed for convergence. The agreement between simulated and observed data was characterized in a least-squares sense by the normalized fit-error:

$s^2=\sum_i^n [(B_i^{sim}-B_i^{obs})/\varepsilon_i]^2$

where i runs over all n data points and ε_i is the uncertainty of a sample set to either 5% or to a minimum of 2 pg/ml (5.8×10^5 HBV genomes/ml) in HBV-DNA determinations. To account for the intrinsic non-linear properties of the model function, confidence boundaries for the parameters B_0 and k_2 were derived from the fractional increase in the sum of squares of residuals[16,17] computed according to:

$(s_{max} / s_{min})^2 = 1 + (p - 1) \cdot (n - p)^{-1} \cdot F_{\{p-1,\, n-p,\, \alpha\}}$

where n and p are the number of HBV-DNA samples and fit parameters, respectively, and F is the Fisher variance ratio. For the varying numbers of degrees of freedom, critical F-values for a two-tailed test of the F-distribution function were obtained from statistical tables.[18] The rejection probability was set to $\alpha = (31.7/2)\%$ associated with one standard deviation based on the assumption of a multi variate Gaussian distribution. The deviations in each of the optimum parameters required to increase the sum of squares of the residuals from s_{min}^2 to the threshold s_{min}^2 were iteratively determined using a modified secant algorithm.[19] The asymmetry of the s^2 isocontour on the error hyper-surface was tested with a bi-directional search using positive and negative deviations in each single parameter,[16] and for each value the larger deviation was given for the confidence interval. Fit significances were tested by analysis of variances (ANOVA). Other data are presented as mean ± S.D.

Minimum virus production and clearance per day was calculated from the initial slope according to $B_0 \times k_2$ multiplied by the extracellular fluid volume, which was estimated from the individual patient's body weight, and assuming that serum and extracellular fluid compartments are in equilibrium.

Results

Within the framework of a three-compartment kinetic model,[8] the hepatitis B virus is produced in infected hepatocytes (compartment [*A*]) and subsequently released into the blood (compartment [*B*]) at a rate constant k_1. Clearance of free virus from the blood, as assessed by total serum HBV-DNA, will occur at rate constant k_2 into a fictitious

degradation compartment ([*C*]). A small percentage of free virions will bind to and enter hepatocytes at a rate constant k_{-1}:

$$[A] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [B] \xrightarrow{k_2} [C]$$

However, virus uptake by hepatocytes previously uninfected (*de novo* infection) or infected (superinfection) cannot be discriminated, and transformation of non-infected into virus-producing hepatocytes cannot be measured *in vivo*.

Administration of lamivudine, which terminates the nascent proviral DNA chain, effectively inhibits hepatitis B virus replication (k_1=0). Since nucleoside analogues, including lamivudine, have no recognized direct effect on the immune system or nonspecific degradation processes,[20,21,22] degradation of HBV-DNA should not be affected, implying a non-vanishing rate for this process (k_2>0). Decline rates of antibody-complexed and uncomplexed virions may be different. Thus, k_2 must be interpreted as a compound rate of antigen-specific and non-specific processes. Rate constants k_{-1} and k_2 are numerically indistinguishable if k_1=0 leading in the limit to identity with a first-order exponential decay as one of the rates k_{-1} or k_2 approaches zero. Such a fit behavior was observed in most cases. Biologically, rate constant k_2 can be anticipated to exceed k_{-1} ($k_2 >> k_{-1}$), and therefore kinetic data are derived from a simple first-order kinetic for HBV-DNA degradation ($d[B]/dt = k_2[B]$).

During a 4-week pretreatment phase serum HBV-DNA levels ranged from 62 to 882 pg/ml (1.8×10^7 to 2.6×10^8 genome equivalents per ml) and varied little in the individual patients, indicating steady-state conditions. Following lamivudine treatment with at least 20 mg once daily, all patients showed a rapid decline in serum viraemia, which continued over the complete 28-day treatment period (figure 1A). The decline in serum HBV-DNA was exponential, as demonstrated by a straight-line fit to the data on a log plot and permitted the determination of a half-life of approximately 2-3 days. The calculated minimum daily production and clearance of HBV ranged from 0.26 to 21.06×10^{11} virions per day (table 1).

The rate of HBV elimination from serum after initiation of antiviral therapy was determined by the first-order decay described above. Results for viral clearance from serum yielded half-lifes of $t_{1/2} = \ln 2/k_2 = 3.02 \pm 3.20$ days. Virus half-lifes in patients treated with 20 mg lamivudine once daily were longer than in patients with doses of 100 to 600 mg once daily (4.99 ± 5.55 vs. 2.67 ± 2.48 days). This may reflect an incomplete inhibition of HBV replication in patients treated with only 20 mg lamivudine once daily. The individual results for all 11 investigated patients (including confidence intervals from *F*-statistics) are summarized in table 1. After termination of the 4-week lamivudine treatment period, serum HBV-DNA concentrations increased rapidly. The mean HBV-DNA concentration before initiation of treatment was 340 ± 293 pg/ml and after termination of treatment was 152 ± 133 pg/ml at week 1, 228 ± 154 pg/ml at week 2, 277 ± 266 pg/ml at week 4, 275 ± 241 pg/ml at week 6 and 298 ± 198 pg/ml at week 8 .

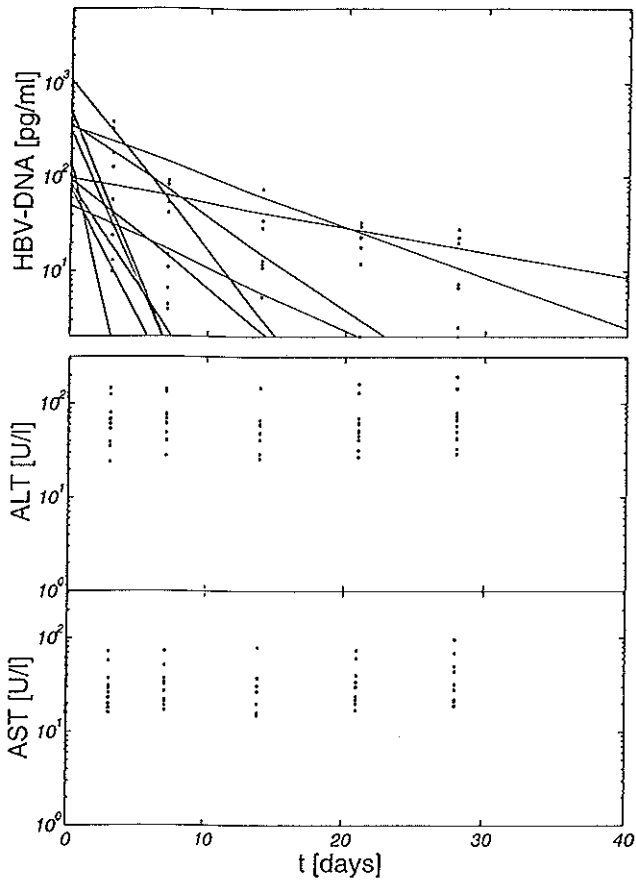

Figure 1. *Serum HBV-DNA and aminotransferase determinations in patients with chronic hepatitis B responding to lamivudine. All patients (n = 11) responding to lamivudine treatment are shown. Treatment with 20 to 600 mg lamivudine once daily was initiated at day 0 and subsequently continued for 4 weeks.* Upper panel: *Dots (•) represent the experimental HBV-DNA values as determined by liquid hybridization techniques. Lines (___) are calculated from the fit parameters given in table 1.* Lower panel: *Dots represent the experimental ALT and AST values determined by enzymatic standard methods. The normal ranges for ALT and AST are 5 - 30 and 5 - 30 IU/l, respectively. The lower edge of each panel indicates the analytical sensitivity.*

The core gene of HBV encodes for both core antigen (HBcAg) and a cleavage product, the e antigen (HBeAg), which is secreted by infected cells. HBeAg is generally a good marker of active HBV replication because the gene product is generated from the template that is

also used for replication. In patients treated with at least 20 mg lamivudine once daily, the amount of HBeAg showed no decline. Compared to the HBeAg levels before treatment, the relative amounts of HBeAg were 1.00 ± 0.16 and 0.96 ± 0.20 at days 22 and 28 of treatment, respectively. Four weeks after termination of lamivudine treatment the relative amount of HBeAg was 1.04 ± 0.19.

Hepatocyte damage and turnover can be estimated only by surrogate parameters such as aminotransferases, which are released due to direct virus-related cytopathic and/or immune-mediated processes. Alanine aminotransferase (ALT) is a cytosolic enzyme mainly present in the liver. Aspartate aminotransferase (AST) is a mitochondrial enzyme present in many organs, which is considered to be a more pronounced surrogate parameter of cell destruction. In none of the patients chronically infected with HBV was a decline in aminotransferases observed during treatment with lamivudine for 28 days (figure 1B).

Table 1. Summary data of HBV turnover during the pretreatment steady state. [a]

Patient	Lamivudine [mg / day]	HBV-DNA			
		B_0		$t_{1/2}$ ($\ln 2/k_2$) [days]	Production and clearance [10^{11} virions /day]
		[pg/ml] [b]	[10^7 / ml] [b]		
01	20	95.2 ± 62.9	2.76 ± 1.82	2.58 ± 2.31	0.86
02	20	98.0 ± 49.7	2.84 ± 1.44	11.34 ± 6.61	0.26
03	20	77.4 ± 49.3	2.25 ± 1.43	1.05 ± 0.95	1.75
04	100	50.3 ± 51.9	1.46 ± 1.51	4.49 ± 2.33	0.33
05	100	1096.7 ± 546.9	31.80 ± 15.86	1.65 ± 0.48	18.17
06	100	150.5 ± 63.2	4.37 ± 1.83	0.47 ± 0.09	8.68
07	300	368.7 ± 320.8	10.69 ± 9.30	3.02 ± 1.32	3.48
08	300	348.8 ± 211.0	10.12 ± 6.12	5.58 ± 2.15	2.15
09	300	317.7 ± 174.3	9.21 ± 5.06	0.90 ± 0.39	8.49
10	600	84.8 ± 58.8	2.46 ± 1.71	1.35 ± 0.58	1.73
11	600	510.6 ± 188.2	14.81 ± 5.46	0.84 ± 0.20	21.06

[a] For definition of kinetic parameters see Methods.

[b] 1 pg HBV-DNA ≈ 2.9×10^5 genome equivalents.[12,13]

Discussion

The hepatitis B virus is a small DNA virus with a circular double-stranded, partly single-stranded genome. Four mRNA transcripts of known function have been identified. The longest (3.5 kB) is the template for the expression of pre-core/core and polymerase

proteins. The hepatitis B *e* antigen (HBeAg) is a cleavage product of the core antigen (HBcAg) and is secreted from infected cells. Since the 3.5 kB genomic RNA also serves as a template for reverse transcription, serum HBeAg generally represents a good marker of active HBV replication because the pre-core/core gene product is generated from the template that is also used for replication. Minus strand DNA synthesis initiates at the 3'-short direct repeats (DR)-1 with the terminal protein of the polymerase as a primer and, as synthesis progresses, the RNA template is simultaneously degraded by RNase H. Plus strand DNA synthesis initiates at the 3' end of the short direct repeats (DR)-2 and synthesis continues until the terminal protein at the 5' end of the minus strand is passed, producing an open circular DNA molecule.[1,2] Lamivudine inhibits DNA synthesis by terminating the nascent proviral DNA chain and apparently does not interfere with transcription from the covalently closed circular HBV-DNA or with translation.[9,10,11]

In a recent study the half-life of the hepatitis B virus *in vivo* has been estimated to be around 1.0 day.[23] In the same study the daily HBV production was calculated to be 2.2×10^{11} particles. This rate is similar to the minimum virus production and clearance per day in the patients of the present study (6.1×10^{11} particles). However, the HBV half-life *in vivo* as determined in the present study is significantly longer (2-3 days) than in the study of Nowak et al.[23] This discrepancy is not readily explained, but may be related to dose-dependent differences in drug efficiency.

Despite a profound effect on virus replication, we observed no effect of lamivudine on HBeAg expression during the 4-week treatment period. The constant expression of HBeAg during the 4-week treatment period with lamivudine was confirmed in three independent assays (radioimmunoassay, Abbott IMx system, and Kodak Amerlite) (data not shown). In a previous study, 4 of 32 patients treated with 25-300 mg lamivudine for 12 weeks showed a sustained clearance of HBeAg.[20] In these four patients the mean HBV-DNA levels were significantly lower at baseline. From a clinical point of view it can be speculated that prolonged treatment periods with complete suppression of HBV replication may be beneficial. Continuous e.g. immune-mediated elimination of HBV-infected hepatocytes and hepatocellular regeneration without subsequent viral infection should lead to increased rates of HBeAg clearance.

Aminotransferases are surrogate parameters of hepatocyte damage and turnover. It has been suggested that the operating mechanism of hepatocellular damage in patients chronically infected with the hepatitis B virus is immune-mediated.[2,24] Therefore, the continuous expression of viral proteins and the insignificant change of aminotransferase levels during a 4-week treatment with lamivudine are in agreement with the proposed model. Similarly, in the study by Dienstag et al. no effect of lamivudine on ALT levels was observed within the initial eight weeks of treatment.[20]

In the study of Nowak et al., an initial decay of HBeAg in patients taking lamivudine was considered to reflect the decay of infected hepatocytes. The half-life of infected hepatocytes was calculated to range between 10 and 100 days with an average of $t_{1/2}$ = 13 days.[23] The constant expression of HBeAg as observed in the present study during a 28-day lamivudine treatment period does not allow the calculation of a definite decay rate for

virus-producing cells. Our data, however, imply that the minimum half-life of infected cells may exceed 100 days. In patients with an infected cell half-life of more than 100 days, one year of treatment would not reduce the number of infected cells to less than 10% of its initial value.

In the present study, kinetic analysis of hepatitis B virus turnover *in vivo* revealed a half-life of approximately 2-3 days. In patients with chronic hepatitis B, the minimum virus production and clearance per day was calculated to be $6.09\pm7.33\times10^{11}$ virions/day. A recent comparison of methods for detection of HBV-DNA using the Eurohep HBV-DNA standards revealed that the results obtained by the Abbott liquid hybridization assay were, on average, 19 times lower than calculated.[25] Thus, the minimum HBV production and clearance per day may be at least one order of magnitude higher.

Viral dynamics have recently also been investigated in patients infected with HIV-1[5,6,7] and in patients chronically infected with the HCV.[8] HIV-1 production *in vivo* was estimated to yield around 10^9 copies per day with a mean virus half-life of 1.2 to 2 days.[5,6,7] Similar data were reported for HCV with an *in vivo* half-life of 2 days and an estimated minimum daily virus production of 6.7×10^{10} virions per day.[8] Compared to these two RNA viruses, the hepatitis B virus replication *in vivo* is even more productive. Similar to HIV-1 and HCV, the initial viral load before initiation of treatment is independent of viral decay slopes (clearance rate constants), indicating that the serum HBV-DNA concentration is largely a function of viral production.

HBV replicates by means of an RNA intermediate. The process is catalyzed by a translation product of the polymerase open-reading frame that has reverse transcriptase activity. The enzyme is found in association with the virion and achieves a high rate of nucleotide misincorporation during transcription because such enzymes lack proofreading activity.[24,26] Due to the compact organization of the HBV genome only few of the mutations that occur during the normal replication cycle permit the entry of a new virus to the pool for natural selection. Nevertheless, patients with chronic hepatitis B have been shown to have viruses with different sequences cocirculating and some regions of the genome are poorly conserved between different isolates. The high HBV turnover rates *in vivo* evidently explain the rapid generation of viral diversity. Although the single-cycle mutation rate for HBV is not known, precedent set by other viruses[27,28,29,30] suggests that it may possibly lie somewhere between 10^{-5} and 10^{-4} per base per cycle, with considerable variation from base to base depending on sequence context.[31,32] Therefore as for HIV-1[4,6] it is conceivable that, on average, every mutation at every position in the HBV genome occurs numerous times each day in an infected patient.

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3.1

LAMIVUDINE THERAPY FOR CHRONIC HEPATITIS B:

A SIX MONTH RANDOMIZED DOSE-RANGING STUDY

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Summary

Background & Aims: Lamivudine inhibits hepatitis B virus replication. This study investigated 6 months of lamivudine treatment at three doses.
Methods: Fifty-one patients (43% white, 49% Asian) with chronic hepatitis B were randomly assigned to receive 25, 100, or 300 mg of lamivudine orally once daily for 24 weeks with 24 weeks' follow-up.
Results: Serum hepatitis B DNA by liquid hybridization decreased in all patients and was undetectable at the end of the treatment in 7 of 12 (58%, 25 mg), 13 of 14 (93%, 100 mg), and 14 of 16 (88%, 300 mg) patients. Of the 36 patients with abnormal alanine aminotransferase (ALT) levels at baseline, 7 of 11 (64%, 25 mg), 5 of 11 (45%, 100 mg), and 5 of 14 (36%, 300 mg) normalized ALT at treatment completion. Quantitative decreases hepatitis B e antigen and hepatitis B surface antigen concentrations were observed at all doses. In most patients, markers of replication returned after treatment. Two patients (4%) were anti-HBe positive at the end of follow-up. Lamivudine was well tolerated. The incidence of adverse events was similar across all dose groups. However, 2 patients developed temporary hepatic decompensation after increase in transaminase levels after treatment. *Conclusions:* Lamivudine was well tolerated and induced sustained suppression of hepatitis B replication during treatment in all patients at all doses. These data support investigation of longer treatment durations of 100 mg once daily.

Introduction

Hepatitis B virus (HBV) infection remains a considerable health problem worldwide and a significant cause of liver disease and liver cancer in humans.[1] The objective of treatment of chronic hepatitis B is to suppress HBV replication to prevent progression to cirrhosis, hepatic decompensation, and hepatocellular carcinoma.[2]

Alpha-interferon (α-IFN) is currently the most effective and the only approved treatment for chronic hepatitis B.[3,4] However, the relative rate of hepatitis B e antigen (HBeAg) disappearance induced by α-IFN in unselected patients ranges from 20% to 30%.[5]

Lamivudine, the negative enantiomer of 2'3'-dideoxy-3'-thiacytidine, is an oral nucleoside analogue that inhibits viral DNA synthesis by terminating the nascent pro-viral DNA chain. Unlike some dideoxynucleosides, lamivudine does not inhibit mitochondrial DNA synthesis or the proliferation of cells at concentrations that block the synthesis of viral DNA[6] and does not include mitochondrial toxicity *in vitro*.[7,8] The compound has been well tolerated in long-term studies of patients with human immunodeficiency virus (HIV) infections at doses of 600 mg/day for more than 1 year.[9] *In vitro* and *in vivo* models of HBV infection showed that lamivudine potently inhibited HBV replication.[6,10] In previous phase I/II trials, lamivudine given for 1-3 months resulted in a marked but temporary reduction in serum HBV-DNA.[11,12]

The objectives of this study were to assess the safety and efficacy of long-term lamivudine therapy in patients with chronic hepatitis B and to establish the optimal dose.

Patients and Methods

Criteria for selection of patients

Patients with biopsy-proven chronic hepatitis B who had previously participated in the 28-day, dose-ranging study of lamivudine[11] were eligible for inclusion if they were serum HBV-DNA positive, had alanine aminotransferase (ALT) concentrations of <300 IU/l, and were serum positive for both hepatitis B surface antigen (HBsAg) and HBeAg for 6 months preceding screening and at the time of screening.

Patients were ineligible for inclusion if they had evidence of the following: decompensated liver disease (bilirubin level more than two times upper limit of normal (ULN), prothrombin time prolonged by >2 seconds, albumin level <30 g/l, or a history of ascites, variceal haemorrhage, or hepatic encephalopathy); abnormal renal function (serum creatinine >130 μmol/l); haemoglobin concentration <6.3 mmol/l, white cell count <3 x 10^9/l, or platelet count <50 x 10^9/l; coinfection with hepatitis C, hepatitis delta, or HIV; a serious, confounding medical illness; or treatment with antiviral, immunomodulatory, or corticosteroid therapy since completing the previous lamivudine study. Women who were pregnant, lactating, or of childbearing potential and not using an effective method of contraception were also excluded from this study.

Table 1. *Characteristics of study participants in the three treatment groups at baseline*

		Lamivudine		
Treatment group (no. randomized)		25 mg (n = 16)	100 mg (n = 16)	300 mg (n = 19)
male/female		10/6	13/3	13/6
mean age (yr ± SEM)		35 ± 4	36 ± 4	37 ± 3
Race	Asian	9	7	9
	Black	0	1	1
	White	6	7	9
	Other	1	1	0
ALT (*IU/l*, mean ± SEM)*		69 ± 9	75 ± 13	91 ± 20
HBV-DNA (*pg/ml*, mean ± SEM)		147 ± 41	123 ± 25	132 ± 20
Previous interferon use		6 (37%)	5 (31%)	8 (42%)
Cirrhosis**		5	1	4

* The ULN at the four centres ranged from 30 to 55 IU/l.
** At time of randomization to preceding lamivudine study.

Informed consent and ethics
The study was conducted according to the Declaration of Helsinki and Good Clinical Practice. The protocol was approved by the local medical ethics committees of the four centres. All patients gave their written informed consent.

Randomization
The patients were randomly assigned to receive 25, 100, or 300 mg of lamivudine (Glaxo Wellcome Research and Development Ltd., Greenford, England) orally once daily for 24 weeks, with a further 24-week follow-up after treatment. The 25- and 100-mg doses were double-blind, but as a result of the three capsule once daily regimen, the 300-mg dose was not double-blind.
Treatment randomization was stratified according to whether the patient had previously received α-IFN.

Clinical evaluation
Safety was monitored by clinical and laboratory evaluations (including the determination of anion gap and lactate) at baseline, 2 weeks and 4 weeks after treatment start, and every 4 weeks thereafter until the end of follow-up. Compliance was assessed by questioning the

patient and by comparing the number of tablets returned with the number of days on treatment.

Viral parameters were assayed centrally by Covance (Harrogate, England) to Good Laboratory Practice standards. Serum HBV-DNA was assessed quantitatively by the Abbott Genostics liquid hybridization assay (Abbott Laboratories, Abbott Park, IL). A validation of the performance of this assay conducted by Covance determined the lower limit of quantification to be 3 pg/ml. HBeAg and anti-HBe were assessed qualitatively by Kodak Amerlite, Kodak Clinical Diagnostics, Amersham (Buckinghamshire, England). HBeAg was also assessed semiquantitatively using a 12-point standard curve calibrated against the Paul Ehrlich Institute standard (Paul Ehrlich Institute, Federal Agency for Sera and Vaccines, Langen, Germany). HBsAg was assessed quantitatively by Kodak Amerlite, and anti-HBs was assessed quantitatively by Abbott IMX (Abbott Laboratories) using the standards provided with the kits.

Statistical analysis

Analyses were performed on a per protocol basis for the proportion of patients serum HBV-DNA negative at the end of treatment. Analyses were performed on an intention-to-treat population, defined as all patients with confirmed chronic hepatitis B infection who received at least one dose of the study medication, for all other parameters. Two-tailed *p* values and *95%* confidence intervals were used.

Time until suppression of HBV-DNA was calculated as the first visit when the patient's serum HBV-DNA concentration decreased below the limit of quantification. Times to suppression were compared pairwise between treatment groups by the log rank test, and stratified by the investigator.

For the purposes of the analysis, all HBsAg concentrations >90,000 IU/ml were set to 90,000 IU/ml, and all HBV-DNA values <3.0 pg/ml were set to 1.5 pg/ml.

Results

Enrollment and baseline characteristics of the patients

Fifty-one patients were enrolled, and their baseline characteristics are shown in table 1. At baseline, the mean (± SEM) serum HBV-DNA of all patients randomized was 134 ± 16 pg/ml and the mean ALT was 79 ± 9 IU/l. Nineteen patients (37%) received at least one previous course of α-IFN, 15 patients (29%) had normal transaminase levels, 25 patients (49%) were Asian, and 22 (43%) were white. Ten patients (20%) were previously reported as having cirrhosis. There were no clinically relevant differences in these parameters between the 25-, 100-, and 300-mg dose groups. However, the mean ALT and serum HBV-DNA concentrations at baseline for Asian patients were 56 ± 9 IU/l and 104 ± 19 pg/ml, respectively, compared with 110 ± 16 IU/l and 161 ± 30 pg/ml for white patients.

Efficacy

Lamivudine therapy induced rapid decreases in serum HBV-DNA concentration in all patients at all doses (figure 1), irrespective of the baseline HBV-DNA concentration. Undetectable serum HBV-DNA was attained by week 2 in 6 of 16 (38%), 11 of 16 (69%), and 11 of 19 (58%) of the patients in the 25-, 100-, and 300-mg groups, respectively. The time until undetectable serum HBV-DNA was significantly shorter in the 100- and 300-mg groups than in the 25-mg group (p = 0.016 and 0.027, respectively).

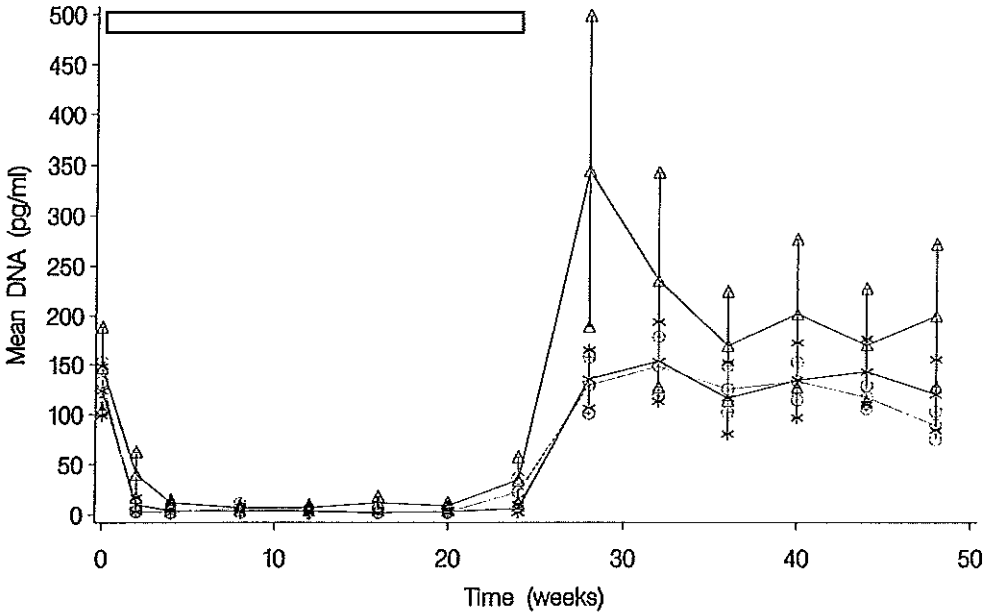

Figure 1. *A comparison of mean serum HBV-DNA concentration by dose (pg/ml ± SEM) in all patients during and after treatment. □ Treatment period. Lamivudine at 25 mg (△), 100 mg (*), and 300 mg (○).*

Serum HBV-DNA was undetectable at the end of treatment in 34 of 42 (81%) patients, and 7 of 12 (58%), 13 of 14 (93%), and 14 of 16 (88%) patients in the 25-, 100-, and 300-mg cohorts, respectively. Serum HBV-DNA decreased precipitously but failed to become negative in 7 patients, 5 receiving 25 mg and 1 in each of the 100-mg and 300-mg cohorts. A further patient in the 300-mg cohort was serum HBV-DNA positive at the week 24 assessment only (8 pg/ml), despite being negative throughout treatment and for 12 subsequent weeks of the posttreatment period.

This per-protocol analysis includes 3 patients (1 from each treatment group) who were borderline negative for HBV-DNA (≤4 pg/ml). Four patients known to have completed their therapy before their end of treatment assessment (and who were HBV-DNA

negative), becoming strongly positive for HBV-DNA by the time of that visit, were excluded from the analysis (1 in each of the 25-mg and 100-mg cohorts, and 2 receiving 300 mg). Also excluded from the analysis were the 5 patients who withdrew from the study during the treatment period, only 2 of whom were serum HBV-DNA positive at the time of withdrawal (1 in each of the 25-mg and 300 mg cohorts).

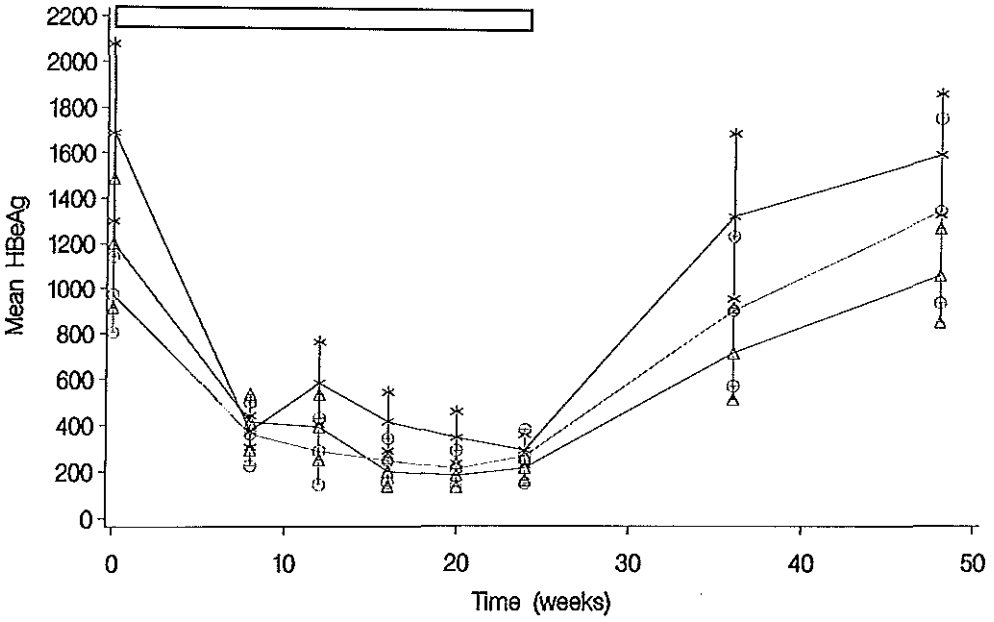

Figure 2. *A comparison of mean serum HBeAg concentration by dose (Paul Ehrlich units per ml ± SEM) in all patients during and after treatment. □ Treatment period. Lamivudine at 25 mg (△), 100 mg (*), and 300 mg (○).*

Mean serum HBeAg concentration (± SEM) decreased across all treatment groups from 1268 ± 166 Paul Ehrlich units per ml at baseline to 256 ± 53 Paul Ehrlich units per ml at the end of treatment. The data for each treatment group are presented in figure 2. The response of HBeAg was not dose dependent with a median percent decrease at week 24 of 78%, 82% and 79% in the 25-, 100-, and 300-mg cohorts, respectively. Similar trends were observed in HBsAg concentration, which decreased from a mean (± SEM) of 41,275 ± 4497 IU/ml at baseline to 19,491 ± 3427 IU/ml at the end of treatment. No difference was seen in response between Asian and white patients.

ALT concentration decreased across all dose groups from a mean (± SEM) of 79 ± 9 IU/l at baseline to 39 ± 3 IU/l at the end of treatment. Of the 36 patients with abnormal ALT at baseline, 17 (47%) had normalized ALT by the end of treatment: 7 of 11 (64%), 5 of 11 (45%), and 5 of 14 (36%) in the 25-, 100-, and 300 mg cohorts, respectively. Eleven

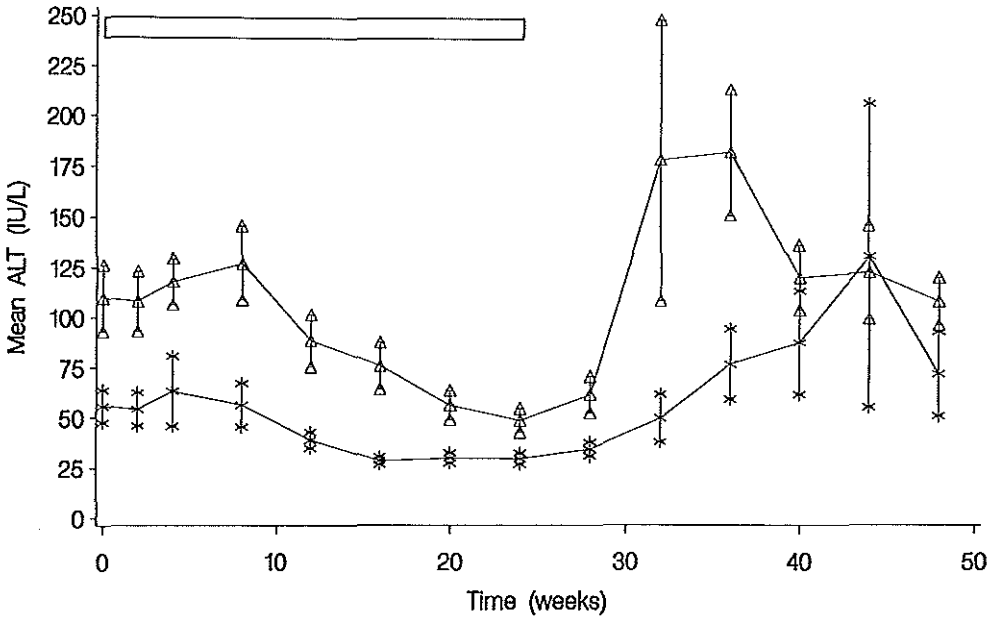

Figure 3. *A comparison of mean ALT concentration (IU/l ± SEM) in white (△) and Asian (*) patients during and after treatment. □ Treatment period.*

patients experienced at least a twofold increase of ALT during the treatment period: 2 of 16 patients (13%) in the 25-mg, 4 of 16 (25%) in the 100-mg, and 5 of 19 (26%) in the 300-mg group. For all patients, the mean time to onset of the ALT elevation was 8 weeks. The mean ALTs ± SEM for both white and Asian patients are given in figure 3. Seven of the 11 patients with at least a twofold increase of ALT during the treatment period were white, and the maximum mean ALT of 181 ± 31 IU/l (median, 150 IU/l; range, 16-534 IU/l) for this cohort occurred 12 weeks after treatment completion. In contrast, the maximum mean ALT for Asian patients of 130 ± 75 IU/l (median, 39 IU/l; range, 16-1460 IU/l) occurred 20 weeks after treatment completion.

After the completion of treatment, serum HBV-DNA became rapidly positive. Within 1 month of completing treatment, the majority of the patients had returned to pretreatment concentrations of serum HBV-DNA with a more gradual return in ALT, HBeAg, and HBsAg concentrations.

Four patients (8%) seroconverted to anti-HBe during the study, 2 during the treatment period, and 2 after treatment. No clear predisposing factors were observed in the patients who seroconverted: 3 patients had received prior interferon treatment; 2 patients were Asian and 2 were white; 2 patients were receiving the 100-mg dose, whereas the remaining 2 patients were receiving 25 and 300 mg, respectively; mean baseline serum HBV-DNA was 73 ± 36 pg/ml, and mean baseline ALT was 70 ± 9 IU/l. At the conclusion of the

follow-up, 2 patients remained anti-HBe positive and 1 patient had reverted to HBeAg and HBV-DNA positivity. The remaining patient had no HBV serology data available.

Safety

In total, 11 patients withdrew over the course of the study. Five patients withdrew during the treatment period: 2 patients because of adverse events (recurrent nausea and headache); 2 patients stopped treatment for personal reasons; and 1 failed to return.

Table 2. *Adverse events during lamivudine treatment.*

	Lamivudine			
Treatment group (no. randomized)	25 mg (n = 16)	100 mg (n = 16)	300 mg (n = 19)	Total (n = 51)
Patients experiencing an adverse event*	5 (31%)	4 (25%)	5 (26%)	14 (27%)
Symptoms				
Nausea and vomiting	2	0	2	4 (8%)
Fatigue	0	1	2	3 (6%)
Abdominal discomfort and pain	1	0	1	2 (4%)
Skin rashes	1	1	0	2 (4%)
Diarrhea	1	1	0	2 (4%)
Dizziness	0	0	1	1 (2%)
Hypoglycemia	0	0	1	1 (2%)
Headache	0	1	0	1 (2%)
Dyspepsia	0	1	0	1 (2%)
Vertigo	0	1	0	1 (2%)
Constipation	0	1	0	1 (2%)
Acne and folliculitis	0	1	0	1 (2%)
Eczema	0	1	0	1 (2%)
Muscle pain	1	0	0	1 (2%)
Pigmentary skin disorders	1	0	0	1 (2%)
Laboratory abnormalities				
Amylase > 2x ULN	1	0	0	1 (2%)
CPK > 5x ULN	0	0	2	2 (4%)

* Considered by the investigator to be at least possibly related to lamivudine treatment.

Adverse events occurring during the treatment period and considered by the investigators to be at least possibly related to lamivudine are listed in table 2. Two patients experienced serious adverse events considered by the investigator to be related to lamivudine (hypoglycaemia in a diabetic patient and elevation of transaminases to 2.5 times the baseline level), but both patients continued lamivudine treatment, and the events resolved.

All other events were mild and also resolved on continued treatment. For all patients, the most common adverse events considered to be related to therapy were gastrointestinal (i.e., nausea, diarrhea) or fatigue. The incidence of all adverse events was similar across all treatment groups. There were no significant changes in laboratory parameters related to potential mitochondrial toxicity (creatinine phosphokinase, amylase, lipase or lactate) during or after the treatment period in any of the three treatment groups.

During the posttreatment period, 8 patients (16%) experienced elevated ALT of more than three times the baseline value. These elevations followed return of measurable serum HBV-DNA and were considered to represent a reactivation of hepatitis B after the period of suppression of replication. All of these cases remained asymptomatic and resolved spontaneously, with the exception of 2 patients whose increases in transaminase levels were temporarily associated with elevations in bilirubin and prolongation of prothrombin time. One patient received a short course of prednisone.[13] Additional monitoring after the conclusion of this study showed that both patients had seroconverted to anti-HBe positive status. During the 1 -year study period, no signs of disease progression were observed.

Discussion

This study confirmed that lamivudine suppresses HBV replication in patients with chronic hepatitis B and is well tolerated. A reduction in serum HBV-DNA concentration was seen in all patients at all doses, with the 100- and 300-mg doses superior to the 25-mg dose in reducing HBV-DNA. In the parameters of efficacy used in this study, the 300-mg dose provided no advantage in antiviral effect (loss of HBV-DNA) over the 100-mg dose.

Of patients receiving the 100- and 300-mg doses, 27 of 35 patients (77%) were HBV-DNA negative at the week 24 assessment. This is in contrast to the 22 of 22 patients (100%) who were negative after 3 months for the same doses reported by Dienstag et al.[12] and may be explained by the higher number of withdrawals in this study, variation near the cutoff of the HBV-DNA liquid hybridization assay, and completion of therapy before the week 24 assessment. Taking these factors into account, only 3 of 35 (9%; 1 patient receiving 100 mg and 2 receiving 300 mg) did not experience total suppression of serum HBV-DNA throughout the treatment period.

Lamivudine also reduced the serum concentration of HBeAg and HBsAg by approximately 80% in the study population, an effect that occurred independently of the development of anti-HBe or anti-HBs. A trend to normalization of ALT was also observed and is suggestive of a concurrent reduction in liver inflammation. This is further supported by data showing improved histology during treatment[14] and with previous reports of a correlation in humans between progressive liver disease and the presence of serum markers for ongoing HBV replication.[15]

Unlike α-IFN, the response to treatment of lamivudine was not dependent on pretreatment characteristics. This study included patients who would normally be considered poor responders to α-IFN; at baseline, 29% of the population had normal ALT levels, 55% had

HBV-DNA concentrations above 100 pg/ml, 49% were of Asian origin, and 37% were nonresponders to a previous course of α-IFN. However, a sustained response to α-IFN is generally achieved through seroconversion from HBeAg to anti-HBe. For the majority of patients, serum HBV-DNA returned rapidly after treatment, with only 2 patients (4%) confirmed anti-HBe positive at the end of the follow-up period. It is unlikely that the large cohort of Asian patients reduced the overall rate of seroconversion observed; the small number of seroconversions in this study occurred in both Asian and white patients.

Chronic HBV infection is sustained by multiple copies of covalently closed circular DNA (cccDNA) in the nucleus, which act as templates for replication.[16] The pool of cccDNA is maintained by an intracellular conversion pathway to ensure that a stable number of copies of cccDNA exist in equilibrium.[17] Inhibition of HBV replication by lamivudine may result in a decline in the total HBV load through two mechanisms: a combination of diminished production of viable virus (permitting new cells to remain uninfected) with hepatocyte turnover, resulting in a decrease in the number of infected cells; and depletion of the number of copies of cccDNA within the infected hepatocyte through inhibition of production of replicative intermediates. However, without host immune response to HBV, the potential for infection remains as long as cccDNA persists.

Despite the potent suppression of replication observed in this study, sustained response to therapy was rare. Considerably longer-term treatment regimens may be required to induce a sustained response. Further studies to assess lamivudine alone and in combination with interferon on the histological evolution of the disease are ongoing.

Lamivudine was well tolerated during the study period. The overall incidence of adverse events was low, and both the type and severity of events reported did not increase with increasing dose. Some patients showed a marked increase in serum HBV-DNA after treatment with a subsequent elevation of ALT 8-20 weeks after treatment. In most patients, the acute hepatitis-like events were asymptomatic and can be related to the return of HBV replication after treatment cessation; the 2 patients with increases in ALT levels after treatment and associated temporary hepatic decompensation both subsequently seroconverted from HBeAg to anti-HBe after the conclusion of this study. However, careful monitoring remains indicated if lamivudine therapy is withdrawn in patients with marginally compensated or decompensated liver disease, because severe reactivation of HBV replication may induce decompensation.[13]

In summary, lamivudine potently suppresses hepatitis B replication in chronic hepatitis B patients irrespectively of race and is very well tolerated. Data from this study suggest that a decline in inflammatory activity accompanies treatment and that long-term treatment durations of 100 mg once daily and/or combination therapy are required.

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3.2

QUANTITATIVE HBV-DNA ASSESSMENT BY THE LIMITING DILUTION POLYMERASE CHAIN REACTION IN CHRONIC HEPATITIS B PATIENTS DURING A 24-WEEK COURSE OF LAMIVUDINE

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Summary

Lamivudine, a novel cytosine analogue exhibits potent antiviral activity against hepatitis B virus *in vitro* and *in vivo*. The standard HBV-DNA hybridization assay used in phase II clinical studies has a low sensitivity, the detection limit of HBV-DNA levels is approximately 10^7 genome equivalents per ml (gen.eq./ml). We used the a semi-quantitative PCR assay (detection limit ~10^3 gen.eq./ml) to determine HBV-DNA levels during a 24 weeks lamivudine study in 51 stable chronic hepatitis B patients (HBsAg and HBeAg positive). Patients were randomly allocated to receive oral doses of 25, 100 or 300 mg of lamivudine once daily.

The median serum concentration of HBV-DNA fell from 10^8 to 10^4 gen.eq./ml, a four log median reduction. A trend towards more profound suppression of viral replication with increasing dose of lamivudine was observed. After 12 weeks of therapy 12% of patients had undetectable HBV-DNA in the PCR assay; this increased to 26% after 24 weeks, while an additional 20% dropped to the level of detection of the assay.

We conclude that a 24-week course of lamivudine decreases serum HBV-DNA to the level of PCR detection in 46% of patients. Such additional viral suppressive activity with higher doses and more protracted lamivudine may be of clinical utility prior to liver transplantation. Further studies are needed to define the degree of virus suppression required in clinical practice, and methods to increase the efficacy of virus suppression.

Introduction

Lamivudine, the (-)enantiomer of 2'3'-dideoxy-3'-thiacytidine, was found to be a non-toxic potent inhibitor of hepatitis B virus (HBV) replication.[1,2] In patients treated at doses of at least 100 mg, lamivudine induced reduction in HBV-DNA levels to below the detection limit of the liquid phase hybridization assay (Genostics; Abbott Laboratories, Chicago, Ill, USA) in the majority of patients.[3,4,5]
The limit of detection of the Abbott liquid hybridization assay used for quantitative measurement of HBV-DNA levels in serum is approximately 10^7 genome equivalents per ml (gen.eq./ml), when the test is calibrated on the well defined Eurohep HBV-DNA standard.[6] The semiquantitative polymerase chain (PCR) method expands the scale of measurement to a detection limit of approximately 10^2-10^3 gen.eq./ml.[7] In view of the scientific and clinical interest in information on HBV clearance from blood during lamivudine therapy, we measured HBV-DNA by the semiquantitative PCR assay during a 24 weeks course of lamivudine in patients with chronic hepatitis B.

Patients and methods

Study design
In a randomized single-blind (patient) study, 51 stable chronic hepatitis B patients (HBsAg (+) and HBeAg(+) for at least 6 months) received oral doses of 25, 100 or 300 mg lamivudine once daily for 24 weeks. Follow-up after treatment lasted 24 weeks. Patients selected for the study were to have serum HBV-DNA levels exceeding 10 pg/ml (Genostics, Abbott) and ALT below 300 IU/l. Results of this study have been reported elsewhere.[5]

Analyzed sera
At the start of the study and the visits at 12 and 24 weeks serum was aliquoted and frozen immediately at -20°C. Serum HBV-DNA was assessed quantitatively by liquid hybridization (Genostics, Abbott) with the lower limit of quantification, according to the manufacturer's standard, defined to be 3 pg/ml. Per protocol analysis excluded one patient who was withdrawn from the study at baseline due to negative HBV-DNA result by hybridization assay, one patient was withdrawn at week 12 and 7 patients at week 24. Of these 5 attended the clinic at least three days behind the calculated week 24 visit, therefore their trial medication was scheduled to be completed at least three days before the week 24 sample was taken. In 40 patients with HBV-DNA levels < 10 pg/ml at week 24, PCR assays were performed in duplo on sera obtained at week 0, 12 and 24 of treatment (108 samples). In the remaining 11 patients HBV-DNA levels were calculated from the results of the liquid hybridization assay (27 samples). According to Kuhns et al[8] 103 pg/ml (Genostics, Abbott) is equal to 3 x 10^7 gen.eq./ml, subsequently we obtained evidence that

the Abbott assay may underestimate the actual number of HBV genomes.[9] In this study PCR results in 46 samples (tested in duplo) that were positive in the hybridization assay result, allowed the calculation of a conversion factor. We observed that 1 pg (Genostics, Abbott) is equal to 5 x 10^5 gen.eq./ml. In case the results of the liquid hybridization assay was negative and no serum was left for PCR analysis (6 samples), the HBV-DNA level result was taken as 10^6 gen.eq./ml. Negative PCR results were taken as 10^1 gen.eq./ml. All samples were assayed in duplicate, the two values of the semi-quantitative PCR exhibited a maximum discrepancy of one log and were both used for analysis.

Table 1. *Characteristics of study participants according to dose of lamivudine.*

Dose group	25 mg	100 mg	300 mg
Total randomized (n)	16	16	19
Male/female	10/6	13/3	13/6
Mean age in years(range)	35 (18-65)	36 (17-66)	37 (18-62)
Race Asian	9	7	9
Caucasian/white	6	7	9
Other	1	2	1
Median ALT IU/l (range)	54 (15-130)	68 (20-184)	54 (23-352)
Median AST IU/l (range)	44 (23-89)	36 (20-135)	39 (18 -255)
Median HBV-DNA pg/ml* (range)	99 (4 -498)	100 (<3 -380)	117 (21-391)

*(Genostics, Abbott)

Polymerase chain reaction and quantitation

HBV-DNA was isolated from serum essentially as described by Boom.[10] Briefly, 100 µl serum was denatured in 800 µl lysis buffer 1 (120 g guanidinium-iso-thiocyanate, 100 ml 0.1 M Tris-HCl (pH 6.4), 22 ml 0.2 M EDTA (pH 8), 2.5 ml Triton X-100) and 40 µl celite (0.2 g/ml) for 10 min at room temperature. After washing twice with lysis buffer 2 (120 g guanidinium-iso-thiocyanate plus 100 ml 0.1 M Tris-HCl, pH 6.4), twice with 80% ethanol and once with acetone, respectively, the celite pellet was dried briefly in a vacuum exsiccator. DNA was released by incubating for 10 min in 100 µl 10 mM Tris-HCl (pH 8) at 56 °C.

Samples were diluted ten-fold in 10 mM Tris-HCl, pH 8, containing 10 µg/ml poly A as a carrier. Dilutions up to 1:100,000 were made. Each sample was isolated twice on separate days. As reference, the Eurohep HBV-DNA standard containing 4.2 .10^9 gen.eq./ml for genotype adw and 3.8. 10^9 for genotype ayw was included in each dilution series.[11] Ten µl were used in a PCR assay with primers directed against the pre S1/S2 region of HBV (primer 1, nucleotide 3044-3067: 5'gtg.gag.ccc.tca.ggc.tca.gg, primer 2, nucleotide 169-188: 5'ggt.cct.agg.aat.cct.gat.gt). The PCR assay was performed in a Biomed 60 thermocycler (Biomed, Theres, Germany) starting with preheating for 4 min at 94°C, followed by 40 cycles for 1 min at 94°C, 1 min at 52°C and 1 min at 74°C with 1 U Taq DNA

polymerase (Promega, Leiden, The Netherlands). The PCR product of 328 bp was analyzed by standard 2% agarose gelelectrophoresis and confirmed after electroblotting (BioRad SemiDry transfer cell, Hercules, CA, USA) with a ^{32}P-labelled oligonucleotide probe (probe: nucleotide 58-87: 5'cct.gct.ggt.ggc.tcc.agt.tcc.gga.aca.tga). The detection limit of our PCR assay is around 500-1000 genome eq per ml, using the Eurohep standard, and has been validated in a quality assurance programme.[7] Positive and negative control samples were included both during sample preparation and during the PCR procedure.

Statistical analysis

The geometric means of HBV-DNA levels were calculated from the different dose groups at week 0, 12 and 24 of treatment. Wilcoxon signed rank test was used to show statistically significant differences ($p<0.05$) between HBV-DNA levels comparing dose groups (unmatched pairs) and duration of treatment (matched pairs).

Table 2. *Per protocol analysis of patients with negative HBV-DNA according to dose of lamivudine. The lower limit of detection of the HBV-DNA PCR assay is 10^3 gen.eq./ml.*

	25 mg	100 mg	300 mg
Number of randomized patients	16	16	19
Genostics negative	0 (0%)	1 (6%)	0 (0%)
Week 12 analysis (n)	16	15	18
Genostics negative (patients)	7/16 (44%)	14/15 (93%)	15/18 (83%)
PCR negative (samples)	1/32 (3%)	4/30 (13%)	7/36 (19%)
Week 24 analysis (n)	13	14	15
Genostics negative (patients)	6/13 (46%)	12/14 (86%)	13/15 (87%)
PCR negative (samples)	3/26 (12%)	8/28 (29%)	11/30 (37%)

Results

The baseline characteristics of all patients randomized to this study are shown in table 1. The geometric means of HBV-DNA levels were calculated from the different dose groups at week 0, 12 and 24 of treatment. HBV-DNA levels were not significantly different at baseline for the three dose groups. All dose groups showed a statistical significant decrease of HBV-DNA levels between week 0 and 12 ($p<0.001$); between week 12 and 24 HBV-DNA levels decreased significantly only for the 300 mg dose group ($p<0.01$). We observed no significant difference in HBV-DNA levels between the 100 and 300 mg dose groups (figure 1).

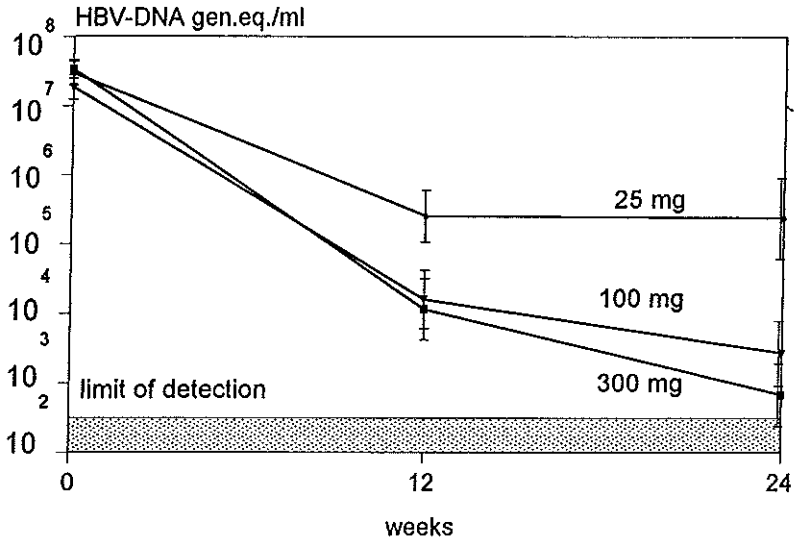

Figure 1. *Serum HBV-DNA levels in the limiting dilution PCR in patients receiving lamivudine once daily according to dose [25 mg (•), 100 mg (▾) and 300 mg (■), geometric mean]. Patients with negative PCR taken as 10^1. Error bars represent SEM.*

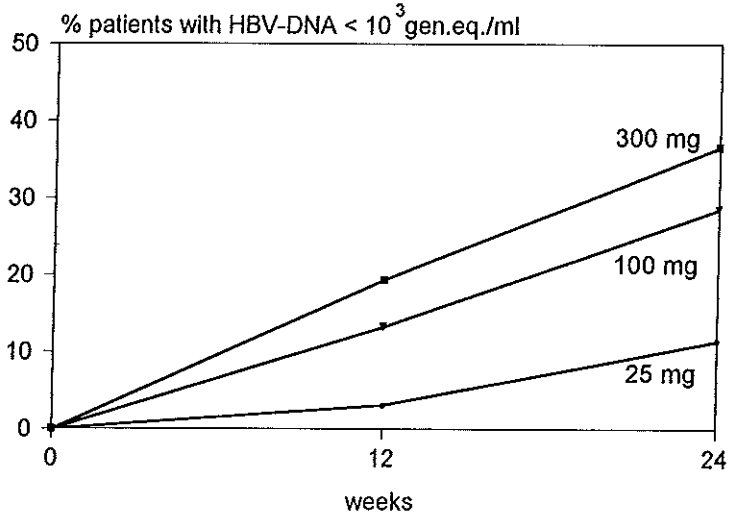

Figure 2. *Percentage of patients who became HBV-DNA negative by limiting dilution PCR at baseline, after 12 and 24 weeks of lamivudine therapy according to dose [25 mg (•), 100 mg (▾) and 300 mg (■)].*

When baseline values were compared to PCR values at week 24, 67% of patients had over 2 log reduction in the amount of HBV-DNA with a median 4 log reduction.
At the week 12 assessment, 1 (3%), 4 (13%) and 7 (19%) of samples (2 per patient) were negative by PCR in the 25, 100 and 300 mg cohorts respectively, while a further 5 (16%), 3 (10%) and 6 (17%) were at the level of detectability of the PCR assay (10^3 gen.eq./ml). At week 12 totally 27% of 49 patients (per protocol analysis) had HBV-DNA levels below or at the level of detection of the PCR assay (figure 2).
At the week 24 assessment, 3 (12%), 8 (29%) and 11 (37%) samples tested by PCR were negative in the 25, 100 and 300 mg cohorts respectively, while a further 1 (4%), 4 (14%) and 12 (40%) samples were at the level of detectability of the PCR assay (figure 2).
Overall 26% of 42 patients (per protocol analysis) were negative for serum HBV-DNA by PCR at the week 24 assessment, in addition 20% of patients had serum HBV-DNA concentrations at the level of detectability of the PCR assay (figure 2).
ALT normalization occurred in 17 (47%) of patients with abnormal ALT at baseline (n=36). No significant correlation was found between ALT levels and HBV-DNA levels measured by PCR.

Discussion

This study shows that suppression of serum HBV-DNA during a 24-week course of lamivudine is continuing below the level of detection of the liquid hybridization assay. Per protocol analysis showed that 46% of patients had HBV-DNA levels below or at the level of detection of the PCR assay ($\leq 10^3$ gen.eq./ml); in comparison to 74% by the less sensitive liquid hybridization assay ($<10^7$ gen.eq./ml). Higher daily doses (100 mg and 300 mg) are more effective in suppressing HBV-DNA compared to the 25 mg (table 2, figure 1, $p<0.05$). These results are in accordance with the findings after a three-month course of lamivudine. In that study a negative HBV-DNA test was observed in 70% of patients of the 25 mg group at 3 months, compared to 100 % in the 100 and 300 mg groups using the same liquid hybridization assay.[4]
Our study showed a more profound suppression of HBV-DNA after 24 weeks compared to 12 weeks of treatment (12% PCR negativity at week 12 and 26% at week 24, table 2, $p<0.01$). The incidence of PCR negativity increases with increasing duration of treatment. Prolongation of treatment is attractive firstly because lamivudine is well tolerated in clinical studies[5,12] and secondly, but probably more important, since a correlation between persistent suppression of viral replication and subsequent improvement in liver histology has been found.[13] The results of the larger phase III study with liver histology as primary outcome variable are eagerly awaited.
However, prolongation of lamivudine monotherapy beyond 6 months may result in the development of a lamivudine resistant mutant virus.[14,15] In order to prevent the development of lamivudine resistant mutants it is an attractive thought to aim for rapid and profound inhibition of HBV replication.

Based on early kinetic studies the standard dose of lamivudine for the treatment of chronic hepatitis B was suggested to be 100 mg daily. Our data suggest a possibly more profound suppression induced by the 300 mg dosage than by the 100 mg dose. The highest daily dose of 300 mg showed the highest percentage of PCR negative patients at week 24 and a statistically significant reduction of HBV-DNA levels between week 12 and 24 (figure 1 and 2). Therefore future studies should be monitored by a sensitive quantitative PCR to confirm the relationship between the degree of virus suppression and outcome measures like the rate of developing mutants. In addition, further standardization of HBV-DNA measurement can be applied since recent publications suggest that our measurement still underestimates the number of HBV genomes.[6]

A much smaller but very important group to whom such a powerful and easily administered antiviral drug could be of immediate value is HBV-DNA-positive patients who face liver transplantation. Antiviral therapy to reduce the level of circulating HBV-DNA before liver transplantation is one of the logical approaches to improvement of the results of liver transplantation for HBV-related liver disease. Transplantation for patients with cirrhosis and hepatic decompensation has been hampered by the lack of treatments with a favourable efficacy and toxicity profile.[16-18] Lamivudine can decrease HBV-viraemia to below the level of detectability by PCR in 27% of all patients randomized to doses of 100 mg or more for 24 weeks, and has been well tolerated in clinical studies to date.[4,5,19]

In conclusion, our results for chronic hepatitis B patients indicate that lamivudine continues to potently suppress viral replication below the limit of detectability of traditional assays. Suppression in viral replication is more profound after 24 weeks of treatment in comparison to 12 weeks, resulting in the loss of detectable HBV-DNA by PCR in 33% of patients after 24 weeks of therapy with 100 or 300 mg lamivudine daily.

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3.3

HISTOLOGICAL IMPROVEMENT IN PATIENTS WITH CHRONIC HEPATITIS B VIRUS INFECTION TREATED WITH LAMIVUDINE

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Liver 1997;17:103-106

Summary

Lamivudine is an oral nucleoside analogue with strong antiviral activity against hepatitis B virus (HBV). In previous clinical studies, a course of lamivudine for 4-12 weeks induced a profound decrease in HBV viraemia with excellent tolerance, but data on histology have not yet been reported. We studied the liver histology of 13 patients with stable chronic HBV infection treated with 25 mg, 100 mg or 300 mg lamivudine daily for 6 months. All patients became HBV-DNA negative during treatment. The paired biopsies taken at entry and during treatment were scored by two independent observers, using the components of the histology activity index (HAI) and fibrosis (modified Knodell). The items scored were piecemeal necrosis, focal necrosis, confluent necrosis, portal inflammation and fibrosis. Before treatment, the biopsies yielded a mean HAI of 4.4 (±0.8), which decreased to HAI 2.8 (±0.5) during treatment. An analysis of the individual components of the classification system showed a significant decrease in piecemeal necrosis from a pre-treatment 1.4 (±0.3) to 0.8 (±0.1) during treatment (p = 0.02). Although a trend was found for the other components, it was not statistically significant, probably due to the number of pairs examined. In conclusion these results suggest that prolonged suppression of viral replication by lamivudine can improve liver histology. In contrast to previously published reports on α-interferon therapy, this study indicates that the improvement in liver histology with lamivudine is independent of HBeAg seroconversion.

Introduction

Lamivudine, the (-) enantiomer of 2'-deoxy-3'-thiacytidine, is an oral nucleoside analogue that strongly inhibits hepatitis B virus (HBV) replication both *in vitro*[1] and *in vivo*[2,3]. The aim of antiviral therapy for chronic hepatitis B is HBeAg seroconversion, since that event is usually followed by biochemical and histological remission of the liver disease.[4] Lamivudine for 6 months does not appear to induce HBeAg seroconversion, but biochemical remission ensues in the majority of patients. We tried to establish whether lamivudine therapy leads to histological improvement.

Table 1. Demographic and baseline characteristics of the patients.

Patient (*n*)	15
Gender (m/f)	12/3
Age (mean, range)	32.4 (18-61) years
HBV-DNA (mean, range)	169 (4-520) pg/ml*
HBeAg (mean, range)	36579 (168-158716) U/l
ALT (mean, range)	66 (32-188) IU/l
α-Interferon non-responder (*n*)	8
Caucasian/Asian/Black (*n*)	11/3/1

* pg HBV-DNA (Genostics, Abbott)

Patients and methods

Study population

Fifteen patients with stable chronic (HBeAg positive) hepatitis B viral infection, who had previously participated in a 28-day lamivudine study, entered the study.[5] All had HBV-DNA >3 pg/ml (Genostics, liquid hybridization assay, Abbott Laboratories, IL, USA) and HBeAg >1 U/l (IMx, Micro particle Enzyme Immunoassay, Abbott Laboratories, IL, USA) detectable in serum and elevated alanine aminotransferase (ALT) levels (>30 IU/l). None of the patients had decompensated liver disease, severe hepatitis (ALT >300 IU/l), co-infection with hepatitis C, hepatitis D or HIV or other serious medical illnesses. Eight patients had previously received alpha-interferon (α-IFN) without response.

These patients, part of a multicentre phase-2 study, had been randomly assigned to receive either 25, 100 or 300 mg lamivudine orally once daily for 24 weeks. The clinical, biochemical and virological results of this multicentre study have been reported elsewhere.[6] A liver biopsy was obtained from thirteen of fifteen patients of our unit before and during treatment. Initially to exclude the occurrence of fialuridine (FIAU) like toxicity, the second biopsy was taken after 8 weeks (*n*=4), 12 weeks (*n*=3), 16 weeks (*n*=4) or 24 weeks (*n*=2) of therapy.

Liver biopsies

Liver tissue was fixed in 4% phosphate-buffered formaldehyde, routinely processed and embedded in paraffin blocks; 5 µm sections were then cut. Liver sections were stained with haematoxylin and eosin (H&E), periodic acid-Schiff (PAS), PAS after diastase digestion and elastica Von Giesson stain. HBcAg was detected on paraffin-fixed slides with polyclonal rabbit anti-HBc (Dako Corp., Santa Barbara, CA, USA) as first antibody and peroxidase-labelled swine anti-rabbit IgG (Dako Corp., Santa Barbara, CA, USA) as second antibody after immersion in 3,3'-diamino benzidine (DAB) solution for 7 min. All liver biopsies (n=26) were scored blindly by two observers using the Desmet modification of the Knodell scoring system.[7,8] The histology activity index (HAI) combines the scores for piecemeal necrosis, fresh confluent necrosis, intralobular degeneration/focal necrosis and portal inflammation. The percentage of hepatocytes found to be positive for HBcAg ranged from none, <1%, 1-5%, 5-30%, 30%-70% to >70%.

Statistical analysis

We tested the hypothesis that within the study group no difference existed between the values before treatment and during treatment. For continuous variables (HAI, ALT, HBcAg), we used the Wilcoxon signed rank test for paired samples. P-values for components of the HAI and fibrosis were calculated using the chi-squared test. Results are given as mean ± standard error of the mean, (±SEM).

Ethics

This study was approved by the institutional review board, and all patients gave written informed consent.

Results

Baseline characteristics of the patients are shown in table 1. All 15 patients who entered the study completed treatment and became HBV-DNA negative according to the standard hybridization technique (Genostics, Abbott) during treatment. In 13 patients, HBV-DNA became detectable within 4 weeks of termination of lamivudine treatment. However, two patients became HBeAg-negative during the same 6 months, one is still HBeAg and HBV-DNA-negative. All patients had elevated serum ALT at entry, mean (±SEM) was 66 (±11) IU/l. Serum-ALT dropped in five patients (33%) to the normal range (ALT ≤30 IU/l). Mean post-treatment ALT was 44 (±6) IU/l, this difference is statistically significant (p=0.02).

Of 13 patients who underwent liver histological studies, histology improved in seven cases (figure 1). Piecemeal necrosis decreased from a mean (±SEM) of 1.4 (±0.3) for pre-treatment biopsies to 0.8 (±0.1) during treatment (p=0.02). An improvement was also found for portal inflammation. The total HAI improved from a mean (±SEM) of 4.4 (±0.8) before treatment to 2.8 (±0.5) during treatment; this difference was not statistically significant

(table 2). The improvement in HAI was most obvious for patients who received a cumulative dose of lamivudine of more than 5 gram (figure 2). No difference in fibrosis was observed. For six patients (46%), the HAI was normal (score≤2) for the second liver biopsy (figure 1). The expression of HBcAg in hepatocytes disappeared in two cases. In one of these patients, a HBeAg seroconversion occurred. In the other patient very low levels of HBeAg remained detectable during treatment and HBeAg relapsed after the end of treatment. For all patients, the decrease in HBcAg expression during treatment was not statistically significant.

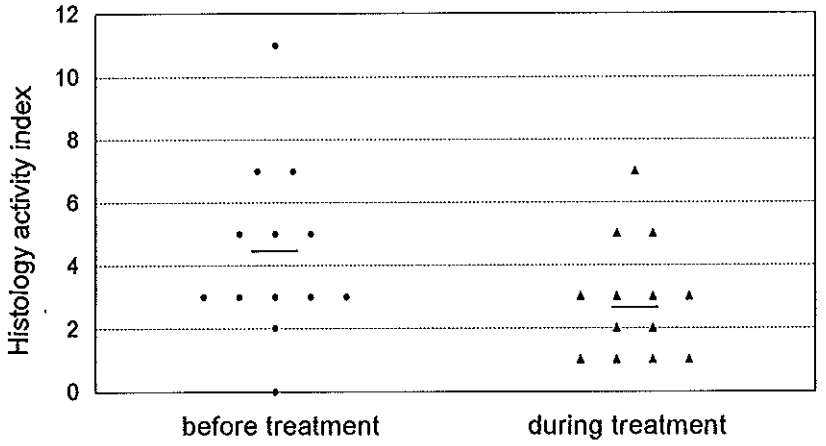

Figure 1. *Individual histology activity index before and during treatment with lamivudine.*

Discussion

The results of this study suggest that prolonged viral suppression without HBeAg seroconversion can induce histological improvement. The suppression of viral replication inactivates the disease process as reflected by significant improvement in serum-ALT and piecemeal necrosis. Moreover, the total HAI score improved during therapy.
The liver biopsies were taken at different time-points during lamivudine therapy to determine whether duration of therapy is an important factor, but this issue could not be clarified in this study. In contrast, the cumulative dose of lamivudine seems to be more important, the critical limit being 5 grams (100 mg for 50 days) (figure 2). From previous studies, we know that 25 mg is a suboptimal dose;[6] however, in the present study the four patients who received 25 mg daily became HBV-DNA negative, but in two of them the HAI score worsened during treatment. Overall no differences were detected between the three doses because of the small numbers. In addition, we tested HBV-DNA negative patients by polymerase chain reaction (HBV-DNA PCR), out of the three patients who

became HBV-DNA negative by PCR during treatment two had a marked improvement in HAI (5 points).

This study covered a small group of patients treated with lamivudine for 6 months: histological improvement was already evident in the majority of patients. At present, no reports on patients treated for more than 6 months are available, so we still do not know the outcome of long-term viral suppression by lamivudine. From other studies we know that piecemeal necrosis in particular is the histological finding that predicts the development of cirrhosis.[9] This suggests that long-term viral suppression may eventually prevent the development of cirrhosis.

Especially interesting is the fact that patients with a high HAI score before therapy exhibited the most pronounced improvement. This suggests that patients with severe hepatitis, who were actually excluded from this phase 2 study, would benefit the most from prolonged viral suppression.

Table 2. *Components of HAI and fibrosis before and during treatment in 13 patients treated with lamivudine.*

		Before treatment	During treatment
1	Piecemeal necrosis	1.4	0.8*
2	Confluent necrosis	0	0
3	Focal necrosis	1.2	1.2
4	Portal inflammation	1.8	1
5	Fibrosis	1.4	1.4
(1-4)	Total HAI	4.4	2.8
(1-5)	Knodell score	5.8	4.2

Results are given as mean.

* $p = 0.02$, chi squared test.

The primary goals of therapy for chronic HBV infection are to diminish infectivity, improve the quality of life and inactivate the liver disease. These objectives are usually achieved when HBV is completely abolished, defined as elimination of all viral markers, or active viral replication is terminated, characterized as sustained elimination of HBeAg and HBV-DNA and normalization of ALT levels.[4,10,11] When achieved, these factors, after α-IFN therapy, lead to sustained histological improvement.[12] Lamivudine has been shown to be a potent inhibitor of viral replication. Six months of lamivudine therapy is not sufficient to induce a permanent response through HBeAg seroconversion and raises the issue of treatment outcome with this class of compounds. This outcome can be cessation of histological progression of the disease after persistent viral suppression.

On the basis of our findings for this small group of patients, we suggest that our primary treatment goal should be redefined as improvement of the histological standard, in contrast

to the treatment goals defined for α-IFN therapy.
In conclusion, lamivudine is a very effective inhibitor of HBV replication. This results in improvement of serum transaminase levels and histological activity, especially piecemeal necrosis, also in the absence of HBeAg seroconversion.

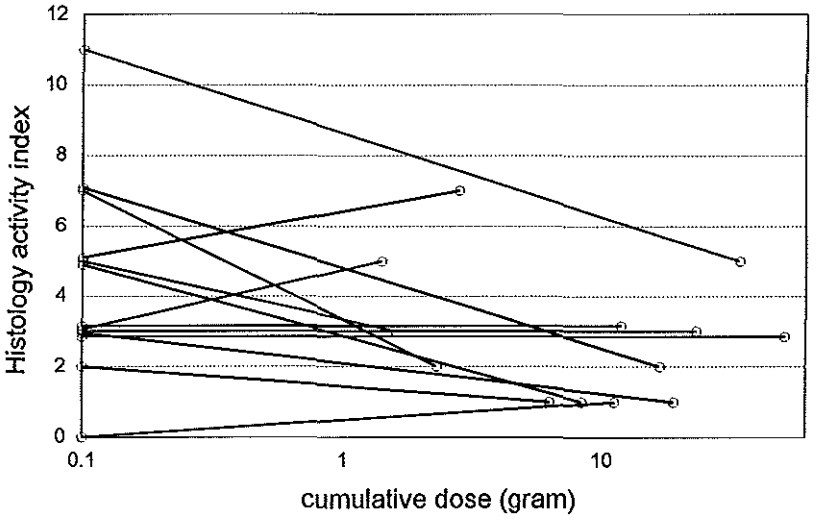

Figure 2. *Individual histology activity index before and during treatment. HAI is plotted against cumulative dose (duration x dose) of lamivudine at the time of the second liver biopsy.*

Acknowledgments

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4

COMBINATION ALPHA-INTERFERON AND LAMIVUDINE THERAPY FOR ALPHA-INTERFERON RESISTANT CHRONIC HEPATITIS B INFECTION: RESULTS OF A PILOT STUDY

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Summary

Alpha-interferon (α-IFN) achieves seroconversion in about one third of naive patients; attempts to achieve seroconversion in patients who have previously failed α-IFN have proven disappointing. Combination chemotherapy (α-IFN with a nucleoside analogue) might provide a treatment alternative for these patients. We have undertaken a phase 2 study in 20 patients who had previously failed at least one course of α-IFN. All patients were treated for 16 weeks with α-IFN in combination with 12 or 16 weeks of lamivudine (3'TC). This study was designed to assess the safety, tolerability and efficacy of the combination. In addition, pharmacokinetic studies were performed to identify/exclude significant pharmacokinetic drug interaction.

The combination was well tolerated, and side-effects of the combination were indistinguishable from the recognized side-effects of α-IFN. Pharmacokinetic studies performed on days 1 and 29 did not show any significant interaction. All patients achieved HBV-DNA clearance during treatment, but 19 relapsed after the end of treatment. HBeAg/anti-HBe seroconversion was observed for 4 patients, but was sustained for a single patient (who also had sustained HBV-DNA clearance).

In conclusion, combination therapy with α-IFN and lamivudine given for 16 weeks appears safe and is well tolerated. However, for this group of patients who had previously failed interferon monotherapy, the efficacy of combination interferon/lamivudine therapy appears disappointing; other treatment strategies should be investigated.

Introduction

Lamivudine, the negative enantiomer of 2'3'-dideoxy-3'-thiacytidine, is an oral nucleoside analogue that inhibits viral DNA synthesis by terminating the nascent proviral DNA chain. Unlike some dideoxynucleosides, lamivudine does not inhibit mitochondrial DNA synthesis[1] and does not suppress haemopoiesis at concentrations that inhibit the synthesis of viral DNA.

Lamivudine has been used to treat more than 30,000 HIV-infected patients, and has been well tolerated in long-term HIV studies at 600 mg/day.[2]

Suppression of hepatitis B virus (HBV) replication was observed when HIV/HBV co-infected patients were treated with lamivudine.[3] In dose-ranging studies of HBV-infected patients, lamivudine was prescribed for 28 days to 6 months at doses from 5 mg to 600 mg.[4,5] Marked reduction of serum HBV-DNA was observed for all doses greater than 20 mg/day, though levels returned to pre-treatment values at conclusion of treatment for most patients.

Currently, α-IFN is the only agent licensed for the treatment of chronic HBeAg-positive infection, but only one third of treated patients achieve HBeAg seroconversion.[6] Further attempts to suppress viral replication in patients who previously failed interferon have proven disappointing.[7,8,9,10,11]

Recently, Dienstag et al reported the results of lamivudine therapy (at 3 different doses) for patients with chronic hepatitis B infection.[12] Thirty two patients were treated, and the cohort included 17 patients who had previously failed interferon monotherapy. HBeAg seroconversion was achieved for 3/17. Therapy of chronic HBV infection might combine the antiviral properties of lamivudine with the immunomodulatory properties of α-IFN. Interferon-induced HBeAg/anti-HBe seroconversion may be favoured by lamivudine-induced reduction of serum HBV-DNA.

This multicentre study was designed to assess the safety, tolerability and efficacy of combination lamivudine/interferon therapy for the treatment of patients with chronic HBeAg and DNA-positive HBV infection who have previously failed to seroconvert with α-IFN monotherapy.

Patients and methods

Eligible patients were aged 18-70 and had compensated liver disease due to chronic HBV infection. All were HBsAg, HBeAg and HBV-DNA positive (>10 pg/ml according to the Abbott Genostics assay). Patients had failed to seroconvert (from HBeAg to anti-HBe) with at least one prior course of α-IFN at a minimum dose of 13.5 MU per week for at least 16 weeks. At the time of screening, at least 6 months had elapsed since most recent interferon treatment. Exclusion criteria included the presence of co-infection with HDV, HCV or HIV.

Eligible patients were randomized to one of two treatment regimens. All patients received alpha 2b interferon (Intron A, Schering-Plough Inc, USA) 10 MU three times weekly for 16 weeks. In addition, patients randomized to group A received placebo (orally) for 4 weeks, followed by lamivudine 100 mg/day for 12 weeks.
Patients in group B received lamivudine 100 mg/day for 16 weeks. Randomization was in the ratio 1:2, treatments A:B. Randomization was double-blind and all patients were followed for 16 weeks post-treatment (figure 1).

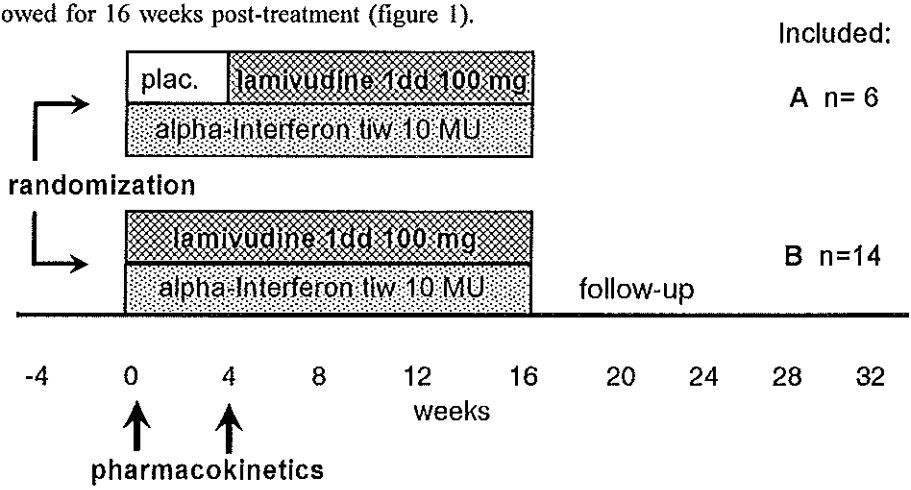

Figure 1. Design of the study

This design enabled the study to assess the tolerability of the combination therapy. α-IFN monotherapy is associated with significant and predictable side-effects. These side-effects are most pronounced during the initial weeks of therapy. The inclusion of a (double-blind) placebo group enabled the study to distinguish morbidity associated with the interferon/lamivudine combination therapy from the morbidity associated with α-IFN alone. All patients were followed after treatment for 16 weeks.
Safety and tolerability were measured by clinical and laboratory evaluations at each clinic visit (baseline, weeks 1, 2, 3, 4, 6, 8, 12 & 16 of treatment, then every 4 weeks until the end of follow-up). An ALT "flare" was defined as a 3-fold rise in serum ALT to a value in excess of ten times the upper limit of normal.
HBV markers were assayed centrally from stored serum by Corning Hazleton, Harrogate, UK. Serum HBV-DNA was measured quantitatively by the Abbott Genostics assay (Abbott, Chicago, Illinois, USA). HBeAg/anti-HBe was assessed in a semi-quantitative assay (Kodak Amerlite, Kodak Clinical Diagnostics, Amersham, UK). HBsAg and anti-HBs were measured by ELISA (Abbott, Chicago, Illinois, USA).

Pharmacokinetics
Lamivudine and α-IFN pharmacokinetics were assessed on days 1 and 29. Blood samples

for α-IFN pharmacokinetic analysis were taken before the first dose and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 10, 12, 18 and 24 hours after dosing. Additional blood samples were taken pre-dose and 1, 2, 4, 8, 12 and 24 hours post-dose for lamivudine assay. Urine samples for the assay of lamivudine concentration were pooled in the following fashion: post-dose 0-6 hours, 6-12 hours and 12-24 hours.

Plasma samples were analyzed for α-IFN concentration by ELISA or IRMA at the Schering-Plough Research Institute, Kenilworth NJ, USA. Serum and urine samples were analyzed for lamivudine concentration by a validated high pressure liquid chromatography method with UV detection.[13]

The non-compartmental pharmacokinetic analysis was performed using TOPFIT version 2.0 software.[14] The following parameters were determined for both α-IFN and lamivudine: C_{max}, t_{max}, AUC, AUC_{last}. In addition, oral clearance (Cl/F), Ae_t (amount excreted in urine over time t), Cl_r (renal clearance) and AUC_∞ were estimated for lamivudine.

The study was approved by the local research ethics committees of the three participating centres, and all patients gave written informed consent.

Table 1. *Basic demography and pre-treatment characteristics of 20 patients.*

	Group A (n=6)	Group B (n=14)	Total (n=20)
Patient characteristics			
Age (yrs)	39(7)	39(13)	39(11)
Sex (m/f)	5/1	14/0	19/1
Ethnic origin (asian/caucasian)	3/3	4/10	7/13
Baseline variables			
Weight (kg)	76(9)	78(11)	77(10)
Pre-treatment ALT (IU/l)	66(46)	81(63)	77(58)
Pre-treatment HBV-DNA (pg/ml)*	92(75)	187(156)	158(142)
*Pre-treatment histology***			
cirrhotic	0	2	2
non-cirrhotic	5	12	17
minimal/mild inflammatory activity	4	8	12
moderate/severe inflammatory activity	1	6	7

Data are presented as mean (standard deviation).

* HBV-DNA (Genostics, Abbott)

** 1 patient did not underwent a liver biopsy.

Results

Efficacy

Six patients were randomized to group A, and 14 patients were randomized to group B. Baseline characteristics with regard to age, sex, weight, ethnic origin, pre-treatment ALT and HBV-DNA are given in table 1.

In all patients serum HBV-DNA dropped to below the level of detection of the Abbott assay during treatment (table 2). For patients in group A, this was achieved on median day 57 (end of week 8, 4 weeks after introduction of lamivudine; figure 2). For patients in group B, serum HBV-DNA clearance was achieved on median day 15 (figure 2). For 19 patients, serum HBV-DNA relapsed when treatment was stopped. Sustained HBV-DNA clearance was observed for a single patient. This patient was randomized to group B, HBV-DNA clearance was achieved by the end of treatment week 2, and HBeAg/anti-HBe seroconversion was achieved during therapy. DNA clearance and seroconversion were associated with a transaminase flare at the end of treatment week 8. At the end of follow-up, anti-HBe was sustained and serum ALT was normal.

Table 2. *Cumulative number of patients with HBV-DNA, HBeAg negativity and ALT normalization during therapy.*

		wk 0	wk 4	wk 8	wk 12	wk 16
HBV-DNA	A (n=6)	0	0	4	5	6
	B (n=14)	0	13	14	14	14
HBeAg	A (n=6)	0	0	0	0	0
	B (n=14)	0	1	2	2	4
ALT	A (n=6)	0	2	3	4	5
	B (n=14)	3	4	6	9	11

HBeAg/anti-HBe seroconversion was achieved by 4 patients (all in group B; table 2 and figure 3). For 3 patients, seroconversion was not sustained, and all were HBeAg and HBV-DNA positive at end of follow-up. Serum ALT rose during therapy for 2 of these patients (though not fulfilling the definition of a "flare" for either patient).

Serum ALT declined during therapy to reach lowest values 4 weeks after completion of therapy for both treatment groups ($p<0.05$, Wilcoxon test). In ten patients normalization of ALT was achieved during treatment (table 2). After stopping treatment values rose to achieve pre-treatment levels by the end of follow-up (figure 4).

ALT flares were observed for 3 patients. A flare at 8 weeks was associated with sustained seroconversion for one patient. For 2 other patients, the ALT flare was observed during the second post-treatment month. This flare followed the post-treatment rise in serum HBV-

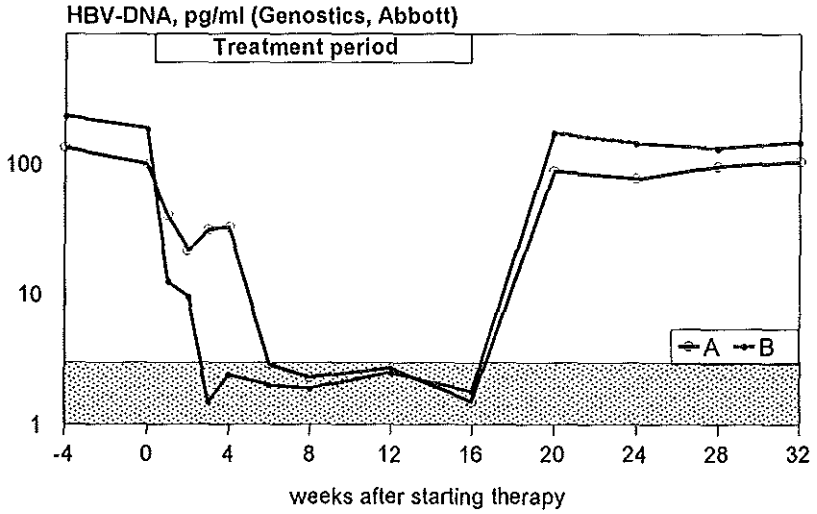

Figure 2. *Response of serum HBV-DNA to combination therapy, showing response for 6 patients in group A, and for 14 patients in group B. The delayed decline observed for group A suggests that inhibition was principally due to lamivudine treatment. HBV-DNA was measured by the Abbott Genostics assay which has a minimum sensitivity of 3 pg/ml. Values are expressed as mean.*

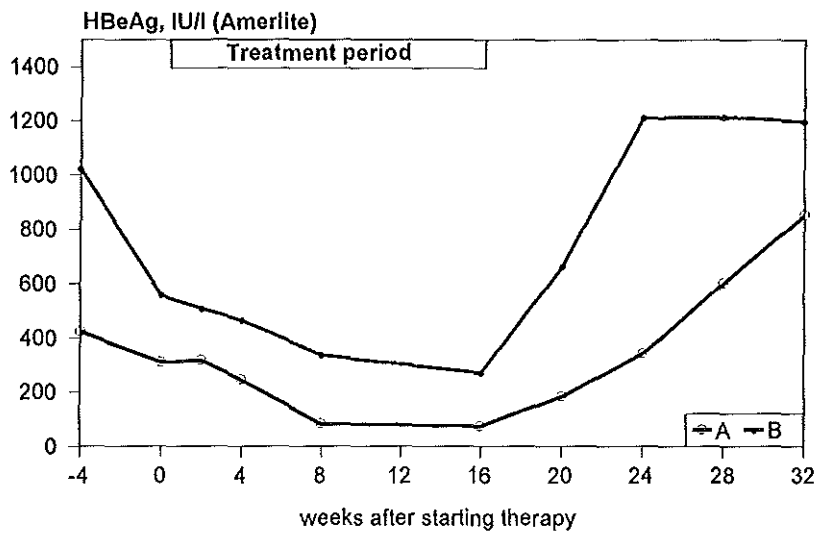

Figure 3. *Response of serum HBeAg to combination therapy. HBeAg was measured by Amerlite assay. Values are expressed as mean.*

DNA, and was associated with a decline in serum HBV-DNA. For these 2 patients, the flare was not associated with HBeAg/anti-HBe seroconversion.

Safety

The combination therapy was well tolerated.

Seventeen patients completed 16 weeks of α-IFN therapy at full dose, and three required dose reduction. For the patient who achieved sustained HBeAg seroconversion, α-IFN dose was reduced at week 6 then stopped at week 12 in response to the ALT rise. This ALT rise was associated with seroconversion.

For one patient, interferon dose reduction was required for malaise and fatigue, and symptoms responded to dose reduction.

For another patient, interferon (and lamivudine) was stopped after 15 weeks of treatment. This patient developed myopathy during treatment. CPK was elevated before treatment (323 U/l, normal <110) rose further during therapy, and peaked (967 U/l) just after treatment withdrawal. At that time, a diagnosis of hepatocellular carcinoma was established. Muscle biopsy did not show ragged red fibers, the typical sign of mitochondrial dysfunction. All other patients completed placebo/lamivudine therapy without interruption or dose reduction.

Serum CPK was measured at each outpatient attendance. For 12/20 patients CPK was elevated prior to antiviral treatment. For 14/20 patients, at least 1 CPK elevation was documented during therapy (table 3).

For 4 patients, CPK exceeded 3 times the upper limit of the normal reference range, for one patient at baseline and for one patient at the end of one week's therapy (patient was receiving interferon and placebo) and for one patient, CPK was normal throughout the treatment period, but rose during follow-up, the other patient developed hepatocellular carcinoma as mentioned before.

Table 3. *Abnormal laboratory values before, during and after treatment.*

	group A + B n = 20 week -4-0	group A n = 6 week 1-4	group B n = 14 week 1-4	group A + B n = 20 week 6-16	group A + B n = 20 week 20-32
CPK elevation	12 (60%)	5 (83%)	4 (29%)	8 (40%)	12 (60%)
CPK > 3 ULN	1 (5%)	1 (17%)	0	0	2 (10%)
Amylase elevation	3 (15%)	2 (33%)	3 (21%)	5 (25%)	6 (30%)
ALT > 10 ULN	1 (5%)	0	0	1 (5%)	2 (10%)

Fourteen patients had normal serum amylase during the whole study period. Serum amylase was elevated (but never more than three times the ULN) for 6 patients on at least one occasion. For 3 patients, serum amylase was elevated at baseline, and remained elevated during therapy. No significant increase was observed during therapy for these 3

patients. For 2 patients, amylase was normal at baseline, and fluctuated about the upper limit of the normal range for the duration of treatment and follow-up. One patient had an elevated amylase level at one occasion during follow-up. No patient experienced abdominal pain suggestive of pancreatitis. Serum lipase was not measured.
Blood anion gap was calculated at each outpatient attendance. A widened anion gap, as found in cases of lactic acidosis, was not recorded for any patient.
Adverse events were reported for all patients. Adverse events occurred with similar incidence in both treatment groups, and the incidence was comparable with reported adverse events for patients receiving α-IFN monotherapy. Adverse events reported during the first 4 weeks of therapy (the placebo-controlled phase of the study) did not distinguish group A from group B.

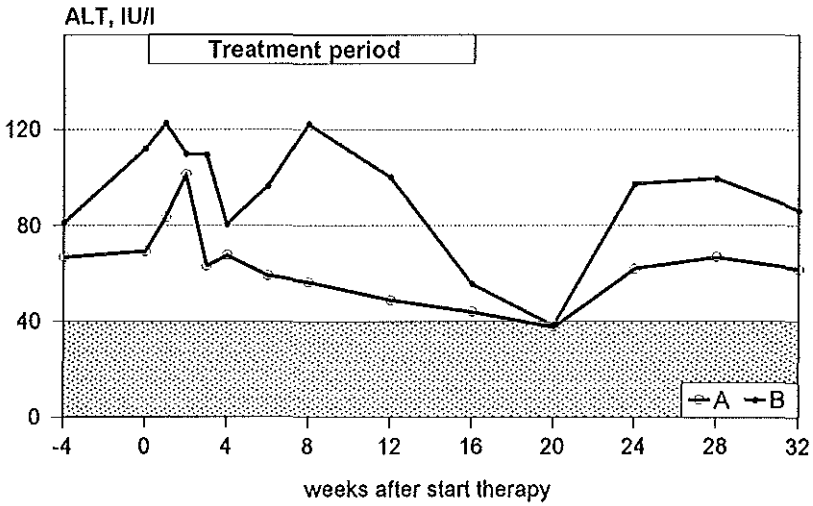

Figure 4. *Serum ALT levels during combination therapy. In group B an early ALT rise was observed; in group A the ALT rise occurred after switching placebo to lamivudine at week 4. In both groups ALT normality was observed 4 weeks after stopping therapy.*

Pharmacokinetics

(a) *α-IFN pharmacokinetics:* after inspection of the log-linear plot of α-IFN against time for each patient, a terminal mono-exponential phase could not be identified, thus the parameters λ_z, $t_{1/2}$, AUC_∞ and %AUC extrapolated were not determined. Data from 8 patients who received α-IFN concomitantly with lamivudine were available for pharmacokinetic analysis on day 1 and day 29. Maximum plasma concentrations (C_{max}) of α-IFN were attained, on average at 10 hours post-dose on day 1, and at 6-7 hours post-dose on day 29 (table 4a and figure 5).

(b) *lamivudine pharmacokinetics:* the pharmacokinetic parameters of lamivudine in

patients administered lamivudine concomitantly with α-IFN on day 1 and day 29 are shown in table 4b and figure 6.
Comparison of α-IFN pharmacokinetics in this study to those obtained in other studies with 10 MU interferon s.c. indicates that co-administration of lamivudine does not alter the pharmacokinetics of α-IFN. Lamivudine pharmacokinetics were unaltered by co-administration of α-IFN.

Table 4a. *α-IFN pharmacokinetics on day 1 and day 29 of combination lamivudine/α-IFN therapy.*

		Day 1	Day 29
Number		8	7
AUC_{last} *(IU.h/ml)*	mean	275	715
	median	252	647
	range	117-491	401-1035
C_{max} *(IU/ml)*	mean	33.8	54.3
	median	29.6	54.7
	range	15.5-75.2	36.8-68.0
t_{max} *(h)*	median	10	7
	range	5-12	4-10

IU = international units, h = hour, ml = millilitre.

Table 4b. *Lamivudine pharmacokinetics in patients receiving combination therapy.*

	Day 1	Day 29
Number	7	8
AUC (ng.h/ml)	4503(3998-5073)	NC
C_{max} *(ng/ml)*	1042(894-1215)	1008(759-1338)
t_{max} *(h)**	1(1-2)	1(1-4)
$t_{1/2}$ *(h)*	4.1(2.7-6.1)	5.0(3.3-7.6)
Cl/F (l/h)	22.2(19.7-25)	19.5(14.2-27)
Cl_r *(l/h)*	14.8(10.6-20.5)	15.8(12.0-20.8)

* values are median (range).

Discussion

For treatment-naive patients with chronic replicative HBV infection, α-IFN is the treatment of choice. α-IFN achieves serum HBV-DNA clearance in 37% of treated patients, and 33% achieve HBeAg clearance.[6] In addition, successful interferon-induced HBeAg clearance is

associated with a diminished risk for the subsequent development of liver failure.[15] Characteristics predicting interferon responsiveness have been identified.[16,17] Further attempts to suppress viral replication in patients who previously failed interferon therapy have proven disappointing, and favourable pre-treatment characteristics are poor predictors of response to retreatment in this patient group.[7]

We have treated 20 patients who had previously failed to seroconvert in response to at least one course of α-IFN therapy. Treatment achieved prompt reduction in levels of serum HBV-DNA. For 20/20 patients, HBV-DNA became undetectable during treatment. The delayed clearance of viral DNA observed for treatment group A suggests that lamivudine, and not interferon, was responsible for reduction of serum viral DNA. Consistent with the observations of Dienstag et al,[12] serum ALT rose during antiviral therapy for group B, and peaked during the second month of treatment. In contrast, serum ALT did not rise during interferon/lamivudine therapy for group A. Instead, ALT rose during the interferon/placebo phase, then declined during combination treatment.

An ALT flare was observed for 3 patients. For one patient, ALT flared during treatment, and this was associated with HBeAg/anti-HBe seroconversion and with subsequent sustained normalization of ALT. For this patient, the pre-treatment HBV-DNA was 98 pg/ml.

For 2 patients, ALT flared after treatment. Treatment withdrawal was followed by a rise in serum HBV-DNA, which in fact provoked an ALT flare. This flare may represent an enhanced immune response to renewed viral replication. This pattern of response has been observed following corticosteroid withdrawal, and the associated decline in serum HBV-DNA may enhance subsequent response to interferon therapy.[17] Unfortunately, the enhanced immune response observed for these 2 patients after combination therapy withdrawal was not associated with HBeAg/anti-HBe seroconversion.

Sustained HBeAg/anti-HBe seroconversion was observed for a single patient. This result compares unfavourably with the success of Dienstag et al.[12] In that study, 32 patients (including 17 who had previously failed interferon monotherapy) were treated with lamivudine. Of 17 interferon failures, 5 achieved sustained HBV-DNA clearance, and 3 achieved HBeAg/anti-HBe seroconversion. Our result suggests that combination lamivudine/interferon therapy is not superior to lamivudine monotherapy for the treatment of patients who have previously failed to respond to interferon monotherapy. Indeed, combination therapy may be associated with an inferior response rate, with more side-effects, and with greater expense than lamivudine monotherapy.

The results of combination lamivudine/interferon therapy appear similar to those achieved by interferon monotherapy retreatment of patients who have failed a previous course of α-IFN.[7] Janssen et al. retreated 18 non-responders to interferon therapy. Despite favourable pre-treatment characteristics (for interferon response in treatment-naive patients), only 2 patients achieved HBeAg/anti-HBe seroconversion.

Interferon has been used in combination with other nucleoside analogues for the treatment of replicative HBV infection.[8,9,10,18] The combinations of interferon/ara-AMP[18] and

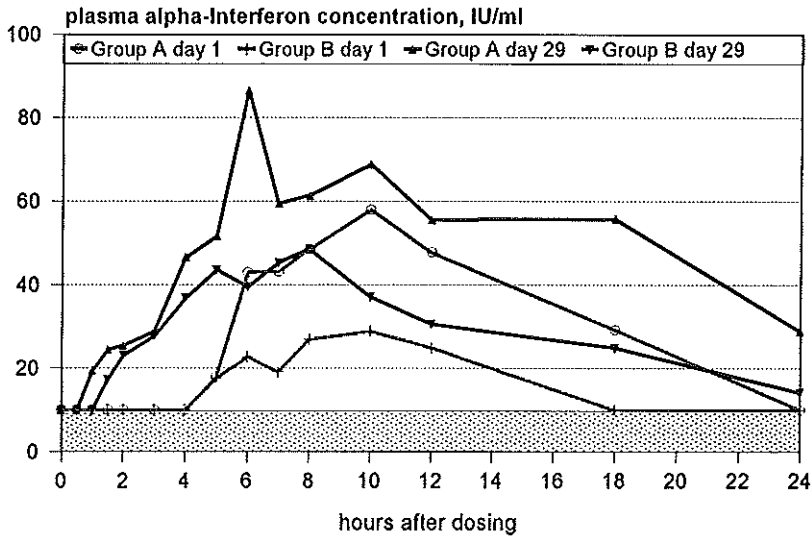

Figure 5. *Pharmacokinetics of α-IFN at day 1 and day 29. Levels of α-IFN tend to be lower in group B, but still fall within range observed in other patients receiving α-IFN.*

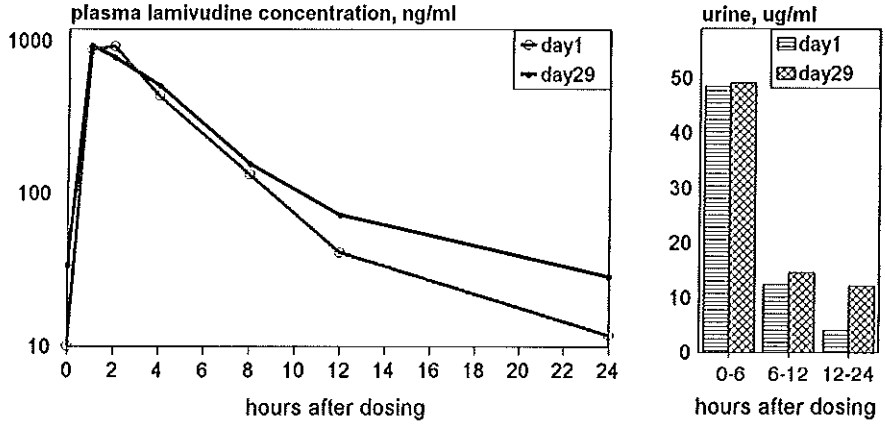

Figure 6. *Pharmacokinetics of lamivudine at day 1 and day 29 in group B. No difference was observed between group A and B in comparison to other studies.*[20]

interferon/ zidovudine[10] have proven toxic, and were associated with more side-effects than was treatment with interferon alone. Kakumu et al. studied the combination of interferon/ribavirin.[9] Ribavirin monotherapy inhibits HBV replication,[9,11] but significantly greater inhibition is observed for interferon monotherapy than is observed for the interferon/ribavirin combination.[9] Janssen et al. found that the addition of zidovudine to interferon did not enhance the inhibition of viral replication observed for interferon monotherapy.[10]

These observations are consistent with the results of our study which suggest that combination interferon/lamivudine therapy is not superior to the results of interferon and lamivudine monotherapy. Pharmacokinetic studies performed as part of this study suggest that disappointing efficacy is not the result of unexpected pharmacokinetic interaction. The rate and extent of systemic exposure to α-IFN (characterized by C_{max}, t_{max}, and AUC) in the presence of lamivudine was similar to published values after administration of α-IFN alone,[19] although the mean serum levels of interferon given in combination with lamivudine seems to be lower than during monotherapy. However, all values fell in the broad range of interferon after subcutaneous administration of 10 MU in individuals not taking lamivudine.

Pharmacokinetic data after administration of lamivudine (on days 1 and 29) in the present study were similar to previous data.[20] This indicates that concomitant administration of α-IFN has no appreciable effect on lamivudine pharmacokinetics.

The combination of α-IFN and lamivudine therapy appeared safe. Interferon treatment is associated with significant morbidity.[21] In particular, flu-like symptoms and lethargy are experienced by most treated patients. Incorporation of an initial placebo treatment period for group A enabled this study to differentiate the side-effects of the lamivudine/interferon combination from the side-effects of interferon alone. Adverse events reported during the first 4 weeks of therapy did not distinguish treatment group A from treatment group B. Adverse events were reported for all patients at some time during the study, but these occurred with similar incidence in both treatment groups. The incidence appears comparable with reported adverse events for patients receiving interferon monotherapy.[21] A relatively high dose of α-IFN was prescribed. Despite the high dose, 17/20 patients completed 16 weeks of therapy at full dose. Two patients required interferon dose reduction but one of them completed 16 weeks of therapy. For one patient, interferon (and lamivudine) was stopped after 15 weeks of therapy. This patient developed myopathy with elevated serum CPK. It is quite possible that CPK elevation and myopathy were associated with an underlying hepatocellular carcinoma, and were not secondary to lamivudine (or interferon) treatment. Serum amylase was slightly elevated on at least one occasion for 6 patients. Serum amylase elevation was not associated with abdominal pain, and was not clearly related to antiviral therapy. Arterial blood lactate was not measured during this study, but blood anion gap was calculated at each outpatient attendance. Widening of the anion gap, reflecting a lactic acidosis, was not observed for any patient. The multisystem toxicity observed in fialuridine-treated patients as a probable consequence of mitochondrial DNA polymerase inhibition and incorporation of FIAU into mitochondrial DNA,[22] was not

observed in lamivudine-treated patients.[23]

Thus, the lamivudine/interferon combination therapy appears safe and well tolerated. Unfortunately, combination therapy given to patients who previously failed interferon treatment achieved seroconversion in only 1 of 20 patients. However, all four patients who became HBeAg negative during treatment received combination therapy from start. In two patients the first HBeAg negative result was at the end of therapy. Therefore, prolongation of therapy after 16 weeks would probably induce sustained response. Future studies with combination therapy should continue α-IFN treatment after 16 weeks in case of significant HBeAg response. Another strategy is to start lamivudine therapy a few weeks before initiating interferon. Following this scheme HBV-DNA levels are low at the time of the first dose of interferon, which is found to be a good predictor of response to interferon treatment.[16]

The best treatment option for treatment-naive patients has yet to be determined. For this patient group, ongoing randomized studies are comparing lamivudine to interferon and to the combination of the two.

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5.1

MITOCHONDRIAL INJURY:

LESSONS FROM THE FIALURIDINE TRIAL

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Summary

Fialuridine is an antiviral agent with potent activity against hepatitis B virus replication *in vitro* and *in vivo*. In a phase II study 7 of 15 patients experienced severe toxicity due to the drug after 9-13 weeks of treatment. Adverse effects included nausea, vomiting and painful paraesthesia; subsequently hepatic failure, pancreatitis, neuropathy, myopathy and lactic acidosis developed, probably due to multi-systemic mitochondrial toxicity. Possible mechanisms of fialuridine toxicity include mitochondrial injury and pyruvate oxidation inhibition. While other nucleoside analogues have shown evidence of inducing mitochondrial injury (zidovudine, didanosine, zalcitabine), others to date have not (lamivudine, famciclovir). Specific recommendations for future study of existing and new nucleoside analogues include testing for toxicity after prolonged incubation, specific investigations to measure mitochondrial function, toxicological tests and well designed clinical trials with appropriate testing to monitor for any adverse effects on mitochondrial integrity and function.

Introduction

Over the past decade, advances in our understanding of the molecular virology of hepatitis B virus (HBV) have resulted in renewed interest in nucleoside analogues for the treatment of chronic hepatitis B. Most of the first generation nucleoside analogues, such as vidarabine, aciclovir, didanosine, zidovudine and ribavirin, were either ineffective, associated with unacceptable toxicity or both when used to treat chronic hepatitis B. The advent of an *in vitro* cell line (human hepatoblastoma, Hep G2) which propagates the virus led to the development of promising new generation nucleoside analogues.[1] One of these drugs was fialuridine, [1-(2'-deoxy-2'-fluoro-1-β-D-arabinofuranosyl-5-iodo)-uracil], an orally administered pyrimidine nucleoside analogue.[2] Preliminary dose-finding studies showed marked suppression of viral replication during treatment for 2 or 4 weeks.[3] However, prolongation of the treatment period beyond 4 weeks resulted in severe multi-organ toxicity characterized by delayed onset and refractory lactic acidosis, pancreatitis, hepatic failure and death.[4] Available evidence suggested that the mitochondria were damaged by the drug. These untoward findings led to both scientific and procedural investigations. The aim of this discussion, in an attempt to prevent future clinical occurrences as experienced in the fialuridine trial, is to summarize the scientific data and mechanisms of mitochondrial damage and provide recommendations for procedures of future clinical trials and monitoring parameters.

Studies on fialuridine

The clinical trial

The phase II fialuridine trial was designed to evaluate the safety and efficacy of a 6-month treatment course in patients with chronic hepatitis B without decompensation or other serious medical illnesses.[4] Fifteen patients received oral fialuridine 0.10 or 0.25 mg/kg of body weight per day in 2 or 3 divided doses; 11 out of 15 patients had already participated in a study of a 4-week treatment course of fialuridine. Virological response was evident, with HBV-DNA levels dropping more than 90% within 4 weeks. During the first 2 months of treatment, only a few adverse effects were reported; subsequently, increasing fatigue, nausea, numbness and tingling of the feet or hands and abdominal cramps developed in the majority of patients. After 13 weeks, 1 patient experienced sudden-onset hepatic failure, shock and lactic acidosis, and the study was then immediately terminated. During the first few weeks of follow-up, 7 patients exhibited varying degrees of hepatic failure, jaundice, lactic acidosis and biochemical signs of pancreatitis: 5 patients died and the other 2 survived after emergency liver transplantation. None of the patients who had received fialuridine for less than 4 weeks (cumulative dose 200 mg) developed obvious clinical or biochemical signs of toxicity.[4]

Histological analysis of liver explants from the patients who underwent liver

transplantation revealed micro vesicular and macro vesicular steatosis and cholestasis. Steatosis appeared to be an early sign of toxicity because 3 out of 6 patients with mild or no evidence of hepatotoxicity exhibited an increase in steatosis in their liver during treatment.[5] Electron microscopic evaluation of explanted livers showed swollen, misshapen mitochondria with fewer cristae and fat droplets of various sizes. The clinical symptoms of the toxicosis suggested multisystem mitochondrial injury. No data were available on either the levels of mitochondrial-DNA (mt-DNA) or the activity of the mitochondrial enzymes.

Other fialuridine studies

In vitro studies of fialuridine in human hepatoblastoma cell lines showed that the 50% inhibitory dose (ID_{50}) for viral replication was 0.90 μmol/L. The 50% cytotoxic dose (CD_{50}) after 5 days of incubation was 344 μmol/L. These data yield a relatively satisfactory therapeutic index (CD_{50} / ID_{50}) for fialuridine of 382.6.[6]

Preclinical studies with laboratory animals did not predict the toxicity. Dogs and monkeys administered fialuridine 3 mg/kg/day for 90 days and 25 mg/kg/day for 30 days, respectively, did not exhibit any significant histological or biochemical abnormalities.[7]

Pilot studies of fialuridine administered for 2 to 4 weeks to patients with chronic hepatitis B infection or HIV infection did not show hepatotoxicity on initial analysis. Among the 67 patients who participated in these pilot studies, 3 died of liver disease and 1 of pancreatitis within 6 months of completing therapy. Re-evaluation of these cases suggests that these events may have been caused by delayed toxicity of fialuridine.[7]

After the emergence of toxicity, additional studies were performed to elucidate the mechanism of the fialuridine-induced hepatotoxicity. Studies performed by the manufacturer of fialuridine were conducted in rats; the animals were given fialuridine up to 510 mg/kg/day, a dosage 1000 times greater than that administered to humans, for a period of 10 weeks. The animals tolerated the high dose better than expected; however, in the last weeks of the study a significant difference in body weight gain was observed. Liver samples showed significantly lower cytochrome *c* oxidase activity while citrate synthetase activity was increased. Plasma lactate levels were increased as were γ-glutamyl transferase (γ-GT) levels, but ALT and AST levels remained normal.[7]

Lessons of the fialuridine studies

Mechanism of mitochondrial toxicity

The mechanism of toxicity of fialuridine is not yet fully understood, but most evidence points to mitochondrial injury. Various *in vitro* experiments have been performed to evaluate and understand more fully the mitochondrial toxicity of fialuridine.

The triphosphate forms of some antiviral nucleoside analogues are substrates for, and can inhibit the activity of, nuclear-DNA (N-DNA) polymerase-α, -β, -δ and -ε as well as DNA polymerase-γ (pol-γ) the replication of mt-DNA. Neither clinical nor laboratory evidence supports inhibition of N-DNA polymerases by antiviral nucleoside analogues such

as zidovudine, didanosine and zalcitabine. The relatively selective inhibition of DNA polymerase-γ by fialuridine may explain in part its mitochondrial toxicity.[8,9] In addition to the inhibition of DNA polymerase-γ activity, some nucleoside analogues (e.g. fialuridine) can be incorporated into the mt-DNA chain by the action of DNA polymerase-γ.[8,10,11]

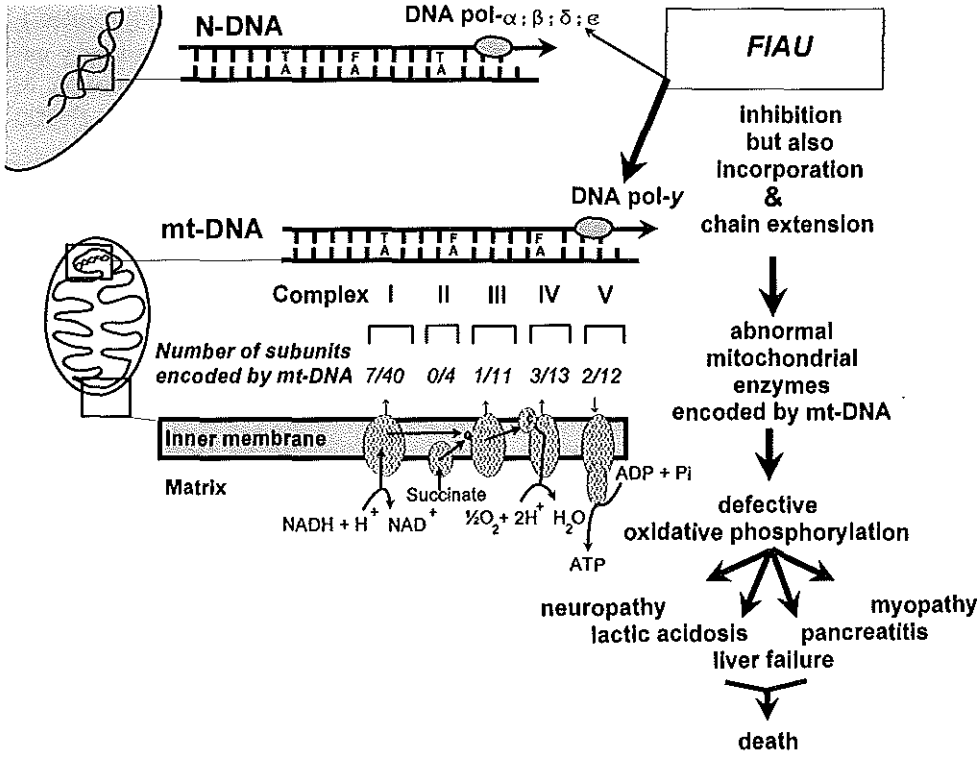

Figure 1. *Hypothesized mechanism by which fialuridine causes mitochondrial dysfunction and related clinical problems. Abbreviations: Complex I= NADH-CoQ reductase; Complex II= succinate-CoQ reductase; Complex III= ubiquinol cytochrome c reductase; Complex IV= cytochrome c oxidase; Complex V= ATPase; mt-DNA= mitochondrial DNA; N-DNA= nuclear DNA.*

Fialuridine replaces thymidine in both N-DNA and mt-DNA. The higher affinity of DNA polymerase-γ for fialuridine, compared with the nuclear polymerases, suggests that fialuridine is incorporated into mt-DNA at a higher rate.[10,12] In contrast to other nucleoside analogues such as zidovudine, fialuridine possesses a 3'-hydroxyl group; therefore, the nascent mt-DNA chain can extend beyond the inserted fialuridine (figure 1). This is probably an essential difference between fialuridine and other nucleoside analogues as far as the induction of severe mitochondrial injury is concerned. This may also explain why the cellular content of mt-DNA remained normal after incorporation of fialuridine, while

the mitochondrial function deteriorated in HepG2 cells.[13,14] Since no mt-DNA editing system is known, mt-DNA damage is considered permanent.[11,15]
It is likely that in the event of prolonged treatment, incorporation of fialuridine into mt-DNA leads to both alterations in the transcription of mt-DNA and defective mitochondrial polypeptide synthesis (figure 1). Most of the subunits in the respiratory chain (complex I, III, IV and V), all located in the mitochondrial inner membrane, are partly encoded by mt-DNA.[16,17] Impaired oxidative phosphorylation by mitochondria leads to stimulation of anaerobic glycolysis to maintain cellular ATP levels. *In vitro* experiments with HepG2 cells showed increased lactate levels after exposure to fialuridine, the increase being dose-dependent.[13,14] The consequence of the defective synthesis of several key mitochondrial enzymes is the development of certain disorders, some of which are similar to those encountered in inherited diseases attributable to mt-DNA mutations (such as myopathy, neuropathy, lactic acidosis and liver failure).[18] The brain remains unaffected in the fialuridine toxicity syndrome while pancreatitis is uncommon in the group of inherited diseases.
Characteristic for mitochondrial mutations is the threshold effect.[18] During accumulation of mutant mt-DNA, the phenotype will be unaffected until the threshold for expression is reached. At that point, major changes in phenotype occur even in the event of small changes in the amount of mutant mt-DNA. Apparently, a low percentage of wild type mt-DNA is enough to maintain adequate synthesis of mitochondrial gene products. It is likely that this threshold effect also plays a role in fialuridine toxicity.[18]
Although current data suggest that this toxicity may be due to DNA polymerase-γ inhibition and nucleoside analogue incorporation, as summarized in figure 1, several studies have provided evidence that other scenarios might also be possible. For example, studies of HepG2 cells have confirmed the effect of fialuridine on mitochondrial function (lactate levels exceeded by 500% those found for normal controls) but this was not caused by a decreased mt-DNA level and complex IV activity.[19] This implies that in these relatively short cell studies the oxidation of pyruvate was inhibited by a mechanism other than depletion of mt-DNA or complex IV deficiency. Likely candidates are N-DNA encoded gene products, such as pyruvate dehydrogenase, or the N-DNA encoded subunits of the respiratory chain. In order to distinguish between these two possibilities, pyruvate should also be assayed because in the former case, the lactate / pyruvate ratio will be much lower.[20,21]

Toxicity of other nucleoside analogues in clinical studies of hepatitis B virus
Investigation of other nucleoside analogues (table 1) revealed examples of mitochondrial toxicity. Long term treatment with zidovudine (AZT, 3'-azido-3'-deoxythymidine) induced myopathy, with a prevalence of up to 20%, which generally improved when treatment was discontinued. Muscle biopsies from these patients showed the hallmark of mitochondrial dysfunction, ragged red fibers. Histochemical staining of complex IV showed a decrease in activity,[22] and the amount of mt-DNA was decreased.[23] Zidovudine also affected mitochondria in the liver, resulting in morphological changes such as steatosis and swollen

mitochondria as demonstrated by electron microscopy.[24] Other nucleoside analogues, such as didanosine (ddI, 2',3'-dideoxyinosine) and zalcitabine (ddC, 2',3'-dideoxycytidine), also affect the mitochondrial system since both caused peripheral neuropathy and occasionally fulminant hepatic failure with steatosis, pancreatitis and lactic acidosis.[25,26]

New nucleoside analogues, such as lamivudine and famciclovir, are now being investigated in clinical studies, investigating the treatment of chronic hepatitis B infection. Famciclovir is the well-absorbed oral form of penciclovir, an acyclic guanine derivative, and is widely used to treat varicella zoster and herpes simplex virus infections. Recently, activity against hepatitis B virus (HBV) replication was observed.[27,28,29] Phase III studies of the efficacy in chronic hepatitis B, as well as the prevention of reinfection after liver transplantation, are ongoing. To date, no signs of mitochondrial toxicity during famciclovir treatment have been observed.[30]

Lamivudine, the (-) enantiomer of 2'-deoxy-3'-thiacytidine, is another new nucleoside analogue that strongly inhibits HBV replication both *in vitro*[31] and *in vivo*.[32,33] Again, thus far neither clinical nor sub-clinical mitochondrial toxicity has been observed during long term treatment.[34]

Table 1. *Nucleoside analogues for treatment of chronic hepatitis B.*

Generic name	Abbreviation	Proprietary name	Toxicity
Vidarabine	ARA-A	Vira-A	myalgia, neuropathy
Acyclovir	ACV	Zovirax	neuropathy
Ganciclovir	DHPG	Cymevene	neutropenia
Zidovudine	AZT	Retrovir	myopathy, steatosis
Ribavirin	RTCA	Virazole	hemolytic anaemia
Didanosine	ddI	Videx	neuropathy, lactic acidosis
Zalcitabine	ddC	Hivid	neuropathy
Fialuridine	FIAU	-	multi organ failure
New nucleoside analogues in clinical testing for chronic hepatitis B			
Lamivudine	3TC/SddC	Epivir	not reported
Famciclovir	FCV	Famvir	nephrotoxicity
Phosphonylmethoxyethyladenine	PMEA	Adefovir	not thoroughly studied
Deoxythiafluorocytosine	FTC	-	not thoroughly studied
Carbodeoxyguanosine	2-CDG	-	not thoroughly studied

Organizational aspects and clinical implications for the future

Several nucleoside analogues with potent activity against HBV are in the phase of experimental administration to patients with chronic hepatitis B. After a review of the fialuridine trial, the Institute of Medicine in the US has made recommendations[7] which allow clinical research to proceed but also minimize the risks inherent in new drug therapies.[7]

One of the recommendations of the US Institute of Medicine was to continue research into the mechanism of fialuridine toxicity. Based on these new findings, some additional recommendations for the preclinical testing of related drugs can be proposed.

General recommendations

The efficacy and toxicity of antiviral agents should be assessed in cell culture systems.[7] In most studies, cytotoxicity is measured after relatively short incubation periods. A recently published study on antiviral agents in HBV-transfected cell lines showed considerable changes for some agents in the CD_{50} between 4 and 12 days of incubation.[35] Cytotoxicity evaluated at day 4 may give an underestimation of the toxic effect on cellular metabolism and thus, an overestimation of the therapeutic index. Therefore, prior to clinical testing, candidate antiviral agents should also be tested for toxicity after prolonged incubation. A therapeutic index of more than 1000 has been suggested as a 'safe' index.[35]

Drugs with possible mitochondrial toxicity

Based on the research that has been done into the mechanism of fialuridine toxicity, in the case of nucleoside analogues which might possibly induce mitochondrial toxicity, cell culture studies should be performed to evaluate the effect of the nucleoside analogue on inhibition of DNA polymerase-γ, incorporation into N-DNA and/or mt-DNA and the total amount of mt-DNA.[10,13,14,19] Probably more important are assays of mt-DNA-encoded enzymes in relation to N-DNA-encoded enzymes and functional mitochondrial tests, such as assays of lactate and pyruvate production from glucose or ATP synthesis from mitochondrial substrates.[36,37]

Prolonged testing in animals remains important; 2 different species should be used.[7] Toxicological studies, using the route of administration intended for patients and a duration of treatment at least as long as that intended for clinical trials, and extended follow-up of at least a subsample of animals, should be key aspects of preclinical testing. All information from *in vitro* and animal studies must always be accessible.[7]

Trial design and performance

When the drug is considered to be safe, on the basis of *in vitro* and *in vivo* tests, clinical studies can be performed in a few experienced centres.[7] Preferably, the study should include patient controls to help differentiate drug effects from changes caused by the underlying disease.[7] Follow-up should last at least 6 months, especially for drugs which can modify nucleic acids and thus, possibly induce late toxicity.[7] During the study period, the trial sponsor should inform the investigators about all known adverse events on a continuing real-time basis. Similarly, the investigators should report all adverse events to the sponsor cumulatively, including all unexpected adverse events as well as all serious adverse events. A careful analysis of all available information (rather than a worst-case assumption) should then determine further actions.[7] It is important that patients included in the study are also made aware of possible and predictable adverse effects that may occur after discontinuation of the drug under study.

Additional testing recommendations

We recommend that during initial studies of new nucleoside analogues, regular assays of plasma lactate levels be performed and that, in case of abnormal results or other signs of mitochondrial dysfunction, a liver biopsy be taken. Histological studies should focus on staining of fat droplets and electron microscopic evaluation of mitochondrial ultrastructure, since the accumulation of fat droplets and abnormal mitochondrial ultrastructure, as well as a decreased number of cristae, were found to be relative early signs of mitochondrial toxicity in the fialuridine trial.[4,5] If possible, complex I and IV and a mitochondrial N-DNA-encoded marker enzyme in liver homogenate should be assayed. Serological markers for mitochondrial myopathy (creatine kinase, lactate, pyruvate, acetoacetate and 3-hydroxy butyrate) and pancreatitis (amylase and lipase) must be assessed in the event of symptoms because they are usually involved in a later stage of the disease.

Conclusion

Laboratory and clinical studies to discover new approaches to the treatment of viral diseases should be continued. At the same time everyone involved in such investigations should be aware of the potential toxicity of these new drugs despite preclinical evidence of safety. By following this approach, important preclinical findings with nucleoside analogues can be transformed into safe clinical studies for the benefit of patients with chronic viral infections.

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5.2

EFFECT OF LAMIVUDINE ON MORPHOLOGY AND FUNCTION OF MITOCHONDRIA IN PATIENTS WITH CHRONIC HEPATITIS B

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Summary

Nucleoside analogues can induce mitochondrial dysfunction leading to severe clinical syndromes. Lamivudine, a new nucleoside analogue, is an active inhibitor of hepatitis B viral replication without apparent clinical toxicity. To assess subclinical mitochondrial toxicity, we studied the morphology and function of the mitochondrial system in 15 patients treated with lamivudine. Morphology was investigated by routine histological evaluation and electron-microscopic studies of mitochondria in liver biopsies. Mitochondrial function was assessed by 2-keto[1-^{14}C] isocaproic acid decarboxylation (KICA breath test) and by measuring the activity in liver biopsy specimens of the mitochondrial enzymes encoded by nuclear and mitochondrial DNA (mt-DNA) (Complex I and IV) as well as a mitochondrial and a cytosolic enzyme both encoded by nuclear DNA only (Complex II and lactic dehydrogenase [LDH]).

All 15 patients underwent a liver biopsy before treatment and a KICA breath test before and during treatment; 13 agreed to undergo a repeat liver biopsy during lamivudine treatment. Liver tissue with no or minimal fibrotic changes from 7 patients treated for 6 months with lamivudine was suitable for assessment of the mitochondrial enzyme activity.

We observed no signs of toxicity by routine histological or electron-microscopic evaluation. KICA breath tests revealed no differences in either peak exhalation or the area under the curve from 0 to 60 minutes between healthy controls (3.0% and 19.3%), untreated patients with chronic hepatitis B (3.4% and 19.3%), and patients treated with lamivudine (3.1% and 20.6%). The activities of the mt-DNA-encoded enzymes remained normal after lamivudine therapy. Unexpectedly a significant decrease in the activity of nuclear-DNA-encoded enzymes in patients with chronic hepatitis B in comparison with normal controls was found. The mean activity of Complex II dropped from 45.3 to 20.0 μmol.min^{-1}, that of lactic dehydrogenase from 106 to 44 μmol.min^{-1} (Wilcoxon rank sum $p<0.05$).

In conclusion: No subclinical signs of mitochondrial toxicity resulting from lamivudine therapy for 6 months were observed.

Introduction

Nucleoside analogues can induce mitochondrial toxicity.[1] The clinical manifestations of mitochondrial toxicity include myopathy, neuropathy, lactic acidosis, pancreatitis, and hepatic failure.[2] Recently *in vitro* and *in vivo* models of hepatitis B viral (HBV) infection led to the identification of new nucleoside analogues with marked inhibitory activity against HBV: fialuridine, lamivudine and famciclovir.[3] A phase 2 trial with fialuridine for patients with chronic HBV infection was terminated because of severe clinical multisystemic toxicity attributed to mitochondrial damage.[4,5] *In vitro* studies indicated that fialuridine had only a minimal effect on the amount of mitochondrial-DNA (mt-DNA).[6,7] Incorporation of fialuridine in mt-DNA led to abnormalities of enzymes encoded by mt-DNA.

Lamivudine, the negative enantiomer of 2'-deoxy-3'-thiacytidine, markedly inhibits HBV replication both *in vitro*[8] and *in vivo*.[9,10] At present clinical toxicity has not been observed during treatment for periods up to 1 year, but its effects on mitochondrial morphology and function have not yet been reported.

The early clinical symptoms of mitochondrial toxicity are nonspecific complaints, such as fatigue, nausea, or muscle weakness. Biochemical markers of mitochondrial dysfunction, such as serum amylase, lipase, creatine phosphokinase (CPK), and lactate levels, only become abnormal when severe organ damage already exists. Histological analysis of patients with nucleoside analogue-induced toxicity (zidovudine, fialuridine) reveals moderate to marked steatosis and cholestasis. During experimental treatment with new nucleoside analogues, it is important to be able to detect mitochondrial dysfunction at an early stage. For this purpose we elected to test the mitochondrial integrity on two levels: mitochondrial function was assessed by the activity of functional mitochondrial enzymes, and morphology by the presence of steatosis and electron-microscopic evaluation of the mitochondria during a 6-month course of lamivudine.

Patients and methods

Study population

Fifteen patients with chronic (hepatitis B e antigen [HBeAg] positive) HBV infection with detectable HBV-DNA (>3 pg/ml, Genostics, Abbott Laboratories, Chicago, IL) and elevated alanine aminotransferase (ALT) levels (>30 IU/l) were treated with lamivudine. They all had mild disease and participated in a 24-week dose ranging (25, 100, or 300 mg/day) study of lamivudine; two patients were treated for only 12 weeks because of unforeseen pregnancy and abdominal discomfort, respectively. All patients underwent a liver biopsy before treatment; for safety purposes another liver biopsy was obtained after 8 weeks (n=4), 12 weeks (n=3), 16 weeks (n=4) or 24 weeks (n=2) of therapy. Two patients refused to undergo repeat liver biopsy during treatment.

Table 1: *Demographic and baseline characteristics of the patients.*

Patients (n)	15
Gender (M/F)	12/3
Age (mean, range)	32.4 (18-61) years
Interferon non-responder (n)	8
Caucasian/Asian/Black (n)	11/3/1
ALT (mean, range)*	66 (32-188) IU/l
HBV-DNA (mean, range)**	169 (4-520) pg/ml
HBeAg (mean, range)***	36579 (168-158,716) U/l

* Uper limit of normal 30 IU/l
** Standard hybridization technique (Genostics, Abbott)
*** Microparticle Enzyme Immunoassay (IMx, Abbott)

Controls

Pretreatment liver biopsy specimens were used as disease controls when possible; otherwise patients with chronic hepatitis B who fulfilled the inclusion criteria but did not enter the lamivudine study for other reasons served as disease controls. Macroscopic and microscopic normal liver tissue from four patients who underwent hemi-hepatectomy for small metastases served as the normal controls.

Morphology

Liver tissue was taken by standard tru-cut needle and fixed in 4% phosphate-buffered formaldehyde, routinely processed and embedded in paraffin blocks; 5 μm sections were then cut. Liver sections were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), with or without previous diastase digestion, and Van Gieson's stain. All liver biopsy specimens were scored blindly by two observers who used the Desmet modification of the Knodell scoring system.[11,12] Steatosis was scored semiquantitatively as none, mild, moderate, or severe.

Electron-microscopic evaluation of the mitochondria in liver biopsy specimens from nine patients was performed during treatment. Four patients with chronic (HBeAg positive) HBV infection were used as controls. Liver tissue was fixed first in glutaraldehyde-formaldehyde and then in 1% (wt/vol) osmium tetroxide at 4 °C. After acetone dehydration, the specimens were embedded in LX 112 (Epon, LADD [Zeiss], Weesp, The Netherlands). Semi-thin plastic sections for light microscopy were stained with a freshly prepared 1% toluidine blue solution in distilled water. Ultrathin sections (ultratome IV, UB, LKB, Bromma, Sweden) were mounted on copper grids (300 mesh) and contrast stained with uranyl acetate (10 minutes at 45 °C) and lead citrate. The preparations were examined with a Zeiss 902 electron microscope (Zeiss, Weesp, The Netherlands). Magnifications of 3,000, 7,000, 12,000 and 20,000 were used to visualize the

mitochondrial morphology. Pictures were evaluated blindly for abnormalities in mitochondrial density, cristae, smooth and rough endoplasmic reticulum (SER, RER), lysosomes and the lateral membranes, as described previously.[13]

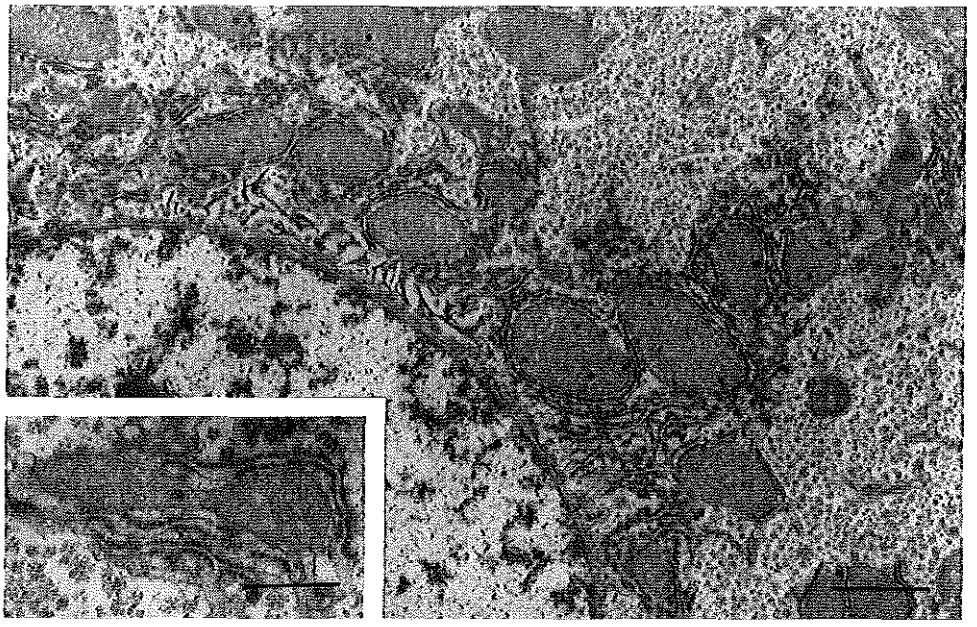

Figure 1. *Electron micrograph of a hepatic parenchymal cell from a patient with chronic HBV infection; at the time of the liver biopsy the patient had received with a cumulative dose of 33.6 g lamivudine (300 mg/day for 16 weeks). Nucleus, mitochondria, smooth endoplasmic reticulum, and rough endoplasmic reticulum show no ultrastructural abnormalities. Bar: 1μm.*
Inset: detail of a mitochondrion of the same patient showing intact cristae. Bar: 0.58μm.

Function

KICA-breath test. For early detection of impaired metabolic capacity of the mitochondrial system we used the 2-keto[1-^{14}C] isocaproic acid (KICA) breath test, as described by Lauterburg et al.[14] This breath test has been used as a noninvasive test of mitochondrial function for patients with alcoholic liver disease. After an overnight fast the subjects, who rested for 30 minutes before as well as during the test, received 1μCi KICA together with 20 mg/kg L-leucine dissolved in 200 ml orange juice. Breath samples were obtained before and at 10-minute intervals during the test for 120 minutes. The exhaled $^{14}CO_2$ was quantified by liquid scintillation spectroscopy. All patients were tested before treatment, at

the time a liver biopsy was taken during treatment, and at the end of lamivudine therapy. To confirm its diagnostic value the test was also administered to seven alcoholic patients as positive controls and eight healthy persons as negative controls. The reproducibility for healthy controls was measured by repeating the test 1 week later. We analyzed the peak exhalation, of $^{14}CO_2$, the time to reach peak exhalation and the area under the $^{14}CO_2$ exhalation curve (AUC) after 60 and 120 minutes (expressed as percent dose exhaled).

Mitochondrial enzyme activities in liver tissue. Seven patients with no or minimal morphological changes (fibrosis stage 0 or 1)[11] at entry were eligible for the study of mitochondrial enzyme activity during lamivudine treatment. All timepoints of biopsy were presented and the cumulative dose of lamivudine at the time of biopsy ranged from 1.4 to 50.4 g (median 11.2 g). Liver tissue was snap-frozen and stored in aluminium tins at -80°C. Liver specimens of 5 to 20 mg were weighed, thawed, and homogenized with 0.5 ml 0.25 mol/l sucrose, 10 mmol/L HEPES-KOH and 1 mmol/l ethylenediaminetetraacetic acid (EDTA) (pH 7.4) in a tight-fitting, motor-driven (800 tpm) Potter-Elvehjem Teflon-glass homogenizer by 10 strokes at 0°C. The homogenates were divided into small batches, frozen, stored at -84°C and thawed immediately before the assays, which were performed at 37°C.

Complex I was measured after two extra freeze-thaw cycles as the rotenone-sensitive reduced form of nicotinamide adenine dinucleotide (NADH) decylubiquinone oxidoreductase.[15] Succinate dehydrogenase (Complex II) was assessed as succinate-ferricenium oxidoreductase in 50 mmol/l potassium phosphate (pH 7.5), 0.2 mmol/L ferricenium (10 μl of 10 mmol/l ferricenium hexafluorophosphate in 10 mmol/l HCl in a volume of 0.5 ml) and 10 mmol/l sodium succinate.[16] The blank contained 10 mmol/l sodium malonate and no succinate. The reaction was started with 5 to 10 μl liver homogenate and absorbancy was measured at 300 nm. Complex IV was measured as horse heart ferrocytochrome *c* oxidase.[17] Lactate dehydrogenase (LDH) was assayed according to Kornberg,[18] but with 1 μmol/l rotenone and total carnitine,[19] after alkaline hydrolysis for 1 hour at 56°C. LDH is a cytosolic enzyme that oxidizes lactate to pyruvate, a reaction enhanced by a low NADH/NAD^+ in the liver. Carnitine, a small zwitter-ion, is an amino-acid essential for transport of activated fatty acids from the cytosol into the mitochondria where oxidation occurs.[20] We also measured the total amount of protein in the homogenate. All enzyme activities are expressed as μmol substrate converted per minute per gram liver tissue. Complex IV is expressed as the first-order rate constant *k* per minute per gram.

Statistical analysis

We tested the hypothesis that within the study group no differences existed between the values for healthy controls and patients with chronic hepatitis B before and during treatment with lamivudine. For continuous variables we used the Wilcoxon signed rank test. Results are given as mean ± standard error of the mean (SEM). For patients who underwent a KICA breath test or a liver biopsy before and during treatment, a subgroup analysis was performed with the Student's *t*-test for paired samples.

Ethics
This study was approved by the institutional review board, and all patients gave written informed consent.

Results

Baseline characteristics of the patients are shown in table 1. All 15 patients became HBV-DNA (Genostics, Abbott) negative during treatment and two patients became HBeAg-negative (Genostics, Abbott). All patients had elevated serum alanine aminotransferase (ALT) levels at entry (mean ± SEM: 66 (±11) IU/l); serum ALT decreased during therapy in five patients (33%) to the normal range (≤30 IU/l).

Morphology.
Standard histological evaluation of pretreatment liver biopsy specimens showed minimal macro vesicular steatosis in three patients that did not change during treatment. The remaining 12 patients did not have appreciable steatosis by liver biopsy either before or during treatment. The mean (± SEM) histology activity index (HAI) was 4.4 ± 0.8 before treatment and decreased to 2.8 ± 0.5 during treatment (table 2); piecemeal necrosis in particular improved significantly during treatment.[21]

Table 2. *Histological grading, staging and morphology in 13 patients before and during treatment with lamivudine.*

	Before treatment	During treatment
Histology Activity Index (mean score)	4.4 ± 0.8	2.8 ± 0.5
Fibrosis (mean score)	1.4 ± 0.4	1.4 ± 0.4
Steatosis (number of patients)*	none	none
Electron-microscopic abnormalities	none	none

* Minimal macro vesicular steatosis was observed in three patients but was not enough to score as mild steatosis (score 1) and did not change during treatment

Electron-microscopic evaluation of mitochondria (including density, cristae, smooth endoplasmic reticulum, rough endoplasmic reticulum, lysosomes and lateral membranes) revealed no differences between untreated patients and those treated with lamivudine. Moreover no individual patient showed significant morphological changes after treatment with lamivudine (figure 1). Virus particles were not detected by electron microscopy in any patient.

Function.

The *KICA breath test* was validated in healthy controls and patients with alcoholic liver disease. For healthy controls peak exhalation and area under the curve at 60 minutes (mean ± SEM) were 3.0 ± 0.16% and 19.3 ± 1.4%, respectively. Reproducibility within the group of healthy controls showed a variation of 5.1% for peak exhalation and 0.8% for area under the curve at 60 minutes. The ability of the KICA breath test to detect abnormal mitochondrial function was tested in seven patients with alcoholic liver disease. In alcoholic patients KICA decarboxylation in the liver was impaired, resulting in a lower peak exhalation and a lower fraction of the dose exhaled after 60 minutes (2.5 ± 0.3% and 13.4 ± 1.8%, respectively). The KICA breath test revealed no differences between the healthy controls and the patients on lamivudine (table 3). There were no significant differences in either the pretreatment, during treatment or posttreatment breath analysis separated according to dose of lamivudine. When the initial KICA was taken as the individual reference value, no patient had an abnormal KICA breath test at the end of treatment.

Table 3. *Results of KICA breath test for healthy controls and patients with chronic hepatitis B before and during treatment with lamivudine.*

	Healthy controls (n=8)	Patients: pre-treatment (n=15)	Patients: end of treatment (n=15)
Peak exhalation (%)	3.0 ± 0.16	3.4 ± 0.26	3.1 ± 0.16
Time of peak (min)	38.7 ± 3.0	42.7 ± 4.6	35.3 ± 3.4
AUC 60 (%)	19.3 ± 1.4	21.5 ± 2.5	20.6 ± 1.5
AUC 120 (%)	24.1 ± 1.4	26.9 ± 2.6	25.4 ± 1.6

Note: Results expressed as % of dosage $^{14}CO_2$ exhaled in 10 minutes.
AUC 60 means area under the $^{14}CO_2$ exhalation curve 0 to 60 minutes after intake.

The *activities of mitochondrial enzymes* are shown in table 4 and figure 2. In patients treated with lamivudine the activities of mt-DNA-encoded enzymes were not different from those of patients with chronic hepatitis B before treatment. Among untreated chronic hepatitis B patients the activities of the enzymes encoded by nuclear-DNA (N-DNA) was lower than those in controls; the activity of Complex II in patients with chronic hepatitis B being 45% of that of normal controls and LDH activity 42% of that of normal controls. Carnitine levels in patients with chronic hepatitis B were 65% of that of normal controls. The activity of Complex I and the total amount of protein were also lower in patients with chronic hepatitis B but the values were not significantly different from those found for normal controls.

Discussion

This study was initiated to evaluate the potential of mitochondrial toxicity as a result of lamivudine treatment. In several phase I and II studies of lamivudine, there were no instances of clinical symptoms which might be related to mitochondrial toxicity (lactic acidosis, pancreatitis or liver failure). To detect mitochondrial toxicity at a subclinical level in patients who were exposed to long-term lamivudine therapy we studied the morphology and function of the mitochondrial system prospectively in a closely followed cohort of 15 patients.

Histological evaluation of liver biopsy specimens showed no signs of mitochondrial dysfunctioning, such as steatosis by light microscopy or abnormalities in the morphology of the mitochondria by electron microscopy. Indeed, liver histology improved during lamivudine therapy. In patients with hepatic failure because of fialuridine, histological analyses revealed moderate to marked steatosis and cholestasis and electron microscopy showed mitochondrial abnormalities and accumulation of small fat droplets.[4] In our group of patients who received up to 6 months of lamivudine these signs of mitochondrial damage were not detected.

The KICA breath test has been used for assessment of mitochondrial dysfunction in patients with chronic alcoholic liver disease. KICA decarboxylation occurs mainly in mitochondria and is catalyzed by mitochondrial branched-chain 2-ketoacid dehydrogenase and thus reflects more than decreased liver cell mass as is assessed by the aminopyrine breath test and the galactose elimination test.[14] In the rat more than 75% of the exhaled $^{14}CO_2$ after KICA administration originates in the liver,[22] suggesting that the KICA breath test reflects hepatic mitochondrial function. The decreased KICA decarboxylation found for patients with chronic alcoholic liver disease is believed to reflect functional impairment of the mitochondrial system as a result of ethanol toxicity. The KICA breath test is one of the few noninvasive tools available to quantify mitochondrial function and was therefore used to exclude impaired capacity of metabolism by the mitochondrial system because of lamivudine therapy. The KICA breath test revealed no differences between healthy controls and patients with chronic hepatitis B either before or during treatment. Using the initial KICA breath test result as an individual control, none of the patients developed abnormalities in KICA breath test at the end of treatment. KICA decarboxylation by the mitochondria was not suppressed during lamivudine treatment.

To further evaluate the possibility of subclinical mitochondrial toxicity, we studied the activity of mitochondrial enzymes involved in oxidative phosphorylation. We chose to do a functional analysis instead of measuring the amount of mt-DNA which can be normal despite of severe toxicity.[6,7,23] The respiratory chain is divided into five functional units or complexes, embedded in the inner mitochondrial membrane. Four of them (Complex I, III, IV and V) are partially encoded by mt-DNA.[24,25]

This biochemical study covered a small group of patients with mild compensated liver disease who received lamivudine for 6 months; all patients with significant fibrosis or

Table 4. *Mitochondrial enzyme activity in patients with chronic hepatitis B with or without treatment with lamivudine in comparison to healthy controls.*

Enzyme, Compound	Location	Number of subunits encoded by mt-DNA	Normal controls (n=4)	Patients: pretreatment (n=7)	Patients: during treatment (n=7)
Complex I (μmol.min^{-1})	Mitochondrial inner membrane	7	5.7 ± 0.8	4.3 ± 0.6	6.4 ± 0.9
Complex IV (k.min^{-1})	Mitochondrial inner membrane	3	16.3 ± 2.6	36.4 ± 11.5	29.3 ± 3.8
Complex II (μmol.min^{-1})	Mitochondrial inner membrane	0	45.3 ± 3.6	20.2 ± 3.0	26.9 ± 2.0*
LDH (μmol.min^{-1})	Cytosol	0	106 ± 16	44 ± 4.5	48 ± 3.9*
Carnitine (μmol)	Mainly cytosol		0.72 ± 0.08	0.47 ± 0.07	0.57 ± 0.07
Protein (mg)			159 ± 5	121 ± 16	143 ± 9

Note: Results are expressed per gram liver tissue.

*Different from normal controls with $p<0.05$ (Wilcoxon ranksum).

cirrhosis were excluded to prevent variations in activity because of fibrotic tissue. The biochemical analysis showed no evidence that lamivudine treatment affected the mitochondrial system, as indicated by the normal activity of the mt-DNA-encoded enzymes (Complex I and IV). Patients with chronic hepatitis B and active viral replication showed subnormal activity of the mitochondrial enzymes, especially enzymes encoded by N-DNA. Protein levels were also decreased in these patients. One explanation for these findings is that active HBV replication, with the production of large amounts of hepatitis B surface antigen (HBsAg) and HBeAg proteins in hepatocytes, competitively inhibited the transcription of other proteins.[26] It was of interest to note that the activity of some mitochondrial enzymes appeared to be restored during lamivudine treatment, especially in the five patients who underwent paired liver biopsies before and during treatment (figure 2), although the differences were not statistically significant, possibly due to the small number of patients (n=5, p=0.08 for Complex I).

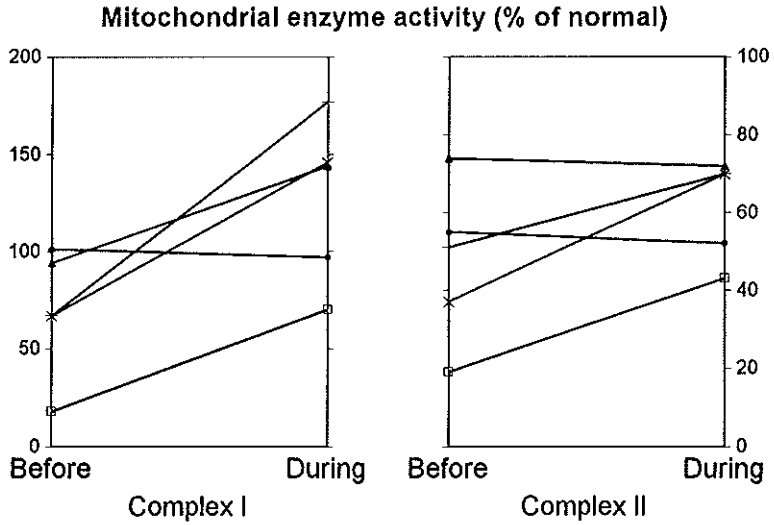

Figure 2. *Improvement in mitochondrial enzyme activity found for the five patients who underwent paired liver biopsies before and during treatment. Shown are the activities of Complex I (encoded by N- and mt-DNA) and Complex II (encoded by N-DNA). Results are expressed as percentage of normal.*

Lamivudine is known to block HBV replication[8] that might result in partial normalization of the mitochondrial function. Additional possibilities are that the activity of the mitochondrial enzymes is decreased as a result of liver cell necrosis following inflammation because of the HBV infection. As a 6-month course of lamivudine resulted in normalization of serum transaminases in 47% to 87% of patients in a multicentre study.[27]

In conclusion, normal mitochondrial morphology, normal KICA breath test results and

normal levels of mt-DNA-encoded enzymes indicate that it is unlikely that lamivudine treatment induces mitochondrial toxicity. In patients with chronic HBV infection and active viral replication, the activities of N-DNA-encoded enzymes were suppressed. Lamivudine treatment appeared to restore mitochondrial enzyme activity, possibly because of a decrease in the viral replication or general hepatocellular injury.

Acknowledgments

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6.1

HBV REACTIVATION WITH HEPATIC DECOMPENSATION AFTER LAMIVUDINE THERAPY FOR CHRONIC HBV INFECTION

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Introduction

Lamivudine is an effective reverse transcriptase inhibitor and also a potent inhibitor of hepatitis B virus replication *in vivo*.[1,2,3,4] In view of its efficacy and remarkable safety profile, large scale phase III trials are ongoing. We report a serious adverse event in a patient 5 months after a 6 months course of lamivudine, which may be associated with drug therapy.

Case

A 29-year-old Chinese man with chronic HBV infection (biopsy stage chronic hepatitis with minimal fibrosis, HBcAg in 30% of hepatocytes, HBeAg positive, HBV-DNA positive, ALT 7x ULN), who had received 1 standard course of alpha-interferon therapy and 2 previous courses of experimental antiviral therapy between 1990-93, started on lamivudine 100 mg/day in January 1994. He completed 6 months of therapy without any signs of side effects; HBV fell below levels of detection (Genostics, Abbott) at 2 weeks after initiation of therapy, HBeAg fell to a level just above detection-limit (IMx, Abbott) and serum aminotransferase levels became normal after 16 weeks of therapy. A liver-biopsy at 16 weeks showed chronic hepatitis with some lobular activity; HBcAg was not detected. Four weeks after discontinuation of lamivudine therapy ALT levels increased and rose exponentially to 100x ULN 4 months later, associated with jaundice and a fall in clotting factors below 50% (incipient liver failure). Laparoscopy with liver-biopsy showed hepatic collapse; no evidence of cirrhosis was found. Coinfection with hepatitis A or superinfection with hepatitis D or hepatitis C was excluded. HBV serology showed a resurgence of active viral replication (HBV-DNA and HBeAg positive) from week 28-32 onwards (figure 1).

Assuming a severe immunological reaction to hepatocytes infected with HBV, prednisone treatment was started with an initial dose of 30 mg/day. A marked drop in bilirubin and ALT followed, clotting factors returned to pre-treatment levels. Prednisone was decreased to 10 mg/day and stopped when HBV-DNA levels rose again. After stopping prednisone, hepatitis activity increased somewhat followed by HBV-DNA and HBeAg clearance; anti-HBe antibodies have appeared. Currently, the patient is well and has completely normal liver-tests.

The temporal relation of increasing viral replication from week 28 and rising serum ALT also from week 28 strongly suggests that the severe hepatitis flare is a consequence of re-infection of hepatocytes with immune reaction, following suppression of HBV replication by lamivudine. To date, 83 chronic hepatitis B patients have been treated with lamivudine for periods of 3 to 6 months. Post-treatment elevation of ALT (an elevation of $\geq$ 3 times baseline) occurred in 16% of patients within 8 to 24 weeks (median 12 weeks) of treatment cessation. Only 3 cases of ALT elevations were associated with hyperbilirubinaemia or jaundice and, with the exception of the case described above, resolved spontaneously.

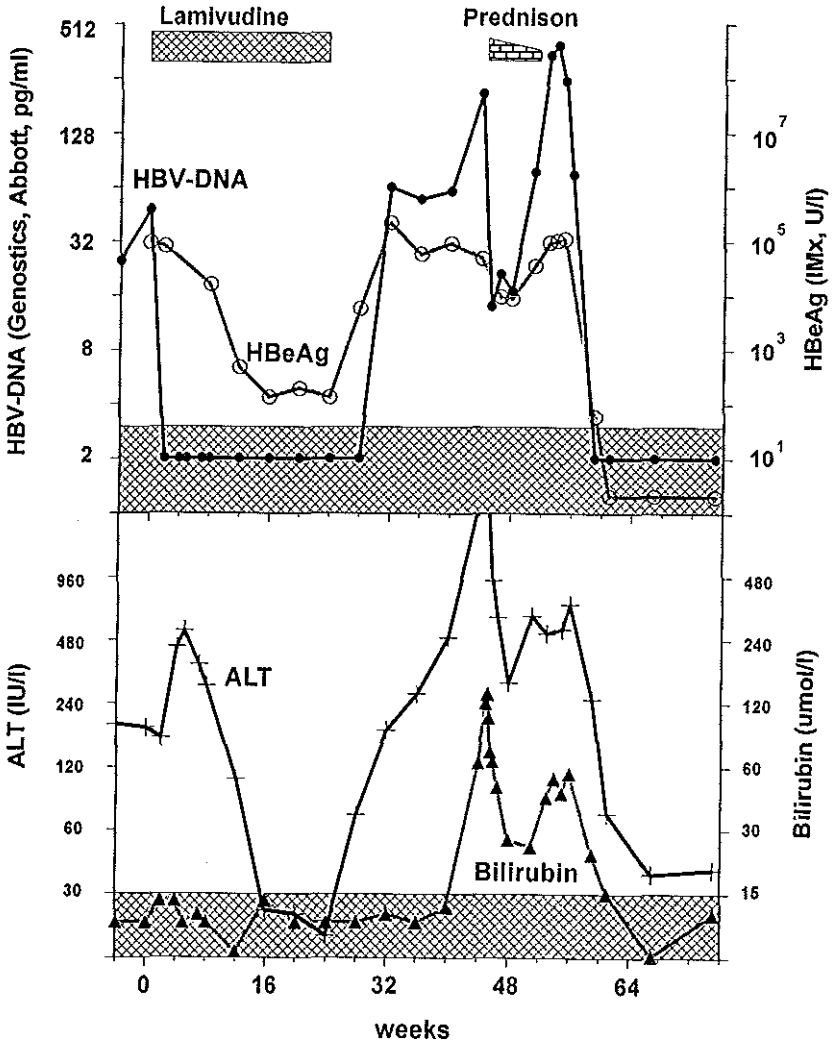

Figure 1. *Changes in ALT, HBV-DNA, HBeAg and bilirubin after withdrawal of lamivudine therapy.*

However, spontaneous hepatitis flare has been described in chronic hepatitis B in particular in patients from East Asia[5] and the relation lamivudine therapy - hepatitis flare cannot be proved. On the other hand, liver-biopsy at the end of therapy showed absence of HBcAg in hepatocytes, and we assume massive reinfection of hepatocytes in the months after stopping lamivudine and an immune reaction similar to that of acute hepatitis subsequently.

In this patient the severe hepatitis flare was not fatal and subsequently led to HBeAg seroconversion, but such an event in a patient with cirrhosis could have grave consequences. Patients participating in lamivudine studies should be monitored carefully after discontinuation of therapy. When clinical signs of hepatitis occur, rapid and thorough investigation in a hepatological unit is advised and immunosuppressive therapy may be indicated. Frequent monitoring should continue until the flare resolves and the replication of HBV is controlled.

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6.2

THE MANAGEMENT OF SEVERE ACUTE EXACERBATION OF CHRONIC HEPATITIS B VIRUS INFECTION AFTER WITHDRAWAL OF LAMIVUDINE

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Submitted for publication

Summary

Acute exacerbations of chronic hepatitis B virus infection occur after withdrawal of lamivudine therapy in about 16% of patients and are considered of little clinical significance. We observed 'lamivudine withdrawal hepatitis' accompanied by jaundice and incipient liver failure, but also followed by viral clearance.

In order to investigate the incidence, severity and virological outcome of 'lamivudine withdrawal hepatitis' we monitored 41 patients for at least 6 months after discontinuation of experimental nucleoside analogue therapy.

The incidence of hepatitis flares, defined as an abrupt elevation of ALT at least three times baseline level and ten times the upper limit of normal, was estimated to be 20%; in 5% of cases, hepatitis flares were associated with jaundice and incipient liver failure. A noticeable feature of 'lamivudine withdrawal hepatitis' flares were the high HBV-DNA levels at the time of the ALT peak.

To minimize risk of liver failure and to enhance elimination of HBV following flares reinstitution of lamivudine therapy was evaluated. In three patients with a severe hepatitis flare with jaundice, lamivudine therapy was resumed, followed by HBV-DNA and HBeAg seroconversion. Such an event did not occur in six other patients with a non-icteric 'lamivudine withdrawal hepatitis', who were not retreated with lamivudine.

Active management of severe hepatitis flares is proposed, assuming that a rapid and effective suppression of viral replication in combination with the activated immune system can induce HBeAg seroconversion accompanied by remission of disease activity.

Introduction

Patients with chronic hepatitis B virus (HBV) infection can suffer acute exacerbations under various conditions. Studies in Taiwan showed an annual incidence of spontaneous acute exacerbation of 27% for HBeAg positive patients and 10% for anti-HBe positive patients.[1] In these studies acute exacerbation was defined as an abrupt elevation of alanine amino transferase (ALT) to more than 300 IU/l in patients with chronic hepatitis B, minimal symptoms, normal bilirubin levels and baseline ALT levels below 150 IU/l (upper limit of normal (ULN) 30 IU/l). Some of these episodes were caused by hepatitis A, hepatitis D or hepatitis C infections,[2,3] but the large majority were apparently due to increased host immunity against HBV-infected hepatocytes.[4] In patients with HBeAg positive chronic hepatitis an acute exacerbation frequently precedes HBeAg clearance.[4,5,6] However, a spontaneous acute exacerbation in HBeAg negative patients is often associated with reactivation of HBeAg.[7,8,9]

Acute exacerbations have also been described after withdrawal of corticosteroid therapy[10] and cancer chemotherapy.[11,12] The mechanism of this syndrome is in all likelihood increased HBV replication and antigen expression on hepatocytes;[13,14] and after withdrawal of therapy rebound immune reactivity and immune-mediated cytolysis of HBV-infected hepatocytes.[12] Finally, acute exacerbation or hepatitis flares are seen regularly during alpha-inteferon (α-IFN) therapy. During the first four weeks of treatment they are regarded as drug-related toxicity; later these flares may be beneficial since flares are the hallmark of impending HBeAg seroconversion.[13,14] Hepatitis flares usually occur without symptoms but are associated with clinical jaundice in 11-16% of cases. After withdrawal of cancer chemotherapy severe acute exacerbation leading to death has been described;[11,17] mortality has also been described after prednisone withdrawal [18] or during α-IFN therapy among patients with cirrhosis and a diminished hepatic reserve.[19]

We previously reported on severe icteric hepatitis associated with reactivation of hepatitis B virus replication five months after withdrawal of lamivudine therapy.[20] In view of the potentially serious consequences of acute exacerbation after nucleoside analogue therapy for patients with cirrhosis and diminished hepatic reserve, we carefully monitored all patients monthly after discontinuation of nucleoside analogue therapy for at least six months. In this paper we report on the incidence, severity, characteristics and virological outcome of posttherapy hepatitis flares and propose a management approach.

Patients and methods

We monitored 41 patients who had received nucleoside analogue treatment for more than six months for at least six months after discontinuation of therapy. We present three patients with an icteric hepatitis flare, with incipient liver failure in two.

HBV-DNA levels were measured with a liquid hybridization assay (Digene, Murex, UK),

quantified with an EUROHEP standard as reference and expressed in genome equivalents (gen eq). Detection limit of the Digene assay is 1.6 x 10^6 gen.eq./ml. HBeAg levels were measured by micro particle enzyme immunoassay technology (IMx, Abbott, Chicago, Ill), quantified with a Paul Ehrlich standard as reference and expressed in Paul Ehrlich units (PEIU). The detection limit of the IMx assay was 0.6 PEIU/ml). For all liver biopsies, the histology activity index (HAI) was calculated using the Desmet modification of the Knodell scoring system.[21,22] The upper limit of normal (ULN) for the level of serum alanine amino transferase (ALT) was 30 IU/l.
Sequence analysis to determine mutations in the highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif was performed amplifying a selected genomic region of the polymerase gene of HBV (nucleotide 56-806), using the primers ACPR (sense) and S3 (antisense). Specifically, the region 459-806 was sequenced on a Vistra labstation (Amersham, UK) using dye terminator chemistry. Analysis was performed on an Applied 373 automated sequencer (Perkin Elmer, USA). The sequences were analyzed and aligned using Geneworks software (Intelligenetics,UK).

Patients

Case A.
A 29-year-old man with chronic HBV infection and active viral replication who did not respond to α-IFN therapy received lamivudine for a period of 6 months. Before treatment the ALT level was 191 IU/l and he was positive for both HBV-DNA (371 x 10^6 gen eq/ml) and HBeAg (7322 PEIU/ml). A liver biopsy showed chronic hepatitis (HAI score 3) with minimal fibrosis; HBcAg expression was detected in 30% of hepatocytes and HBsAg in 5%. During treatment, ALT levels came within the normal range; HBV-DNA fell below the levels of detection and HBeAg dropped to a level just above the detection limit. A liver biopsy taken during treatment showed chronic hepatitis with some lobular activity (HAI score 3); HBcAg was not detected and HBsAg in 50% of hepatocytes. After discontinuation of lamivudine therapy ALT levels increased exponentially to 3000 IU/l (100x ULN) 4 months later, jaundice developed and clotting factors dropped below 50% (incipient liver failure). HBV serology showed a resurgence of active viral replication (HBV-DNA 1893 x 10^6 gen.eq./ml, HBeAg 10545 PEIU/ml). Liver biopsy showed severe activity (HAI score 10), immunohistochemistry showed HBcAg expression only in the cytoplasm and HBsAg was detected in 50% of hepatocytes. Assuming a severe immunological reaction to hepatocytes infected with HBV, prednisone therapy was started at an initial dose of 30 mg/day. A marked drop in bilirubin and ALT followed; clotting factors returned to pre-treatment levels. Prednisone was decreased to 10 mg/day and discontinued when the HBV-DNA levels rose again. After prednisone withdrawal, hepatitis activity increased somewhat, followed by loss of HBV-DNA and HBeAg; HBe-antibodies appeared.[20]

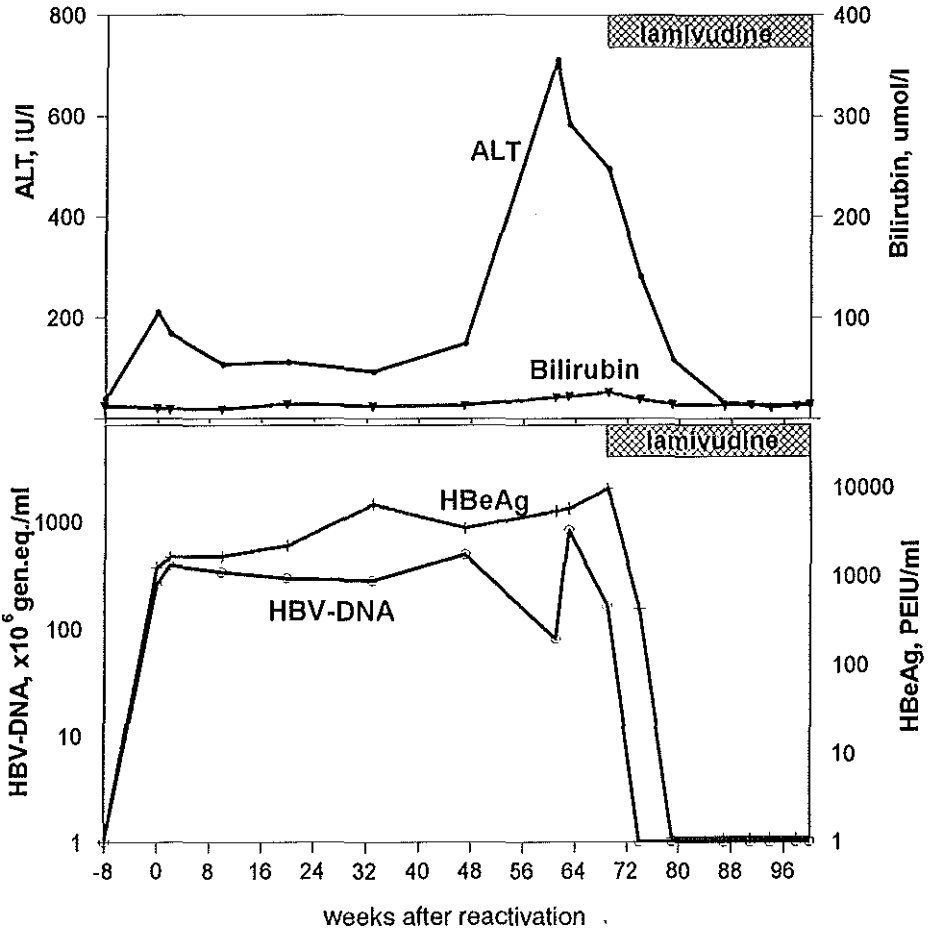

Figure 1. *ALT, Bilirubin, HBV-DNA and HBeAg levels after reactivation and during retreatment with lamivudine, in a patient previously treated with lamivudine (case A).*

Six months later a reactivation of hepatitis activity was observed (HBV-DNA 260 x 10^6 gen eq/ml; HBeAg 1230 PEIU/ml) and ALT rose to levels between 100 and 200 IU/l. Recently (14 months after reactivation) a hepatitis flare was observed (ALT 711 IU/l, bilirubin 26 µmol/l) in combination with high levels of viral replication (figure 1). We resumed lamivudine therapy (150 mg/day); HBV-DNA became undetectable and HBeAg seroconversion occurred within two months after reinstitution of lamivudine therapy. According to the polymerase chain reaction (PCR, detection limit 500 gen.eq./ml) HBV-

DNA is not detectable. The patient is still on lamivudine therapy (4 months after HBeAg seroconversion), since in another patient reactivation occurred when lamivudine was stopped after confirmation of HBeAg seroconversion (case B).

Case B.

A 40-year-old man with chronic hepatitis B, active viral replication (HBV-DNA 617 x 10^6 gen.eq./ml; HBeAg 1975 PEIU/ml) and elevated serum transaminase levels (ALT 113 IU/l) was treated with lamivudine (100 mg/day) for 1 year. Before treatment liver histology showed chronic hepatitis with mild activity (HAI score 2) and cirrhosis; 15% of the hepatocytes exhibited positivity for HBcAg. Prothrombin time and albumin were in the normal range. During therapy, serum transaminase values came within the normal range. Despite good compliance, serum HBV-DNA did not become undetectable during treatment and started to rise again after 24 weeks of therapy. This secondary rise was found to be related to the development of a mutant virus which was resistant to lamivudine. Sequence analysis showed a replacement of Methionine by Isoleucine ($Met_{552} \rightarrow Ile_{552}$) in the YMDD motif of the reverse transcriptase gene. The virus population was cloned and the YMDD motif was sequenced at week 16, 36, 52 of therapy and 8 weeks after discontinuation. The percentages viral populations were calculated and we found 0% mutant virus (YIDD) at week 16, 8% at week 36 and 100% at 52 weeks. Eight weeks after discontinuation of therapy 100% of the viral population was found to be wild type HBV (YMDD).

After 12 months of treatment, the liver biopsy showed cirrhosis with minimal histological activity (HAI score 1) but about 20% of hepatocytes still exhibited nuclear core-expression with additional cytoplasmic expression, HBsAg was shown in 5% of hepatocytes. After discontinuation of treatment serum transaminases initially rose to just above pre-treatment levels (figure 2), but three months later they had increased exponentially to 1270 IU/l. Jaundice and a three-second prolongation of prothrombin time developed. Serum HBV-DNA was 1971 x 10^6 gen.eq./ml, indicating a high level of viral replication; sequence analysis showed resurgence of the wild type hepatitis B virus. Other causes of hepatitis were excluded. In view of the incipient liver failure, intervention was indicated and treatment with lamivudine (100 mg/day) was resumed. This therapy was associated with an immediate drop in serum transaminase values and disappearance of jaundice. Within two weeks, HBV-DNA became undetectable, HBeAg seroconversion was observed, and ALT normalized. Two weeks later seroconversion was confirmed and lamivudine treatment was withdrawn. Unfortunately, reactivation of wild type virus replication was observed four months later, this time without a hepatitis flare.

Case C.

A 49-year-old man with chronic hepatitis B, who previously had refused α-IFN therapy, was included in a placebo-controlled study of nucleoside analogues. Serum amino transferase levels were mildly elevated (ALT 74 IU/l), HBV-DNA was in the low range (32 x 10^6 gen.eq./ml). Liver histology showed chronic hepatitis with mild to moderate

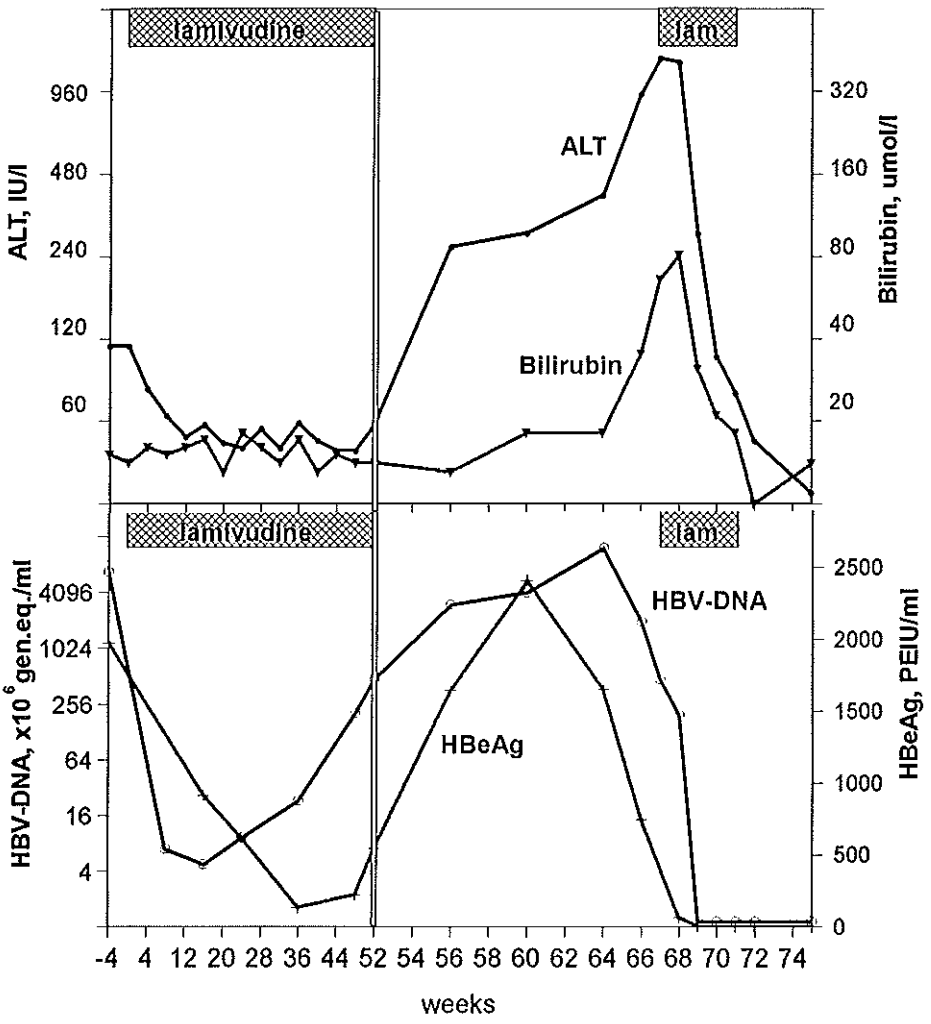

Figure 2. *ALT, Bilirubin, HBV-DNA and HBeAg levels during and after 52 weeks of lamivudine therapy (case B). Hepatitis flare was treated with lamivudine.*

activity (HAI score 3) and no fibrosis. Immunohistochemistry revealed sporadic expression of HBcAg in the liver biopsy, HBsAg expression was shown in about 30% of hepatocytes. Fourteen weeks after discontinuation of the study medication a spontaneous hepatitis flare occurred (ALT 1560 IU/l). Concomitant infections with hepatitis A virus (HAV), hepatitis D virus (HDV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV) were excluded. The patient was monitored

carefully and when jaundice developed (bilirubin 73 μmol/l, prothrombin time 1 sec prolonged) lamivudine therapy (150 mg/day) was started.
A liver biopsy at that time showed chronic hepatitis with severe inflammatory activity (HAI score 11) and minimal fibrosis. Immunohistochemistry revealed sporadic nuclear expression of HBcAg and only cytoplasmic expression in some hepatocytes, HBsAg expression was still observed in 30% of hepatocytes. The randomization code was broken and it appeared that the patient had received the placebo. After initiation of antiviral therapy, serum transaminase levels returned to normal, jaundice disappeared and viral replication dropped to zero in combination with HBeAg seroconversion within 6 weeks. HBV-DNA is undetectable by PCR. We have not yet discontinued lamivudine therapy.

Discussion

Incidence and severity of 'lamivudine withdrawal hepatitis'

After withdrawal of lamivudine therapy, hepatitis flares -defined as an increase in ALT ranging from at least three times baseline levels to more than ten times the upper limit of normal (300 IU/l)- occur regularly. Based on the results in 83 patients from two multicentre studies, 'lamivudine withdrawal hepatitis' occurs in about 16% of patients.[23,24] For our cohort of patients who had been treated with lamivudine we also calculated the risk of the development of a post-treatment hepatitis flare. We observed eight hepatitis flares in 41 patients (20%) who had received treatment for more than one month and had been followed for at least six months after discontinuation of treatment. Mean peak ALT level of those flares was 997 IU/l in comparison to 940 IU/l in the study of Liaw et al.[1] Mortality has occasionally been described due to severe exacerbations of HBV infection, spontaneously,[7] after cytotoxic therapy,[11,12,17] after prednisone withdrawal[18] and after lamivudine withdrawal[25] Two of our patients (5%) developed incipient liver failure (jaundice and clotting factors below 50%). To minimize the risk of liver failure lamivudine retreatment was initiated; both patients recovered.

Virological and histological features during hepatitis flares

Characteristics of hepatitis flares
During the spontaneous hepatitis flares associated with chronic hepatitis B, low levels of HBV-DNA and HBeAg are usually observed at the time of the ALT peak, especially in jaundiced patients.[26,27,28] Hepatocyte damage is preceded by a rise in HBV-DNA levels, the same pattern is observed during acute hepatitis B.[29] It is suggested that increased viral replication can trigger an immune reaction against HBV-infected hepatocytes.[27]
This cellular compound of the immune reactivity was studied in detail by Marinos et al.; in patients after withdrawal of lamivudine an increased T helper-cell response was observed

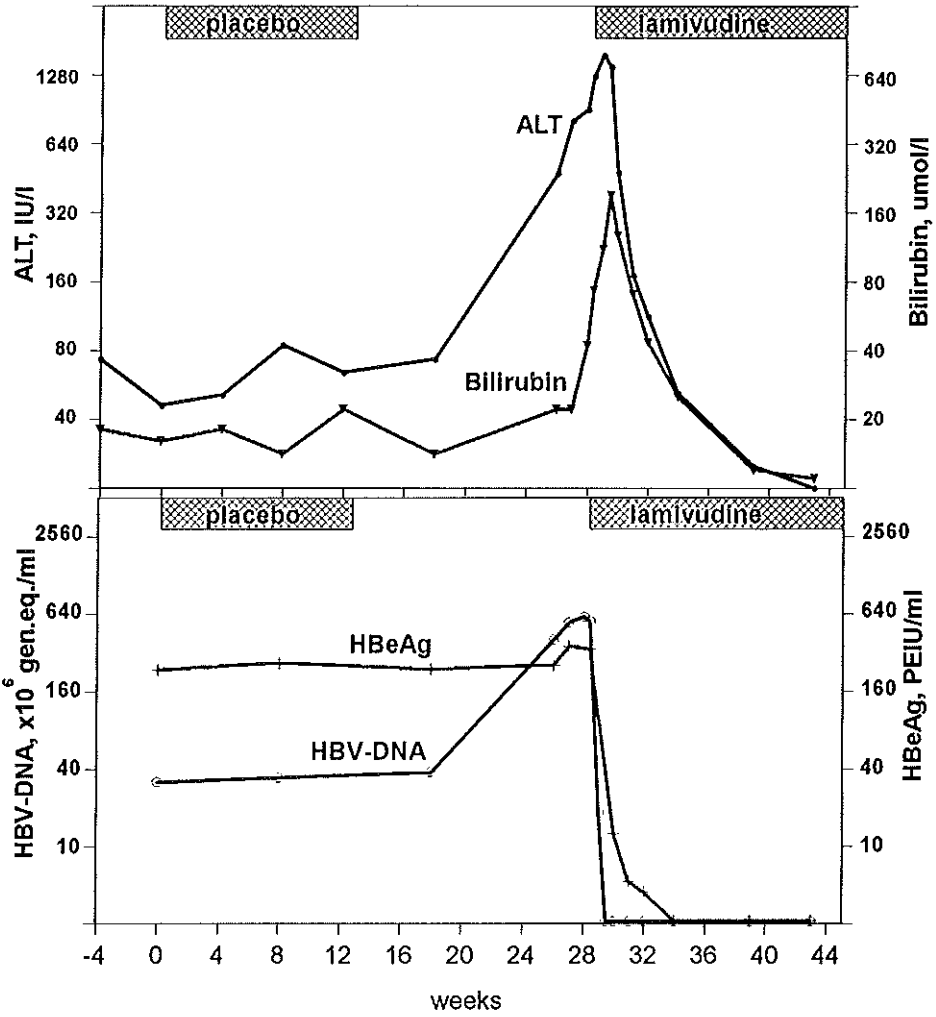

Figure 3. *ALT, Bilirubin, HBV-DNA and HBeAg levels before and after a spontaneous hepatitis flare treated with lamivudine (case C).*

after 1-3 months, in response to rebound viral replication.[30] In our jaundiced patients the HBV-DNA levels were still high (clearly above the cut-off of insensitive hybridization assays) at the time of the ALT peak, therefore spontaneous reduction of hepatitis activity was uncertain. We wondered whether blocking of viral replication with a subsequent decrease of viral antigen display on hepatocytes might reduce the hepatitis activity. Therefore we started lamivudine therapy. The virus-suppressing therapy in combination

with the ongoing immune response resulted in normalization of serum transaminase levels; HBV-DNA levels became undetectable by PCR, and HBeAg seroconversion followed in all three cases within two months.
This course was rewarding but also unexpected since HBeAg seroconversion is unusual during flares with high HBV-DNA levels and also unusual during lamivudine studies in stable chronic hepatitis B patients.[23,24] Virus suppression by lamivudine and the immune reaction associated with icteric flares might thus be synergistic in effective clearance of virus infected cells. In our group of 6 patients with an anicteric hepatitis flare after lamivudine withdrawal, all patients had high HBV-DNA levels at the time of ALT peak. Retreatment was not initiated and all flares subsided spontaneously but no seroconversion was observed (figure 4).

Lamivudine resistance and hepatitis flares
In case B measurement of HBV-DNA and HBeAg indicated that HBV replication was not completely blocked during lamivudine therapy; it increased after 24 weeks of treatment due to the appearance of a lamivudine-resistant mutant virus. Sequence analysis of the HBV genome showed a mutation in the highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif of the reverse transcriptase region, in which methionine was replaced by isoleucine ($Met_{552} \rightarrow Ile_{552}$). This mutation was first described in immunosuppressed HBV-infected patients after liver transplantation[31,32,33] and later in immunocompetent HBV-infected patients.[34] In the present case the percentages viral populations were calculated and we found 0% mutant virus (YIDD) at week 16, 8% at week 36 and 100% at week 52, the end of lamivudine therapy. After discontinuation of lamivudine, HBV-DNA levels increased tenfold due to resurgence of the wild type virus (YMDD). Eight weeks after termination of treatment, 100% of the viral population was found to be wild type HBV. It was the emergence of the wild type HBV that provoked the hepatitis flare in our patient who had already developed liver cirrhosis. The relationship between the development of 'lamivudine-withdrawal hepatitis' and the lamivudine-resistant mutant virus is not yet clear.
In case A and C no mutations were observed in the YMDD motif during hepatitis flares. In 5 out of 6 patients with an anicteric hepatitis flare no YMDD mutation was found at the time of ALT peak. One patient (figure 4, ▪) developed a severe hepatitis flare early after discontinuation of treatment, this immune reactivity was probably triggered by a rapid increase in viral replication during therapy, which in fact was a result of a mutation in the YMDD motif ($Met_{552} \rightarrow Val_{552}$). Three weeks after discontinuation of therapy the major virus population was still the mutant (YVDD).

Histology during hepatitis flares
In case A liver biopsy at the time of the first hepatitis flare showed severe histological inflammatory activity (HAI score 10). Immunohistochemistry showed only cytoplasmic expression of HBcAg. In case C a spontaneous hepatitis flare occurred in a patient with mild liver disease without cirrhosis. During the hepatitis flare, liver histology showed

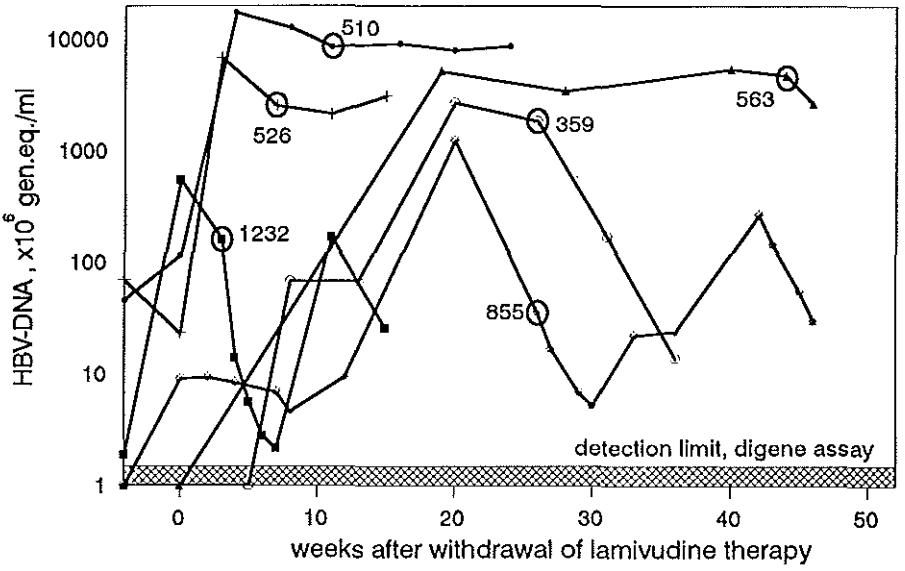

Figure 4. *HBV-DNA levels in six patients with a hepatitis flare after withdrawal of lamivudine therapy. Circles indicate the time of the ALT-peak and the number the peak ALT level (IU/l). HBV-DNA was quantified by the Digene liquid hybridization assay (Murex, UK).*

severe inflammatory activity together with unchanged nuclear HBcAg expression. In contrast to the liver biopsy 6 months prior to reactivation, HBcAg expression was also found in the cytoplasm, which is associated with increased inflammatory activity.[35,36] HBsAg showed a regular spread of HBsAg expression in the cytoplasm, which was unchanged compared to the biopsy 6 months prior to reactivation.

Management of hepatitis flares

Until recently 'lamivudine withdrawal hepatitis' was supposed to be self-limiting and patients were left untreated. Nowadays, the goal of management should be the prevention of liver failure and death, and - after our observation - could also include enhancement of the rate of HBeAg seroconversion. Therefore reinstitution of lamivudine therapy should be considered. Especially if HBV-DNA levels are high at the time of diagnosis of the hepatitis flare, spontaneous seroconversion is unlikely and therefore effective antiviral therapy is a logical choice. Treatment with lamivudine reduces viral replication rapidly but allows the activated cytotoxic T cell response[30] to eliminate hepatocytes that present viral antigens. This approach can also be tried in the event of spontaneous hepatitis flares during chronic hepatitis B. In this way the immune activity evoked by reactivation of hepatitis B viral replication may be used in a therapeutic setting.

In the event of incipient liver failure (jaundice and clotting factors below 50%), the question arises whether also immune reactivity should be tapered. Reports by others[20,37] as well as our case suggest that a short course of prednisone might be helpful. All patients who receive prednisone to control a hepatitis flare should -in our opinion- also receive lamivudine (figure 5).
Hepatitis flares are expected to occur more regularly now that nucleoside analogues are going to be introduced on a large scale. In our group of patients suffering a lamivudine withdrawal hepatitis, HBeAg seroconversion was not observed in the six cases without lamivudine retreatment. We believe that controlled evaluation of the management of hepatitis flares should be initiated in order to minimize morbidity and mortality but also to have the best chance to achieve permanent seroconversion.

In conclusion, severe hepatitis flares occur after withdrawal of lamivudine treatment. At the time that the serum ALT levels are peaking, the level of HBV-DNA is still high, and reinstitution of lamivudine is therefore a logical approach. Since lamivudine therapy rapidly reduces the viral load and viral spread, the activated immune system can more easily eliminate infected hepatocytes and induce HBeAg seroconversion.

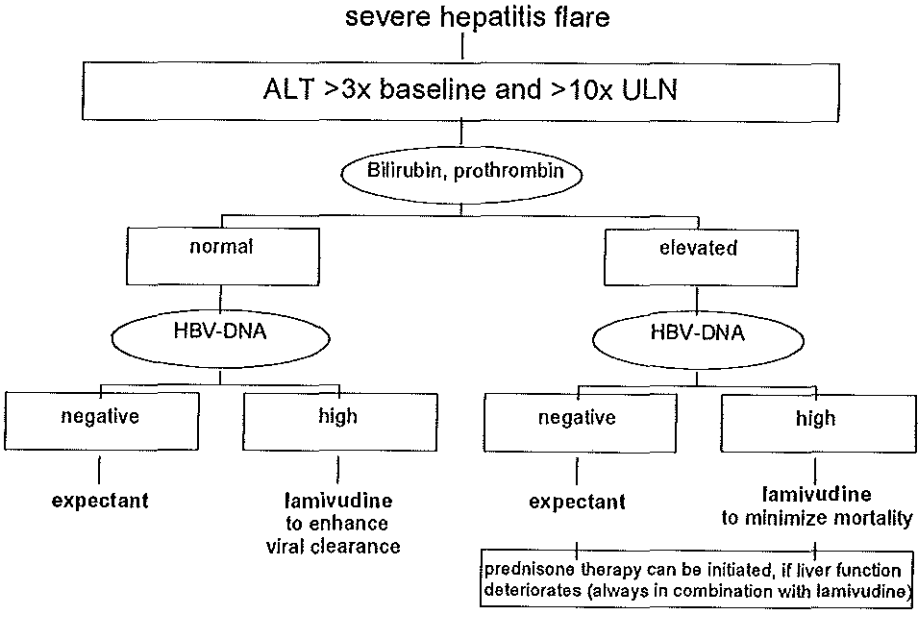

Figure 5. *Flow chart for the management of hepatitis flares for chronic hepatitis B patients.*

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7.1

LAMIVUDINE RESISTANCE IN IMMUNOCOMPETENT CHRONIC HEPATITIS B: INCIDENCE AND PATTERNS

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Summary

Background/Aims: Lamivudine is a non-toxic, potent inhibitor of hepatitis B virus replication. Recently, hepatitis B virus resistance to lamivudine has been described in patients using immunosuppressive drugs after liver transplantation.
Methods: From our cohort of 81 consecutive patients treated with lamivudine, we selected all immunocompetent patients who received lamivudine monotherapy for a period over 26 weeks (n=14).
Results: Lamivudine resistance with the characteristic mutation in the YMDD motif was observed in four patients (actuarial cumulative incidence: 39%). Two patterns of viral resistance were observed: incomplete response (n=2) and viral breakthrough (n=2).
Conclusions: The observed high frequency of lamivudine resistance may have implications for the concept of long-term virus-suppressive therapy of chronic hepatitis B by lamivudine monotherapy.

Introduction

Lamivudine, a non-toxic potent inhibitor of hepatitis B virus (HBV) replication[1] is well absorbed and oral administration of 100-300 mg daily usually reduces HBV-DNA to levels undetectable by liquid hybridization techniques. During a treatment period up to 6 months, no viral breakthrough has been reported in immunocompetent patients.[2,3,4] Lamivudine is an inhibitor of the viral enzyme reverse-transcriptase.[5] In human immunodeficiency virus type 1 (HIV-1) infected patients, lamivudine monotherapy induces development of drug resistance within a few weeks;[6] the loss of inhibitory activity is caused by a mutation in the tyrosine-methionine-aspartate-aspartate (YMDD) motif of the reverse transcriptase.[7] Recently, cases of lamivudine resistance *in vivo* have been described in immunosuppressed liver transplantation patients.[8,9] Here, we report lamivudine resistance due to mutation in the YMDD motif in a population of immunocompetent HBV infected patients, occurring as incomplete response or breakthrough with a higher than expected incidence in therapy of 6-12 months' duration.

Material and Methods

Patients

We analyzed a cohort of 81 consecutive patients treated with lamivudine for chronic hepatitis B. Eleven patients coïnfected with HCV, HDV or HIV, and three patients receiving immunosuppressive drugs after transplantation were excluded. From the remaining 67 immunocompetent patients, we selected all 14 patients who received lamivudine monotherapy for a minimum period of 6 months; all received a dose of 100 mg daily. At entry patients were HBV-DNA positive by liquid hybridization (Digene, Murex, UK) and HBeAg positive (Abbott, USA) and had elevated serum transaminases. All patients were tested for HBV-DNA quantitatively at least every 8 weeks during treatment. If HBV-DNA was positive in the last sample collected during treatment (n=5), we sequenced the YMDD motif region in the DNA-polymerase gene.

HBV nucleotide sequence analysis

If HBV-DNA remained detectable or reappeared during therapy, the latest serum sample under therapy was analyzed. To determine mutations in the YMDD motif, a selected genomic region of the polymerase gene of HBV was amplified (nucleotide 56-806), using the primers ACPR and S3 (ACPR, sense: 5'-CCT.GCT.GGT.GGC.TCC.AGT.CCC.GGA.-ACA.GTA-3'; S3, antisense 5'-TTG.GTA.ACA.GCG.GTA.TAA.AGG-3'). Specifically, the region 459-806 was sequenced on a Vistra labstation (Amersham, UK) using dye terminator chemistry. Analysis was performed on an Applied 373 automated sequencer (Perkin Elmer, USA). The sequences were analyzed and aligned using Geneworks software (Intelligenetics, UK).

Table 1. *Patient characteristics*

Patient	Age	Cirrhosis	Previous lamivudine treatment	YMDD analysis at week	YMDD mutation	HBV-DNA during treatment
1	35	No	Yes	52	Met→Val	incomplete response
2	41	Yes	No	52	Met→Ile	incomplete response
3	18	No	No	52	Met→Ile	breakthrough
4	17	No	No	52	Met→Ile	breakthrough
5	20	No	No	46	None	incomplete response

Results

In 14 consecutive immunocompetent patients with chronic hepatitis B treated with 100 mg of lamivudine daily for a period over 26 weeks, HBV-DNA levels initially decreased 2 log during the first 2 months of treatment (figure 1). However, after this initial drop in HBV-DNA levels, three patients remained positive and two others became repeatedly HBV-DNA negative but showed a breakthrough after 48 weeks of treatment. In these five patients we sequenced the *YMDD* motif of the DNA-polymerase gene and identified a mutation in four of these patients (table 1). Methionine was replaced by isoleucine (Ile, n=3) or valine (Val, n=1). In one patient (patient 5) no mutation was found but this patient admitted to poor compliance. The actuarial cumulative incidence of YMDD mutation during 52 weeks of lamivudine treatment was estimated to be 39% (standard error 16%). We observed that viral resistance is also related to liver inflammation; in the three patients with incomplete HBV-DNA response, serum transaminases remained elevated during treatment. The two patients with breakthrough showed initially normalization of serum transaminases, which then started to rise when the mutant virus became detectable.

Discussion

This study shows the development of lamivudine resistance in immunocompetent patients with chronic hepatitis B virus infection during prolonged lamivudine monotherapy. The actuarial cumulative incidence of lamivudine resistance in our patient group treated for more than 6 months amounted to 39% at 1 year. All cases had a mutation in the YMDD motif of the DNA-polymerase gene.

In HIV-1 infected patients lamivudine resistance developed rapidly after initiating therapy.[6] In HBV infected patients, phase I and II studies usually showed inhibition of HBV

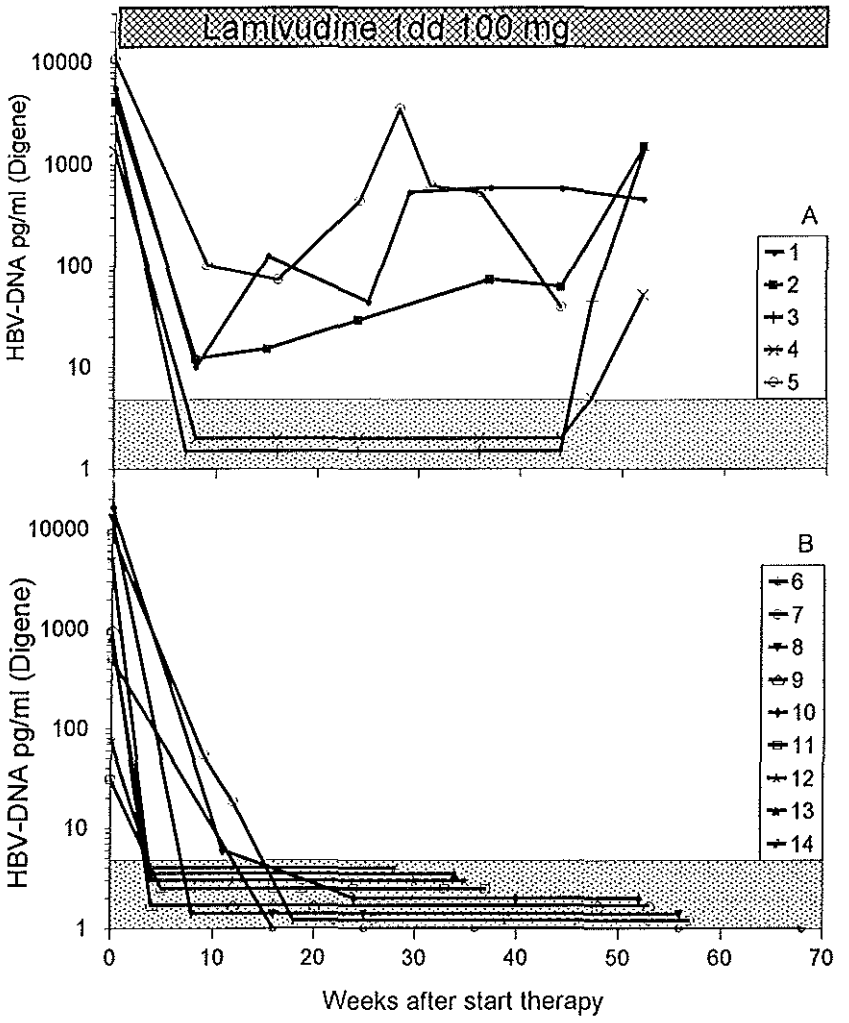

Figure 1A. *HBV-DNA levels during lamivudine treatment in five patients suspected of developing lamivudine resistance due to viral mutations. YMDD analysis revealed characteristic mutation in patients 1 and 2 (incomplete response) and patients 3 and 4 (breakthrough). Patient 5 admitted to not taking medication for several weeks (non-compliance).*

Figure 1B. *HBV-DNA levels during lamivudine treatment in nine other patients with sustained response to lamivudine.*

replication until the end of the treatment.[2,3,4] However in the study with the longest treatment period reported (24 weeks),[4] only about 75% of patients, treated with 100 mg or 300 mg daily, were HBV-DNA negative at the end of treatment. No analysis of HBV-DNA in the non-responders has been published.

Recently, viral resistance to lamivudine has been reported in patients using immunosuppression after liver transplantation.[8,9] Sequencing of the YMDD motif in these cases showed replacement of methionine by valine or isoleucine, the same mutation as described in HIV-1 infected patients. The development of resistance in patients after liver transplantation was thought to be related to immunosuppression and the associated high levels of viral replication. This study reports for HBV resistance to lamivudine in a cohort of immunocompetent patients.

We observed two types of viral responses in which resistance occurred. The first type showed an initial decline in HBV-DNA but subsequently an incomplete response to lamivudine treatment (patients 1 and 2) and selection of a mutant virus during continued treatment. The second type of viral response (patients 3 and 4) showed prolonged undetectable HBV-DNA levels and after 11 months of treatment emergence of a mutant virus. The relatively frequent occurrence of the latter response type is particularly disappointing since it diminishes hopes for simple long-term virus suppressive therapy in chronic hepatitis B. The results of the ongoing phase III studies will point out more accurately the incidence of lamivudine resistance during long-term monotherapy and lamivudine/interferon combination therapy.

A 24-weeks dose ranging study showed that 100 mg of lamivudine daily should be as effective as higher doses.[4] This conclusion is based on the observation that HBV-DNA levels became undetectable by liquid hybridization technique in the 100 and 300 mg dose groups. However, quantitation of HBV-DNA levels by PCR technique suggests a more profound drop in HBV-DNA levels in the 300 mg group.[10] Lamivudine doses over 100 mg per day may induce an effective block in viral replication more rapidly, which would delay the development of viral mutations.

In conclusion, lamivudine resistance of HBV can also occur in chronic hepatitis B without immunosuppression. The mutation identified is located in the highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif of the DNA-polymerase. As in HIV-1, the relatively easy development of drug resistance following lamivudine monotherapy calls for studies with combination therapy for long-term virus suppression.

Acknowledgments

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7.2

IDENTIFICATION OF MORE THAN ONE MUTATION IN THE HEPATITIS B VIRUS POLYMERASE GENE ARISING DURING PROLONGED LAMIVUDINE THERAPY

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Summary

Lamivudine has been shown to be a potent and non-toxic inhibitor of hepatitis B virus (HBV) replication in chronically infected patients. Upon prolonged treatment drug resistance may develop, related to a mutation of methionine (Met) to valine (Val) or isoleucine (Ile) in the $YM_{552}DD$ motif of the HBV-DNA polymerase gene.
Analysis of the HBV-DNA polymerase gene from eight chronic HBV patients suspected of resistance to lamivudine, showed that in addition to a mutation in the $YM_{552}DD$ motif, a second mutation located in the B domain of this gene, a Leu_{528} to Met_{528} change, was consistently and exclusively found in four patients showing the $YV_{552}DD$ motif. This suggests a functional or structural relationship between these domains. Since the presence of both the $YI_{552}DD$ and $YV_{552}DD$ motif sometimes preceded the exclusive presence of the $YV_{552}DD$ motif, we conclude that the $YI_{552}DD$ motif could occur as a temporal intermediate. After cessation of therapy, the wild type sequences re-emerged.

Introduction

New therapeutic approaches, including the use of nucleoside analogues like (-)2',3'-dideoxy-3'-thiacytidine (lamivudine) and 9-(4-acetoxy-3-acetoxymethyl-butyl)-2-aminopurine (famciclovir), have recently been explored in chronic hepatitis B.[1] Most information is presently available about the therapeutic use of lamivudine. It has been shown to be relatively non-toxic and to reduce HBV-DNA levels in the serum profoundly, often to levels undetectable by polymerase chain reaction (PCR).[2,3] During treatment periods lasting up to six months no breakthrough has been observed. However, after cessation of treatment, serum HBV-DNA levels to return to pretreatment values and even enhanced replication of HBV has been reported.[4]
Recently, Ling et al.[5] described resistance to lamivudine in liver transplant recipients, which was confirmed by other groups.[6,7] We reported the high frequency of resistance in chronic hepatitis B infected patients who received a prolonged therapy with lamivudine.[8] This resistance proved to be related to mutations in the YMDD motif of the DNA polymerase gene: a mutation of Met to Val or Ile at position 552 appeared to be involved.[9,10] Also in HIV-1, changes in the same motif of the reverse transcriptase gene proved to be related in lamivudine resistance.[11-14]
Here we present the results of a longitudinal analysis of HBV polymerase gene sequences from eight chronically infected HBV patients treated with lamivudine, showing that a second mutation in this gene may be involved in the development of resistance to this compound.

Materials and Methods

Patients.
From a cohort of 60 chronically HBV infected patients, treated with lamivudine for more than three months with a daily dose of at least 100 mg of lamivudine orally, eight patients were selected on basis of either an incomplete response or a breakthrough (table 1). Patients with an "incomplete response" to therapy were defined as individuals showing an initial drop in serum of HBV-DNA levels, measured by liquid hybridization techniques (Digene, Murex, Breukelen, The Netherlands), which however did not decline to undetectable levels. Patients with a "breakthrough" were defined as individuals with an initial response leading to undetectable HBV-DNA serum levels, which later during treatment became detectable again. Two of the patients (# 7 and #8) had undergone liver transplantation with subsequent immunosuppressive therapy, and lamivudine therapy was started after transplantation. Of the other six patients one (#1) had previously been treated with lamivudine for 24 weeks with 300 mg daily, and another (#6) proved to be co-infected with HIV-1 and received co-medication with zidovudine. At entry all patients were HBV-DNA positive by liquid hybridization and had elevated serum transaminase

levels.

Sequence analysis.
A selected genomic region of the polymerase gene as well as the HBsAg gene was amplified and sequenced using primers ACPR and S3 (ACPR, nucleotide 56-85, sense: 5'-CCT.GCT.GGT.GGC.TCC.AGT.CCC.GGA.ACA.GTA-3'; S3, nucleotide 806-786, antisense 5'-TTG.GTA.ACA.GCG.GTA.TAA.AGG-3'). The PCR products were sequenced on a Vistra Labstation (Amersham, Buckinghamshire, United Kingdom) using dye terminator chemistry and analyzed on an Applied 373 automated sequencer (Perkin Elmer, Nieuwerkerk, The Netherlands). Both strands were sequenced and analyzed using Geneworks software (Oxford Molecular, Oxford, United Kingdom). To confirm the sequencing results, two consecutive serum samples were analyzed.
For further analysis, the PCR products were directly cloned into pGEM-T (Promega, Leiden, The Netherlands) or pCRII vectors (InVitrogen, Leek, The Netherlands). The obtained colonies were prescreened by PCR to confirm the size of the insert. DNA was isolated and sequenced on the Vistra Labstation using Energy transfer dye primer chemistry.

Results

Kinetics of serum HBV-DNA levels upon lamivudine treatment.
The kinetics of serum HBV-DNA levels after starting lamivudine therapy are shown in figure 1. From the eight patients analyzed, five (#1 to #5) belonged to a group of chronically infected HBV patients, who had a pretreatment viral load ranging from 1363 to 10610 pg per ml (Digene, Murex). After initiating the treatment, the viral loads decreased in three out of the five patients to levels undetectable with the hybrid capture assay (detection level 5 pg/ml). However, after 48 to 56 weeks a breakthrough occurred, with viral loads remaining below the pretreatment levels (median 11.3%, range 2.1-33.6).
In one patient (#6), who was co-infected with HIV-1, serum HBV-DNA levels remained undetectable by hybrid capture assay for 95 weeks, after which the HBV-DNA level under therapy reached pretreatment levels. Finally, the HBV-DNA levels of the two liver transplant recipients (#7 and #8) under lamivudine treatment were analyzed. One of them (#7) showed an incomplete response. Viral load decreased with 98.1%, but after 53 weeks, the viral load increased despite continued treatment to about seventy times the pretreatment level. In the other liver transplant recipient (#8), serum HBV-DNA levels became undetectable. Breakthrough occurred after 40 weeks of treatment, but the serum HBV-DNA load remained less than 6% of pretreatment levels.

Sequence analysis of the HBV-DNA polymerase gene.
To study the appearance of lamivudine resistance, PCR products covering nucleotide 56 to 806 and encoding the YMDD motif (position 733 to 745), were sequenced. In all the eight

patients, Met at position 552 had been replaced by either a Val (patients #1, #5, #6 and #8) or an Ile (patients #2, #3, #4 and #7) as has been documented previously in the development of HBV resistance to lamivudine (table 1). In the former four patients, who all exhibited theMet_{552} to Val_{552} mutation, a second mutation - Leu_{528} to Met_{528} - was observed. Cloning and sequencing of individual PCR products from each pretreatment and resistance time pair, confirmed an absolute linkage between the Leu_{528} to Met_{528} mutation on the one hand, and the Met_{552} to Val_{552} mutation on the other hand in these patients. No additional changes between the two time points were observed.
Since in HIV-1 lamivudine resistance it has been shown that the Met_{552} to Val_{552} mutation is preceded by a Met_{552} to Ile_{552} mutation,[13] we wondered whether this sequence of events also takes place in the development of HBV resistance to lamivudine. Therefore additional cloned PCR products from two patients (#1 and #7), generated towards the end of lamivudine therapy, were cloned and sequenced. Sequence analysis was performed on six and eight independently generated clones, respectively. Analyses of sequences obtained from liver transplant patient (#7) showed that all these clones exhibited the $YI_{552}DD$ mutation only. Analysis of a chronically infected HBV patient with a $YV_{552}DD$ mutation (#1), revealed that from the six clones analyzed, four had a $YV_{552}DD$ mutation, while two had an $YI_{552}DD$ mutation. In these $YV_{552}DD$ containing clones, again the linkage of Met_{528} with Val_{552} was demonstrated.
Finally, in four patients (#1 to #4), therapy was discontinued. Sequence analyses of samples collected after cessation of therapy showed viruses with the HBV polymerase gene wildtype sequences, Met_{552} and Leu_{528} had replaced the mutated viruses.

Discussion

In the present paper we have confirmed earlier findings from our group and others, showing that the development of HBV resistance to lamivudine is associated with a mutation in the $YM_{552}DD$ motif of the HBV-DNA polymerase gene into either $YI_{552}DD$ or $YV_{552}DD$. In addition we have found that in contrast to the $YI_{552}DD$ motif, the $YV_{552}DD$ motif is consistently linked to a Leu_{528} to Met_{528} mutation. This second mutation is located in the B domain of the reverse transcriptase part of the HBV-DNA polymerase gene, while the YMDD motif is located in the C domain. Analyzing individual clones from a patient with a chronic HBV infection and the YVDD motif, indicated that during therapy both the YIDD and the YVDD mutations could be present at the same time. The latter finding is reminiscent of the same sequence of mutations occurring in the YMDD motif of the reverse transcriptase gene of HIV-1 upon lamivudine treatment, in which the YIDD motif is a temporal intermediate.[11] Again a linkage of Val_{552} with Leu_{528} was observed.
Although HBV as a member of the Hepadnaviridae family is a DNA virus, it replicates through a RNA intermediate with a replication strategy similar to retroviruses. By comparing the RNA dependent DNA and RNA polymerases, four conserved motifs or elements with the same linear arrangements were indentified, in which altogether four

Table 1. *Patients characteristics and mutations arising in the HBV-DNA polymerase gene during lamivudine therapy of chronical hepatitis B infected patients.*

Patient	Previous lamivudine treatment	Documented co-infection	Immuno-suppressive therapy (LTx)	Arising mutations	Response to therapy	Reduction of HBV-DNA under therapy
#1	Yes		No	Met →Val Leu →Met	Incomplete response	72.8 %
#2	No		No	Met → Ile	Incomplete response	66.4 %
#3	No		No	Met → Ile	Breakthrough	97.9 %
#4	No		No	Met → Ile	Breakthrough	97.1 %
#5	No		No	Met →Val Leu →Met	Breakthrough	97.7 %
#6	No	HIV-1	No	Met →Val Leu →Met	Breakthrough	62.0 %
#7	No		Yes	Met → Ile	Incomplete response	0% (increase 69 fold)
#8	No		Yes	Met →Val Leu →Met	Breakthrough	94.5%

* *LTx: liver transplant patient.*

** *sequences determined on PCR products. Sequence differences are related to the pretreatment sequences.*

strictly and eighteen relatively conserved amino acids are present.[15] One of these domains (domain C) contains the YMDD motif, and it is suggested that one conserved amino acid is placed in, or proximal to, turn structures which are crucial for the catalytic activity of the enzyme. In the YMDD motif, most likely the $D_{553}D_{554}$ are crucial, since they are largely invariable, except in minus strand RNA viruses. The Tyr_{551} (Y) residue is highly conserved, but can be replaced by chemically related amino acids. The Met_{552} residue is conserved among the DNA viruses group, but has been replaced by Ile and Val in some retroviruses. The Leu_{528} residue is located in a highly conserved region of the B domain, but is only present in the DNA viruses and hepatitis A virus. The direct linkage between the Leu_{528} into Met_{528} mutation coupled with the YVDD motif, suggests that these two regions are mutually under functional or structural constraints.

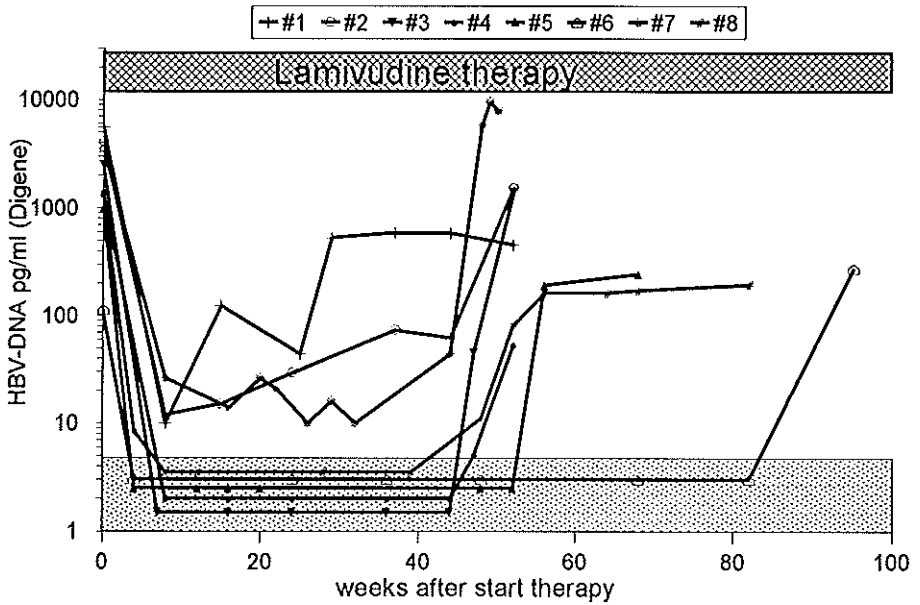

Figure 1. *HBV-DNA levels during lamivudine therapy in five chronically infected patients (#1 to #5), one HBV-HIV co-infected patient (#6) and two liver transplant patients (#7 and #8) all developing lamivudine resistance. Viral DNA levels were measured with a hybrid capture assay (Digene, Murex), which has a detection level of 5 pg/ml.*

The limited data available on serum HBV-DNA levels in lamivudine treated individuals and mutated virus indicate that in immunocompetent HBV chronic carriers, the DNA level of the mutated virus, does not rebound to pretreatment levels. A reduction up to 98% may be achieved. This indicates that in the patients, the mutated virus has a reduced capacity to replicate. However, our data and the data presented by Tipples et al.[10] suggest that in liver transplant patients, the HBV-DNA levels under lamivudine may become higher than before

transplantation. This may be related to the immunosuppressive therapy that is routinely practiced after transplantation. This by itself may lead to relatively high serum HBV-DNA levels. Stopping of lamivudine therapy may lead to reactivation of the HBV infection,[4] which may be related to the observation that a virus with the YMDD motif returns, probably due to its higher replication capacity.

The time needed before an increase of viral load related to the observed mutations can be detected appears to be different in HIV-1 and HBV infected individuals. In HIV-1, resistance to lamivudine is usually observed within a few weeks.[14] In our study and those of others on the development of hepatitis B virus resistance to lamivudine,[5,6,7,10] increase of viral load was observed after up to 80 weeks. The difference between HIV-1 and HBV in this respect might be explained by the fact that HBV uses two open reading frames simultaneously, which may restrict the emergence of viable mutations.

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7.3

CLINICAL IMPACT OF LAMIVUDINE RESISTANCE IN CHRONIC HEPATITIS B

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Submitted for publication

Introduction

Lamivudine is an effective virostatic drug for the treatment of chronic hepatitis B virus infections. However, after prolonged treatment periods lamivudine resistance has been observed in both immunosuppressed and immunocompetent patients.[1,2] One year after the emergence of this entity incidence, patterns and molecular biology have been described.[3] We present our data on the clinical impact of lamivudine resistance in chronic hepatitis B patients.

Patients and Methods

We assessed virology and biochemistry data before, during and after the emergence of a lamivudine resistant viral mutant in 13 patients from our centre. Ten were immunocompetent chronic hepatitis B patients, and three were so called immunodeficient (two liver transplant recipients with allograft reinfection and one HIV-HBV coinfection).
Ten patients continued lamivudine therapy after the emergence of a mutant virus, while three stopped lamivudine at the time of the detection of a viral mutant; two additional patients stopped five months later (per protocol). Four patients that continued lamivudine therapy after the diagnosis of resistance had additional therapy with famciclovir (1500 mg daily for 1 month).
HBV-DNA was assessed by liquid hybridization assay (Digene, Murex, UK) with a detection limit of 1.5×10^6 genome equivalents per ml (gen.eq./ml), according to the Eurohep standard. Sequence analysis of the genomic region (nucleotide 56-806) of the polymerase of HBV (including the $YM_{552}DD$ motif) was performed on a Vistra labstation (Amersham, UK) using dye terminator chemistry. Analysis was performed on an automated sequencer (Perkin Elmer, USA). The sequences were analyzed and aligned using Geneworks software (Intelligenetics, UK).

Results

During lamivudine therapy in 10 out of 13 patients no HBV-DNA was detectable. Three patients remained positive at levels between 4.3 and 18×10^6 gen.eq./ml. ALT levels normalized during therapy in 10 patients.
After the development of the lamivudine resistant mutant, all patients had detectable serum HBV-DNA, but HBV-DNA levels remained below pretreatment levels in 5 out of 9 patients that continued lamivudine therapy. However, in all three immunosuppressed patients higher HBV-DNA levels were observed after the emergence of a mutant virus. After the diagnosis of lamivudine resistance ALT levels became abnormal in 11 out of 13 patients (8 patients had an ALT relapse), ALT levels remained below pretreatment values

in 8 out of 9 patients after 5 months.

We observed 2 hepatitis flares in 9 patients that continued lamivudine therapy in 5 months of follow-up. One patient died of a severe hepatitis flare within four weeks after the emergence of a mutant virus, this patient used immunosuppression after liver transplantation.[4]

In four patients after the diagnosis of resistance additional therapy with famciclovir (1500 mg daily for 1 month) in combination with continued lamivudine therapy had no effect on HBV-DNA and ALT in three. In one patient with rapid increasing HBV-DNA levels and an ALT flare, HBV-DNA became negative during famciclovir and remained negative after discontinuation of famciclovir.

After withdrawal of lamivudine, HBV-DNA levels rebounded to pretreatment values, associated with more marked hepatitis activity than during lamivudine therapy. In two patients hepatitis flares were observed within 6 months of discontinuation, both became icteric but recovered. HBV-DNA reverted back to wild type in all cases.

Table 1. *Virological and biochemical results in 13 patients before, during and after the emergence of a lamivudine resistant viral mutant*

		During lamivudine therapy			
	Pre-treatment (n=13)	3 months lamivudine (n=13)	At resistance (n=13)	5 months thereafter (n=9)	After withdrawal (n=5)
HBV-DNA (x10^6 gen.eq./ml, Digene, Murex)					
Virus type	wild type Met_{552}	wild type Met_{552}	Ile_{552} (n=7) Val_{552} (n=6)	Ile_{552} (n=5) Val_{552} (n=5)	wild type Met_{552}
Median level (range)	398 (6.6-2836)	0.5 (0.5-18)	87 (9.1-2395)	142 (0.5-834)	578 (233-2602)
ALT level (IU/l)					
Median level (range)	79 (40-306)	40 (24-71)	56 (29-1145)	59 (31-97)	131 (54-211)
Flare*	n=0	n=0	n=1, died	n=2	n=2

* Hepatitis exacerbations exceeding ten times the upper limit of normal (300 IU/l)

Discussion

The clinical impact of lamivudine resistance in chronic hepatitis B needs to be assessed. Especially since rapidly increasing use of this drug in the prevention of HBV reinfection after liver transplantation, and in the treatment of various forms of chronic hepatitis B is anticipated. In most of such conditions lamivudine will be used as a virostatic drug, and persistence of the clinical benefit can only be expected if the antiviral effect can be maintained for years. The incidence of lamivudine resistant HBV is reported to be 14 % of patients after one year,[5] but in our centre 50% of patients developed resistance to lamivudine within two years, suggesting a time dependent cumulative problem.

Continuation of lamivudine therapy was associated with lower HBV-DNA and ALT-levels than before the start of therapy in the majority of patients.

Withdrawal of lamivudine led to reappearance of the wild type virus and an increase in HBV-DNA levels and marked ALT elevation; in 2 out of 5 an icteric hepatitis flare was observed.

In this situation antiviral combination therapy with famciclovir although attractive, is of unproven benefit.

Currently, continuation of lamivudine monotherapy appears the best approach for most patients, in spite of increase in HBV-DNA and ALT levels. This preliminary conclusion on short term consequences needs a firm database to be assembled in 1998.

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8

FUTURE CLINICAL STUDIES ON LAMIVUDINE IN CHRONIC HEPATITIS B

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Background

Chronic hepatitis B can give rise to serious conditions like cirrhosis, hepatocellular carcinoma and liver failure. Positivity of HBsAg in serum will be followed by additional testing of HBeAg and serum transaminase levels. In case one of these is abnormal further evaluation of liver disease is required. When active viral replication is demonstrated standard treatment with alpha-interferon (α-IFN) has to be considered, since longitudinal studies on the natural history of chronic hepatitis B have shown that ongoing viral replication and liver inflammation are features which predict unfavourable evolution of the disease[1]. Alpha-interferon therapy will induce viral latency in approximately 30-40% of patients.[2] High levels of viral replication (HBV-DNA) and mild elevation of serum transaminase levels (ALT) have shown to be associated with non-response.[3] Therefore, the majority of patients still do not qualify for α-IFN therapy either will not respond to therapy. Therefore potent antiviral nucleoside analogues are a welcome approach in the treatment of chronic hepatitis B.
The studies described in this thesis focussed on several aspects of the nucleoside analogue lamivudine in the treatment of chronic hepatitis B virus infection. In this chapter we would like to discuss how we think our clinical research opens new horizons for additional treatment strategies in patients with chronic hepatitis B.

Clinical use of lamivudine

During the initial 28 days clinical trial, lamivudine was well tolerated and doses above 20 mg once daily resulted in marked suppression of HBV-DNA. Dynamics of HBV replication during therapy gave us the opportunity to calculate viral turnover *in vivo*. The minimum virus production and clearance per day was calculated to be over 10^{11} virions per day. This showed the extreme efficacy of one daily tablet of lamivudine that will result in undetectable HBV-DNA levels (as tested by the conventional liquid hybridization assay) within four weeks in the majority of patients.[4] In view of the universal reactivation after withdrawal, prolongation of therapy up to 6 months was offered to the patients. This study showed that beside undetectable HBV-DNA by conventional assays also normalization of serum transaminase levels was observed in the majority of patients.[5] Liver biopsies during treatment gave the first clue that also histological activity improved.[6] Especially piecemeal necrosis which is thought to be a predictor for progression of cirrhosis[7] improved significantly. In spite of the classical markers of successful antiviral therapy (HBV-DNA negativity and ALT normalization) reactivation occurred in almost all patients after discontinuation of lamivudine.[5,8] However, lamivudine demonstrated to be a non-toxic, and potent virostatic drug, deprived of mitochondrial toxicity.[9]
The question remains how to use the drug in an optimal way to prevent progression of chronic liver disease in hepatitis B patients? Two different strategies are emerging:

1. Long-term suppression with potent antiviral drugs will result in long-term suppression of disease activity which will result in a better prognosis.
2. Short-term virus suppression combined with immunostimulation will give the host immune response the opportunity to induce definite viral clearance, with the development of antibodies and thereby improvement of life expectancy.

For chronic hepatitis B virus infection both strategies are justifiable and we would like to expand on these in more detail.

Long-term virus suppression

In the past some virus-suppressive nucleoside analogues induced a decrease in HBV-DNA levels during therapy, however, in the majority of patients HBV-DNA did not became negative. Early lamivudine studies *in vitro* and *in vivo* showed that over 99% reduction of viral replication was feasible. Since lamivudine induces probably a complete blockade of HBV replication, prolongation of therapy resulted in improvement of liver disease without immune mediated viral clearance (table 1).[4,5,6,8,10,11]

Table 1. Improvement of disease activity during lamivudine therapy (25, 100 or 300 mg)

Duration of lamivudine therapy	1 month	3 months	6 months	12 months
Loss of HBV-DNA (hybridization)	65%[4]	73%[5]	81%[5]	-
Loss of HBV-DNA (PCR)	-	12%[10]	26%[10]	-
HBeAg negativity	0%[4]	4%[5]	8%[5]	14%[11]
ALT normalization	6%[4]	28%[5]	47%[5]	68%[11]
Histological improvement	-	-	46%[6]	67%[11]

However, in view of the very large number of viral particles produced each day, the emergence of mutant viruses with resistance to lamivudine seems to be unavoidable. Especially in case of lamivudine monotherapy one single mutation in the lamivudine sensitive part of the genome of the virus is enough to escape the antiviral pressure.

Lamivudine resistance

Lamivudine resistance was first observed in patients using immunosuppressive therapy after liver transplantation,[12,13,14] but soon thereafter also in immunocompetent patients.[15] Until now we observed lamivudine resistance in 13 patients and calculated a risk of 32% after 12 months of lamivudine therapy (figure 1). Recently Lai et al. reported that lamivudine resistance was detected in 14% of patients after 52 weeks of therapy in an Asian population.[16] This difference in incidence of lamivudine resistance between our

European centre and the Asian population needs further exploration. Currently lamivudine resistance has not been observed during the initial 6 months of treatment and thereafter lamivudine resistant mutants will occur in 3-5% of patients every month.
The clinical implication of lamivudine resistance is poorly known. After the development of the lamivudine resistant mutant HBV-DNA levels remained below pretreatment values in 5 out of 9 patients that continued lamivudine therapy. However, in all three immunosuppressed patients higher HBV-DNA levels were observed after the emergence of a mutant virus. After the diagnosis of lamivudine resistance serum transaminase levels became abnormal in 11 out of 13 patients, ALT levels remained below pretreatment values in 8 out of 9 patients after 5 months. In our group of 13 patients, 5 experienced an ALT flare over ten times the upper limit of normal (300 IU/l), three during continued therapy and two after withdrawal of lamivudine. Therefore relapse of disease activity is usual after the emergence of a lamivudine resistant viral mutant. At the moment it is uncertain if lower HBV-DNA levels are associated with delayed disease progression, however, continuation of lamivudine therapy appears the best approach. If rapid progression of disease activity is observed, the addition of other antiviral drugs may be required.

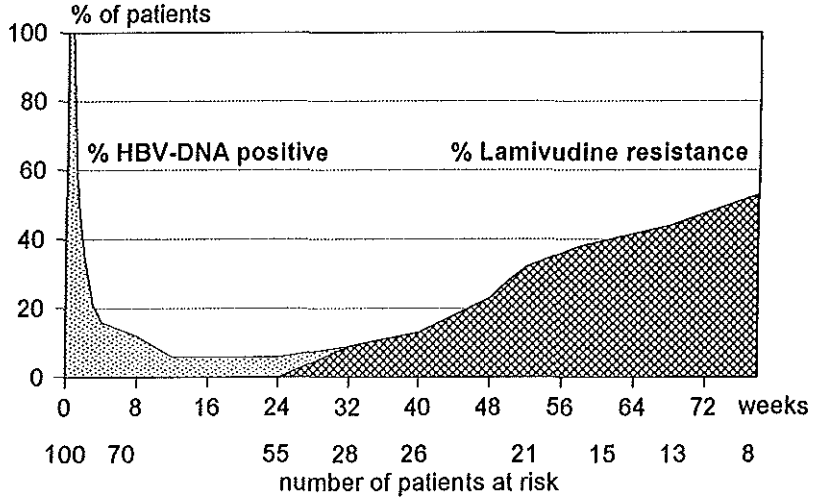

Figure 1. *Percentage of patients becoming HBV-DNA negative (hybridization assay) and the percentage of patients developing the lamivudine resistant mutant.*

Future studies should be performed to know how lamivudine resistance can be delayed or even prevented. In our opinion higher dosages of lamivudine should also be considered. The standard dose of 100 mg daily was found to be optimal based on HBV-DNA levels measured by liquid hybridization technique. However, viral replication is still ongoing below the limit of detection of this assay, which allows the development of viral mutations. Therefore sensitive methods as PCR are required to show more properly the

level of virus suppression during lamivudine therapy. By PCR with a detection limit of 10^3 gen.eq./ml, we observed a trend towards superior suppression of viral replication by the 300 mg group in comparison to the 100 mg dose group. During early phase II studies, 600 mg of lamivudine was not associated with more side-effects in comparison to lower doses. Therefore 600 mg lamivudine can be considered during the initial 4 weeks of therapy, followed by 100 mg maintenance period.

Also combination with other nucleoside analogues can be helpful to prevent the development of lamivudine resistant mutants. Recently Locarnini et al. observed that lamivudine and famciclovir act synergistically during *in vitro* experiments.[17] In conclusion, comparable with the observation during nucleoside analogues studies in HIV infected patients, maximal antiviral induction therapy might be the best way to minimize the emergence of mutants during therapy.

Table 2. *Golden rules of antiviral therapy*

- Prolong survival
- Low side effects
- Maximal antiviral induction therapy
- Sensitive assays for monitoring
- Delay or prevent viral evolution

Discontinuation of therapy

The question at what time antiviral therapy can be discontinued based on what parameters still remains unanswered. When viral replication is completely blocked by antiviral therapy, infection of new hepatocytes will not occur and the chronic infection might extinguish. Therapy can theoretically be discontinued at the time the whole liver is regenerated in the presence of antiviral drugs. This approach will take several years since whole liver regeneration has been calculated to occur in a period of 4-10 years. To determine the time of discontinuation of therapy beside serological tests liver biopsy with immunohistochemical staining of HBsAg and HBcAg will be necessary. At the moment data on the effect of lamivudine on the the most resistant viral DNA species (cccDNA) are lacking. This DNA will remain able to resume the viral replication cycle.

Immune mediated viral clearance

Patients chronically infected with the hepatitis B virus still have the opportunity to eliminate the virus. Therefore we should not treat them life-long with antiviral drugs in case they have a real chance to control the virus by their own immune-system. Previous studies with combination therapy of α-IFN and nucleoside analogues were disappointing.[18,19,20] Since the antiviral effect of lamivudine was found to be superior, combined approaches with α-IFN were evaluated. In this thesis we described the first study on lamivudine and α-IFN combination therapy in previous interferon non-responders.[21]

Combination therapy was well tolerated, pharmacokinetic interaction of both drugs was not observed and HBV-DNA levels decreased dramatically during therapy. However only 1 out of 20 patients had a sustained HBeAg seroconversion. In our opinion this combined approach needs to be studied in more detail because 4 patients (previously not responding to interferon) became HBeAg negative at the end of treatment. This is unusual in lamivudine monotherapy, therefore prolongation of the treatment period may induce sustained response as is shown in several patients when interferon monotherapy was prolonged beyond 16 weeks.[22] Another attractive approach is to start lamivudine therapy a few weeks before initiating interferon. Following this scheme HBV-DNA levels are low at the time of the first dose of interferon, which is found to be a good predictor of response to interferon treatment.[3] In our opinion combination therapy with a strong antiviral drug and an immune stimulator is likely to offer the patient the best chance of disease remission. However, at the moment we still have to learn how to combine these approaches.

Other immune modulators

Recently some other immune modulating agents showed promising *in vitro* results, for example Tucaresol and IL-12 are both candidates to stimulate the immune system which in combination with the antiviral effect of lamivudine may induce seroconversion.[23,24] Especially since IL-12 seems to play a crucial role during seroconversion at least during interferon therapy.[25]

After liver transplantation when the infected liver is replaced by a donor liver, reinfection can be prevented by polyclonal hepatitis B immunoglobulin (HBIg). Since lamivudine is able to decrease viral replication below the limit of detection of the PCR assay, passive vaccination with monoclonal or polyclonal antibodies should be evaluated also in patients chronically infected with HBV.

Hepatitis flares

Acute exacerbations in chronic hepatitis B have been described after withdrawal of corticosteroids[26,27] and cancer chemotherapy.[28,29] The mechanism of this syndrome is in all likelihood increased HBV replication and antigen expression on hepatocytes.[30] After withdrawal of therapy rebound immune reactivity and immune-mediated cytolysis of HBV-infected hepatocytes occurs.[31] Spontaneous hepatitis exacerbations have been described both in HBeAg negative and positive patients.[32] The majority of these hepatitis flares were presumed to represent increased host immunity against HBV-infected hepatocytes. Some of these flares will precede HBeAg seroconversion and others will not.[33] Monthly monitoring of patients with chronic hepatitis B revealed the serological patterns preceding the hepatitis exacerbations.[34,35,36] Hepatocyte damage was found to be preceded by a rise in HBV-DNA levels, the same pattern as observed during acute hepatitis B.[37] During the spontaneous hepatitis flares associated with chronic hepatitis B, low levels of HBV-DNA (and HBeAg) at the time of the ALT peak are associated with a subsequent HBeAg seroconversion.[34,35,36]

A rapid increasing viral replication also occurs after discontinuation of potent antiviral therapy and can be followed by hepatocyte damage. We observed acute hepatitis exacerbations in 20% of cases after withdrawal of lamivudine.[38] All patients demonstrated high levels of viral replication at the time of the ALT peak. Therefore, subsequent HBeAg seroconversion was unlikely and we started lamivudine retreatment in the icteric cases which resulted in normalization of serum transaminase levels; HBV-DNA levels became undetectable by PCR, and HBeAg seroconversion followed in these cases within two months. Virus suppression by lamivudine in combination with the activated cytotoxic T cell response[39] might thus be synergistic in effective clearance of virus infected cells. In this way the immune activity evoked by reactivation of hepatitis B viral replication may be used in a therapeutic setting.

Based on these observations reinstitution of lamivudine therapy should be considered in all cases with acute exacerbation of hepatitis B virus infection, either to prevent further liver damage or to enhance the rate of HBeAg seroconversion. Especially if HBV-DNA levels are high at the time of diagnosis of the hepatitis flare, spontaneous seroconversion is unlikely and therefore effective antiviral therapy is a logical choice. Furthermore in patients with incipient liver failure as a result of an acute hepatitis exacerbation, immune reactivity can be tapered by a short course of prednisone. However, all patients who receive prednisone to control a hepatitis flare should -in our opinion- also receive lamivudine

Table 3. Clinical indications for lamivudine therapy

◆ Contra-indication for alpha-interferon	• Decompensated liver cirrhosis • High level of viral replication • Low level of immune reactivity
◆ Non-response to alpha-interferon ◆ Pre-treatment before liver transplantation ◆ Reinfection after liver transplantation ◆ Fulminant hepatic failure ◆ Severe acute exacerbation	

Summary clinical impact of lamivudine

Lamivudine is a strong inhibitor of hepatitis B virus replication without significant toxicity. This gives us the opportunity to offer chronic hepatitis B patients one single daily tablet to induce significant disease remission. However viral clearance is feasible in chronic hepatitis B and the first choice antiviral approach still is interferon-alpha, in the near future probably combined with lamivudine. Since the majority of patients will not qualify for or will not respond to interferon therapy, a major role for lamivudine will be put aside in the treatment of chronic hepatitis B.

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9

SUMMARY

SAMENVATTING

DANKWOORD

CURRICULUM VITAE

Summary

The new potent nucleoside analogues have generated important changes in the therapeutic approach in the treatment of chronic hepatitis B. The studies presented in this thesis focused on lamivudine which was found to be one of the most effective inhibitors of HBV replication both *in vitro* and *in vivo*. At least an advance for the majority of patients in need for antiviral therapy, that will not qualify for, or will not respond to standard interferon-alpha therapy (chapter 1). Viral dynamics during the initial 28 days of therapy showed a dramatic drop of HBV-DNA levels in patients treated with a single daily tablet of lamivudine. These complete blockade of viral replication allowed us to calculate the minimum virus production and clearance which was found to be over 10^{11} virions per day.

Based on these results prolonged treatment up to six months was offered to the patients. In chapter 3 we discussed the biochemical, virological and histological remission of disease activity. During 6 months of lamivudine therapy ALT levels normalized in 47% of patients. This was accompanied by improvement of inflammatory activity in liver biopsy specimens taken during treatment. Virological response was obvious and we observed continued viral suppression below the detection limit of the liquid hybridization assay. By the sensitive PCR assay HBV-DNA became undetectable in 26% of patients, with evidence for continued suppression with longer duration and higher dose of lamivudine.

Since HBeAg seroconversion is not observed during lamivudine monotherapy, the combination with interferon-alpha appears logic. In chapter 4 we presented the first combination study on lamivudine and interferon-alpha. This combined approach was well tolerated and we could not detect any pharmacological interaction of both drugs. However, in this group of patients who had previously failed interferon therapy, efficacy was not superior to monotherapy. Therefore other treatment schedules should be investigated.

In chapter 5 we evaluated morphology and function of mitochondria in patients during lamivudine treatment, since severe clinical problems due to mitochondrial injury were observed in studies with a related nucleoside analogue (fialuridine). We discussed the mechanism of toxicity and observed that lamivudine did not induce mitochondrial toxicity.

After withdrawal of lamivudine therapy HBV replication will reappear in almost all cases, which can be accompanied by a an acute exacerbation of hepatitis activity. This so called 'lamivudine withdrawal hepatitis flare' will occur in about 20% of patients and may result in incipient liver failure in 5% of patients. However, the activated immune system during the exacerbation, in combination with reinstitution of lamivudine have been shown to eliminate infected hepatocytes and induce definite HBeAg seroconversion (chapter 6).

In case lamivudine therapy is continued beyond 6 months, the emergence of a viral mutant with resistance to lamivudine have been described (chapter 7). The mutation found is located in the highly conserved YMDD motif of the HBV polymerase gene, the same mutation as described in HIV-1 infected patients during lamivudine therapy. One single mutation is responsible for a 45-fold decrease in lamivudine susceptibility and will result in increased HBV-DNA and ALT levels. However, in most immunocompetent patients HBV-DNA and ALT levels during continued lamivudine therapy were below pretreatment values. Therefore, continuation of lamivudine appears the best approach for most patients.

Samenvatting

De nieuwe effectieve nucleoside analogen hebben belangrijke veranderingen veroorzaakt in de therapeutische mogelijkheden voor een patiënt met chronische hepatitis B. De studies in deze dissertatie richtten zich op de meest effectieve remmer van HBV replicatie, lamivudine. Dit is een aanwinst aangezien de meerderheid van de patiënten geen baat heeft bij de standaard therapie met interferon-alpha (hoofdstuk 1). De dynamiek van de virusdeling gedurende de eerste 28 dagen van de behandeling met één enkele tablet' lamivudine, lieten een dramatische daling zien van de hoeveelheid virus in het bloed. Door deze complete remming van virusactiviteit kon berekend worden dat iedere dag meer dan 10^{11} virusdeeltjes worden geproduceerd bij een chronisch geïnfecteerde patiënt.
Op basis van deze effectiviteit is de behandelingsperiode verlengd tot 6 maanden (hoofdstuk 3). Dit resulteerde in zowel biochemische, virologische als histologische verbetering van ziekteactiviteit. Serum transaminasen normaliseerden in 47% van de patienten, dit ging gepaard met een verbetering van histologische ontstekingsactiviteit tijdens therapie. Bij 90% van de patiënten was niet langer HBV-DNA aantoonbaar met behulp van hybridisatie technieken. Zelfs met behulp van de zeer gevoelige PCR techniek werd bij 26% van de patienten geen HBV-DNA aangetoond, met aanwijzingen voor een betere virus onderdrukking bij hogere dosering en langere duur van de behandeling.
HBeAg seroconversie wordt zelden waargenomen tijdens lamivudine monotherapie, daarom is de combinatie met standaard therapie logisch. In hoofdstuk 4 wordt de eerste combinatie studie beschreven met lamivudine en interferon-alpha. Deze combinatie werd goed verdragen en er werden geen farmacologische interacties waargenomen. Echter in de behandelde groep patienten die eerder niet op interferon-alpha hadden gereageerd was het succes matig. Daarom moeten ook andere behandelingsschema's onderzocht gaan worden.
Aangezien er ernstige klinische problemen als gevolg van mitochondriale schade werden waargenomen bij gebruik van een gerelateerd nucleoside (fialuridine), werd in hoofdstuk 5 de morfologie en functie van de mitochondriën in patiënten tijdens lamivudine behandeling onderzocht. Het mechanisme werd geëvalueerd en wij konden geen mitochondriale schade als gevolg van lamivudine gebruik aantonen.
Na het stoppen van de lamivudine behandeling zal in de meerderheid van de patienten de virusdeling reactiveren, dit kan gepaard gaan met een acute exacerbatie van de hepatitis activiteit. Deze zogenaamde 'lamivudine onttrekkings hepatitis' zal bij 20% van de patiënten voorkomen, in 5% zelfs met dreigend leverfalen. Echter, het op dat moment geactiveerde immuunsysteem kan, in combinatie met een opnieuw ingestelde lamivudine behandeling de geïnfecteerde hepatocyten opruimen en leiden tot een definitieve HBeAg seroconversie (hoofdstuk 6).
Lamivudine monotherapie voor een periode langer dan 6 maanden kan resulteren in de ontwikkeling van een HBV mutant die resistent is tegen lamivudine (hoofdstuk 7). De gevonden mutatie is gelokaliseerd in de YMDD regio van het HBV polymerase gen, overigens dezelfde mutatie zoals beschreven is bij HIV-1 geïnfecteerde patiënten tijdens

lamivudine gebruik. Een enkele mutatie is verantwoordelijk voor een factor 45 verminderde gevoeligheid van het virus voor lamivudine en zal resulteren in een stijging in HBV-DNA en serum transaminasen. Echter, in de meeste niet immuun-gecompromitteerde patiënten zullen de HBV-DNA en ALT waarden bij continuering van de behandeling lager zijn dan voor aanvang van de therapie. Daarom lijkt voortzetten van de lamivudine op dit moment de beste optie voor de patiënt.

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Curriculum Vitae

De auteur van dit proefschrift werd op 2 september 1969 geboren te Kampen. Na het V.W.O. aan het Van Lodenstein College in Amersfoort gevolgd te hebben werd in 1987 begonnen met de studie Geneeskunde aan de Erasmus Universiteit Rotterdam. In 1991 was hij 3 maanden werkzaam in het Nkandla hospitaal, Kwazulu, Rep. Zuid-Afrika (E. Thalmeyer MD.) In 1992 werd gedurende 6 maanden een post-doctoraal onderzoek verricht, op de afdeling Immunologie, naar het effect van cytokine behandeling in een huidtransplantatie model bij muizen (Prof. dr R. Benner). Op 18 maart 1994 werd (Cum Laude) het artsexamen afgelegd. Van april 1994 tot december 1997 was hij werkzaam op de afdeling Hepato-gastroenterologie van het Academisch Ziekenhuis Rotterdam-Dijkzigt (Prof. dr S.W. Schalm). Tijdens deze periode werd onder begeleiding van Dr R.A. de Man onderzoek verricht naar antivirale therapie bij patienten met chronische hepatitis B, hetgeen de basis vormde voor dit proefschrift. Sinds januari 1998 volgt hij de opleiding to internist in het Ikazia Ziekenhuis te Rotterdam (opleider Dr R.J.Th. Ouwendijk).

Abbreviations

ACV	acyclovir
ALT	alanine aminotransferase
ARA-A	adenine arabinoside
AST	aspartate aminotransferase
AUC	area under the curve
AZT	zidovudine
cccDNA	covalently closed circular DNA
CMV	cytomegalovirus
CPK	creatine phosphokinase
ddI	didanosine
ddC	zalcitabine
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
ER	endoplasmic reticulum
FIAU	fialuridine
gen.eq.	genome equivalents
HAI	histology activity index
HAV	hepatitis A virus
HBcAg	hepatitis B core antigen
HBeAg.	hepatitis B envelop antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCV	hepatitis C virus
HDV	hepatitis delta virus
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
α-IFN	interferon-alpha
IU	international units
Ile	isoleucine
KICA	α-keto-isocaproic acid
Leu	leucine
LTx	liver transplantation
Met	methionine
mt-DNA	mitochondrial-DNA
MU	mega-units
NA	nucleoside analogue
N-DNA	nuclear-DNA
PCR	polymerase chain reaction
PEI	Paul Ehrlich Institute
RNA	ribonucleic acid
RT	reverse transcriptase
SddC	lamivudine
SD	standard deviation
SEM	standard error of the mean
ULN	upper limit of normal
Val	valine