IMMUNE MODULATION

IN CHRONIC HEPATITIS B PATIENTS

A.B. van Nunen

Printed by University Press Facilities, Eindhoven

This study was performed at the department of Gastroenterology & Hepatology of the University Hospital Rotterdam, The Netherlands. Financial support for this thesis was kindly given by Schering-Plough, GlaxoSmithKline, Altana Pharma, Bristol-Myers Squibb and AstraZeneca.

IMMUNE MODULATION IN CHRONIC HEPATITIS B PATIENTS

(IMMUUN MODULATIE IN CHRONISCHE HEPATITIS B PATIENTEN)

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR AAN DE
ERASMUS UNIVERSITEIT ROTTERDAM OP GEZAG VAN DE

Rector Magnificus

Prof. dr. ir. J.H. van Bemmel

EN VOLGENS BESLUIT VAN HET COLLEGE VOOR PROMOTIES.

DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 23 OKTOBER 2002 OM 15.45 UUR

DOOR

Andeltje Berenice van Nunen
geboren op 29 mei 1971 te Eindhoven

Promotiecommissie

Promotor:	Prof. dr. S.W. Schalm
Overige leden:	Prof. dr. J.M.W. Hazes
	Prof. dr. Th. Stijnen
	Dr. R.A. Heijtink
Co-promotor:	Dr. R.A. de Man

Voor Bas

Contents

		Page
Chapter I	**General Introduction**	
	Treatment of chronic hepatitis B: Immune modulation in chronic hepatitis B patients.	3
Introduction to Chapter II		17
Chapter II	**Anti-HBs in chronic hepatitis B.**	
	1. Efficacy and safety of an intravenous monoclonal anti-HBs in chronic hepatitis B patients.	23
	2. Passive immunization of chronic hepatitis B patients on lamivudine therapy: a feasible issue?	35
	3. Administration of a human monoclonal antibody (TUVIRUMAB) to chronic hepatitis B patients pre-treated with lamivudine: monitoring of serum TUVIRUMAB in immune complexes.	49
Chapter III	**Immunologic mechanisms in chronic hepatitis B.**	
	1. Suppression of hepatitis B virus replication mediated by hepatitis A-induced cytokine production.	65
Chapter IV	**IFN-Lamivudine combination therapy in chronic hepatitis B.**	
	1. Efficacy of lamivudine monotherapy followed by interferon-lamivudine combination therapy in chronic hepatitis B patients.	79
	2. Lamivudine and interferon combination therapy in chronic hepatitis B: a study of viral kinetics.	91
	3. Durability of HBeAg seroconversion following antiviral therapy for chronic hepatitis B: relation to type of therapy and pretreatment serum HBV DNA and ALT.	107
	4. Is combination therapy with lamivudine and interferon-alpha superior to monotherapy with either drug?	119
Chapter V	**Discussion**	
	Discussion and future immune modulatory strategies in the treatment of chronic hepatitis B.	133
Chapter VI	**Summary**	146
	Samenvatting	149
	Dankwoord	153
	Curriculum vitae	155
	Bibliography	157
	List of abbreviations	159

Chapter I

General Introduction

Treatment of chronic hepatitis B: Immune modulation in chronic hepatitis B patients.

Hepatitis B virus morphology and replication

The hepatitis B virus (HBV) is a 42 nm viral particle and member of the hepadnaviridae family. Its double-shelled structure consists of an outer envelop composed of surface proteins (HBsAg) and an inner capsid formed by core-proteins (HBcAg) surrounding the partially double stranded DNA (1). The HBV DNA has 4, partially overlapping, open reading frames (ORF´s) encoding for the viral antigens.

ORF P is the longest one and has four contiguous regions: one encoding for the terminal protein (a covalently bound protein necessary for minus strand syntheses priming (primase)), a second non-specific domain (spacer), a third for the reverse transcriptase and a fourth domain encoding for RNaseH.

ORF S encodes for the pre-S1, pre-S2 proteins and the HBs-antigen and is completely located within ORF P. In particular pre-S1 is suggested to be important for viral attachment (2). HBsAg contains the major antigenic a-determinant. HBsAg is produced at the membrane of the endoplasmic reticulum (ER); complete virions, the non-infectious filaments (containing small, middle and large HBsAg proteins) or spheres (consisting of small and middle-sized HBsAg proteins) circulate in serum in excess.

ORF C encodes for the (pre-) core proteins, HBeAg and HBcAg. The core particles are postulated to be necessary for the assembly of the virus. HBcAg is synthesized in the cytosol and can be stored in the nucleus. In serum however, it is undetectable in contrast to the secretory form of the core protein, HBeAg.

ORF X expresses the X-gene, the putative transcriptional activator of several genes. Both ORF C and ORF X partially overlap with ORF P (1).

After attachment of HBV to the host cell membrane, the viral DNA is uncoated and translocated to the cell nucleus where it is repaired and fully double-stranded covalently closed circular DNA (ccc-DNA) is formed. The ccc-DNA acts as a template for the transcription of mRNA´s among which the RNA pre-genome regulated by host RNA polymerase. These transcripts are transported to the cytoplasm. The core particles aggregate and encapsidize at least one HBV polymerase protein and the pre-genomic RNA to form the core particle. In this replicating complex a minus DNA strand will be formed after priming of the reverse transcriptase by HBV polymerase. RNaseH degrades the pre-genomic RNA, with the exception of a small region at the 5' end, which is the primer of the plus-strand formation. The small, middle and large HBsAg transcripts are transported to the ER and can envelop core particles after budding into the lumen of the ER, followed by release of the virion in serum by exocytosis (1, 3).

Chronic Hepatitis B virus infection

Chronic HBV infection is defined as persistence of HBV replication for more than 6 months after the acute infection. Worldwide, approximately 350 million people are chronic HBV carriers. Chronic HBV can progress to liver failure, portal hypertension and hepatocellular carcinoma (4). Complications of HBV infection are accountable for the death of around one million people each year.

Active viral replication is reflected by detectable serum HBV DNA and HBeAg levels, except in patients infected with a pre-core mutant in whom HBeAg is undetectable. Viral replication is often accompanied by fluctuating serum transaminase values. HBeAg seroconversion is usually followed by normalization of serum transaminases and improvement of liver histology (5-8). In patients with cirrhosis clinical outcome and survival in chronic HBV is significantly improved after HBeAg seroconversion, especially biochemical disease remission seems to be an important factor (5, 9-10). Therefore achieving sustained HBeAg seroconversion is the major goal of antiviral therapy in HBV.

Antiviral therapy in chronic hepatitis B virus infection

Two drugs are registered for the treatment of CHB: interferon-alpha (IFN), since the early nineties, and lamivudine (Zeffix®), since the end of 1999. Several new immune modulating drugs and nucleoside analogues are under investigation.

I. Immune modulating drugs

1.Alpha-Interferon

IFN-α has direct and indirect antiviral properties, which are of major importance in the host defense system against viral infections (11). After binding to its cell membrane receptor, IFN-α stimulates the transcription of a wide variety of proteins. Some have antiviral or immunomodulatory effects, however the function of most proteins is unknown. IFN-α acts as an immunostimulator by enhancing the expression of HLA class I antigens, which will probably facilitate the NK cell and cytotoxic T-cell mediated killing of infected hepatocytes (11-12). In addition IFN acts by blocking of entry and uncoating of the virus and inhibiting of the production of pre-genomic m-RNA (11-13).

Loss of HBeAg following IFN therapy is reported in 20 to 40% of patients, this response is sustained in 80 to 90% of responders (5, 7, 9, 14-15). Prolongation of IFN therapy to 32 weeks may enhance the efficacy and in those patients who failed to a standard course re-treatment given in an adequate dose may be beneficial (16-17). In general the HBeAg seroconversion

rate strongly depends on baseline ALT levels and IFN increases the spontaneous response rate by a factor 1.8 (18). IFN responders are more likely to completely clear HBV DNA (by PCR) and HBsAg compared to spontaneous HBeAg-seroconverters (5, 7, 9). Depending on the duration of follow up (4 to 9 years), eventually 15-55% of patients loose HBV DNA (PCR) from serum and 23-86% become HBsAg negative (6-7, 9,19-20). Patients clearing HBeAg following IFN therapy have a significantly lower risk to develop cirrhosis and an improved survival rate (5, 10, 21-22).

Pegylated interferon-alpha (IFN covalently binded to a polyethylene glycol molecule) has been developed increasing the half-life of IFN significantly. This allows dosing once weekly instead of three times weekly. Efficacy may be enhanced, side effects may be reduced. Pegylated IFN is currently evaluated in chronic HBV and HCV. The limited efficacy and the numerous serious side effects of standard IFN have initiated the search for other therapeutical approaches such as new immune modulating drugs and nucleoside analogues.

2. Other immunostimulatory drugs

a. The use of *Therapeutic vaccination* in chronic HBV patients to induce an immune response has been a subject of investigation for years. Although pilot studies show encouraging results, no clear benefit has been shown yet. Roughly three groups of specific vaccines have been investigated so far: the recombinant HBV vaccines, T-cell vaccines and genetic DNA vaccines.

Several pilot studies on HBs-vaccines showed encouraging results (23-26). One study showed a $CD4^+$ mediated (Th1) response in part of the patients (7/27) after vaccination with HBsAg (23). In another pilot study using a vaccine consisting of the HBV envelope antigen together with a new adjuvant system sustained loss of HBeAg was obtained in 15% of patients (24). However in this study no placebo group was included. One large controlled trial has been published so far, including 118 patients treated with a PreS2/S vaccine (GeHevac B), an S vaccine (Recombivax) or placebo. Vaccination with either vaccine was followed by a significant decrease in HBV DNA. Although an increased HBeAg seroconversion rate was observed compared to placebo (13.3% versus 3.6% respectively) this did not reach statistical significance (25).

The Hepatitis B core antigen based vaccine Theradigm-HBV™, is a single-epitope vaccine consisting of a cytotoxic T cell (CTL) epitope derived from the HBV core protein incorporated into a vaccine comprising a T-helper cell epitope and two palmic acid molecules. It has been used to induce a CTL response to HBV. Although the vaccine was able to induce a CTL-response, the magnitude of this response was below the level observed following a resolved acute HBV infection (27-28).

DNA-based vaccines (consisting of plasmid DNA encoding HBV envelop proteins) have been only evaluated in mouse models so far.

b. Thymosine alpha 1 (Talpha1) is an immunomodulatory peptide. In Asians a significant increased HBeAg loss at the end of follow-up was shown in patients following Talpha1 therapy compared to placebo. However these favorable results could not be confirmed in another large multi-center trial in chronic HBeAg positive HBV patients (29-30).

c. Cytokine therapy. The efficacy of IL12 has been investigated in a phase I/II trial including 116 chronic HBV patients who received 0.03 μg, 0.25 μg or 0.50 μg rHuIL12 once weekly for 12 weeks. Follow-up was 12 weeks. Although a significant decrease in HBV DNA was observed at the end of therapy, sustained HBeAg seroconversion was limited to the 2 groups receiving 0.25 μg and 0.50 μg rHuIL12. At the end of follow-up 16% (5/31) showed loss of HBeAg and gain of anti-HBe (31).

Cytokine therapy with IFN-β -or γ or TNF-α is under investigation.

d. Anti-HBs is used after liver transplantation in HBV patients to prevent re-infection of the graft, in newborns of HBV infected women to prevent perinatal transmission and as post-exposure prophylaxis. In the treatment of chronic HBV the use of anti-HBs as an immune modulator has only been studied in 2 small studies. Passive immunization with anti-HBs in 6 chronic HBsAg positive patients induced a (temporary) decrease or clearance of serum HBsAg (32). Monoclonal anti-HBs given in chronic HBV patients with active disease caused a (temporary) drop in HBV DNA and HBsAg levels (33). In addition in 2 patients with hypogammaglobulinemia the clinical, serological and histological profile improved after infusion of monoclonal anti-HBs (34). Furthermore HBsAg-anti-HBs complexes have been used as therapeutic vaccines in chronic hepatitis B patients resulting in an enhanced HBsAg specific T cell response (35).

II. Virus suppressive drugs

1. Lamivudine

Lamivudine is a cytidine dideoxynucleoside analogue which acts as a chain terminator after incorporation in the viral DNA during formation of the (-) and (+) DNA strand. Lamivudine is a strong virus suppressive agent that is well tolerated showing minimal side effects during therapy for up to 3 years (36-37). Antiviral therapy with lamivudine aims primarily at long-term virus suppression leading to reduction of necrosis and inflammation preventing further liver damage. Furthermore the strong viral load reduction during lamivudine therapy may restore the T-cell response to HBcAg and HBeAg in HBV patients, although this has been found in one study only up to date (38).

Lamivudine monotherapy significantly reduces HBV DNA levels, normalizes ALT levels and improves liver histology (36, 39-41). Between 15 and 40% of patients at 1-3 yeas of therapy obtain the endpoint of seroconversion (36, 39-42). Elevated baseline ALT is the strongest

predictor of HBeAg seroconversion (40,43). Although prolongation of therapy up to 3 years increases the HBeAg seroconversion rate to 40% a similar increased occurrence rate of lamivudine resistant mutants is observed up to 57% at 3 years (37). A point-mutation in the YMDD motif of the HBV polymerase gene, replaces the methionine by either a isoleucine (M552I) or valine (M552V), which considerably reduces the sensitivity of HBV to lamivudine (44). The YMDD motif is located in the highly conserved domain C, encoding for the triphosphate binding and catalytic region of the viral polymerase. A third mutation, with cross-resistance to famciclovir, has been described in the C domain replacing leucine to methionine (L528M) and is linked to M552V (45-46).

Although the durability of HBeAg seroconversion following lamivudine monotherapy is suggested to be comparable to IFN therapy, reports on this are contradictory (47-51). Whereas in some reports a sustained response is seen in around 80% of patients, single-centre studies show a relapse in nearly 50% of patients during long-term follow up (49-50). Since lamivudine is unable to reduce the amount of ccc-DNA *in vitro*, the persistence of ccc-DNA in the nuclei of the hepatocytes during therapy is probably responsible for relapse of viral replication after cessation of therapy (52).

2. New nucleoside analogues

a. Famciclovir, a deoxyguanosine nucleoside analogue, is the oral pro-drug of penciclovir and has been investigated in a phase 3 clinical trial. Monotherapy for 1 year results in a significant histologic improvement. However famciclovir has only a modest HBV suppressive activity which is clearly inferior to lamivudine monotherapy (53). Famciclovir may have a role in combination therapy with other nucleoside analogues in HBV (54).

b. Lobucavir is a guanosine analogue, showing a 2-4 log reduction in HBV DNA after 4 weeks of therapy. However clinical studies in HBV have been terminated prematurely because of potential carcinogenic effects of lobucavir after a prolonged exposure in small animal models (55).

c. Adefovir-dipivoxil (bis-POM PMEA), is a acyclic nucleotide analogue of dAMP, showing potent antiviral activity against HBV (55). Twelve weeks of Adefovir causes a 4 log reduction of HBV DNA levels and enhances the HBeAg seroconversion rate compared to placebo (56). Moreover Adefovir shows antiviral activity against lamivudine resistant strains and may be effective in the treatment of lamivudine resistant HBV patients (57). However renal toxicity, probably dose-dependent, has become a safety problem after a prolonged period of time. This has made it necessary to reduce the dose of Adefovir from 60 to 30 and finally 10 mg daily.

d. Entecavir is a carbocyclic deoxyguanosine analogue currently under investigation in phase III trials. It shows potent anti-HBV activity at a low dose, a 2.42 mean log reduction in HBV

DNA levels at 4 weeks of therapy was observed (58). Entecavir is the only nucleoside analogue with potential effect on ccc-DNA (59).

e. Emtricitabine, 5 fluorothiacytidine ((-) FTC), is the 5-fluorinated derivate of lamivudine and is currently under investigation in phase I/II trials. In a dose escalating study therapy for 24 weeks was well tolerated and the maximal dose (200 mg) showed a median 3 log reduction of HBV DNA levels at week 24 (60).

III. Combination therapy

Combining immunostimulatory and virus suppressive drugs may theoretically increase the degree of virus suppression and elimination. The efficacy of combination therapy of standard IFN and lamivudine in HBV has been investigated in several trials in the past (40, 61-63). A study on combination therapy in previous non-responders showed a more profound suppression of HBV replication during combination therapy, however no therapeutic merit was obtained (61). Three large randomised controlled trials on combination therapy in chronic HBV have been performed (40, 62-63). In the first 2 studies, evaluating 226 IFN naive and 238 IFN non-responders, no significant benefit of combination therapy was observed (40, 62). However a per-protocol analysis performed by Schalm et al revealed a significant higher HBeAg seroconversion rate following combination therapy (36%) compared to lamivudine monotherapy (19%) or IFN monotherapy (22%). Subgroup analysis based on baseline ALT levels showed a benefit of combination therapy especially in patients with moderately elevated serum ALT levels (40). These encouraging results where confirmed in a more recent study in 151 HBeAg positive patients treated with 24 weeks of lamivudine-IFN combination therapy or 52 weeks of lamivudine monotherapy (63). Combination therapy significantly increased the HBeAg seroconversion rate compared to lamivudine monotherapy (33% versus 19% respectively) (63).

A more detailed study on combination therapy of lamivudine and IFN in HBV seems to be worthwhile. In addition we have studied the effect of addition of anti-HBs in lamivudine treated patients.

Objectives of the study:

1. To evaluate the effect of combination therapy of virus suppressive (lamivudine) and (possible) immuno-modulatory agents (anti-HBs or recombinant alpha-IFN) in chronic HBV on virus suppression and elimination.
2. To study the influence of baseline host immune reactivity (marker serum ALT) on response and durability of response following mono- and combination therapy of IFN and lamivudine in chronic HBV.

References

1. Gerlich W. Structure and molecular virology. Viral Hepatitis: Scientific basis and clinical management. Zucherman and Thomas 1993.
2. Le Seyec J, Chouteau P, Cannie I, Guguen-Guillouzo C, Gripon P. Infection process of the hepatitis B virus depends on the presence of a defined sequence in the pre-S1 domain. J Virol 1999;73:2052-7.
3. Colacino JM, Staschke KA. The identification and development of antiviral agents for the treatment of chronic hepatitis B virus infection. Prog Drug Res 1998;50:259-322.
4. Fattovich G. Natural course and prognosis of chronic hepatitis type B. Viral Hep Rev 1996;2:263-76.
5. Niederau C, Heintges T, Lange S, Goldmann G, Niederau C, Mohr L, Haussinger D. Long-term follow-up of HBeAg-positive patients treated with interferon alpha for chronic hepatitis B. N Engl J Med 1996;334:1422-27.
6. Koorenman, J, Baker B, Waggoner J, Everhart JE, Bisceglie AM, Hoofnagle JH. Long-term remission of chronic hepatitis B after alpha-interferon therapy. Ann Int Med 1991;114:629-34.
7. Carreno V, Castillo I, Molina J, Porres JC, Bartolome J. Long-term follow-up of hepatitis B chronic carriers who responded to interferon therapy. J Hepatol 1992;15:102-6.
8. Lok AS, Chung HT, Liu VW, Ma OC. Long-term follow-up of chronic hepatitis B patients treated with interferon alfa. Gastroenterology 1993;105:1833-8.
9. Lau DT, Everhart J, Kleiner DE, Park Y, Vergalla J, Schmid P, Hoofnagle JH. Long-term follow-up of patients with chronic hepatitis B treated with interferon alfa. Gastroenterology 1997;113:1660-7.
10. Fattovich G, Giustina G, Realdi G, Corrocher R, Schalm SW and the European Concerted Action on Viral Hepatitis (EUROHEP). Long-term outcome of hepatitis B e antigen-positive patients with compensated cirrhosis treated with interferon-alpha. Hepatology 1997;26:1338-42.
11. Foster GR. Interferons in host defense. Semin Liver Dis 1997;17:287-95.
12. Thomas HC. Pathogenesis of chronic hepatitis HBV infection and mechanisms of action of antiviral compounds. Viral hepatitis and liver diseases ed. by Hollinger FB, Lemon SM 1990;612-615.
13. Peters M. Mechanisms of action of interferons. Semin Liver Dis 1989;9:235-9.
14. Wong DK, Cheung AM, O'Rourke K, Naylor CD, Detsky AS, Heathcote J. Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B. A meta-analysis. Ann Intern Med 1993;15:312-23.
15. Brook MG, Karayiannis P, Thomas HC. Which patients with chronic hepatitis B virus infection will respond to alpha-interferon therapy? A statistical analysis of predictive factors. Hepatology 1989;10:761-3.
16. Carreno V, Marcellin P, Hadziyannis S, Salmeron J, Diago M, Kitis GE, Vafiadis I, Schalm SW, Zahm F, Manzarbeitia F, Jimenez FJ, Quiroga JA. Retreatment of chronic hepatitis B e antigen-positive patients with recombinant interferon alfa-2a. The European Concerted Action on Viral Hepatitis (EUROHEP). Hepatology 1999;30:277-82.
17. Janssen HLA, Gerken G, Carreno V, Marcellin P, Naoumov NV, Craxi A, Ring-Larsen H, Kitis G, Hattum J van, Vries RA de, Michielsen PP, Kate FJW ten, Hop WCJ, Heijtink RA, Honkoop P, Schalm SW. Interferon alfa for chronic hepatitis B infection: increased efficacy of prolonged treatment. The European Concerted Action on Viral Hepatitis (EUROHEP). Hepatology 1999;30:238-43.
18. Krogsgaard K, Bindslev N, Christensen E, Craxi A, Schlichting P, Schalm S, Carreno V, Trepo C, Gerken G, Thomas HC, et al. The treatment effect of alpha interferon in chronic hepatitis B is independent of pre-treatment variables. Results based on individual patient data from 10 clinical controlled trials. European Concerted Action on Viral Hepatitis (EUROHEP). J Hepatol 1994;21:646-55.
19. Krogsgaard K. The long-term effect of treatment with interferon-alpha 2a in chronic hepatitis B. The long-term follow-up investigator group. The European Study Group on Viral Hepatitis (EUROHEP). Executive team on anti-viral treatment. J Viral Hepat 1998;5:389-97.
20. Honkoop P, Hanssen BE, Ashruf RZ, Man de RA, Schalm SW. Long-term follow-up of chronic hepatitis B patients after interferon treatment. J Hep 1998;1:112A.
21. Lin SM, Sheen IS, Chien RN, Chu CM, Liaw YF. Long-term beneficial effect of interferon therapy in patients with chronic hepatitis B virus infection therapy in patients with chronic hepatitis B virus infection. Hepatology 1999;29:971-5.
22. Liaw YF, Tai DI, Chu CM, Chen TJ. The development of cirrhosis in patients with chronic type B hepatitis: a prospective study. Hepatology 1988;8:493-6.
23. Couillin I, Pol S, Mancini M, Driss F, Brechot C, Tiollais P, Michel ML. Specific vaccine therapy in chronic hepatitis B: induction of T cell proliferative responses specific for envelope antigens. J Infect Dis 1999;180:15-26.

24. Van Hattum J, Rash Mc, Boland GJ, Leroux-Roels G, Vandepapeliere P. Early evaluation of two schedules of administration of a novel therapeutic vaccine for chronic hepatitis B. Hepatology 2000;32:458A.
25. Pol S, Nalpas B, Driss F, Michel ML, Tiollais P, Denis J, Brecho C. Efficacy and limitations of a specific immunotherapy in chronic hepatitis B. J Hepatol 2001;34:917-21.
26. Pol S, Couillin I, Michel ML, Driss F, Nalpas B, Carnot F, Berthelot P, Brechot C. Immunotherapy of chronic hepatitis B by anti HBV vaccine. Acta Gastroenterol Belg 1998;61:228-33.
27. Heathcote J, McHutchison J, Lee S, Tong M, Benner K, Minuk G, Wright T, Fikes J, Livingston B, Sette A, Chestnut R. A pilot study of the CY-1899 T-cell vaccine in subjects chronically infected with hepatitis B virus. The CY1899 T Cell Vaccine Study Group. Hepatology 1999;30:531-6.
28. Vitiello A, Ishioka G, Grey HM, Rose R, Farness P, LaFond R, Yuan L, Chisari FV, Furze J, Bartholomeuz R, et al. Development of a lipopeptide-based therapeutic vaccine to treat chronic HBV infection. Induction of a primary cytotoxic T lymphocyte response in humans. J Clin Invest 1995;95:341-9.
29. Mutchnick MG, Lindsay KL, Schiff ER, Cummings GD, Appelman HD, Peleman RR, SilvaM, Roach KC, Simmons F, Milstein S, Gordon SC, Ehrinpreis MN. Thymosin alpha1 treatment of chronic hepatitis B: results of a phase III multicentre, randomized, double-blind and placebo-controlled study. J Viral Hepat 1999;6:397-403.
30. Chien RN, Liaw YF, Chen TC, Yeh CT, Sheen IS. Efficacy of thymosin alpha1 in patients with chronic hepatitis B: a randomized, controlled trial. Hepatology 1998;27:1383-7.
31. Zeuzem S, Carreno V. Interleukin-12 in the treatment of chronic hepatitis B and C. Antiviral Res 2001;52:181-8.
32. Reed WD, Eddleston AL, Cullens H, Williams R, Zuckerman AJ, Peters DK, Williams DG, Maycock WA. Infusion of hepatitis-B antibody in antigen-positive active chronic hepatitis. Lancet 1973;15:1347-51.
33. Galun E, Eren R, Safadi R, Ashour Y, Terrault N, Keeffe EB, Matot E, Mizrachi S, Terkieltaub D, Zohar M, Lubin I, Gopher J, Shouval D, Dagan S. Clinical evaluation (phase I) of a combination of two human monoclonal antibodies to HBV: safety and antiviral properties. Hepatology 2002;35:673-9.
34. Lever AM, Waters J, Brook MG, Karayiannis P, Thomas HC. Monoclonal antibody to HBsAg for chronic hepatitis B virus infection with hypogammaglobulinaemia. Lancet 1990;23:1529.
35. Wen YM, Qu D, Zhou SH. Antigen-antibody complex as therapeutic vaccine for viral hepatitis B. Int Rev Immunol 1999;18:251-8.
36. Lai CL, Chien RN, Leung NWY, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray DR. A one-year trial of lamivudine for chronic hepatitis B. N Engl J Med 1998;339:61-8.
37. Leung NW, Lai CL, Chang TT, Guan R, Lee CM, Ng KY, Lim SG, Wu PC, Dent JC, Edmundson S, Condreay LD, Chien RN. Extended lamivudine treatment in patients with chronic hepatitis B enhances hepatitis B e antigen seroconversion rates: results after 3 years of therapy. Hepatology 2001;33:1527-32.
38. Boni C, Bertoletti A, Penna A, Cavalli A, Pilli M, Urbani S, Scognamiglio P, Boehme R, Panebianco R, Fiaccadori F, Ferrari C. Lamivudine treatment can restore T cell responsiveness in chronic hepatitis B. J Clin Invest 1998;1:968-75.
39. Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, Crowther L, Condreay LD, Woessner M, Rubin M, Brown NA. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med 1999;341:1256-63.
40. Schalm SW, Heathcote J, Cianciara J, Farrell G, Sherman M, Willems B, Dhillon A, Moorat A, Barber J, Gray F. Lamivudine and alpha interferon combination treatment of patients with chronic hepatitis B infection: a randomised trial. Gut 2000;46:562-8.
41. Lau DT, Khokhar MF, Doo E, Ghany MG, Herion D, Park Y, Kleiner DE, Schmid P, Condreay LD, Gauthier J, Kuhns MC, Liang TJ, Hoofnagle JH. Long-term therapy of chronic hepatitis B with lamivudine. Hepatology 2000;32:828-34.
42. Liaw YF, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Chien RN, Dent J, Roman L, Edmundson S, Lai CL. Effects of extended lamivudine therapy in Asian patients with chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. Gastroenterology 2000;119:172-80.
43. Chien RN, Liaw YF, Atkins M. Pretherapy alanine transaminase level as a determinant for hepatitis B e antigen seroconversion during lamivudine therapy in patients with chronic hepatitis B. Asian Hepatitis Lamivudine Trial Group. Hepatology 1999;30:770-4.
44. Allen MI, Deslauriers M, Andrews CW, Tipples GA, Walters KA, Tyrrell DL, Brown N, Condreay LD. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. Hepatology 1998;27:1670-7.

45. Niesters HG, Honkoop P, Haagsma EB, de Man RA, Schalm SW, Osterhaus AD. Identification of more than one mutation in the hepatitis B virus polymerase gene arising during prolonged lamivudine treatment. J Infect Dis 1998;177:1382-5.
46. Batholomeusz A, Locarnini S. Mutations in the hepatitis B virus polymerase gene that are associated with resistance to famciclovir and lamivudine. J Antivir News 1997;5:123-4.
47. Perrillo RP, Schiff ER, Davis GL, et al. A randomized, controlled trial of interferon alfa-2b alone and after prednisone withdrawal for the treatment of chronic hepatitis B. The Hepatitis Interventional Therapy Group. N Engl J Med 1990;323:295-301.
48. Dienstag JL, Schiff ER, Mitchell M, Casey DE Jr, Gitlin N, Lissoos T, Gelb LD, Condreay L, Crowther L, Rubin M, Brown N. Extended lamivudine retreatment for chronic hepatitis B: maintenance of viral suppression after discontinuation of therapy. Hepatology 1999;30:1082-7.
49. Schiff E, Cianciara J, Karayalcin S, Kowdley K, Woessner M, McMullen S, Pearce M, Brown N. Durable HBeAg and HBsAg seroconversion after lamivudine for chronic hepatitis B (CHB). J Hep 2000;2:99A.
50. Song BC, Suh DJ, Lee HC, Chung YH, Lee YS. Hepatitis B e antigen seroconversion after lamivudine therapy is not durable in patients with chronic hepatitis B in Korea. Hepatology. 2000;32:803-6.
51. Fontaine H, Driss F, Lagneau JL, Zylberbeg H, Brechot C, Pol S, Necker CHU. Hepatitis B virus reactivation after lamivudinediscontinuation. Hepatology 1999;30:349A.
52. Moraleda G, Saputelli J, Aldrich CE, Averett D, Condreay L, Mason W. Lack of effect of antiviral therapy in nondividing hepatocyte cultures on the closed circular DNA of woodchuck hepatitis virus. J Virol 1997;9392-99.
53. de Man RA, Marcellin P, Habal F, Desmond P, Wright T, Rose T, Jurewicz R, Young C. A randomized, placebo-controlled study to evaluate the efficacy of 12-month famciclovir treatment in patients with chronic hepatitis B e antigen-positive hepatitis B. Hepatology 2000;32:413-7.
54. Shaw T, Locarnini S. Combination chemotherapy for hepatitis B virus: the final solution? Hepatology 2000;32:430-2.
55. Torresi J, Locarnini S. Antiviral chemotherapy for the treatment of hepatitis B virus infections. Gastroenterology 2000;118:S83-103.
56. Heathcote E, Jeffers L, Wright T, Sherman M, Perillo R, Sacks S, Carithers R, et al. Loss of HBV DNA and HBeAg and seroconversion following short-term (12 weeks) adefovir dipivoxil therapy in chronic hepatitis B: two placebo-controlled studies. Hepatology 1998;28:317A.
57. Perrillo R, Schiff E, Yoshida E, Statler A, Hirsch K, Wright T, Gutfreund K, Lamy P, Murray. Adefovir dipivoxil for the treatment of lamivudine-resistant hepatitis B mutants. Hepatology 2000;32:129-34.
58. de Man RA , Wolters L, Nevens F, Chua D, Sherman M, Lai CL, Thomas N, DeHertogh D. A study of oral entecavir given for 28 days in both treatment naive and pre-treated subjects with chronic hepatitis. Hepatology 2000;32:376A.
59. Innaimo SF, Seifer M, Bisacchi GS, Standring DN, Zahler R, Colonno RJ. Identification of BMS-200475 as a potent and selective inhibitor of hepatitis B virus. Antimicrob Agents Chemother 1997;41:1444-8.
60. Gish RG, Wright TL, Wang C, Corey L, Leung N, Chan FKL, Fried MW, Sacks S, Fang L, Wang LH, Rousseau FS, Delahanty J. Emtricitabine (FTC): results of a 24-week dose-escalating trial in patients with chronic HBV infection (FTCB-102). Hepatology 2000;32:378A.
61. Mutimer D, Naoumov N, Honkoop P, Marinos G, Ahmed M, de Man R, McPhillips P, Johnson M, Williams R, Elias E, Schalm S. Combination alpha-interferon and lamivudine therapy for alpha-interferon-resistant chronic hepatitis B infection: results of a pilot study. J Hepatol 1998;28:923-9.
62. Schiff ER, Karayalcin S, Grimm IS, Perillo RP, Dienstag JL, Husa P, de Man RA, Goodman Z, Condreay LD, Crowther LM, Woessner MA, McPhillips PJ, Brown NA and an International Lamivudine Investigator Group. Lamivudine and lamivudine/interferon combination therapy in patients with hepatitis B who previously failed interferon: an international controlled trial. Ann Int Med 2002, accepted for publication.
63. Barbaro G, Zechini F, Pellicelli AM, Francavilla R, Scotto G, Bacca D, Bruno M, Babudieri S, Annese M, Matarazzo F, Di Stefano G, Barbarini G. Long-term efficacy of interferon alpha-2b and lamivudine in combination compared to lamivudine monotherapy in patients with chronic hepatitis B. An Italian multicenter, randomized trial. J Hepatol 2001;35:406-11.

Introduction

To Chapter II

Considering the limited efficacy of the current drugs registered for chronic hepatitis B (HBV) lamivudine and interferon-alpha (IFN), the search for new anti-virals or combination therapy aiming at different antiviral targets is ongoing. In chapter II we report on the use of antibodies to HBV-proteins (anti-HBs) either alone or in combination with lamvudine or interferon. Anti-HBs has proven its value in the prevention of post-liver transplantation HBV reinfection of the graft, post-exposure prophylaxis after needle stick injuries and prevention of vertical transmission in neonates of HBV positive mothers. The formation of HBsAg-Ab complexes neutralizes circulating HBsAg, preventing the transfer to other hepatocytes. In addition, theoretically, the formation of HBsAb-HBsAg complexes may enhance the antibody-dependent cellular cytotoxicity. We studied two different antibodiesl, the polyclonal hepatitis B immunoglobulin (HBIg) and Tuvirumab, a human monoclonal preparation of the IgG_1 subclass, directed against the confirmational epitope the *a*-determinant, produced by a transfectoma cell line (MAb). The advantages of the MAb over HBIg are the reduced costs, unlimited availability and higher affinity for HBsAg shown in preclinical pharmacological studies, on the other hand the risk of selecting immune escape mutants is increased.

We participated in a large multicenter phase II trial investigating the safety and efficacy of the MAb as monotherapy or in combination with standard IFN. This trial was stopped prematurely due to side effects that were suspected to be immune complex mediated. Only 15 patients were included at that time of which ten had received the MAb as mono or in combination therapy. The results are presented in the first part of this chapter. No conclusions about efficacy could be drawn, although various degrees of HBsAg clearance were shown.

Reducing the HBsAg load before administration of anti-HBs could theoretically significantly increase the neutralizing efficacy of anti-HBs. We designed two phase II pilot studies using lamivudine pretreatment to obtain maximal virus suppression (HBV DNA negativity by hybridization assay) before adding anti-HBs (MAb or HBIg). The studies aimed to investigate the tolerability and neutralizing efficacy of both preparations in chronic HBV patients with low level viral replication. The functional activity or "neutralizing efficacy" of both preparations *in vitro* was measured with an inhibition-neutralization assay. In the first study six lamivudine treated HBV DNA negative patients received HBIg (chapter II.2). The dosing was based on the amount of HBIg given to prevent allograft reinfection after livertransplantation. We encountered difficulties and low reproducibility with the standard HBsAg and anti-HBs quantification tests. Experimental EIA's were designed for more reliable quantification.

In the second study, started subsequently, the MAb was given in a dose-escalating schedule to reduce the risk of immune complex mediated side effects to nine lamivudine treated chronic HBV patients. In two out of nine patients repeated infusions with MAb led to serum sickness

like syndrome. One was withdrawn form the study, the other eight were evaluable. EIA procedures were designed to accurately measure free MAb levels, free HBsAg and MAb in MAb-HBsAg complexes. Chapter II.3 focuses on these laboratory methods.

Chapter II

Anti-HBs

in chronic hepatitis B

II.1 EFFICACY AND SAFETY OF AN INTRAVENOUS MONOCLONAL ANTI-HBS IN CHRONIC HEPATITIS B PATIENTS

A.B. van Nunen[1], M. Baumann[2], M. P. Manns[3], J. Reichen[4], U. Spengler[5], J-P. Marschner[2], R. A. de Man[1] on behalf of an international study group.

[1]Dept. of Hepatogastroenterology, University Hospital Rotterdam, The Netherlands

[2]Dept. of Clinical Development, Boehringer Mannheim GmbH, Mannheim, Germany

[3]Dept. of Hepatogastroenterology; Medizinische Hochschule Hannover, Germany

[4]Dept. of Internal Medicine, Inselspital Berne, Switzerland

[5]Dept. of Internal Medicine, University of Bonn, Germany

Liver 2001;21:207-12.

Abstract

Background: In this study the safety and efficacy of a monoclonal anti-HBs, Tuvirumab (MAb), were investigated. Tuvirumab is a human monoclonal antibody recognizing the stable 'a'-determinant of the HBsAg.

Methods: We included 10 chronic hepatitis B patients: four received monotherapy, and six combination therapy with interferon alpha 2b.

Results: Because the development of insoluble [HBsAg-HBsAb] complexes led to adverse events, the MAb dose had to be reduced in seven patients. In nine patients treatment was stopped prematurely because of lack of efficacy, i.e. neutralization of HBsAg in serum. However, temporary HBsAg levels were reduced by at least 50% in all patients, in three patients receiving combination therapy, background levels of HBsAg in serum were reached. A loss of serum HBV-DNA was seen in three patients in the combination group, followed by HBeAg seroconversion in two patients.

Conclusions: We conclude that MAb was not effective in achieving primary efficacy as assessed by neutralization of circulating HBsAg. Whether a combination of Mab with an antiviral agent that reduces the HBsAg load -and therefore minimizes the risk of adverse events - may result in clinical efficacy should be investigated.

Introduction

Only a minority of hepatitis B patients benefits from current treatment strategies. Standard alpha interferon (IFN) therapy is effective in about one third of the patients; prolongation increases the response rate to a maximum of 40% of patients (1, 2). The respond rate depends mainly on baseline characteristics (3). Although lamivudine results in serological, biochemical and histological improvement in 60-70% of patients, viral clearance is achieved in only 16% of patients at 1 year of therapy (4, 5). Prolongation of lamivudine therapy beyond 1 year is hampered by the increasing occurrence of lamivudine-resistant mutants, reaching 50% after 2 years (4, 6). Although lamivudine and interferon monotherapy clearly has a role in antiviral therapy for chronic hepatitis B (CHB), their present therapeutic achievements leave sufficient room for the development of new therapeutic strategies.

The efficacy of hepatitis B immunoglobulin (HBIg) in the passive immune prophylaxis of orthotopic liver transplant (OLT) patients has been reported (7, 8). In a vaccination trial it has been shown that [HBsAb-HBsAg] complexes enhance the proliferative response of human HBsAg-specific T-cell clones, resulting in a therapeutic effect on chronic hepatitis B infection (9). Since immunotherapy in CHB with passive immunization with anti-HBs, was attempted by Reed et al using polyclonal HBIg (1973) and with monoclonal antibodies by Lever et al (1987), no trials of anti-HBs have been performed for the treatment of CHB (10, 11). We therefore attempted immunotherapy with MAb in the ten patients reported here.

Tuvirumab (MAb) is a human monoclonal antibody of the IgG_1 subclass. It is produced by a transfectoma cell line, a recombinant mouse cell line (SP2/0) which is transfected with the genetic material of a human B lymphocyte of a HBV-vaccinated patient. Regarding binding site and binding affinity with HBsAg, Tuvirumab is identical to OST 577, produced by a "trioma" cell line, for which clinical pilot studies were performed in OLT patients (12, 13). Both antibodies have the same genetic code from the same peripheral blood lymphocyte. MAb recognizes the 'a'-determinant of the hepatitis B surface antigen. The 'a'-determinant is the most stable part of the hepatitis B virus and one of the major neutralizing epitopes. Anti-'a' antibodies are protective against almost all known subtypes of the hepatitis B virus (14, 15). The binding activity of MAb is 200-fold higher compared with serum-derived polyclonal antibodies (HBIg) (16).

In a phase II trial, the safety and efficacy of MAb were investigated. The trial was stopped prematurely after 10 patients had been treated with the monoclonal antibody for at least 4 weeks. The results are presented here.

Patients and Methods

Patients

Fifteen patients, eight IFN nonresponders and seven IFN naive, were included in this trial after giving their informed consent. All patients were above 18 years of age and suffered from chronic hepatitis B, defined as HBsAg and HBeAg positivity in serum lasting for at least 6 months, increased ALT (50-400 U/l) for the last 6 months before study entry, detectable serum HBV-DNA (liquid hybridization test), and a histological diagnosis of chronic hepatitis (portal inflammation, hepatocyte necrosis).

Exclusion criteria were: Asian origin, pregnant or lactating women, detectable serum HBeAb, co-infection with HAV, HCV, HDV, HIV, fulminant, alcoholic or drug-induced hepatitis or decompensated liver disease. For safety reasons, patients were not included if there was a history of periarteriitis nodosa, serum sickness, psoriasis, creatinine >1.5 times upper limit of normal, proteinuria, micro-hematuria, major organic dysfunction or contraindication for interferon.

Medication

Patients were stratified for previous IFN therapy and randomly assigned to one of the following groups:

- MAb treatment group (*n*= 4)
- MAb + IFN treatment group (combination group) (*n*= 6)
- IFN treatment group (only IFN naive) (*n*= 3)
- Untreated control group (only IFN nonresponders) (*n*= 2)

Interferon α-2b (Intron™ A, Schering-Plough) was given according to the standard regimen, i.e. 5-10 MU tiw intracutaneously for 16 weeks, and was not administered on the same day as MAb in the combination group.

MAb is a lyophilisate, which has to be reconstituted with 10-ml sterile water before administration. After reconstitution, the drug was given intravenously in 250 ml 0.9% NaCl over a 30-min period.

During the first 2 weeks (escalation phase) MAb was given every 2-3 days according to a fixed dosing schedule: 20mg-20mg-40mg-40mg-80mg-80mg (20 mg Mab corresponds to about 360.000 IU anti-HBs HBIg). The dosing schedule was based on safety and efficacy data collected in a pilot study with OST-577 (14). The dose escalation should result in a step-wise reduction of the HBsAg serum level and should therefore minimize potential adverse events caused by the formation of insoluble [HBsAg-HBsAb] complexes. The aim of this escalation phase was to neutralize circulating HBsAg, resulting in free circulating anti-HBs.

In week 3, the maintenance phase, dosing was individualized in order to maintain free anti-HBs levels in the blood. In this phase anti-HBs serum levels were measured every 2 weeks. Further MAb doses were administered depending on the anti-HBs levels, according to the following schedule:

- serum anti-HBs level >5µg/ml (corresponds to about 90 000 IU/l HBIg): no further MAb administration for the next 2 weeks;
- serum anti-HBs level >1µg/ml (corresponds to about 18 000 IU/l HBIg) but <5µg/ml: 1 single dose of 80 mg MAb or of the individual maximum tolerated dose (MTD, see critical events) for the next 2 weeks;
- serum anti-HBs level <1µg/ml: 5 doses (every 2 days) of 80 mg MAb or of the MTD for the next 2 weeks. If free serum anti-HBs levels were not reached after this frequent dosing, treatment was stopped for feasibility reasons.

Critical events

According to the above-mentioned pilot study with OST-577, it was anticipated that the development of adverse events in patients receiving MAb would be mainly related to the formation of insoluble [HBsAg-HBsAb] complexes. For safety reasons critical events were defined: anaphylactic reactions type I, cardiovascular reaction during or within 3 hours after infusion (shock index >1.2, hypertension), generalized dermatological reactions, severe arthralgia, shaking chills/rigors, increase in body temperature >1.5°C (2.5°F). The following laboratory parameters were considered critical adverse events: micro-hematuria, proteinuria, serum creatinine and urea >150% of initial value, a relevant decrease in serum albumin or an increase of serum ALT 5x higher initial value or >800 IU/L.

If critical events occured during the therapy, MAb dosing was stopped until event resolved, and afterwards therapy was continued with a 50% reduced dose, which was considered the individual maximum tolerated dose (MTD). Frequency of dosing of MTD was as described above.

Laboratory Methods

The primary endpoint of the study was the development and maintenance of free anti-HBs levels. Secondary, reduction of HBsAg and changes in HBV DNA and ALT were measured.

All serum samples were stored at -20°C, and HBV markers were assessed centrally. HBV-DNA was quantified by liquid hybridization (Digene-Murex, UK). HBeAg and anti-HBe (qualitatively) were measured using ENZYMUN™ tests (Boehringer Mannheim, Germany). Anti-HBs (µg/ml) was detected with a specific double-antigen ELISA (enzyme-linked

immunosorbent assay) using biotinylated wild-type HBsAg, which was immobilized on a streptavidin-coated tube, as the solid phase. The bound antibodies were detected by binding to a horseradish peroxidase conjugated wild-type HBsAg catalyzing the formation of a colored product (ENZYMUN™ tests, Boehringer Mannheim, Germany). HBsAg (U/ml) was measured by a standard MEIA (microparticle enzyme immuno-assay) (Abbott IMx System). A monoclonal HBsAg-antibody that specially binds HBsAg from serum is immobilized to microparticles. The bound HBsAg is detected with biotinylated polyclonal goat anti-HBs and a polyclonal rabbit anti-biotin antibody conjugated with alkaline phosphatase is catalyzing the formation of a fluorescent product.

The study was performed according to good clinical practice guidelines and the Declaration of Helsinki. All local Medical Ethics Committees approved the protocol.

Results

Fifteen patients were included in the trial: ten received MAb (four monotherapy and six combination therapy with interferon). Baseline characteristics are listed in table 1.

Table 1

Baseline characteristics of patients. Data are expressed as median (min-max).

	MAb treatment group	MAb + IFN treatment group	IFN treatment group	Untreated control group
IFN naive/ nonresponders	1 / 3	3 / 3	3 / 0	0 / 2
Age (yr)	55 (39-63)	39 (19-46)	37 (24-48)	29 (28-30)
Gender (m/f)	4 / 0	4 / 2	2 / 1	1 / 1
HBsAg (U/ml)	36,750 (1,740-72,000)	2,835 (2,200-22,200)	43,200 (6,200-73,000)	3,550 (900-6,200)
HBV DNA (pg./ml)	2,149 (467-6,130)	152 (8-5,065)	3,755 (2,641-33,220)	556 (67-1,045)
ALT (U/l)	90 (65- 116)	100 (27*-199)	166 (144-199)	132 (34*-230)

*protocol violator

Safety

No drug-related serious adverse event was observed. A summary of all adverse events probably or possibly related to MAb is demonstrated in table 2. Eight out of ten patients experienced adverse events probably related to the administration of MAb. In seven patients, four patients in the MAb group and three patients in the combination group, critical adverse

events occurred. Shaking chills/rigors partially combined with fever was reported 5 times for three patients, one in the combination group and two in the MAb group. Proteinuria and micro-hematuria were reported for three patients, two in the combination group and one in the MAb group. Flu-like syndrome was reported twice for one patient in the combination group. Shaking chills/rigors, fever and flu-like syndrome resolved within hours after infusion, one patient received an H_2-antagonist and in some cases paracetamol was used. Proteinuria and micro-hematuria resolved within a few days after withdrawal of therapy. According to the study protocol, all seven patients were re-challenged with a 50% reduced MAb dose (MTD) after the events resolved. No further critical event was observed.

Table 2

Summary of adverse events probably or possibly related to MAb.

	AE (no measures)	critical AE (dose reduction)	critical AE (temp. stop of treatment + dose reduction)	total AE
Chills	1	3	0	4
Fever	0	2	0	2
Abdominal pain	1	0	0	1
Tinnitus	1	0	0	1
Arthralgia	1	0	0	1
Syncope	1	0	0	1
Dyspnoea	2	0	0	2
Flu-like syndrome	0	2	0	2
Lower back pain	1	0	0	1
Leucopenia	1	0	0	1
Proteinuria	1	0	3	4
Micro-hematuria	0	0	1	1

Efficacy

MAb was administered over a period of 27-59 days. In eight out of ten cases MAb was discontinued prematurely according to the study protocol, because a frequent treatment of 5 infusions in 2 weeks during one cycle in the maintenance phase did not result in free anti-HBs

serum levels. One patient was withdrawn from further treatment as scheduled prematurely; no free anti-HBs was detectable. Seven of these eight patients received a reduced MAb dose (MTD) due to critical events: 40 mg (*n*=3) and 20 mg (*n*=4). In two patients the administration of Mab was withdrawn prematurely because the study was stopped, in one patients anti-HBs was detectable.

Thus, none of the patients had a sustained clearance of serum HBsAg with the described dose regimen. In nine out of ten patients treated with MAb the treatment was stopped prematurely because free anti-HBs levels could not be achieved.

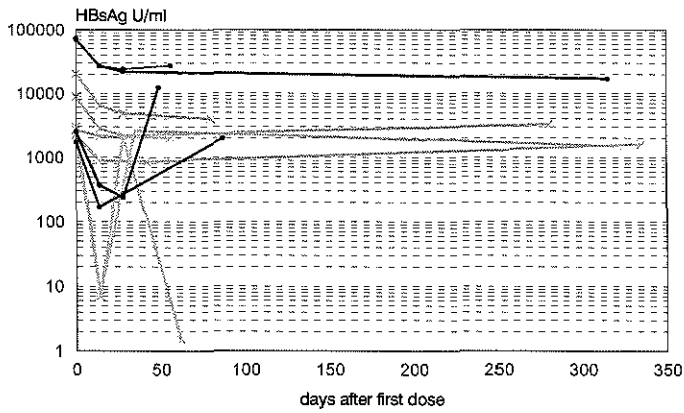


Figure 1 HBsAg levels (U/ml) measured before start of therapy, at day 14, 27, the end of treatment and end of follow up after administration of MAb in the monotherapy (•) or combination therapy (x) group.

HBsAg levels were reduced by at least 50% in all ten patients during MAb treatment in both groups (monotherapy and combination). Figure 1 shows the individual HBsAg curves. In three out of six patients in the combination group HBsAg levels were transiently decreased to background levels on various occasions, coinciding with free anti-HBs levels in the serum. A relapse of HBsAg above pre-treatment levels after stopping treatment was observed in two patients in the MAb group. The relative changes of HBsAg serum levels for these ten patients at the end of therapy and end of follow-up (EFU) are demonstrated in figure 2.In the IFN and untreated control group HBsAg levels fluctuated considerably. A temporary drop by at least 50% was observed in two out of five patients, while in another patient HBsAg increased by 100%.

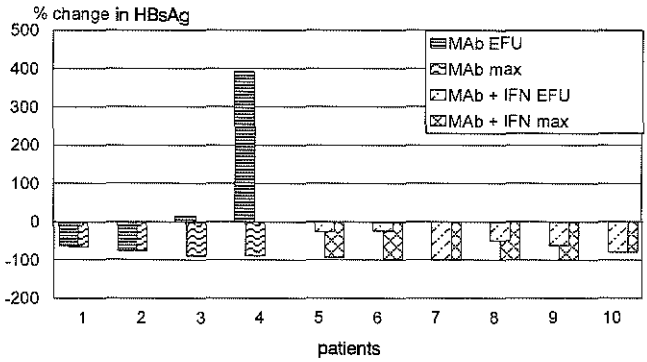


Figure 2 The percentage change in HBsAg at the end of follow-up (EFU) and the maximal (max) change in HBsAg (U/ml) per patient in the monotherapy (MAb) and combination (MAb +IFN) group. In 7 patients HBsAg levels increased again at the EFU, in 3 patients minimal HBsAg levels were reached at the EFU.

With respect to the HBV-DNA levels, three patients in the combination group exhibited a loss of HBV-DNA after about 1 week, which was maintained until the end of follow-up, i.e. 8-46 weeks after start of treatment, IFN was continued in all cases during this period. HBeAg seroconversion was seen in two of these three patients. More than 50% reduction of HBV DNA was observed in the other three patients treated with combination therapy. In the other treatment groups no clinically relevant changes in HBV DNA levels were observed.ALT decreased by 30% in the patients treated with MAb, which implies normalization of ALT in two out of ten patients, one patient treated with combination therapy group and one with monotherapy. No clinically relevant changes in ALT were observed in the untreated and IFN treated patients, which was expected considering the short observation period.

Discussion

The way of action of passive immunization in hepatitis B patients is only partially known. Monoclonal or polyclonal antibodies bind HBsAg, i.e. virions, filaments and spheres that are circulating as free particles in the blood or that are presented at the surface of cells. Consequently, the infection of non-infected cells via circulation should be inhibited. This assumption is supported by results generated during the administration of HBIg in OLT patients and by results of HBIg treatment for interruption of vertical transmission of the virus in neonates of HBsAg-positive mothers and after needle-stick injuries (7-8, 17, 18).

Antibodies might have an indirect effect on cellular immune response via ADCC (antibody dependent cellular toxicity) by binding and activation of NK cells, or other so far only incompletely understood mechanisms. This hypothesis is supported by results from a vaccination trial, in which the administration of [HBsAg-HBsAb] complexes resulted in enhanced proliferative response of human HBsAg specific T-cell clones, producing a

therapeutic effect in CHB patients (9). The concept of immune complexes acting as a strong immune stimulus for humoral immunity is further supported by experimental work (19). Furthermore, coating of HBV-infected hepatocytes may stimulate the leucocyte-mediated phagocytosis (opsonization) (20). *In vivo* and *in vitro* (mice model) IgG-anti-HBs can stimulate T-helper cells to produce lymphokines, which promote antibody synthesis by autologous B-lymphocytes (21). In chronic hepatitis B patients, excess of circulating HBsAg may paralyze the immune system, enabling the virus to survive (high dose tolerance induction). A reduction of serum HBsAg therefore might result in a reactivation of the endogenous immune system (15).

In this trial the most important finding as to efficacy was that sustained clearance of serum HBsAg was not achieved in any of the MAb-treated patients using the described dose regimen. In nine out of ten patients treated with MAb the treatment was stopped prematurely because the primary goal to reach and maintain a status of free anti-HBs levels could not be achieved. On the other hand, it could be shown that MAb recognized and cleared HBsAg from the serum to various degrees. Only in the combination therapy group serum HBsAg decreased transiently to background levels in three patients. Theoretically, this may also reflect a lower production of HBsAg particles because of a reduction of infected hepatocytes in these patients. However, in case of stimulation of the cellular immune system, one would expect an elevation of serum transaminases. None of the ten patients treated with MAb showed a peak in transaminase activity during the treatment or follow-up.

Only in the combination group serum HBsAg was decreased transiently to background levels in three patients. However, it has to be noted that these three patients had relatively low pretreatment HBsAg levels. In general, patients with lower baseline HBsAg levels had a better response. Therefore, an additional effect of IFN on HBsAg serum levels in the combination group, especially in the first weeks of therapy, is unlikely.

With respect to HBV-DNA, however, it seemed that the combination of MAb with IFN is clearly superior to each compound alone. Only in the combination group three patients showed a sustained loss of HBV-DNA. In two of these, this was even combined with a HBeAg-seroconversion when the study was stopped, about 8 to 46 weeks after start of treatment. Since IFN treatment was continued in all cases, even if MAb treatment was stopped prematurely, it is not possible to discern the relative contribution of the two compounds to this positive development, i.e. it cannot be ruled out that this simply reflects the well known effect of IFN.

The critical adverse events seem to be caused by the development of insoluble [HBsAg-HBsAb] complexes after MAb infusion. This indicates again that the antibody is highly effective in binding the antigen and furthermore that the chosen dosing schedule was not sufficient in terms of a safe neutralization of HBsAg. There was no predictor for the development of these

critical events. However, it is likely that after reduction of HBsAg following MAb administration the amounts of antigen and antibody draw near an "equivalence range", combined with an increased risk of development of insoluble antigen-antibody complexes.

With respect to the very limited sample size and treatment/observation period, it is obviously not possible to judge whether the described effects on serum markers of CHB could potentially be translated into clinically meaningful results if the treatment could be maintained by the use of an alternative regimen. The most appropriate approach appears to be a substantial reduction of HBsAg before initiating the MAb treatment and a maintained blocking of viral transcription, e.g. by pre- and concomitant treatment with nucleoside analogues. This may prevent the occurrence of insoluble [HBsAg-HBsAb] complexes and may facilitate the complete clearance of HBsAg and viral particles by the monoclonal antibody.

Acknowledgements

This study was supported by a grant from Boehringer Mannheim GmbH, Germany. Participants in the international study group were: Prof. Katz, Long Beach, CA, Prof. Manns, Hannover, Prof. Murphy, Savannah, GA, Prof. Pruitt, Nashville, TN, Prof. Reichen, Bern, Prof. Reindollar, Charlotte, NC, Prof. Schalm, Rotterdam, Prof. Shiffman, Richmond VA, Prof. Spengler, Bonn, Prof. Warnes, Manchester.

References

1. Wong DK, Cheung AM, O'Rourke K, Naylor CD, Detsky AS, Heathcote J. Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis. Ann Intern Med 1993;119:312-23.
2. Janssen HLA, Gerken G, Carreno V, Marcellin P, Naoumov NV, Craxi A, Ring-Larsen H, Kitis G, Hattum J van, Vries RA de, Michielsen PP, Kate FJW ten, Hop WCJ, Heijtink RA, Honkoop P, Schalm SW. Alpha interferon for chronic hepatitis B infection: increased efficacy of prolonged treatment. Hepatology 1999;30:238-43.
3. Krogsgaard K, Bindslev N, Christensen E, Craxi A, Schlichting P, Schalm SW, Carreno V, Trepo C, Gerken G, Thomas HC, et al. The treatment effect of alpha interferon in chronic hepatitis B is independent of pre-treatment variables. Results based on individual patient data from 10 clinical controlled trials. J Hepatol 1994;21:646-55.
4. Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J. A one-year trial of lamivudine for chronic hepatitis B. N Engl J Med 1998;339:61-8.
5. Schalm SW, Heathcote J, Cianciara J, Farrel G, Sherman M, Stephenson SL, Gray DF. Lamivudine and alpha-interferon combination treatment of patients with chronic hepatitis B infection: a randomized trial. Gut 2000;46:562-8.
6. Honkoop P, Niesters HGM, Man RA de, Osterhaus AD, Schalm SW. Lamivudine resistance in immunocompetent chronic hepatitis B: Incidence and patterns. J Hepatol 1997;26:1393-5.
7. Man RA de, Metselaar HJ, Heijtink RA, Schalm SW. Long-term application of human hepatitis-B immunoglobulin to prevent hepatic allograft infection. Neth J Med 1993;43:74-82.
8. Samuel D, Müller R, Alexander G et al. Liver transplantation in European patients with the hepatitis B surface antigen. N Engl J Med 1993;329:1842-7.
9. Wen YM, Wu X-H, Hu D-C et al. Hepatitis B vaccine and anti-HBs complex as approach for vaccine therapy. Lancet 1995;345:1575-6.
10. Reed WD, Eddlstone ALW, Cullens H et al. Infusion of hepatitis B antibody in antigen-positive active chronic hepatitis. Lancet 1973;5:1347-51.
11. Lever AML, Waters J, Brook MG, et al. Monoclonal antibody to HBsAg for chronic hepatitis B virus infection with hypogammaglobulinaemia. Lancet 1990;335:1529.
12. Östberg L, Pursch E. Human x (Mouse x Human) hybridomas stable producing human antibodies. Hybridoma 1983;4:361-7.
13. Fung J, Östberg L, Shapiro R, Todo S, Demetrius A, Starzl T. Human monoclonal antibody against hepatitis B surface antigen in preventing recurrent hepatitis B following liver transplantation. In: Hollinger BF, Lemon SM, Margolis H eds. Viral hepatitis and liver disease. Baltimore: Williams & Wilkins, 1990: 651.
14. Östberg L. Human monoclonal antibodies in transplantation. Transplant Proc 1992;24:26-30.
15. Gerlich W. Structure and Molecular Virology. In: Zuckermann AJ, Thomas HC eds. Viral hepatitis. New York: Churchill Livingstone, 1993:83-112.
16. Ehrlich PH, Moustafa ZA, Justice JC, Harfeldt KE, Kelley RL, Ostberg L. Characterization of human monoclonal antibodies directed agianst hepatitis B surface antigen. Human Antibody Hybridomas 1992;3:2-7.
17. Ip HM, Lelie PN, Wong VC, Kuhns MC, Reesink HW. Prevention of hepatitis B virus carrier state in infants according to maternal serum levels of HBV DNA. Lancet 1989;8635:406-10.
18. Beasley RP, Hwang LY, Lin CC, Stevens CE, Wang KY, Sun TS, Hsieh FJ, Szmuness W. Hepatitis B immune globulin (HBIg) efficacy in the interruption of perinatal transmission of hepatitis B virus carrier state. Lancet 1981;8234:3888-93.
19. McCluskie MJ, Wen YM, Di Q, Davis HL. Immunization against hepatitis B virus by mucosal administration of antigen-antibody complexes. Viral Immunol 1998;11:245-52.
20. Wawrzynczak EJ. Antibody therapy. Oxford, UK: Bios Scientific Publishers, 1995.
21. Celis E, Abraham KG, Miller RW. Modulation of the immunological response to hepatitis B virus by antibodies. Hepatology 1987;7:563-8.

II.2 PASSIVE IMMUNIZATION OF CHRONIC HEPATITIS B PATIENTS ON LAMIVUDINE THERAPY: A FEASIBLE ISSUE?

A.B. van Nunen[1], R.A. de Man[1], R.A. Heijtink[2], A.C.T.M. Vossen[2], S.W. Schalm[1]

Departments of [1]Hepatology & Gastroenterology and [2]Virology, Erasmus Medical Center Rotterdam, The Netherlands

Journal of Viral Hepatitis 2002;9:221-8.

Abstract

Background: In view of the limited efficacy of lamivudine monotherapy for chronic hepatitis B (HBV), combination with other drugs seems logical. Intravenous neutralization of circulating HBsAg by specific hepatitis B immunoglobulin (HBIg) has been shown to protect hepatocytes against (re-)infection with HBV in the setting of liver transplantation and post-exposure prophylaxis. Large controlled vaccination trials have revealed that HBV can be prevented by HBIg therapy in the majority of newborns after perinatal infection. A benefit of anti-HBs in HBV patients has so far only been investigated in 3 small studies. In this pilot study we investigated the effects of polyclonal i.v. HBIg (Hepatect®, Biotest) administration in HBV patients.

Methods: Six liver biopsy-proven HBV patients, all on lamivudine treatment and HBV DNA-PCR negative, were investigated. Pre-treatment HBsAg levels varied between 120 and 9760 ng/ml. On day 1 10.000 I.U. HBIg was given, followed by 10.000 I.U. once, twice or three times on day 29. Long-term follow-up lasted at least 4 months. HBsAg and anti-HBs were measured quantitatively by standard MEIA and also by an experimental EIA. *In vitro* neutralization of HBsAg by Hepatect was mimicked in an "inhibition in solution assay".

Results: Complete neutralization of HBsAg by HBIg *in vitro* was possible, 50% inhibition concentrations varied between 100-250 IU/l HBIg with HBsAg levels of 68 and 120 ng/ml. No HBIg-related side effects were observed. In 2 patients with low pretreatment HBsAg levels HBsAg reached levels below the detection limit of the assay, which persisted a maximum of 31 and 7.5 hours, respectively. Peak anti-HBs concentrations were 5100 and 4648 IU/l. In the other 4 patients, with higher pre-treatment HBsAg levels, HBsAg concentrations in serum hardly changed. For the whole population, the drop in HBsAg did not reach statistical significance. However, in 4/6 patients a further decrease in HBsAg (18%-66%) was observed.

Conclusions: HBIg was well tolerated; however, efficacy was limited due to high HBsAg levels in spite of maximum inhibition of virion production. "Neutralization" was achieved only in 2 patients with low HBsAg levels. Passive immunization in HBV DNA negative patients is not a feasible option. This strategy seems only feasible if agents inhibiting both the production of viral proteins and Dane particles more selectively, become available.

Introduction

Current treatment strategies for chronic hepatitis B virus infection (HBV) including alpha interferon and lamivudine monotherapy, have limited efficacy. During 16 weeks of interferon monotherapy, around 30% of patients lose HBeAg, which can be increased to almost 40% by prolongation of therapy to 32 weeks (1-3). With lamivudine monotherapy sustained HBeAg seroconversion is achieved in maximal 20% of patients after 52 weeks of treatment (4, 5). This seroconversion is claimed to be durable after withdrawal of therapy, however results are inconsistent (6, 7). In general, withdrawal of therapy is accompanied by a relapse of viral replication. Prolongation is hampered by the occurrence of lamivudine-resistant mutants in up to 50% of patients after 3 years of therapy (7-9). Combination therapy of lamivudine and interferon may lead to a higher seroconversion rate among patients with elevated baseline transaminases, but the therapeutic benefit has not been established as yet (4).

Intravenous immunoglobulins have anti-infectious and immunomodulatory effects and are used both as prophylaxis against certain infectious diseases and as treatment of various autoimmune diseases (10). Hepatitis B-specific immunoglobulins (HBIg) effectively prevent post-exposure infection. Furthermore HBIg interrupts perinatal transmission and a protective antibody titre develops in the majority of newborns after repeated doses (11-14). Long-term passive anti-HBs immunoprophylaxis is part of the standard therapy following liver transplantation in chronic HBV patients (15-18). Complete virus neutralization with a free antibody level in serum prevents clinical reinfection by circulating virions.

Small studies in HBV patients have shown therapeutic benefit of HBIg (HBV clearance) for patients with low HBsAg levels (19). Newly developed monoclonal antibodies significantly reduced viral load in a trimera-mouse model, indicating active binding of HBV particles and removal from serum (20). The infusion of these 2 monoclonal antibodies in HBV patients lead to a (temporary) decrease in HBV DNA load and a reduction in HBsAg (21). The administration of monoclonal antibodies to HBsAg in 2 patients with hypogammaglobulinemia improved the clinical, serological and histological profile of these patients (22).

Antibody-antigen complexes have been shown to enhance viral clearance during long-term follow-up by increasing the uptake of complexed HBsAg by macrophages mainly through the Fc receptor. This modulation of the antigen presentation by the immune-complexes leads to increased T-cell responses and induction of production of pro-inflammatory cytokines (23-27). Encouraging in vitro results in hepatoma cells showed the internalization of IgG antibodies in hepatocytes, independent of the Fc receptor, influencing the assembly and secretion of HBsAg (28).

Infusions of hepatitis C virus immunoglobulins (Cicavir®) in chronically hepatitis C virus (HCV) infected chimpanzees influenced the HCV replication and in vitro HCV specific antibodies seemed to mediate antibody-dependent cellular cytotoxicity (ADCC) (29, 30).

Based on these small studies we investigated the feasibility, determined mainly by high costs, the toxicity and the effectivity of anti-HBs to obtain a sustained decrease in HBsAg levels in 6 HBV patients, in a phase II study. To optimize the therapeutic effect of anti-HBs, in this pilot study patients were pre-treated with lamivudine to decrease the number of complete virions. In case maximum viral suppression was achieved, polyclonal i.v. HBIg (Hepatect^{R}, Biotest) was added.

Patients and methods

Study population

From a cohort of 25 chronic immunocompetent HBV patients treated with lamivudine, 6 patients were selected on the basis of their therapeutic response to lamivudine (HBV DNA levels). All patients had a liver biopsy-proven HBV and HBV DNA negativity (PCR) in combination with HBsAg-positivity during lamivudine monotherapy. Reasons for exclusion of patients were other acquired or inherited causes of liver disease, other significant medical illnesses or conditions which might interfere with the study and contraindications for the use of immunoglobulin therapy such as hypersensitivity or allergy to human immunoglobulins. The study protocol was approved by the Medical Ethical Committee; all patients gave written informed consent.

Administration of HBIg

Hepatect is a human polyclonal hepatitis-B-immunoglobulin preparation containing human polyclonal IgG antibodies against HBsAg with at least 50 I.U. of anti-HBs per ml, mainly of the IgG1 and 2 subclass. Before administration 200 ml of the antibody are dissolved in 250 ml of 5% glucose solution. On day 1 10.000 I.U. (200 ml) was administered intravenously over a 30-minute period. The dose is based on the amount of HBIg given to prevent liver allograft reinfection after orthotopic liver transplantation. If patients tolerated the first dose of Hepatect they were randomized to receive 1, 2 or 3 doses of 10.000 I.U., on day 29, to exclude a carry-over effect of the first dose on day 1. In case of 2 or 3 repeated infusions the doses were given 12 respectively 8 hours apart. Follow-up lasted at least until 4 months after the last dose of Hepatect.

Detection of HBsAg and anti-HBs

At screening, on day 1 and day 29 blood samples for assessment of HBsAg and anti-HBs were taken at start, at the end of the Hepatect infusion and 1, 2, 3, 4, 5, 6, 7, 8, 12, and 24 hours thereafter. In case of 2 or 3 infusions this cycle was repeated after 12 respectively 8 and 16 hours. Additional samples were taken after the first and second infusions on days 3, 4, 5, 8, 15

and 22 and on days 31, 32, 33, 36, 43, 50, 57 and 64 after the first dose. The last sample for long-term follow-up was taken 4 to 5 months after the last infusion.

HBsAg was measured in serum quantitatively by a standard Microparticle Enzyme Immuno assay (MEIA) (AxSYM, Abbott) using a set of standard samples based on the PEI standard (Paul Ehrlich Institute). The MEIA uses microparticles coated with mouse monoclonal anti-HBs to detect HBsAg. The detection limit of the test is 0.01 PEU/ml, but below 3.13 PEU/ml quantification could not be considered reliable.

In addition HBsAg was quantitated in an experimental EIA performed in Costar EIA/RIA 96-wells flat bottom plates (high binding). The plates were coated with monoclonal antibodies according to standard procedures. The solid phase of the HBsAg-EIA consisted of human monoclonal antibody F4-7B (Biotest Pharma, Dreieich, Germany) and mouse monoclonal antibody HBs.OT39 (Organon Teknika, Boxtel, The Netherlands) was used as conjugate. Both antibodies, F4-7B and HBs-OT39, are not influenced by changes in the major antigenic region ("a"-determinant). Wells were incubated with a 100 µl sample for one hour at 37°C. Samples were diluted in buffer (EB) containing PBS with 3% NaCl, 0.05% Tween 20, 0.2% milk powder, and 1% normal goat serum. After incubation, wells were washed four times with PBS/Tween (0.05% Tween 20). Subsequently, incubation was continued with biotin-labeled monoclonal anti-HBs (1 hour 37°C), avidine-labeled horseradish peroxidase (30', 37°C), and TMB substrate (10', room temperature) in succession. Between incubations wells were washed extensively with PBS/Tween. The reaction was stopped with 1N H_2SO_4. Extinction was read at 450 nm. Quantification was performed with an alternative standard related to the PEI standard for HBsAg. HBsAg levels were expressed in ng/ml, 1 ng/ml corresponds to about 1 PEU/ml.

Longitudinal screening for HBsAg and human Ig/HBsAg immune complexes during therapy was performed with solid phase HBs.OT40 and HB.OT42 (mouse monoclonal anti-HBs, Organon Teknika, Boxtel, The Netherlands); F9H9, F7-4B (human monoclonal anti-HBs, Biotest Pharma, Dreieich, Germany) and sheep anti-Hu-Ig conjugate (Amersham) were used for detection. All monoclonal antibodies were directed against epitopes on the small S-protein.

AxSYM MEIA (Abbott, AUSAB) was used for the quantification of anti-HBs. Solid phase HBsAg in this assay is recombinant HBsAg (adw2 and ayw) expressed in mouse L cells (31, 32).

For comparison of HBsAg levels, samples were tested in the same run. Pharmacokinetic samples were tested in the same run per patient.

Inhibition-neutralization assay

Efficacy of serum anti-HBs to achieve neutralization was mimicked in an "inhibition in solution" assay according to the method described by Heijtink et al (33). Inhibition assays were performed with solid phase mouse monoclonal HB.OT40 and HB.OT42, and human monoclonal F4-7B as a conjugate.

Results

Baseline characteristics of the six patients and randomization on day 29 are shown in table I. In all 6 patients HBV DNA (PCR, detection limit 400 genome equivalents/ml (geq/ml) based on the Eurohep HBV standard) was negative at screening; however at inclusion HBV DNA was $3.77x10^3$ geq/ml (Roche, Amplicor, detection limit $1x10^3$ geq/ml) in one patient without evidence of a lamivudine-resistant mutant. In spite of the fact that all patients were HBV DNA negative (PCR) during lamivudine treatment HBsAg levels showed a wide variability unrelated to the duration of lamivudine therapy; HBsAg at baseline varied from 120 to 9760 ng/ml (EIA).

Safety

The administration of Hepatect was well tolerated and there were no drug-related side-effects, in particular there were no symptoms of immune-complex disease. One patient with a pre-existent low blood pressure experienced mild hypotension while sleeping without any complaints before administration of the third infusion on day 29. Other observed adverse events were headache (n=2), flu (n=2), anaemia (n=1), cystitis (n=1) and eczema (n=1). They were not considered to be drug-related.

Table 1

Baseline characteristics

Pt.	race	gender	Age (yrs)	lamivudine (months)	HBV DNA[1] geq/ml	HBsAg ng/ml
1	Chinese	m	50	115	Neg	123
2	Chinese	m	31	8	Neg	120
3	African	m	37	3	Neg	2480
4	African	f	21	4	Neg	4800
5	Turkish	m	27	5	$3,77x10^3$	9760
6	Indonesian	m	37	6	Neg	2320

[1]HBV DNA at screening (within 6 weeks before start of therapy) measured by PCR, detection limit 400 geq/ml (based on the Eurohep standard) and if positive by Quantitative PCR, detection limit 1000 geq/ml (patient 5 before start of therapy).

Neutralization of HBsAg by HBIg in vitro

Pre-treatment serum from 3 patients (patients 1, 4 and 6), representing low (215 ng/ml), high (4200 ng/ml) and moderate (2880 ng/ml) HBsAg levels, respectively, at screening were tested with the inhibition assay. Pre-treatment samples of these patients were diluted and 68, 120 and 68 ng/ml HBsAg respectively were pre-incubated with increasing amounts of Hepatect, up to 5000 IU/l. At HBsAg serum concentrations between 50-100 ng/ml, the 50% inhibition concentrations (IC_{50}) per patient, which can be seen in figure 1, varied between 100 and 250 IU/l of Hepatect. In vitro nearly 100% inhibition of HBsAg-binding to solid phase anti-HBs in EIA was observed in all three cases. However 1250, 5000 and 2500 IU/l respectively were needed to neutralize 68, 120 and 68 ng/ml HBsAg in this assay (Fig 1). Theoretically between 240 and 544 ng/ml HBsAg can be neutralized with 10.000 IU/l Hepatect, according to these measurements.

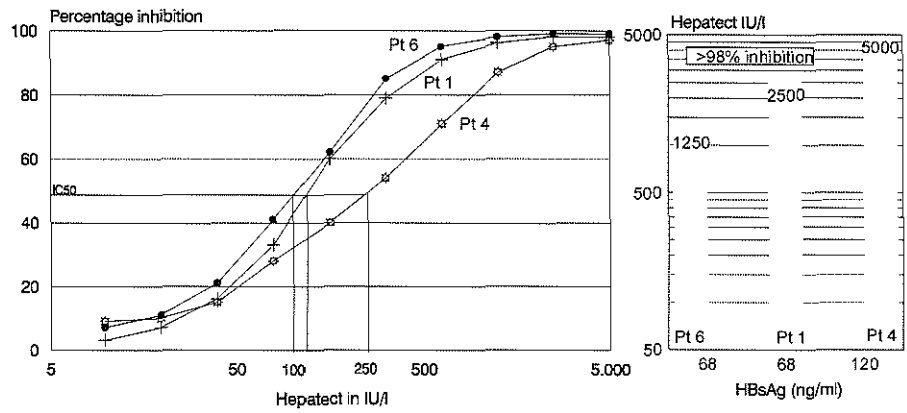


Figure 1 Dose-response curve of neutralizing efficacy of HBIg in three chronic hepatitis B patients (left part). Pretreatment serum samples were diluted and the concentration of HBsAg in the assay was 68 (•), 68 (+) and 120(¤) ng/ml. The figure illustrates that between 100 and 250 IU/l of HBIg are needed to have 50% inhibition of 68 and 120 ng/ml HBsAg (IC_{50}) in this assay. The right part shows the amount of HBIg (IU/l) needed for nearly complete inhibition.

Pt.	neutralization after first dose (HBsAg negativity in hrs)	peak anti-HBs IU/l after first dose	randomization day 29 (no. of doses)	neutralization after second dose(s) (HBsAg negativity in hrs)	peak anti-HBs IU/l after second dose(s)
1	0	464	3	7,5	4.648
2	4,5	328	3	3,5 and 31	5.100
3	0	156	2	0	324
4	0	185	1	0	179
5	0	14	2	0	23
6	0	10	1	0	9

Table 2

Neutralizing effects of Hepatect in hours and peak anti-HBs levels per patient after the first dose on day 1 and after the dose(s) on day 29.

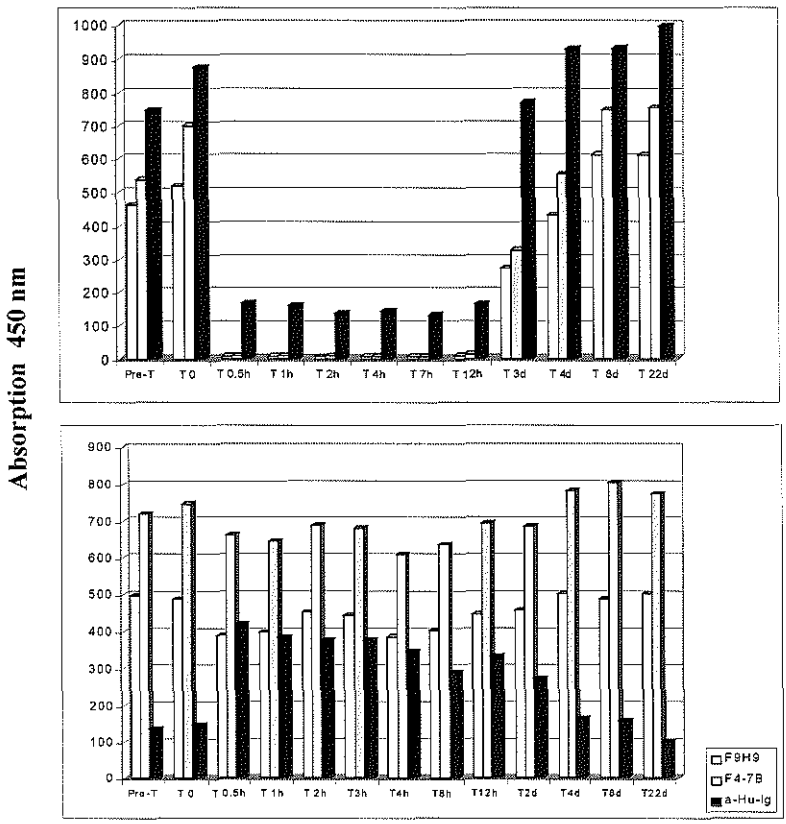


Figure 2 The effect of HBIg on HBsAg levels and the loading of anti-HBs on HBsAg after the first dose are shown. HBsAg (first two bars in both figures) and anti-HBs (third striped bar in both figures) quantification for two patients with low (upper panel) and medium (lower panel) pretreatment HBsAg levels after the first dose of HBIg. All samples were tested in the same dilution. Epitopes corresponding to F9-H9 and F4-7B are located within and outside the major antigenic region of S-protein ("a"-determinant). In patient 2 HBsAg reached background levels after the first HBIg infusion for 12 hours. In contrast HBsAg levels in patient 3 hardly changed, the loading of HBsAg particles with anti-HBs (Hu-Ig) however was clearly detectable at the end of infusion (t=0.5h) and decreased to baseline on day 4. Pre-T=pre-treatment, h=hours, d=days.

Neutralizing efficacy during treatment

Table II summarizes the effects on HBsAg and peak anti-HBs levels per patient after the first dose on day 1 and the other dose(s) on day 29. Only in cases 1 and 2, both patients with very low pretreatment HBsAg levels (123 and 120 ng/ml, respectively) was neutralization possible. Thirty minutes after start of the infusion background levels of HBsAg were reached and maintained in patient 2 on day 1 for 4.5 hours and on day 29 for 3.5 hours, 31 hours after the first and the second infusion; in patient 1 neutralization was maintained for 7.5 hours after the second infusion on day 29. Maximum peak concentrations of anti-HBs in the two patients were 5.100 IU/ml and 4.648 IU/ml, respectively (MEIA, AxSYM).

The short-term effects of HBIg in patients 3, 4, 5 and 6 were minimal; no significant changes in HBsAg levels could be detected after the Hepatect infusions. Although HBsAg was not neutralized, free antibody, probably heterotypical antibody, was detectable in patients 3 and 4 with peak levels of 324 IU/l and 184 IU/l. On day 64 a very low antibody level was still detectable in patient 3.

Figure 2 illustrates in two patients with low (upper panel) and medium (lower panel) pre-treatment HBsAg levels, the changes in HBsAg and the loading of anti-HBs on the surface of particles that still bind to the solid phase of the experimental assay (EIA). Although no change in HBsAg was observed in patient 3, who had a medium pre-treatment HBsAg level, an increase in human IgG (detected with a-Hu-Ig) on HBsAg particles was detected simultaneously with HBsAg. In the patient with low HBsAg levels, a drop of HBsAg to background levels was observed.

Long-term effects of HBIg

One month after the last infusion no significant decline in HBsAg levels was observed (Wilcoxon signed rank), although HBsAg dropped in 4/6 patients. The maximum decrease in HBsAg was 34% compared with baseline values. HBsAg levels, measured at the end of follow up (EFU) 4 to 5 months after the last infusion, were substantially lower in 4 patients compared to pretreatment levels, with a maximum decline of 66%, however this difference was not significant (Wilcoxon signed rank) (Fig 3). Only in the 2 patients with very high HBsAg levels no change was observed. Serum transaminases were normal during lamivudine therapy in 5 out of 6 patients and were not influenced by the HBIg infusions.

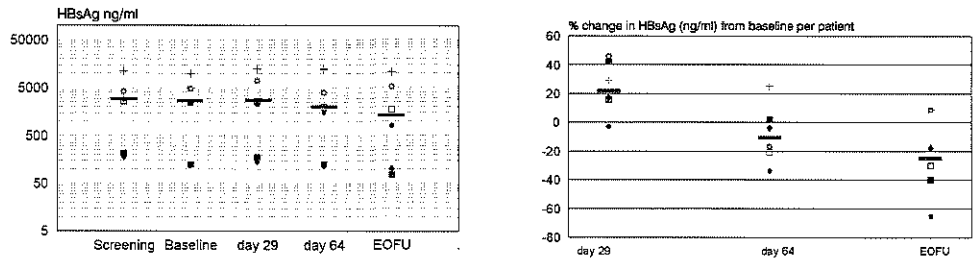


Figure 3 Absolute (left) and percentage (right) change in HBsAg ng/ml per patient before first dose of HBIg (baseline), second dose of HBIg (day 29), at the end of follow-up (day 64) and 4-5 months after the last infusion with HBIg (EOFU) (median —). All samples were measured in the same run with the experimental EIA. No significant changes were observed.

Discussion

HBIg can effectively interrupt the transmission of viral particles between hepatocytes and theoretically may have immunostimulatory effects by ADCC. The administration of anti-HBs was beneficial in 3 small trials, which included 6 chronic carriers, 15 HBV patients and one with 2 HBV patients with hypogammaglobunaemia (19, 21, 22). However these promising results could not be confirmed in this pilot study, encompassing 6 HBV patients.

Lamivudine rapidly suppresses HBV replication and about 26% of patients are HBV DNA negative by PCR after 6 months of therapy (34). At that time viral protein production of HBeAg is reduced by about 80% and a similar trend is observed for HBsAg (35). This is comparable to the reduction of HBsAg in IFN responders in whom HBsAg levels are halved after HBeAg seroconversion (36).

We used lamivudine to decrease the amount of circulating HBV and then tried to neutralize the remaining circulating virions with HBIg in order to prevent infection of regenerating hepatocytes. We realized to be hampered by the enormous bulk of non-infectious particles despite maximal virus suppression by lamivudine pre-treatment, which significantly hampered the efficacy of anti-HBs to obtain and maintain lower HBsAg levels. Furthermore, due to depressed viral production hepatocyte turnover seems to be reduced as deducted from normalization of serum transaminases.

Although HBsAg levels obtained in our patients during lamivudine monotherapy were similar to patients who have responded to IFN therapy, *in vivo,* HBsAg neutralization could only be reached in 2 out of 6 patients (36). Only in those with very low pre-treatment HBsAg levels, HBsAg was undetectable which was maintained only for a relatively short period of time even after 30.000 IU of HBIg. After a maximum of 31 hours HBsAg levels rapidly increased

simultaneously with a decreasing anti-HBs level. *In vitro*, HBIg was capable of complete inhibition of HBsAg in sera from three different patients. The inhibition assay demonstrated nearly complete neutralization of HBsAg levels between 50 and 100 ng/ml with 5000 IU HBIg/l. However, very large amounts of HBIg would be needed theoretically to cover all epitopes in the other 4 patients, with HBsAg levels between 25 to 200 times higher than patients 1 and 2. In these patients HBsAg levels showed little variation, even after multiple infusions within 24 hours. Different subtypes may play a role as well as a high production rate of HBsAg. We measured incomplete binding of HBsAg in one patient with medium pre-treatment HBsAg levels; anti-HBs/HBsAg complexes were detectable until 4 days after HBIg without any change in HBsAg load.

Although on day 64 there was only a small percentage decrease in HBsAg in 4 patients, at the end of follow-up a more pronounced although not significant, drop in HBsAg was found for 4 out of 6 patients. In one case a 66% decrease was reached. Probably this effect is due to ongoing suppression of HBV replication during continuous use of lamivudine or natural fluctuations of HBsAg titers (35). It may also be an effect on HBsAg secretion as has been shown in the hepatoma cells (28). Theoretically, the drop in HBsAg may be explained by (non-) specific immunostimulatory effects of antibodies or antigen-antibody complexes, such as antibody-dependent cellular cytotoxicity (ADCC), opsonization and stimulation of T-cell lymphokines (19, 22, 23, 25, 26, 37). However, despite the formation of immune complexes and reduction of serum HBsAg, biochemically there were no signs of enhanced immune activation, in contrast to the patients treated with anti-HBs HBsAg-complexes and HBsAg (+) bone marrow transplant patients, transplanted with antiHBs positive marrow, who experienced a hepatitis flare before viral clearance (23, 38).

In conclusion the role of passive immunization by HBIg in HBV patients pretreated with lamivudine seems to be limited due to the relatively high HBsAg levels in the majority of patients, despite maximum reduction of viral replication. Although in some of the patients a reduced HBsAg level was observed during long-term follow-up, we did not found evidence for immune stimulation by HBIg. This strategy seems only feasible if agents that inhibit both the production of viral proteins and Dane particles more selectively, become available.

ACKNOWLEDGEMENTS

We acknowledge P. van Bergen for skillful technical assistance. Mouse monoclonal antibodies were kindly provided by Dr. W.P. Pauly (Organon Teknika, Boxtel, The Netherlands).

References

1. Wong DK, Cheung AM, O'Rourke K, Naylor CD, Detsky AS, Heathcote J. Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B. A meta-analysis. Ann Intern Med 1993;119:312-23.
2. Krogsgaard K, Bindslev N, Christensen E, Craxi A, Schlichting P, Schalm SW, Carreno V, Trepo C, Gerken G, Thomas HC, Andersen PK, Ring-Larsen H and the European Concerted Action on Viral Hepatitis (Eurohep). The treatment effect of alpha interferon in chronic hepatitis B is independent of pre-treatment variables. Results based on individual patient data from 10 clinical controlled trials. J Hepatol 1994;21:646-55.
3. Janssen HLA, Gerken G, Carreno V, Marcellin P, Naoumov NV, Craxi A, Ring-Larsen H, Kitis G, Hattum J van, Vries RA de, Michielsen PP, Kate FJW ten, Hop WCJ, Heijtink RA, Honkoop P, Schalm SW and the European Concerted Action on Viral Hepatitis (Eurohep). Interferon alfa for chronic hepatitis B infection: increased efficacy of prolonged treatment. Hepatology 1999;30:238-43.
4. Schalm SW, Heathcote J, Cianciara J, Farrell G, Sherman M, Willems B, Dhillon A, Moorat A, Barber J, Gray DF. Lamivudine and alpha interferon combination treatment of patients with chronic hepatitis B infection: a randomised trial. Gut 2000;46:562-8.
5. Lai CL, Chien RN, Leung NWY, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray DR. A one-year trial of lamivudine for chronic hepatitis B. N Engl J Med 1998;339:61-8.
6. Song BC, Suh DJ, Lee HC, Chung YH, Lee YS. Hepatitis B e antigen seroconversion after lamivudine therapy is not durable in patients with chronic hepatitis B in Korea. Hepatology 2000;32:803-6.
7. Dienstag JL, Schiff ER, Mitchell M, Casey DE Jr, Gitlin N, Lissoos T, Gelb LD, Condreay L, Crowther L, Rubin M, Brown N. Extended lamivudine retreatment for chronic hepatitis B: maintenance fo viral suppression after discontinuation of therapy. Hepatology 1999;30:1082-7.
8. Liaw YF, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Chien RN, Dent J, Roman L, Edmundson S, Lai CL. Effects of extended lamivudine therapy in Asian patients with chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. Gastroenterology 2000;119:172-80.
9. Leung NWY, Lai CL, Chang TT, Cwan R, Lee CM, Ng KY, Wu PC, Dent JC, Edmundsen S, Liaw YF. Three year lamivudine therapy in chronic HBV. J Hepatol 1999;30:59A.
10. Wolf HM, Eibl MM. Immunomodulatory effect of immunoglobulins. Clin Exp Rheumatol 1996;14 (suppl. 15):S17-25.
11. Ip HMH, Lelie PN, Wong VCW, Kuhns MC, Reesink HW. Prevention of hepatitis B virus carrier state in infants according to maternal serum levels of HBV DNA. Lancet 1998;406-10.
12. Beasley RP, Hwang LY, Lin CC, Stevens CE, Wang KY, Sun TS, Hsieh FJ, Szmuness WS. Hepatitis B immune globulin (HBIg) efficacy in the interruption of perinatal transmission of hepatitis B virus carrier state. Lancet 1981;388-93.
13. Beasley RP, Hwang LY, Stevens CE, Lin CC, Hsieh FJ, Wang KY, Sun TS, Szmuness W. Efficacy of Hepatitis B Immune Globulin for prevention of perinatal transmission of the hepatitis B virus carrier state: final report of a randomised double-blind, placebo-controlled trial. Hepatology 1983;135-141.
14. Canho R del, Grosheide PM, Mazel JA, Heijtink RA, Hop WCJ, Gerards LJ, Gast GC de, Fetter WPF, Zwijneberg J, Schalm SW. Ten-year neonatal hepatitis B vaccination program, the Netherlands, 1982-1992: protective efficacy and long-term immunogenicity. Vaccine 1997;15:1624-30.
15. Samuel D, Muller R, Alexander G, Fassati L, Ducot B, Benhamou JP, Bismuth H. Liver transplantation in European patients with the hepatitis B surface antigen. N Engl J Med 1993;329:1842-7.
16. Man RA de, Metselaar HJ, Heijtink RA, Schalm SW. Long-term application of human polyclonal hepatitis-B immunoglobulin to prevent hepatic allograft infection. Neth J Med 1993;43:74-82.
17. Muller R, Gubernatis G, Farle M, Niehoff G, Klein H, Wittekind C, Tusch G, Lautz HU, Boker K, Stangel W, Pichlmayr R. Liver transplantation in HBs antigen (HBsAg) carriers. Prevention of hepatitis B virus (HBV) recurrence by passive immunization. J Hepatol 1991;13:90-6.
18. Konig V, Hopf U, Neuhaus P, Bauditz J, Schmidt CA, Blumhardt G, Bechstein WO, Neuhaus R, Lobeck H. Long-term follow-up of hepatitis B virus-infected recipients after orthotopic liver transplantation. Transplantation 1994;58:553-9.
19. Reed WD, Eddleston ALWF, Cullens H, Williams R, Zuckerman AJ, Peters DK, Williams DG, Maycock WA. Infusion of hepatitis-B antibody in antigen-positive active chronic hepatitis. Lancet 1973;1347-51.
20. Eren R, Ilan E, Nussbaum O, Lubin I, Terkieltaub D, Arazi Y, Ben-Moshe O, Kitchinzky A, Berr S, Gopher J, Zauberman A, Galun E, Shouval D, Daudi N, Eid A, Jurim O, Magnius LO, Hammas B, Reisner Y, Dagan S. Preclinical evaluation of

two human anti-hepatitis B virus (HBV) monoclonal antibodies in the HBV-trimera mouse model and in HBV chronic carrier chimpanzees. Hepatology 2000;32:588-96.

21. Galun E, Eren R, Matot E, Shouval D, Terrault N, Keeffe E. Clinical evaluation (phase I) of a combination of two monoclonal antibodies to HBV: safety and antiviral properties. Hepatology 2000;32:240A.
22. Lever AML, Waters J, Brook MG, Karayiannis P, Thomas HC. Monoclonal antibody to HBsAg for chronic hepatitis B virus infection with hypogammaglobulinaemia. Lancet 1990;335:1529.
23. Wen YM, Wu XH, Hu DC, Zhang QP, Guo SQ. Hepatitis B vaccine and anti-HBs complex as approach for vaccine therapy. Lancet 1995;345:1575-6.
24. McCluskie MJ, Wen YM, Di Q, Davis HL. Immunization against hepatitis B virus by mucosal administration of antigen-antibody complexes. Viral Immunol 1998;11:245-52.
25. Celis E, Abraham KG, Miller RW. Modulation of the immunological response to hepatitis B virus by antibodies. Hepatology 1987;7:563-8.
26. Wen YM, Xiong SD, Zhang W. Solid matrix-antibody-antigen complex can clear viraemia and antigenaemia in persistent duck hepatitis B virus infection. J Gen Virol 1994;75:335-9.
27. Wen YM, Qu D, Zhou SH. Antigen-antibody complex as therapeutic vaccine for viral hepatitis B. Int Rev Immunol 1999;18:251-8.
28. Schilling R, Ljaz S, Williams R, Naoumov NV. Internalisation of monoclonal and polyclonal hepatitis B immunoglobulins in human hepatoma cells and their effect on the assembly and secretion of HBsAg. Hepatology 1999;30:246A.
29. Krawczynski K, Fattom A, Culver D, Kamili S, Spelbring J, Basham L, Sapan C, Naso R, Purdy M, Carson D. Experimental immune treatment of hepatitis C virus (HCV) infection: passive anti-HCV (HBIg) transfer in chronically infected chimpanzees. J Hepatol 2000;2:37A.
30. Schneiders AM, Schulz M, Leifeld L, Duesberg U, Koopman J, Matz B, Inchauspe G, Sauerbruch T, Spengler U. E2 antibodies mediate antibody dependent cellular cytotoxicity in HCV. J Hepatol 2000;2:37A.
31. Ostrow DH, Edwards B, Kimes D, Macioszek J, Irace H, Nelson L, Bartko K, Neva J, Krenc C, Mimms L. Quantitation of hepatitis B surface antibody by an automated microparticle enzyme immunoassay. J Virol Methods 1991;32:265-76.
32. Mimms L, Goetze A, Swanson S, Floreani M, Edwards B, Macioszek J, Okasinski G, Kiang W. Second generation assays for the detection of antibody to HBsAg using recombinant DNA-derived HBsAg. J Virol Methods 1989;25:211-31.
33. Heijtink RA, Kruining J, Weber YAM, Man RA de, Schalm SW. Anti-hepatitis B virus activity of a mixture of two monoclonal antibodies in an "inhibition in solution" assay. Hepatology 1995;22:1078-83.
34. Honkoop P, Man RA de, Niesters HGM, Main J, Nevens F, Sulivan M, Gray F, Thomas HC, Fevery J, Tyrell DL, Schalm SW. Quantitative hepatitis B virus DNA assessment by means of the limiting dilution polymerase chain reaction in chronic hepatitis B patients during a 24-week course of lamivudine. J Viral Hep 1998;5:307-12.
35. Nevens F, Main J, Honkoop P, Tyrell D, Barber J, Sullivan M, Fevery J, Man RA de, Thomas HC. Lamivudine therapy for chronic hepatitis B: a six-month randomized dose-ranging study. Gastroenterology 1997;113:1258-63.
36. Janssen HLA, Kerkhof-Los CJ, Heijtink RA, Schalm SW. Measurement of HBsAg to monitor hepatitis B viral replication in patients on alpha-interferon therapy. Antiviral Res 1994;23:251-7.
37. Wawrzynczak EJ. Antibody therapy. Oxford, UK: Bios Scientific Publishers, 1995.
38. Lau GKK, Liang R, Lee CK, Yuen ST, Hou J, Lim WL, Williams R. Clearance of persistent hepatitis B virus infection in Chinese bone marrow transplant recipients whose donors were anti-hepatitis B core - and anti-hepatitis B surface antibody - positive. J Infect Dis 1998;178:1585-91.

II.3 ADMINISTRATION OF A HUMAN MONOCLONAL ANTIBODY (TUVIRUMAB) TO CHRONIC HEPATITIS B PATIENTS PRE-TREATED WITH LAMIVUDINE: MONITORING OF SERUM TUVIRUMAB IN IMMUNE COMPLEXES

R.A. Heijtink[1], A.B. van Nunen[2], P. van Bergen[1], L.Östberg[3], A.D.M.E Osterhaus[1], R.A.de Man[2].

[1]Departments of Virology and [2]Internal Medicine II, Erasmus University Rotterdam, Rotterdam, The Netherlands.

[3] Protein Design Labs, Inc., Fremont, California, U.S.A.

Journal of Medical Virology 2001;64:427-34.

Abstract

Background: A human monoclonal anti-hepatitis B antibody preparation (TUVIRUMAB (MAb)) was administered 6 times over a 2-week period in a dose-escalating scheme to chronic hepatitis B patients pre-treated with lamivudine.

Methods: We investigated the capacity of the TUVIRUMAB antibody to "neutralize" hepatitis B surface antigen in the circulation by means of experimental enzyme-immunoassays. Monoclonal antibody conjugates enabled the detection of HBsAg, TUVIRUMAB and HBsAg/TUVIRUMAB complexes.

Results and Conclusions: The results showed that a) TUVIRUMAB was partially able to "neutralize" in vitro and in vivo, b) HBsAg/TUVIRUMAB complexes can be traced by assays that capture the complex at either its HBsAg or its TUVIRUMAB component, c) the final concentration of TUVIRUMAB at the end of therapy varied greatly but seemed to be related to HBsAg production at the start of therapy, d) for at least 14 days after discontinuation of therapy a minimal HBsAg level could be maintained in the presence of a declining TUVIRUMAB titer in patients with less than 3 µg/ml HBsAg before the start of therapy, e) three months after therapy all HBsAg levels had returned to pre-treatment levels and TUVIRUMAB had disappeared. This study has set the limits for administration of TUVIRUMAB in chronic hepatitis B patients.

Introduction

In the majority of patients with chronic hepatitis B and active viral replication, anti-hepatitis B surface antigen antibodies cannot be detected by commercial anti-HBs assays. Low levels of anti-HBs antibodies were only observed in long-term carriers (1-2).

In anti-HBs assays of the ELISA type, anti-HBs antibodies are captured by solid phase antigen (HBsAg) and detected by enzyme-labeled HBsAg. Alternatively, solid phase anti-HBs antibodies, e.g. mouse monoclonal anti-HBs antibodies, may be used in experimental assays to capture HBsAg. In a next step, serum anti-HBs antibodies may be bound to this antigen and subsequently monitored with an anti-human-Ig conjugate. Unfortunately, in some cases naturally adhering Ig molecules may give rise to an increased background signal at low serum dilutions. On the other hand, this test system may be exploited to monitor anti-HBs antibodies in HBsAg/anti-HBs complexes by direct application of the HBsAg positive (serum) sample under study to solid phase anti-HBs.

The functional activity of anti-HBs antibodies to cover HBV and HBsAg particles can be measured in vitro in an "inhibition in solution assay" (3). In this case the HBsAg and anti-HBs antibodies are pre-incubated and subsequently applied to solid phase coated anti-HBs antibodies. By measuring residual HBsAg with an anti-HBs conjugate, the inhibiting activity of anti-HBs antibodies can be studied. Similarly, artificial HBsAg/anti-HBs complexes may be monitored for HBsAg by capturing these complexes with anti-HBs specific antibodies (anti-idiotypic antibodies).

In the present study a human monoclonal antibody (TUVIRUMAB) was administered to HBsAg-carrier patients with a low level of virus replication due to treatment with lamivudine. The infused anti-HBs was expected to bind to plasma HBsAg. HBsAg/anti-HBs complexes will remain in the circulation until they are removed by the reticuloendothelial system. An assay for TUVIRUMAB enabled us to monitor the fate of HBsAg and TUVIRUMAB in the circulation.

Patients, Materials and Methods

Patients

We studied serum samples from eight patients. All patients had HBV-DNA levels below the detection limit (1.5×10^6 geq/ml) of the Hybrid Capture assay (Digene Murex) due to lamivudine monotherapy. Three patients were HBV-DNA positive (200-5.000 geq/ml) according to quantitative PCR (Roche Amplicor). The patients received TUVIRUMAB according to the following schedule: 20 mg on days 1 and 3, 40 mg on days 5 and 8 and 80 mg on days 10 and 12. TUVIRUMAB was dissolved in 250 ml of 0.9% NaCl solution and administered by infusion intravenously over at least 30 minutes. Blood samples were taken before and during therapy and up to 3 months after therapy.

This study was performed in accordance with the principles of Good Clinical Practice. The protocol was approved by the institutional review board and all patients had given their written informed consent.

Materials

A murine monoclonal anti-idiotypic antibody specific for TUVIRUMAB was used for coating microtiter plates (Costar HIG EIA/RIA; 1 µg/ml IgG) and as conjugate (biotin/avidin-HRP labeling, Amersham). Reference TUVIRUMAB (TUVIRUMAB-r, 16.1 mg/ml) was titrated in standard dilutions to obtain a reference curve for quantitation. For control of experiments with sera from the TUVIRUMAB treated chronic hepatitis B patients, we used the same batch of TUVIRUMAB material that was administered to these patients (stock TUVIRUMAB-p, 2 mg/ml).

As solid phase antibodies, mouse monoclonal anti-HBs (HBs.OT39, HBs.OT40; Organon Teknika, Boxtel, The Netherlands) and human monoclonal anti-HBs (F4-7B; Biotest Pharma, Dreieich, Germany) were used. Sheep anti-Hu-Ig horse-radish peroxidase was purchased from Amersham (Bucks, UK).

As reference HBsAg we used Mat.O as in earlier experiments (3-4). As reference HBIG preparation we used Hepatect (Biotest Pharma, Dreieich, Germany).

All samples were diluted in EB-buffer (PBS with 3% NaCl, 0.01% Tween 20, 1% normal goat serum and 0.2% milk powder).

Methods

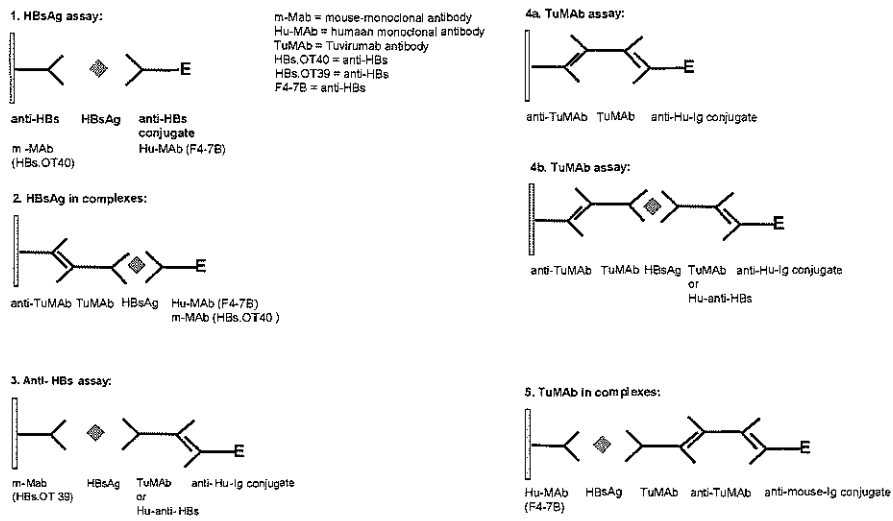
The types of experiments were restricted to EIA- procedures with solid phase antibodies (monoclonal anti-HBs and anti-TUVIRUMAB). The first incubation was performed with TUVIRUMAB, HBsAg or putative HBsAg/TUVIRUMAB complexes. Detection was by monoclonal anti-HBs conjugates (biotin/avidin-HRP labeling), polyclonal anti-human-Ig-HRP (direct monitoring) or anti-mouse-Ig-HRP (indirect monitoring) (see figure 1).

Results

Anti-HBs quantitation

The dilution characteristics of the TUVIRUMAB monoclonal antibody preparations on HBsAg were similar to but distinct from those of the two polyclonal antibody preparations, Hepatect and WHO reference anti-HBs (figure 2). This complicates the quantitation of TUVIRUMAB and conversion to IU/l, which relates anti-HBs to the WHO standard curve.

Figure 1 Schematic presentation of test procedures.



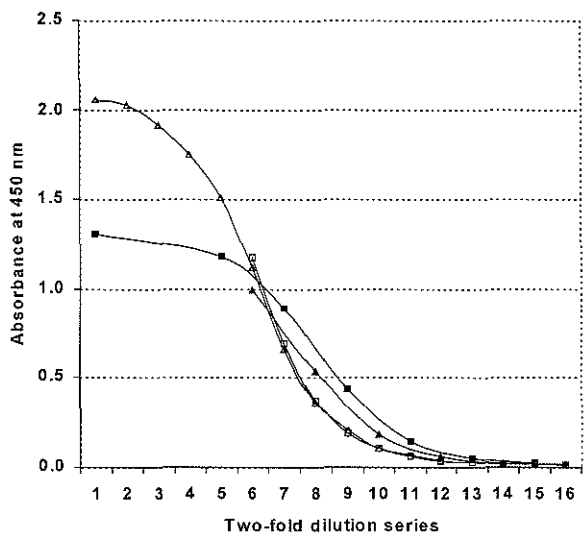


Figure 2

Titration of anti-HBs preparations (test procedure 3) on HepG2 2.2.15 HBsAg/ayw3:

a. TUVIRUMAB-p (▲, start at 2 µg/ml),

b. TUVIRUMAB-r (■, start at 16 µg/ml),

c. Hepatect (Δ, 5.000 IU/l) and

d. WHO reference anti-HBs (□, 200 IU/l).

Artificial HBsAg/TUVIRUMAB complexes in the HBsAg assay

In a pre-incubation step HBsAg was complexed with TUVIRUMAB in titrated amounts. Residual HBsAg was detected in the HBsAg assay ("inhibition assay"). Figure 3 illustrates the inhibition activity of TUVIRUMAB (80% inhibition) compared to Hepatect (100% inhibition). Complete inhibition (100%) was observed readily with polyclonal anti-HBs (Hepatect) but a lower inhibition percentage was found with monoclonal anti-HBs (3). In the latter case the particles were not completely shielded by antibodies and thus may bind to solid phase antibodies with still accessible epitopes.

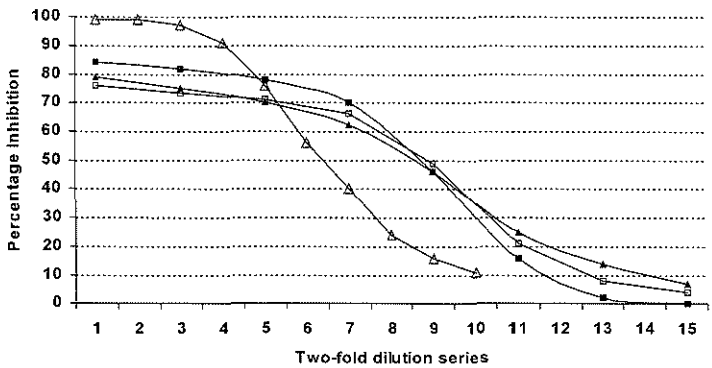


Figure 3 Inhibition in solution of reference HBsAg with TUVIRUMAB (■), Hepatect (Δ) and two patient pre-treatment samples (patients B □, and C ▲) (test procedure 1). Solid phase mouse monoclonal anti-HBs (HBs.OT40) and conjugated human monoclonal F4-7B were used for assaying. Start concentration of Hepatect was 5,000 IU/l; start concentration of TUVIRUMAB was 4 µg/ml.

Monitoring TUVIRUMAB in artificial HBsAg/anti-HBs complexes

In an earlier experiment we observed that the residual HBsAg in the "inhibition assay" was loaded with anti-HBs (4). However, our efforts to confirm the presence of TUVIRUMAB in a more specific way using biotinylated anti-TUVIRUMAB antibody were not successful, probably due to steric hindrance of biotin. As an alternative we monitored TUVIRUMAB antibodies on HBsAg particles with mouse anti-TUVIRUMAB and rabbit anti-mouse-Ig conjugate (procedure 5).

Partially neutralized HBsAg or HBsAg/TUVIRUMAB complexes with different HBsAg-to-TUVIRUMAB ratios were captured with their TUVIRUMAB site on solid phase anti-TUVIRUMAB (test procedure 2; test procedure 4b). In a pre-incubation step equal volumes of HBsAg and TUVIRUMAB antibody (in titrated amounts) were combined. Results as represented in figure 4 may be interpreted as follows: In the complex I samples the HBsAg/anti-HBs ratio was lowest. The solid phase anti-TUVIRUMAB is saturated with free TUVIRUMAB (procedure 4a) and a small fraction of the complexed-HBsAg is bound to anti-

TUVIRUMAB (test procedure 4b). In the complex II sample, half of the HBsAg particle surface is covered with TUVIRUMAB. Due to lowering of the TUVIRUMAB concentration there is not sufficient TUVIRUMAB to saturate the solid phase. More complexes are bound which gives rise to an increased HBsAg signal. In the complex III and IV samples the HBsAg/TUVIRUMAB ratios reached their highest levels and complexed-HBsAg began to decrease.
An increase or decrease in the detectable amount of HBsAg may thus be explained by participation of HBsAg/TUVIRUMAB complexes in competition with free TUVIRUMAB for the solid phase anti-TUVIRUMAB antibody.

Monitoring HBsAg and TUVIRUMAB in patient serum during therapy
Pre-treatment HBsAg levels were compared with end of treatment (day 15; 3 days after last 80 mg dose) and end of follow-up levels (day 29) by EIA using a non-competing antibody (F4-7B) (5). Substantially decreased HBsAg levels were observed at the end of therapy in all patients and at the end of follow-up in half of them (table 1). These latter four patients presented with low HBsAg levels at the start of therapy. All patients had returned to pre-treatment HBsAg levels 3 months after treatment (results not shown).
Using the TUVIRUMAB assay with solid phase anti-TUVIRUMAB antibody and anti-human-Ig as conjugate (test procedure 4) showed that the TUVIRUMAB antibody titer increased gradually during treatment. The increase came earlier in time and reached higher levels in those with lower pre-treatment HBsAg levels and strongest reduction of HBsAg at end of herapy (table 1).
One of the patients (C) with a low level of HBsAg in pre-treatment serum presented only a 36% reduction of HBsAg on day 29. This aberrant reduction corresponds well with the limited amount of free and complexed TUVIRUMAB antibody detected on day 12-29 with the anti-TUVIRUMAB assay. It could well be that the substrain of this Chinese patient was responsible for an underestimate of the amount of HBsAg detected by our HBsAg assay TUVIRUMAB/HBsAg complexes were studied in samples obtained just after infusion of TUVIRUMAB had been completed (30' samples). The TUVIRUMAB/HBsAg complexes as well as free TUVIRUMAB were bound to solid phase anti-TUVIRUMAB and monitored with anti-human-Ig conjugate (for TUVIRUMAB) and F4-7B-conjugate (for HBsAg) (figure 5). The highest level of complexed-HBsAg was observed in patient H (day 1) who also had the highest level of HBsAg in pre-treatment serum in this group of patients. The decrease with time in detectable immune complexes in this assay is ascribed to competition between the excess TUVIRUMAB and HBsAg-complexed-TUVIRUMAB for binding to solid phase anti-TUVIRUMAB antibody. Examples of the TUVIRUMAB load of HBsAg particles captured by solid phase anti-HBs (HBs.OT40) are illustrated in figure 6.
Table 1

Serum TUVIRUMAB content during therapy in the anti-Tuvirumab assay*.

						Tuvirumab dose and content in serum							
						20 mg		40 mg		80 mg			
Pat. code	HBsAg Pre	HBsAg	Perc. Red.	HBsAg	Perc. Red.	1st	2nd	1st	2nd	1st	2nd	EOT	EOF
A[a]	0.49	0.02	96	**0.02**	**96**	-	2.40	7.70	10.90	25.60	46.10	36.60	**8.80**
B	1.12	0.01	99	**0.01**	**99**	-	0.02	1.00	7.60	11.50	28.20	35.04	**7.20**
C	1.76	0.05	97	1.12	36	-	0.02	0.02	0.05	1.30	11.20	18.52	0.04
D	2.00	0.08	96	**0.18**	**91**	-	0.14	0.54	3.02	10.22	29.42	35.55	**8.60**
E[a]	2.48	0.04	98	**0.07**	**97**	-	0.03	1.23	5.76	9.60	25.60	33.56	**8.00**
F	3.84	0.31	92	3.20	17	-	0.07	0.04	0.22	0.58	7.94	12.93	0.07
G	8.32	1.31	84	7.36	12	-	0.11	0.19	0.24	0.79	7.67	16.38	0.21
H[a]	19.84	3.93	80	14.08	29	-	0.27	0.27	0.17	0.35	3.81	8.98	0.18
Day	1	15		29		1	3	5	8	10	12	15	29

*Samples were taken just before administration of each dose of TUVIRUMAB, at the end of therapy (day 15) and at the end of follow-up (day 29). HBsAg quantitation (µg/ml) was performed in EIA using F4-7B human monoclonal anti-HBs as capture and as monitoring antibody (test procedure 1). Anti-HBs(TUVIRUMAB) in µg/ml (test procedure 4). At the end of follow-up (day 29) more than 90% reduction of HBsAg was observed in half of the patients (HBsAg and corresponding antibody levels in bold). EOT= end of therapy, 3 days after last dose. EOF= end of follow-up. [a] HBV-DNA positive by PCR.

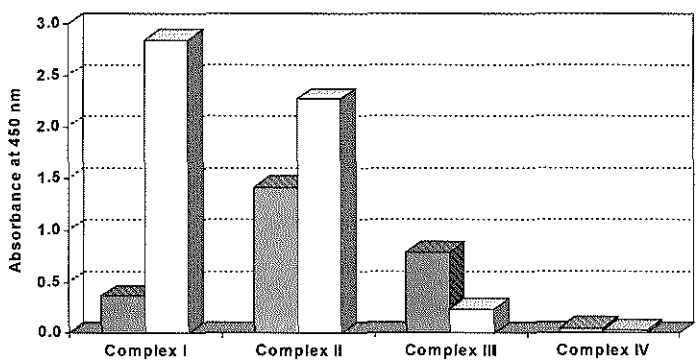


Figure 4 Monitoring of reference HBsAg in artificial TUVIRUMAB/HBsAg complexes with different HBsAg/TUVIRUMAB ratios: complex I: 1/10.000; complex II: 1/1.000; complex III: 1/100: complex IV: 1/10. TUVIRUMAB antibodies and TUVIRUMAB/HBsAg complexes were captured with solid phase anti-TUVIRUMAB antibodies. HBsAg detection in TUVIRUMAB/HBsAg complexes was with human monoclonal F4-7B conjugate (dark grey, test procedure 2); TUVIRUMAB was detected with anti-Hu-Ig conjugate (licht grey, test procedure 4).

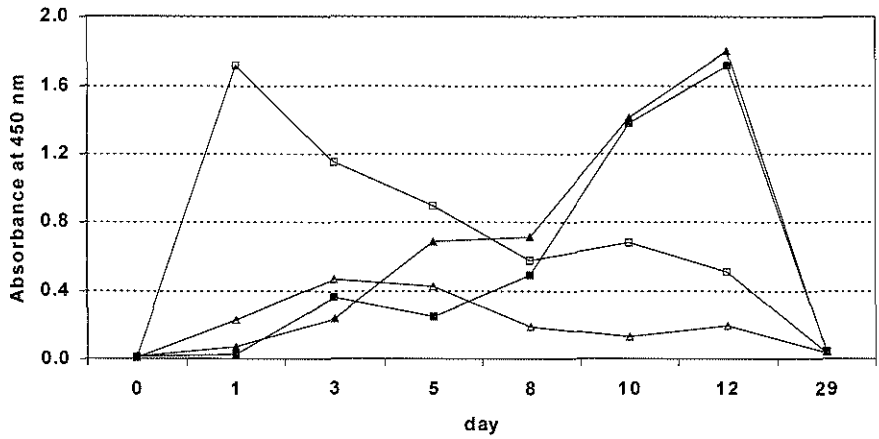


Figure 5 Monitoring of HBsAg in HBsAg/TUVIRUMAB complexes in serum samples from two patients (patient F and H) treated with TUVIRUMAB in a dose escalating schedule. Samples were taken 30 minutes after each intravenous dose administration was started. TUVIRUMAB and TUVIRUMAB/HBsAg complexes were captured with solid phase anti-TUVIRUMAB antibodies in EIA and detected with anti-Hu-Ig conjugate (test procedure 4). Complexed HBsAg was detected with monoclonal anti-HBs (F4-7B) conjugate (test procedure 2). The last sample was taken at the end of follow-up (day 29). Patient F, HBsAg/TUVIRUMAB complexes Δ; TUVIRUMAB ▲; Patient H, HBsAg/TUVIRUMAB complexes □, TUVIRUMAB ■)

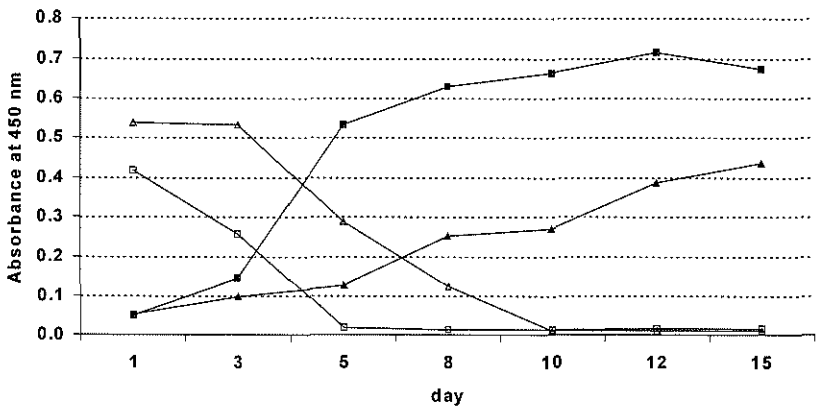


Figure 6 TUVIRUMAB detection in HBsAg/TUVIRUMAB complexes formed after infusion of TUVIRUMAB antibodies as seen in patient sera (patients B and C) taken just before infusion of TUVIRUMAB antibodies and at the end of therapy (day 15). HBsAg was detected by mouse monoclonal anti-HBs conjugate (HBs.OT40) (test procedure 2). TUVIRUMAB detection was with anti-TUVIRUMAB antibodies and anti-mouse Ig conjugate (test procedure 5).(Patient B, HBsAg/TUVIRUMAB complexes □; TUVIRUMAB ■; patient C, HBsAg/TUVIRUMAB complexes Δ , TUVIRUMAB▲).

Discussion

One of the strategies of immunoglobulin therapy is the formation of antigen-antibody complexes that will prevent infectious agents, such as viruses, from entering target cells. Furthermore, the formation of complexes promotes the opsonization of these complexes by macrophages and other scavenger cells. Hepatitis B immunoglobulin preparations consisting of polyclonal anti-HBs are widely used to prevent recurrence of hepatitis B in liver transplant recipients (6) and spread of the virus after accidental exposure. Reports on the application of immunoglobulin preparations in the therapeutic setting of a chronic hepatitis B carrier are scarce (4, 7-10).

In contrast to the polyclonal antibodies that may act largely as complex forming agents, monoclonal antibodies may also react with epitopes with defined functions such as receptor binding on cells, penetration into cells, complement fixation and neutralization of infectivity by specialized mechanisms.

In our study population, the replication rate and production of viral particles were depressed by long-term use of lamivudine. However, this was not accompanied in all cases by a low level of HBsAg at the start of the immune therapy.

The aim of the present clinical study was to investigate the potency of a monoclonal antibody preparation to block hepatitis B epitopes in the circulation in order to prevent the spread of the virus to non-infected hepatocytes. The lack of an easily accessible infection system and the limited knowledge of the role of small S-antigen-located epitopes in infecting hepatocytes (11) hinder the extrapolation of in vitro results to the vivo situation. However, administration of human monoclonal antibodies and the search for an immune modulating effect in the patient's circulation is one step closer to patient-adapted therapy for prevention of relapse or in cases of pending HBeAg seroconversion. An assay that specifically monitors the human monoclonal anti-HBs antibodies in the circulation of our patients under treatment enabled us to directly detect the antibody in HBsAg complexes. This has not been described before.

The total period of administration of TUVIRUMAB antibodies was less than 15 days. The largest amounts of TUVIRUMAB were given in the last 5 days of therapy (2x80 mg). In two cases we found final concentrations of TUVIRUMAB corresponding to the total amount of TUVIRUMAB administered in the whole period of therapy. This was observed in patients with low pre-treatment levels of HBsAg. This result may be explained by the uncertainty in quantitation of TUVIRUMAB at dilutions of 1 to 10 million, the relatively short time in the circulation of the larger dose of TUVIRUMAB, the accessibility of TUVIRUMAB in the HBsAg/TUVIRUMAB complexes and the long circulatory half-life of a human monoclonal antibody in the absence of strong antigen-driven consumption.

Since over 80% of HBsAg reduction was observed in all patients at the end of treatment and a 90% reduction was found for at least 14 days after stop of therapy in half of them, these

experiments suggest that the present schedule of TUVIRUMAB administration is suitable for optimal blocking of HBsAg and with free TUVIRUMAB in the circulation in patients with less than 3 µg/ml HBsAg.

The use of a TUVIRUMAB-specific assay to monitor HBsAg and TUVIRUMAB has clarified the simultaneous presence of HBsAg and TUVIRUMAB. We now know that the presence of TUVIRUMAB, as detected by the anti-HBs assay, may indicate that the antibody is free in the circulation or is bound to HBsAg. Similarly, HBsAg reduction as measured in HBsAg assays does not exclude the binding of partially complexed HBsAg to the solid phase in our assay. In practice, remaining functional groups of importance in the infection process may determine whether these antibodies will really block infection or spread of the virus. As long as we are unaware of the in vivo neutralizing efficacy of TUVIRUMAB, this study may have helped to set the limits of TUVIRUMAB antibodies in relation to the HBsAg load in our chronic hepatitis B patients.

It is expected that under a long-term regimen of lamivudine treatment virus production and virus transfer to uninfected hepatocytes is limited. Therefore, criteria for subjecting patients to immunoglobulin therapy should not only include a low level of HBsAg production but also the regeneration of non-infected hepatocytes. Use of high concentrations of monoclonal antibody might create the conditions needed for expansion of uninfected liver cells and prevention of virus transfer.

References

1. Heijtink RA, Van Hattum J, Schalm SW, Masurel N. 1982. Co-occurrence of HBsAg and anti-HBs: Two consecutive infections or a sign of advanced chronic liver disease. J Med Virology 10:83-90.
2. Zhang YY, Norderfelt E, Hansson BG. 1996. Increasing hetrogeneity of the "a" determinant of HBsAg found in the presumed late phase of chronic hepatitis B virus infection. Scand J Infect Dis 28: 9-15.
3. Heijtink RA, Kruining J, Weber YAM, De Man RA, Schalm SW. 1995. Anti-hepatitis B virus activity of a mixture of two monoclonal antibodies in an "inhibition in solution assay". Hepatology 22: 1078-1083.
4. Heijtink R, Paulij W, Van Bergen P, Van Roosmalen M, Rohm D, Eichentopf B, Muchmore E, De Man R, Osterhaus A. 1999. In vivo activity of a mixture of two human. Monoclonal antibodies (anti-HBs) in a chronic hepatitis B carrier Chimpanzee. J Gen Virology 80: 1529-1535.
5. Paulij W, De Wit PLM, Sünnen CMG, Van Roosmalen MH, Petersen-van Ettekoven A, Cooreman MP, and Heijtink RA. 1999. Localisation of a unique human HBV epitope sheds new light on the structure of HBsAg. J Gen Virology 80: 2121-2126.
6. Samuel D, Muller R, Alexander G, Fassati L, Ducot B, Benhamou JP, Bismuth H. 1993. Liver transplantation with hepatitis B surface antigen. N Eng J med 329: 1842-1847.
7. Reed WD, Eddleston ALWF, Cullens H, Williams R, Zuckerman, AJ, Peters DK, Williams DG and Maycock Wd'A. 1973. Infusion of hepatitis B antibody in antigen-positive active chronic hepatitis. The Lancet II: 1347-1351.
8. Lever AML, Waters J, Brook MG, Karayiannis P, Thomas HC. 1990. Monoclonal antibody to HBsAg for chronic hepatitis B infection with hypogammaglobulinaemia. The Lancet 335: 1529-1530.
9. Östberg L, Queen C. 1995. Human and humanized monoclonal antibodies: preclinical studies and clinical experience. Biochemical Society Transactions 23: 1038-1043.
10. Wen Y-M, Wu X-H, Hu D-C, Zhang Q-P, Guo S-Q. 1995. Hepatitis B vaccine and anti-HBs complex as approach for vaccine therapy. The Lancet 345: 1575-1576.
11. De Meyer S, Depla E, Maertens G, Soumillion A, Yap SH. 1999. Characterization of small hepatitis B surface antigen epitopes involved in binding to human annexin V. J Viral Hepat 6:277-285.

Chapter III

Immunologic mechanisms

in chronic hepatitis B

III.1 SUPPRESSION OF HEPATITIS B VIRUS REPLICATION MEDIATED BY HEPATITIS A-INDUCED CYTOKINE PRODUCTION

A.B. van Nunen[1], O. Pontesilli[2], F. Uytdehaag[2], A. D.M.E.Osterhaus[2], R.A. de Man[1]

Departments of [1]Hepatogastroenterology and [2]Virology, Erasmus University Hospital Rotterdam, Rotterdam, The Netherlands.

Liver 2001;21:45-9.

Abstract

Background: Acute hepatitis A virus (HAV) infection can cause severe hepatitis, especially in patients with underlying chronic liver disease. In patients with pre-existing chronic hepatitis B (HBV), acute HAV infection can suppress HBV replication. The exact mechanism of HBV suppression during acute HAV infection is still a subject of debate. One mechanism may be the production of HAV infection-induced cytokines, leading to suppression of HBV replication and viral clearance.

Objective: To evaluate cytokine production and HBV-specific lympho-proliferative responses (LPR) during acute HAV infection in a patient with chronic HBV infection-clearing markers of active HBV replication.

Methods: Early detection of a case of acute HAV infection in an HBeAg-positive, HBV DNA-positive chronic HBV patient treated with lamivudine. *Setting:* Erasmus University Hospital Rotterdam; tertiary referral center.

Results: At the time of HAV infection a sharp peak in the gamma-interferon (IFN-γ) level occurred just before the rise in serum transaminase activity. This was subsequently followed by a decrease in HBV DNA and HBeAg below the limit of detection of the assay. However, the HBV-specific T-cell response was not modified. After resolution of the acute HAV infection and withdrawal of antiviral therapy, HBV replication relapsed.

Conclusion: The sharp rise in IFN-γ production mediated by the acute HAV infection may be pivotal in the suppression of HBV replication in chronic hepatitis B.

Introduction

After acute hepatitis B virus (HBV) infection 5-10% of all adult patients become chronically infected; the mechanism of the viral persistence in this minority of patients remains unclear. In patients with an acute self-limiting HBV infection a vigorous T-lymphocyte response to the core antigen and a weaker response to the surface antigen are observed (1, 2). It is suggested that the production of Th1-type pro-inflammatory cytokines (gamma-interferon (IFN-γ), interleukin-2 (IL-2) and tumour necrosis factor-alpha (TNF-α)) is mainly instrumental in inducing viral clearance (3). Viral persistence on the other hand is associated with T-cell hyporesponsiveness, possibly due to exhaustion or paralysis of the immune system due to overwhelming antigen presentation (1, 4). Recombinant alpha interferon therapy successfully inhibits viral replication in about 30% of the patients with chronic hepatitis B by means of the direct antiviral effects of the drug or by inducing a HBV-specific immune response (5).

Hepatitis A virus (HAV) is transmitted enterally. In highly endemic areas it causes a mild self-limiting hepatitis among young children. In countries of low endemicity infection occurs at an older age. This is associated with a much higher case fatality rate, especially among adults over 40 years of age (6). In patients with underlying chronic liver diseases, such as chronic hepatitis C, HAV infection can lead to fulminant hepatitis and death (7, 8).

Data on the impact of HAV infection on chronic HBV are contradictory (7-9). HAV superinfection has been reported to have an inhibitory effect on HBV replication during the acute phase of HAV. Several anti-viral mechanisms have been postulated to explain the drop in HBV protein production: a direct effect of the reduction in target cells following hepatic necrosis or viral interference, or an indirect effect due to HAV-induced cytokine production (9-13).

During an HAV outbreak among homosexual men in the Rotterdam area we studied a chronic hepatitis B patient during an early phase of acute HAV infection in detail.

Materials and methods

Viral serology

Diagnosis of acute HAV infection was based on a highly positive IgM-anti HAV test (IMX, Abbott, Chicago, Ill). Co-infections with CMV, EBV, HDV and HIV were excluded by standard serology. Acute HCV infection was excluded by PCR.

HBV DNA was measured by liquid hybridization (Digene II, Murex, UK) and, if negative, by PCR (detection limit 400 genome equivalents per ml (geq/ml) based on the Eurohep standard). HBeAg (IMX, Abbott, Chicago, Ill, USA) and HBsAg (AxSYM HBsAg, Abbott, Chicago, Ill, USA) were measured quantitatively and expressed in Paul Ehrlich Units.

Measurement of serum cytokine levels

Sequential serum samples before and during the acute hepatitis episode have been tested for levels of circulating IFN-α, IFN-γ, IL10, IL12, and TNF-α by means of standard sandwich ELISA technique. Antibodies for coating and detection, and ELISA reagents were obtained from HyCult biotechnology, Uden, The Netherlands (anti-IFN-α), Medgenix Diagnostics, Fleurus, Belgium (anti-IFN-γ), Pharmingen, San Diego CA, USA (anti-IL10), R&D Systems, Minneapolis MN, USA (anti-IL12), and CLB, Amsterdam, The Netherlands (TNF-α). Lower detection limits in each assay were: IFN-α, 50 pg/ml; IFN-γ, 10 pg/ml; IL10, 20 pg/ml; IL12, 80 pg/ml; TNF-α, 4 pg/ml.

Antigens

Recombinant C-terminal truncated HBcAg particles (146aa) expressed in *E.coli* (American Research Products, Belmont MA, USA; cat.n.12-3069, batch r-HBcAg-e-CIT-0070) was used in lymphocyte stimulation assays. As control antigen, recombinant HCV NS3 protein, also expressed in *E.coli* (American Research Products, cat.n.12-00117, batch 9812ns3) was used. Recombinant pre-S_1- and pre-S_2-containig HBsAg particles, expressed in Chinese hamster ovary (CHO) cells, were obtained from Dr. Reinhard Gluck (Swiss Serum and Vaccine Institute Berne, Berne, Switzerland).

In vitro lymphocyte proliferation assays

Ag-specific lymphocyte proliferative response (LPR) were measured retrospectively culturing 10^5 thawed peripheral blood mononuclear cells (PBMC) in 4 replicate round-bottom microtiter wells for 7 days in 0.1 ml of RPMI-1640 supplemented with antibiotics and 10% human pooled serum in the presence of one of the above described Ag's. Optimal Ag concentration was determined as 2 μg/ml according to titrations performed on PBMC from patients with acute hepatitis B and healthy blood donors, either HBsAg-vaccinated or not. [^{3}H]-Thymidine (specific activity 5mCi/mmol) was added for the last 20h of culture and the incorporated radioactively measured in a β-scintillation counter. Results were expressed as counts per minute (cpm) and the lymphocyte stimulation index (LSI) was calculated as the ratio between median cpm in the HBcAg- or HBsAg-containing cultures and median cpm of cultures containing HCV NS3 or no added Ag, respectively. The 99th percentile of HBcAg-specific SI in 10 healthy blood donors was 2.29. For practical purposes, specific LPR was scored positive when LSI>3.

Results

A 33-year-old homosexual man was known to have been HBeAg-positive since 1995. He was a non-responder to a previous 4-month course of alpha interferon in 1996. Lamivudine (150

mg once daily) was started in August 1997 and the patient was included in a standardised monthly follow-up protocol. The initial drop in HBV DNA ceased after 8 weeks of treatment but HBV DNA remained detectable at levels around 10^8 geq/ml (Fig 1).

After 24 weeks of lamivudine therapy ALAT, ASAT and bilirubin rose to peak levels of 3061 U/l, 1764 U/l and 202 umol/l, respectively. He was jaundiced and complained of malaise, lack of appetite and fatigue. There was neither alcohol nor drug abuse. The differential diagnosis included lamivudine resistance, drug toxicity, spontaneous clearance of hepatitis B, a post-lamivudine flare due to non-compliance or super-infection with HAV, HCV or HDV.

Compliance with his lamivudine medication was demonstrated excluding a lamivudine-withdrawal flare. He did not use nor had ever used other drugs. No evidence of HIV or HDV was found. A YMDD showed wild-type hepatitis B virus.

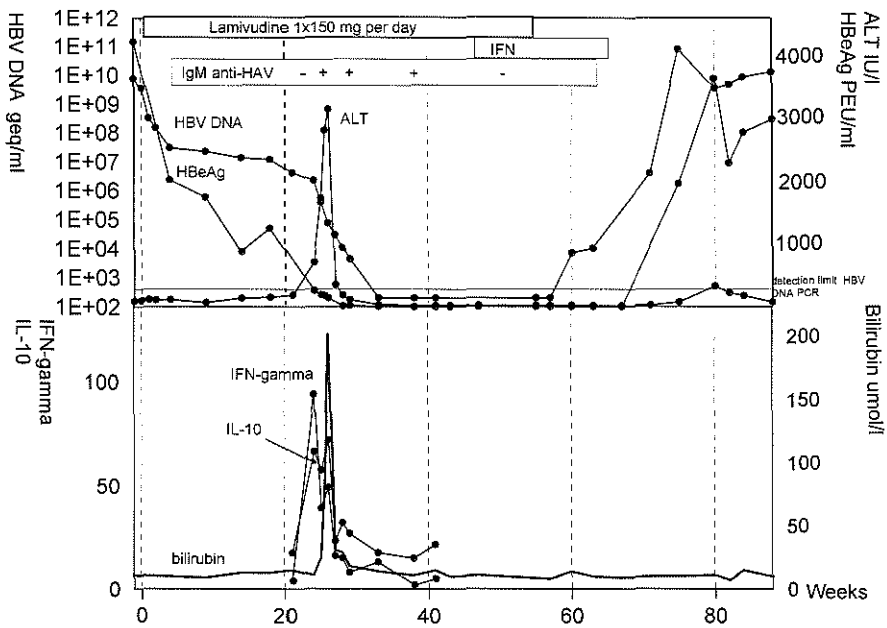


Figure 1 A chronic hepatitis B patient infected with HAV during lamivudine treatment. A sudden drop in HBV DNA was observed in combination with a hepatitis flare followed by loss of HBeAg. An interferon-gamma peak preceded the rise in serum transaminase activity.

Extensive virological testing documented an acute HAV superinfection. IgM anti-HAV was detectable in serum at the moment serum transaminase activity started to increase. Peak serum levels of naturally produced IFN-γ, but also IL-10, were measurable just before the rise in serum transaminase activity and subsequent drop in HBV DNA levels (Fig 1). In these samples the levels of IFN-α, TNF-α and IL-12 were below the level of detection. The proliferative response of PBMC's against the hepatitis B core antigen was already detectable before the acute hepatitis, after lamivudine therapy was started and showed no significant change after the acute HAV infection (Table I). No HBsAg-specific response could be measured in these samples.

During the next 2 months HBV DNA dropped further below the level of PCR detection. HBeAg and anti-HBe reached borderline values, HBsAg remained detectable at very low levels and serum transaminases dropped to normal.

After seroconversion alpha interferon therapy was started to induce a sustained HBeAg seroconversion with detectable anti-HBe antibodies. After 8 weeks of combination therapy lamivudine was withdrawn; subsequently HBV DNA became detectable. After 12 weeks of IFN monotherapy anti-HBe antibodies were detectable; IFN was given for a total period of 20 weeks. However, after its withdrawal, HBeAg relapsed.

Table 1

HBcAg-specific LPR of the control population versus the LPR over time of the acute HAV-infected CHB patient.

		HBc-LSI	LSI SE
Controls:	Healthy donors (n=10)	1.37	0.14
	AHB (n=11)	4.21	0.97
	CHB (n=16)	2.14	0.29
Patient	Wk 0 (start lamivudine)	2.86	
	Wk 4	3.76	
	Wk 48 (after acute HAV)	4.90	

AHB: acute hepatitis B
SE: standard error
CHB: chronic hepatitis B

Discussion

Acute hepatitis A infection has been reported to suppress hepatitis B viral replication in several cases (9-13). In acute hepatitis A lysis of infected hepatocytes is mainly a T-cell-mediated cytotoxic mechanism rather than a direct cytopathic effect of the virus (16-18). It has been suggested that massive necrosis of infected hepatocytes leads to the decrease in HBV DNA (11-13). Hepatitis A caused massive necrosis of infected hepatocytes in our patient, resulting in elevated transaminase activity which was followed by clearance of HBV DNA and HBeAg. However, it is unlikely that cell necrosis of HBV-infected hepatocytes mediated by HAV-specific cytotoxic T lymphocytes eliminates all HBV-infected hepatocytes.

The contribution of noncytolitic antiviral effects may be of major importance for viral clearance in HBV (19). HAV-induced cytokine production is one of the mechanisms which might cause HAV-induced suppression of HBV replication (11, 15). IgM anti-HAV positivity coincided with the high production of IFN-γ followed by inhibition of HBV DNA replication. *In vitro* data suggest that IFN-γ is the main effective mediator in acute HAV infection. The effects occur at three levels: stimulation of HLA antigen expression, direct virus-specific antiviral effects and immunomodulatory effects (14). Acute self-limited HBV is probably also mediated by predominantly Th1 cytokine production, especially of IFN-γ (3). Furthermore, increased IL-12 levels are associated with HbeAg seroconversion (20).

In this case HAV-specific IFN-γ focused on infected hepatocytes probably caused the drop in HBV DNA. An IFN-α peak, as found by Davis et al., could not be confirmed; TNF-α and IL-12 were also below the level of detection. At the same time the production of IL-10, an immune-regulating and anti-inflammatory Th2-cytokine, was stimulated probably contributing to the self-limitation of the response.

Induction of an HBV-specific response would possibly prevent relapse of the HBV infection. Chronic HBV is associated with a weak and ineffective antiviral T-cell response to nucleocapside antigens (HBcAg and HBeAg) in contrast to the strong response in acute HBV infection (1, 3). In acute exacerbations, spontaneous clearance and response to alpha interferon therapy, a re-activated T cell response is measurable, necessary for viral elimination (21-23). Restoration of nucleocapside-specific T cell response has been described also during lamivudine treatment (4), but the underlying mechanism and its clinical meaning are still unclear. Studies in a transgenic mouse model suggest a relevant role for non-cytolytic IFN-γ and TNF-α-mediated suppression of HBV genome expression exerted by HBsAg-specific CTLs (24).

In our patient there was already a pre-existing weak LPR against HBcAg when lamivudine therapy was started. As expected no response to the surface antigen was detectable. The LPR did not increase after the acute HAV infection, when measured in peripheral blood. This does

not exclude an increased response because of the possible compartmentalization of stimulated T-cells in the liver (2). However this is not very likely in our patient in view of his relapse after withdrawal of therapy.

HAV-induced production of IFN-γ probably induced HBV DNA and HBeAg negativity. However this was not combined with stimulation of an HBV-specific T-cell memory which is probably necessary to maintain HBe-seroconversion and protection against HBV relapse. In conclusion we think that our observations may contain important implications for specific immunotherapy for chronic HBV patients on nucleoside analogue therapy.

References

1. Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. Ann Rev Immunol 1995;13:29-60.
2. Ferrari C, Penna A, Bertoletti A, Valli A, Degli Antoni A, Giuberti T, Cavalli A, Petit MA, Fiaccadori F. Cellular immune response to hepatitis B virus-encoded antigens in acute and chronic hepatitis B infection. J Immunol 1990;145:3442-9.
3. Penna A, Del Prete G, Cavalli A, Bertoletti A, Elios MM, Sorrentino R, Amato M, Boni C, Pilli M, Fiaccadori F, Ferrari C. Predominant T-helper 1 cytokine profile of hepatitis B virus nucleocapside-specific T cells in acute self-limited hepatitis B. Hepatology 1997;25:1022-7.
4. Boni C, Bertoletti A, Penna A, Cavalli A, Pilli M et al. Lamivudine treatment can restore T cell responsiveness in chronic hepatitis B. J Clin Invest 1998;102:868-75.
5. Wong DKH, Cheung AM, O'Rourke K, Naylor CD, Detsky AS, Heathcote J. Effect of alfa-interferon treatment in chronic hepatitis B e antigen-positive chronic hepatitis B. Ann Intern Med. 1993;119:312-23.
6. Hadler SC. Global impact of hepatitis A virus infection: changing patterns. In: Hollinger FB, Lemon SM, Margolis H, eds. Viral hepatitis and liver disease. Baltimore: Williams & Williams, 1991:14-20.
7. Vento S, Garofano T, Renzini C, Cainelli F, Casali F, Ghironzi G, Ferraro T, Concia E. Fulminant hepatitis associated with hepatitis A virus superinfection in patients with chronic hepatitis C. N Engl J Med 1998;338:286-90.
8. Keeffe E. Is hepatitis A more severe in patients with chronic hepatitis B and other chronic liver diseases? Am J Gastroenterol 1995;90:201-5.
9. Tassopoulos N, Papaevangelou G, Roumeliotou-Karayannis A, Kalafatas P, Engle R et al. Double infections with hepatitis A and B viruses. Liver 1985;5:348-53.
10. Viola LA, Coleman JC, Barrison IG, Murray-Lyon IM. The clinical course of acute type A hepatitis in chronic HBsAg carriers: A report of three cases. Postgraduate Med J 1982;58:80-1.
11. Davis G, Hoofnagle J, Waggoner J. Acute type A hepatitis during chronic hepatitis B virus infection: association of depressed hepatitis B virus replication with appearance of endogenous alfa interferon. J Med Vir 1984;14:141-7.
12. Conteas C, Kao H, Rakela J, Weliky B. Acute type A hepatitis in three patients with chronic HBV infection. Dig Dis Sci 1983;28:684-6.
13. Wang J-Y, Lee S-D, Tsai Y-T, Lo K-J, Chiang BN. Fulminant hepatitis A in chronic HBV carrier. Dig Dis Sci 1986;31:109-11.
14. Maier K, Gabriel P, Koscielniak E, Stierhof YD, Wiedmann Kh, Flehmig B, Vallbracht A. Human gamma interferon production by cytotoxic T lymphocytes sensitized during hepatitis A virus infection. J Vir 1998;62:3756-63.
15. Tsiquaye K, Harrison TJ, Portmann B, Hu S, Zuckerman AJ. Acute hepatitis A infection in hepatitis B chimpanzee carriers. Hepatology 1984;4:504-9.
16. Vallbracht A, Maier K, Stierhof YD, Wiedmann KH, Flehmig B, Fleischer B. Liver -derived cytotoxic T cells in hepatitis A virus infection. J Inf Dis 1989;160:209-17.
17. Lemon SM, Ping LH, Day S, Cox E, Jansen R, Amphlett E, Sanger D. Immunobiology of hepatitis A virus. Viral hepatitis and liver disease. Baltimore: Williams & Williams, 1991:14-20.
18. Kurane I, Binn LN, Bancroft WH, Ennis FA. Human lymphocyte responses to hepatitis A virus-infected cells: interferon production and lysis of infected cells. J Immunol 1985;135:2140-4.
19. Guidotti LG, Rochford R, Chung J, Shapiro M, Purcell R, Chisari FV. Viral clearance without destruction of infected cells during acute HBV infection. Science 1999;284:825-9.
20. Rossol S, Marinos G, Garucci P, Singer MV, Williams R, Naoumov NV. Interleukin-12 induction of TH1 cytokines is important for viral clearance in chronic hepatitis B. J Clin Invest 1997;99:3025-33.
21. Tsai SL, Chen PJ, Lai PM, Yang PM, Sung JL, Huang LH, Hwang LH, Chang TH, Chen DS. Acute exacerbations of chronic type B hepatitis are accompanied by increased T cell responses to hepatitis B core and e antigens. J Clin Invest 1992;89:87-96.
22. Jung MC, Diepolder HM, Spengler U, Wierenga EA, Zachoval R, Hoffmann RM, Eichenlaub D, Frosner G, Will H, Pape GR. Activation of a heterogenous hepatitis B (HB) core and e antigen specific CD4+ T-cell population during seroconversion to anti-HBe and anti-HBs in hepatitis B virus infection. J Virol 1995;69:3358-68.
23. Rehermann B, Hoofnagle JH, Chisari FV. Cytotoxic T lymphocyte responsiveness after resolution of chronic hepatitis B virus infection. J Clin Invest 1996;97:1655-65.

24. Guidotti LG, Ando K, Hobbs MV, Ishikawa T, Runkel L, Schreiber RD, Chisari FV. Cytotoxic T lymphocytes inhibit hepatitis B virus gene expression by a noncytolytic mechanism in transgenic mice. Proc Natl Acad Sci 1994;91:3764-8.

Chapter IV

Lamivudine-IFN combination therapy

in chronic hepatitis B

IV.1 EFFICACY OF LAMIVUDINE MONOTHERAPY FOLLOWED BY INTERFERON-LAMIVUDINE COMBINATION THERAPY IN CHRONIC HEPATITIS B PATIENTS

A.B. van Nunen[1], R.A. de Man[1], H.G.M. Niesters[2], H.L.A. Janssen[1], S.W. Schalm[1].

Departments of [1]Hepatogastroenterology and [2]Virology, Erasmus University Hospital Rotterdam, The Netherlands

Submitted for publication

Abstract

Background: In chronic HBeAg-positive hepatitis, alpha-interferon (IFN) and lamivudine monotherapy lead to HBeAg seroconversion and low-level viral replication in a minority of patients. To enhance the HBeAg seroconversion response, we used lamivudine to suppress viral replication before starting IFN therapy.

Methods: In a single centre cohort study 24 HBeAg-positive chronic hepatitis B patients received lamivudine monotherapy for a minimum of 16 weeks. IFN therapy was added for at least 16 weeks in a dose of 30 MU/week or more. Lamivudine therapy was withdrawn after at least 8 weeks of combination therapy. Post-treatment follow-up after stop of interferon therapy was 16 weeks.

Results: A response (loss of HBeAg and HBV DNA from serum) was observed in 4 patients. However, this response was sustained in only 2 patients after withdrawal of therapy. Median HBV DNA levels decreased during lamivudine monotherapy by 3.2 logs (5-95 percentile 1.4 to 6.7). IFN therapy further decreased levels of HBV DNA by 1.4 logs (-0.1 to 2.7). Withdrawal of lamivudine therapy resulted in an increase in HBV DNA levels by 3.5 logs (0 to 4.4). After cessation of IFN, HBV DNA increased further by 0.9 logs (-1.8 to 5.6) until the end of follow-up. HBV DNA negativity by quantitative PCR (HBVDNA < 1 x 10^3geq/ml) was obtained in 6 out of 24 patients.

Conclusions: Lamivudine monotherapy followed by IFN-lamivudine combination therapy resulted in strong virus suppression. However a sustained response was obtained in less than 10% of these difficult-to-treat patients with the current treatment schedule. No benefit of lamivudine pretreatment to a course of IFN therapy could be documented in this cohort of patients.

Introduction

Interferon-alpha (IFN) and lamivudine are both licensed for the treatment of chronic hepatitis B virus (HBV) infected patients. Loss of HBeAg from serum, either spontaneously or following IFN therapy, is correlated with improved survival in chronic HBV (1-4). Therefore antiviral therapy in chronic HBV aims at obtaining sustained HBeAg seroconversion.

IFN therapy for up to 6 months leads to HBeAg seroconversion in 20-40% of patients, depending on baseline ALT levels (1, 3, 5). No more than 20% of patients respond with HBeAg seroconversion, at one year of lamivudine monotherapy. These low response rates emphasise the need for new therapeutic modalities. Combination of interferon with lamivudine is one such approach.

Patients with low baseline HBV DNA and high ALT levels are most likely to respond to IFN therapy whereas in lamivudine therapy baseline ALT and HAI score are the most important positive predictors of response (3, 6-8).

Several studies have demonstrated that the overall benefit of lamivudine-IFN combination therapy over IFN alone for 16 weeks in chronic HBV is limited (9-11). Efficacy was highest in patients with moderately elevated baseline ALT levels (10). Recently, it was shown that prolonged lamivudine-IFN combination therapy for 6 months significantly increased the HBeAg seroconversion rate compared to standard lamivudine monotherapy for 1 year (12). In a small-scale study by Serfaty et al sequential administration of lamivudine and IFN in previous IFN non-responders induced sustained HBeAg seroconversion in 5 out of 11 patients (13).

Whether simultaneous or sequential dosing of IFN and nucleoside analogues should be preferred has not been elucidated yet. In this study we added IFN to lamivudine monotherapy in chronic HBeAg-positive patients in order to enhance HBeAg seroconversion. In addition we extensively studied HBV-DNA kinetics during different treatment phases of mono- and combined IFN-lamivudine therapy.

PATIENTS AND METHODS

Patients

In this single center cohort study eligible patients were HBsAg and HBeAg positive at least 6 months before start of lamivudine. All patients were above 18 years of age. Patients were excluded for the following reasons: other acquired or inherited causes of liver disease (HCV, HDV, Wilson's disease, auto-immune disease, hemochromatosis, alcohol or drug abuse); significant systemic illness (e.g. cardiovascular or pulmonary dysfunction, HIV, transplantation); pregnancy or breast-feeding, antiviral or immunosuppressive treatment in previous 6 months; and contra-indications for IFN therapy.

Treatment schedule

Patients received lamivudine monotherapy 150 mg p.o. daily for at least 16 weeks. Subsequently, interferon-alpha 2b (Intron A) was added initially at a dose of 10 MU daily for 4 weeks, followed by a standard dose, 10 MU s.c. thrice weekly. After 8 - 14 weeks of combination therapy, lamivudine was withdrawn. IFN monotherapy was continued for more than 8 weeks thereafter. IFN therapy was prolonged beyond 16 weeks based on a combination of HBeAg levels and HBV DNA levels (5). The follow-up period after discontinuation of IFN was 16 weeks.

Measurements

Patients were evaluated weekly during high-dose IFN therapy and monthly thereafter. At all visits blood was withdrawn for virological markers and serum aminotransferases (ALT, upper limit of normal (ULN): 31 IU/l). HBV DNA was measured quantitatively by Hybrid Capture plate assay I (Digene, Murex, Abbott UK; detection limit 1.5×10^6 genome equivalents per ml (geq/ml) followed by quantitative PCR (PCRQ, Roche Amplicor, Almere, The Netherlands, lower detection limit 1×10^3 geq/ml) and if negative by standard PCR (lower detection limit 4×10^2 geq/ml). All assays were calibrated on the Eurohep standard. HBeAg and anti-HBe were tested by AxSYM (Abbott Laboratories, IL, USA).

Statistics

All results are presented as median and 5-95 percentiles. The primary end-point was a response to therapy defined as loss of HBeAg and HBV DNA negativity (Hybrid Capture assay I). Secondary the HBV DNA levels during the different stages of therapy were compared by use of the Wilcoxon signed rank test for paired samples. Each time point was compared with that of the previous sample.

Ethics

The local medical ethics committee approved this study, all patients gave their written informed consent.

Results

Twenty-four immunocompetent chronic HBeAg-positive patients were enrolled. Table 1 summarises the baseline characteristics of the study population. In 4 patients IFN therapy was stopped prematurely, primarily for reasons of side effects. The reasons for withdrawal were collapse at week 3 of combination therapy in 1 patient, flu-like symptoms at 1.5 weeks of combination therapy in 2 patients, and unknown at week 4 of combination therapy in 1 patient. To assess an optimal analysis of the HBV decay, these 4 patients with early discontinuation of combined therapy were excluded from analysis of viral kinetics (per protocol analysis). Lamivudine monotherapy was given for a period of 16 to 74 weeks.

Combination therapy was given for 8 to 14 weeks. After lamivudine was withdrawn, IFN monotherapy was continued for an additional 7 to 28 weeks.

Viral kinetics

Median HBV DNA levels after logarithmic transformation and ALT levels during therapy and follow up are shown in the upper panel of figure 1, the 5-95 percentiles are added in the lower part.

Phase 1: Lamivudine monotherapy. During lamivudine therapy HBV DNA levels showed a median (5-95 percentiles) drop of 3.2 (1.4 to 6.7) logs ($p< 0.001$). In the initial 4 weeks of therapy 66% (2.1 logs) of the reduction was obtained, followed by a gradual decline of 5-9% per 4 weeks (0.2-0.3 logs/4 weeks). In the second lamivudine treatment period (beyond week 4) only 16% of patients (3/19) showed a reduction in HBV DNA levels of more than 0.5 log/ 4 weeks. One patient was already HBV DNA negative by PCR at week 4.

Phase 2: IFN-lamivudine combination therapy. After addition of high-dose IFN therapy a 1.4 (-0.1 to 2.7) log reduction was observed ($p= 0.002$). Eighty-six percent of the total reduction in HBV DNA (1.2 logs) was obtained in the initial 4 weeks of combination therapy. In 94% of patients (17/18) who were HBV DNA positive by PCR at start of IFN therapy, more than 0,5 log reduction of HBV DNA was observed during the first 4 weeks of combination therapy. A reduction of 2 logs or more was observed in 6/17 patients (35%) during combination therapy.

Phase 3+4: IFN monotherapy and folllow up. Withdrawal of lamivudine was accompanied by a significant increase in HBV DNA levels of 3.5 (0 to 4.4) logs ($p< 0.001$) followed by a further increase of 0.9 (-1.8 to 5.6) logs ($p< 0.001$) after stop of IFN therapy.

Treatment response

Results of HBeAg seroconversion, HBV DNA negativity and ALT normalisation after different treatment phases are shown in table 2. A response (loss of HBeAg and HBV DNA from serum by Hybrid Capture assay) was obtained in 1 patient during lamivudine monotherapy. This patient remained HBV DNA positive by PCR before start of IFN. During combination therapy another 3 patients responded. A relapse following withdrawal of antiviral therapy was observed in 2/4 patients. Thus, in 2/24 (8%) patients a sustained response was observed.

HBV DNA became negative by PCRQ in 3 patients during lamivudine monotherapy. In another 3 patients HBV DNA became undetectable by PCRQ during combination therapy. Cessation of therapy was followed by an HBV DNA relapse in 5 out of 6 patients while in 1 patient HBV DNA levels remained detectable around 2×10^3 geq/ml.

Table 1

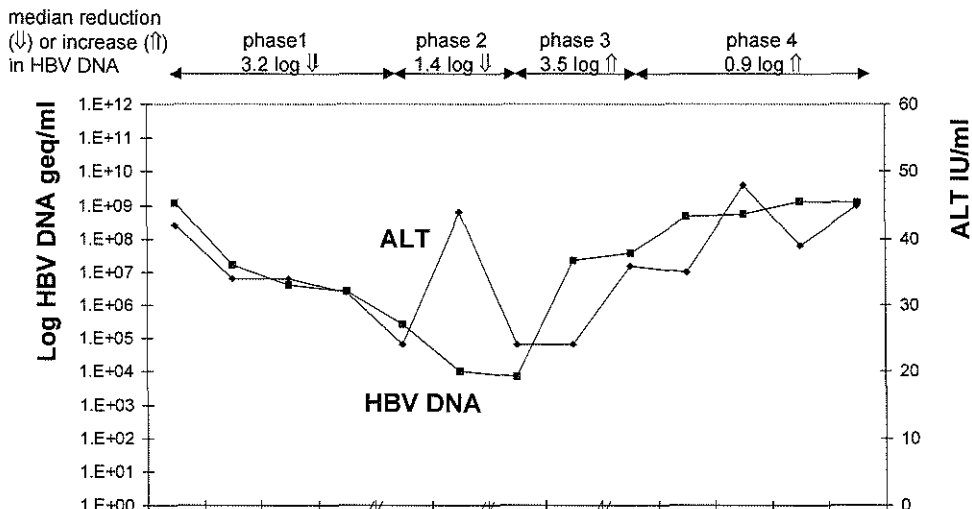
Baseline characteristics expressed as median (5-95 percentiles).

	n=24
sex (m/f)	12 / 12
Age (yrs)	28 (20-59)
Race	Caucasian : 12
	Asian : 9
	Other : 3
previous IFN therapy	10
HBV DNA (geq/ml)	1.3×10^8 ($1.5 \times 10^7 - 5.2 \times 10^{10}$)
ALT IU/l*	39 (13-198)
Cirrhosis (yes/no)	0/24

* ALT upper limit of normal 31 IU/l

Table 2

response	start lamivudine	start IFN	stop lamivudine	stop IFN	end of follow up
virological:					
HBeAg negative	0	3 (13%)	5 (21%)	5 (21%)	2 (8%)
HBV DNA < 1.5×10^6 geq/ml	1 (4%)	15 (63%)	21(88%)	5 (21%)	3 (13%)
HBV DNA < 1×10^3 geq/ml	0	3 (13%)	6 (25%)	2 (8%)	1 (4%)
biochemical:					
ALT normal	8 (33%)	13 (54%)	14 (58%)	11(46%)	6 (25%)
virological and biochemical:	0 (0%)	1 (4%)	4 (17%)	2 (8%)	2 (8%)



	start lamivudine	start IFN	stop lamivudine	stop IFN	end of follow up
HBV DNA	1.2 x 10^9 ($3x10^6$– $3x10^{10}$)	2.7 x 10^5 ($4x10^2$–$2x10^8$)	7.2 x 10^3 ($4x10^2$– $6x10^7$)	3.8 x 10^7 ($4x10^2$ –$1x10^{10}$)	1.2 x 10^9 ($4x10^2$– $6x10^{10}$)
ALT	42 (19-219)	24 (12-51)	24 (14-107)	36 (16-126)	45 (10-634)

Figure 1 In the upper panel median HBV DNA (geq/ml) and ALT levels (upper limit of normal 31 IU/l) during therapy and follow up measured monthly for the per protocol population (n=20) are shown. The 5-95 percentiles calculated at the key points during therapy and follow up are added in the lower panel. Lamivudine monotherapy shows a fast initial decline of HBV DNA caused by strong inhibition of viral replication followed by a more gradual decline with a wide distribution. Addition of IFN causes a further decline of HBV DNA, however after withdrawal of lamivudine viral replication increases and after withdrawal of IFN HBV DNA levels returned to baseline levels.

Hepatitis flares

During lamivudine monotherapy an episode of hepatitis flare (ALT > 10 above normal values and 3 x baseline level) was observed in 2 patients. After addition of IFN 2 patients experienced a hepatitis flare, in one of them the flare was accompanied by a (non-sustained) HBeAg seroconversion. In 4 patients withdrawal of IFN therapy was followed by a hepatitis flare. These flares occurred 9 to 20 weeks after discontinuation of IFN and 25 to 40 weeks after discontinuation of lamivudine. ALT levels increased to levels ranging from 338 to 679 IU/L. All these patients were retreated with lamivudine monotherapy.

Discussion

Pretreatment with lamivudine for 16 weeks followed by 8 weeks of IFN-lamivudine combination and 8 weeks of IFN did not lead to a high rate of sustained HBeAg seroconversion. The major effect of lamivudine on drop of HBV DNA levels was in the first 4 weeks of therapy, thereafter a slow decrease was observed. Adding IFN to lamivudine had significant effect on serum levels of HBV DNA and further decreased HBV DNA by 1.2 logs. Four out of 24 patients (17%) HBeAg seroconverted during therapy. Sustained HBeAg seroconversion was found in only 8%.

In addition to many immune stimulating effects, the antiviral effects of IFN include blocking of entry and uncoating of the virus as well as inhibiting the production of pre-genomic m-RNA; these actions probably also lead to the increased blockage of viral replication in patients on nucleoside-analogue treatment (14-16). Lamivudine has a potential direct antiviral effect and may increase the HBcAg and HBeAg mediated T-cell response suggesting pre-treatment may be beneficial (17).

The benefit of lamivudine-IFN combination therapy in chronic HBV has not yet been elucidated, as has the optimal duration and treatment schedule. The overall benefit of simultaneous dosing of lamivudine and IFN for 16 weeks has been debated for both IFN naive and IFN non-responders (9-11). Subgroup analysis showed most benefit in patients with moderately elevated ALT. A prolonged course of lamivudine-IFN combination therapy showed to enhance the HBeAg seroconversion rate (12). In that study group, harbouring patients with relatively high baseline transaminase values, HBeAg seroconversion was 15% for those treated with lamivudine and 33% for those treated with lamivudine-IFN combination. Promising results were obtained in a pilot study including 14 previous IFN non-responders, 11 HBeAg positive patients, using an add-on therapy with IFN following lamivudine monotherapy (13). Lamivudine monotherapy for 24 weeks was followed by 4 weeks of lamivudine-IFN combination therapy, after which lamivudine was withdrawn and IFN continued for another 24 weeks. A sustained HBeAg-response was observed in nearly 50% (5/11) of patients.

The limited sustained response rate in this study is related to both low initial response as to high relapse rates. Low initial response is primarily related to low baseline ALT and high HBV DNA levels in our population. Baseline characteristics, especially ALT, are important predictors of a sustained response (7, 10). ALT levels below 2 times ULN are associated with a decreased response rate during mono -or combination therapy with lamivudine and IFN (7, 10). The relatively high relapse rate in our study could also be dependent of high pre-treatment HBV DNA and low pre-treatment ALT levels (18). These negative response-predictive factors are in particular important following lamivudine-induced HBeAg seroconversion. Early withdrawal of lamivudine, a few weeks following HBeAg seroconversion, may be another factor that contributed to high relapse rate in this study. The period of continuation of lamivudine after HBeAg seroconversion is correlated with the post-treatment relapse rate. Unlike IFN-induced HBeAg seroconversion, which is stable in over 85% of patients, there is disagreement about the durability of lamivudine-induced HBeAg seroconversion (19). By some it is claimed to be as durable as IFN induced seroconversion, while others reported a relapse rate in up to 49% of patients (19-22).

In conclusion IFN had a significant additional effect to lamivudine monotherapy on inhibition of viral replication. This favourable kinetic profile did not result in high sustained HBeAg seroconversion rates. Sequential withdrawal of therapies was followed by a relapse of viral activity in the majority of patients. Whether simultaneous dosing of both drugs will lead to a better virus suppression and whether prolonged therapy might lower relapse rates has to be investigated in future studies.

References

1. Niederau C, Heintges T, Lange S, Goldmann G, Niederau CM, Mohr L, Haussinger D. Long-term follow-up of HBeAg-positive patients treated with interferon alfa for chronic hepatitis B. N Engl J Med 1996;334:1422-7
2. Fattovich G, Giustina G, Realdi G, Corrocher R, Schalm SW. Long-term outcome of hepatitis B e antigen-positive patients with compensated cirrhosis treated with interferon alfa. European Concerted Action on Viral Hepatitis (EUROHEP). Hepatology 1997;26:1338-42.
3. Krogsgaard K, Bindslev N, Christensen E, Craxi A, Schlichting P, Schalm SW, Carreno V, Trepo C, Gerken G, Thomas HC, Andersen PK, Ring-Larsen H and the European Concerted Action on Viral Hepatitis (Eurohep). The treatment effect of alpha interferon in chronic hepatitis B is independent of pre-treatment variables. Results based on individual patient data from 10 clinical controlled trials. J Hepatol 1994;21:646-55.
4. Lin SM, Sheen IS, Chien RN, Chu CM, Liaw YF. Long-term beneficial effect of interferon therapy in patients with chronic hepatitis B virus infection. Hepatology 1999;29:971-5.
5. Janssen HLA, Gerken G, Carreno V, Marcellin P, Naoumov NV, Craxi A, Ring-Larsen H, Kitis G, Hattum J van, Vries RA de, Michielsen PP, Kate FJW ten, Hop WCJ, Heijtink RA, Honkoop P, Schalm SW and the European Concerted Action on Viral Hepatitis (Eurohep). Interferon alfa for chronic hepatitis B infection: increased efficacy of prolonged treatment. Hepatology 1999;30:238-43.
6. Brook MG, Karayiannis P, Thomas HC. Which patients with chronic hepatitis B virus infection will respond to alpha-interferon therapy? A statistical analysis of predictive factors. Hepatology 1989;10:761-3.
7. Chien RN, Liaw YF, Atkins M. Pretherapy alanine transaminase level as a determinant for hepatitis B e antigen during lamivudine therapy in patients with chronic hepatitis B. Asian Hepatitis Lamivudine Trial Group. Hepatology 1999;30:770-4.
8. Perillo RP, Ochsner AL, Schalm SW, Schiff E, Brown NA, Woessner MA, Sullivan M. Predictors of HBeAg seroconversion in chronic hepatitis B patients treated with lamivudine. Hepatology 1999;30:317(A).
9. Mutimer DJ, Naoumov N, Honkoop P, Marinos G, Ahmed M, Man de RA, McPhillips P, Johnson M, Williams R, Elias E, Schalm SW. Combination alpha-interferon and lamivudine therapy for alpha-interferon resistant chronic hepatitis B infection: results of a pilot study. J Hepatol 1998;28:923-9.
10. Schalm SW, Heathcote J, Cianciara J, Farrell G, Sherman M, Willems B, Dhillon A, Moorat A, Barber J, Gray DF. Lamivudine and alpha interferon combination treatment of patients with chronic hepatitis B infection: a randomised trial. Gut 2000;46:562-8.
11. Schiff ER, Karayalcin S, Grimm IS, Perillo RP, Dienstag JL, Husa P, de Man RA, Goodman Z, Condreay LD, Crowther LM, Woessner MA, McPhillips PJ, Brown NA and an International Lamivudine Investigator Group. Lamivudine and lamivudine/interferon combination therapy in patients with hepatitis B who previously failed interferon: an international controlled trial. Ann Int Med 2002, accepted for publication.
12. Barbaro G, Zechini F, Pellicelli AM, Francavilla R, Scotto G, Bacca D, Bruno M, Babudieri S, Annese M, Matarazzo F, Di Stefano G, Barbarini G. Long-term efficacy of interferon alpha-2b and lamivudine in combination compared to lamivudine monotherapy in patients with chronic hepatitis B. An Italian multicenter, randomized trial. J Hepatol 2001;35:406-11.
13. Serfaty L, Thabut D, Zoulim F, Andreani T, Chazouilleres O, Carbonell N, Loria A, Poupon R. Sequential treatment with lamivudine and interferon monotherapies in patients with chronic hepatitis B not responding to interferon alone: results of a pilot study. Hepatology 2001;34:573-7.
14. Peters M. Mechanisms of action of interferons. Semin Liver Dis. 1989 Nov;9(4):235-9.
15. Foster GR. Interferons in host defense. Semin Liver Dis 1997;17:287-95.
16. Thomas HC. Pathogenesis of chronic hepatitis HBV infection and mechanisms of action of antiviral compounds. Viral hepatitis and liver diseases 1990. ed. by Hollinger FB, Lemon SM; 612-615.
17. Boni C, Bertoletti A, Penna A, Cavalli A, Pilli M, Urbani S, Scognamiglio P, Boehme R, Panebianco R, Fiaccadori F, Ferrari C. Lamivudine treatment can restore T cell responsiveness in chronic hepatitis B. J Clin Invest 1998;102:968-75.
18. A.B. van Nunen, B.E. Hansen, D.J. Suh, H.F. Löhr, H. Fontiane, L. Chemello, B.C. Song, H.L.A. Janssen, J Heathcote, R.A. de Man, S.W.Schalm and the International Meta-Analysis of Individual Patient Data group. Durability of HBeAg seroconversion following antiviral therapy relates to type of therapy and pretreatment HBV DNA and ALT. (submitted).
19. Song BC, Suh DJ, Lee HC, Chung YH, Lee YS. Hepatitis B e antigen seroconversion after lamivudine therapy is not durable in patients with chronic hepatitis B in Korea. Hepatology 2000;32:803-6.
20. Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, Crowther L, Condreay LD, Woessner M, Rubin M, Brown NA. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med. 1999;341:1256-63.

21. Chang TT, Lai CL, Liaw YF, Leung NW, Lim SG, Lee CM, Ng KY, Edmundson S, Stevenson G, Dent J. Enhanced HBeAg seroconversion rates in Chinese patients on lamivudine. Hepatology 1999;30:420A.
22. Fontaine H, Driss F, Lagneau J, Zylberberg H, Brechot C, Pol S, Necker C. Hepatitis B virus reactivation after lamivudine discontinuation. Hepatology 1999;30(4);349A.

IV.2 LAMIVUDINE AND INTERFERON COMBINATION THERAPY IN CHRONIC HEPATITIS B: A STUDY OF VIRAL KINETICS

A. B. van Nunen[1], B. E. Hansen[2], D. J. Mutimer[3], H. L.A. Janssen[1], H. G.M. Niesters[4], R. A. de Man[1], S. W. Schalm[1]

[1]Dept. of Hepatology & Gastroenterology, Univ. Hospital Rotterdam, The Netherlands;
[2]Dept. of Epidemiology & Biostatistics, Erasmus University Rotterdam, The Netherlands;
[3]Liver and Hepatobiliary Unit, Queen Elizabeth Hospital, Birmingham, United Kingdom;
[4]Dept. of Virology, University Hospital Rotterdam, The Netherlands

Submitted for publication

Abstract

Background: Whether the combination of lamivudine and alpha-interferon (IFN) increases the effectivity of antiviral therapy in chronic hepatitis B is unclear. To determine a potentially increased antiviral effect of combination therapy, we studied the viral kinetics of hepatitis B during the first 16 weeks of lamivudine monotherapy, IFN monotherapy and lamivudine-IFN combination therapy.

Patients and Methods: Forty-nine patients (40 males, 9 females), treated in 3 randomized trials, were included. Twenty-one patients were treated with lamivudine monotherapy (150-600 mg daily), twenty received IFN monotherapy (10 MU tiw) and eight patients received lamivudine (100 mg daily) combined with IFN (10 MU tiw). Sequential sera, taken at week 0, 1, 2, 3, 4, 8, 12, and 16 of therapy, were tested for HBV DNA by Hybrid Capture assay followed by quantitative and qualitative PCR (detection limit 400 geq/ml). The decay of viral load was described by a piecewise linear regression analysis (model 1), and in addition, by non-linear regression analysis according to Neumann (model 2). Further analysis was performed taking into account variations in baseline factors, especially serum ALT.

Results: The three treatment groups were comparable regarding baseline characteristics, with the exception of ALT being significantly higher in the IFN treated patients. Lamivudine containing therapies showed the largest decline in the first phase of the viral decay curve, whereas the second phase appeared mainly influenced by IFN containing therapies. After correction for baseline ALT levels, the second phase of viral decline was significantly more rapid for combination therapy compared to IFN and lamivudine monotherapy, which were similar. Nearly all patients receiving combination therapy are theoretically expected to reach viral negativity compared to 55-65% for monotherapy with either drug. The estimated median time needed to obtain viral elimination from serum was 39 weeks for combination therapy, but 84 and 89 weeks for IFN and lamivudine monotherapy respectively.

Conclusions: In comparison with monotherapy, combination of interferon and lamivudine reduces the estimated time to viral negativity, and increases the percentage of patients reaching that endpoint. Prolongation of combination therapy to 1 year might enhance viral clearance in patients chronically infected with the hepatitis B virus.

Introduction

The limited efficacy of standard treatment courses with either alpha-interferon (IFN) or lamivudine, and the time-dependent emergence of viral resistance during prolonged lamivudine monotherapy emphasize the need for new approaches for therapy in chronic hepatitis B. Combination therapy of interferon with lamivudine is one such approach.

Two large studies on combination therapy in HBV have been performed, both with HBeAg seroconversion and HBV DNA negativity (by conventional hybridization assay) as an end-point (1-2). For treatment-naive patients an enhanced HBeAg seroconversion rate was reported for combination therapy in comparison to IFN or lamivudine monotherapy, particularly in patients with elevated pre-treatment ALT; overall the additional benefit was limited (1). For previously IFN treated non-responder patients no benefit of combination therapy over lamivudine monotherapy could be demonstrated (2).

Combination of both drugs in the two large studies was for 16 weeks. However, the decay curve of hepatitis B virus during combination therapy points to the need for longer therapy than 16 weeks to reach virus elimination (3). The curve during therapy with nucleoside analogues exhibits a bi-phasic decline (4), as in hepatitis C (5). The initial fast phase represents the clearance of free viral particles from serum, its magnitude and duration are believed to relate to the effectiveness of antiviral therapy in blocking viral production; the slower second phase is thought to represent the immune-mediated clearance of infected hepatocytes. To further investigate the antiviral effect of combination therapy versus monotherapy, we measured HBV DNA in stored serum samples of patients who completed lamivudine therapy, IFN monotherapy, or combination therapy, analyzed the (biphasic) decline and calculated both the effectiveness of therapy in inhibiting viral production as well as the clearance of cells infected with hepatitis B virus.

Patients and Methods

Patients

Forty-nine compensated chronic hepatitis B patients who participated in 3 randomized trials were included.

Group A consisted of 21 patients participating in a viral kinetics study of lamivudine monotherapy (6). All patients received lamivudine at least 150 mg daily for more than 16 weeks. Eleven patients were treated with 600 mg of lamivudine daily during the first 4 weeks; since a dose higher than 150 mg did not affect the viral kinetics, patients receiving either 150 mg or 600 mg of lamivudine were considered as one group (6).

Group B consisted of 20 patients that were all treated with IFN 10 MU tiw during the first 16 weeks of a randomized controlled trial (7). In 96 of 162 patients stored serum on 5 specific

time points (*vide infra*) was available; out of these 96, 20 patients were randomly selected by a computer generated program.

Group C were all 8 patients from Rotterdam and Birmingham who received IFN 10 MU tiw in combination with lamivudine 100 mg daily for 16 weeks in a randomized trial on lamivudine-IFN combination therapy in previous IFN non-responders (8).

In addition, we analyzed HBV DNA levels in a new Rotterdam cohort of 5 consecutive HBeAg-positive patients who were treated with long-term combination therapy of lamivudine (150 mg daily) and IFN (10 MU tiw) after closure of entry to the controlled trial.

Virologic measurements

Sequential serum samples for HBV DNA measurements were taken before start of therapy, at week 1, 2, 3, 4, 8, 12 and 16. For IFN monotherapy samples at week 1 and 3 were not available. During combination therapy an additional sample was taken at week 6. HBV DNA was measured quantitatively by Digene Hybrid Capture II plate assay (detection limit $2x10^5$ copies/ml) and quantitative PCR (Roche Amplicor, Almere, The Netherlands, detection limit 10^3 copies/ml) and if negative by qualitative PCR (lower detection limit 400 copies/ml). All 3 assays were calibrated on the Eurohep standard and interchangeable (9).

Mathematic modeling and statistics

Viral kinetics were fitted in 2 ways. In the first method we calculated and compared viral kinetics by piecewise linear regression (model 1). In the second method we used non-linear regression analysis (model 2) which has previously been described by Neumann et al. (5) for hepatitis C and modified by Tsiang (4) for hepatitis B.

Model 1: piecewise linear regression The piecewise linear regression model with one breakpoint at week t_{break} is given by:

$$V(t) = \begin{cases} V_0 + b_1 t & \text{for } t < t_{break} \\ (V_0 + (b_1 - b_2)t_{break}) + b_2 t & \text{for } t > t_{break} \end{cases}$$

V= log transformed viral load (HBV DNA)

V_0 = initial viral load (log transformed)

b_1= slope of first phase

b_2= slope of second phase

t= treatment time (week)

The piecewise linear regression model was fitted in SAS 8.00 with PROC MIXED with random intercept V_0 and slopes of viral decline b_1 and b_2 per individual, using an unstructered covariance matrix for the random effects. The break point of the lines t_{break} was fitted seperately for each therapy using non-linear regression analysis (macro NLINMIX in

SAS 8.00) (including random effects per individual): for LAM: t_{break} = week 1; for IFN + LAM: t_{break} = week 1; and for IFN: no break point was found because measurements within the first 2 weeks were not available. Based on the assumption that IFN therapy, as in HCV infection (5), induces a biphasic HBV DNA decay the theoretical breakpoint was set at week 1: t_{break} = week 1.

In addition we used model 1 in adaptive versions to correct for differences in baseline ALT. A correction factor was added to each parameter V_0, b_1, b_2 . Each parameter is written as a sum of two factors. The first factor represents the level of the reference (ALT<2x ULN), the second reflects the difference between the reference and the corrected value (ALT 2-5x ULN or ALT > 5xULN). For example: $V(t) = (V_0 + V_{0\ corrected}) + (b_1 + b_{1corrected})t$

Model 2: non-linear regression The non-linear regression model based on the work of Neumann (5) and Tsiang (4) yields a biphasic curve. Mathematically the model is as follows:

$V(t) = V_0 + \log\{A\exp(-\lambda_1 t) + (1-A)\exp(-\lambda_2 t)\}$

where V= log transformed viral load (HBV DNA), V_0 = initial viral load (log transformed), A is correlated to the duration of the first phase, λ_1 the slope of the first phase and λ_2 the slope of the second phase. These parameters have been transformed to biological parameters: c (clearance rate of free virus from serum), ε (effectiveness of blocking virion production), η (efficacy of blocking the *de novo* infection of uninfected cells) and δ (death rate of infected cells) (5):

$$A = (\varepsilon c - \lambda_2) / (\lambda_1 - \lambda_2)$$

$$\lambda_{1,2} = \tfrac{1}{2}\{(c+\delta) \pm [(c-\delta)^2 + 4(1-\varepsilon)(1-\eta)c\delta]^{\frac{1}{2}}\}$$

Model 2 was fitted with mixed-effects theory using the macro NLINMIX in SAS 8.00, with two exponential curves. With this technique the entire data set can be analyzed and therapy specific dynamic parameters can be estimated. The individual deviation from the therapy specific curves are estimated by including random effects for each subject specific parameter set.

The results of the mathematical calculations for each therapy group were compared using the Log likelihood test, the level of significance was set to $p < 0.05$.

Time to viral negativity Assuming that the slope of the viral decline of the second phase can be extrapolated, the viral load at time t beyond 16 weeks can be predicted using model 1 or model 2. Furthermore the probability of the viral load V(t) reaching some detection limit D at time t: Prob(V(t)<D), can be calculated: under the fitted model 1 V(t) is described by a normal distribution mean given by model 1 and with standard deviation SD(V(t)). The standard deviation of the viral load at time t: SD(V(t)) can be estimated by the fitted covariance matrix

of the random effects (model 1 is here extended with a block diagonal covariance matrix, existing of blocks corresponding to the therapies). It then follows that

Prob (V(t)<D)= $\phi\{(D-V(t))/ SD(V(t))\}$,

where ϕ is the standard normal cumulative density function.

Other statistics For comparison of baseline nominal variables the χ^2 were used, continuous variables were compared with the one-way Anova or Kruskal-Wallis test.

Results

The baseline demographics of the 3 therapy groups are shown in table 1. Significant differences are observed for age (lamivudine), previous antiviral therapy (interferon), and baseline ALT (interferon). In view of the documented effect of serum ALT on both viral kinetics (6) and outcome of antiviral therapy (1), we repeated the comparison of therapy groups after correction for baseline ALT.

Table 1

Baseline characteristics expressed as median value (5^{th} - 95^{th} percentiles).

	LAM	IFN	LAM+IFN	p-value
n=	21	20	8	
Age (yrs)	29 (18 - 53)	47 (31 - 61)	43 (34 - 68)	0.00
Sex (m/f)	18/3	13/7	8/0	0.08
Previous AVT†				0.00
IFN	9	0	8	
Race				0.17
Caucasian	11	17	5	
Asian	7	2	3	
Other	3	1	0	
ALT (IU/l)††	62 (18 - 596)	103 (27 - 332)	48 (25 -108)	0.01
<2 x ULN	10	4	5	
2-5 x ULN	9	11	3	
>5 x ULN	2	5	0	
HBV DNA (geq/ml)*	1.2×10^9 (3.1×10^7–1.3×10^{10})	1.4×10^9 (1.5×10^7–2.5×10^{10})	1.7×10^9 (7.4×10^7–7.1×10^9)	0.62

† AVT = antiviral therapy

†† upper limit of normal (ULN): 30 IU/l

* measured by Digene Hybrid Capture assay (Digene, Diagnostics)

Kinetic results per therapy group: Figure 1 illustrates the average decline of the HBV DNA values per therapy group fitted by both models. A bi-phasic decline was observed during lamivudine monotherapy and combination therapy. In patients treated with IFN monotherapy a breakpoint in the viral decay curve could not be calculated (no HBV DNA measurements in the first 2 weeks) and a theoretical breakpoint was set at week 1. Both models (piecewise linear regression analysis and non-linear regression analysis) showed a similar fit according

to the log likelihood ratio and Akaike's information criteria (AIC) (table 2). Independently of the model, viral decline during the first phase is largest in lamivudine-containing therapies whereas viral decline during the second phase is more pronounced during IFN therapy with or without lamivudine.

Viral kinetic parameters (by piecewise linear regression analysis and non-linear regression analysis) are shown in table 2. Viral kinetics of the first rapid phase shows a 2.6-2.7 log drop of HBV DNA during the first week of combination therapy. This viral decline was significantly faster compared to the 1.5-1.8 log of lamivudine monotherapy ($p \leq 0.001$) and 0.4-0.5 log of IFN monotherapy ($p < 0.0001$). The initial fast drop was followed by a much slower second phase. However, the viral decline remained significantly faster during combination therapy (0.17-0.18 log/week) compared to lamivudine monotherapy (0.08-0.10 log/week) ($p < 0.045$). The viral decline of second phase during IFN monotherapy (0.11-0.13 log/week, $p > 0.10$) did not significantly differ from combination therapy.

The results for combination therapy in patients derived from randomized controlled trials could be confirmed in a subsequent cohort of 5 consecutive patients with chronic HBeAg positive hepatitis B who were treated after closure of entry to the controlled trials. The viral decline pattern was similar to the 8 patients included in the study (median decline of HBV DNA 2.82 log/1st week in the first phase and 0.16 log/week in the second phase (piecewise linear regression)).

The results of the viral dynamics analysis by non-linear regression for the different therapeutic regimens are given in table 3. The effectiveness of a drug in blocking viral replication (ε), calculated from the viral clearance observed during the first phase, was 99% for lamivudine, 99.8% for combination therapy and 83% for IFN; the latter figure is only a rough estimate of the true value due to the absence of first week serum samples. The death rate of the infected cells (δ), calculated from the viral decay curve during the second phase, was higher with combination therapy compared to lamivudine monotherapy (half-life of the infected cell 12.7 versus 26.7 days for combination and lamivudine therapy, respectively). The half-life of infected cells with IFN monotherapy (16.1 days), did not significantly differ from combination therapy.

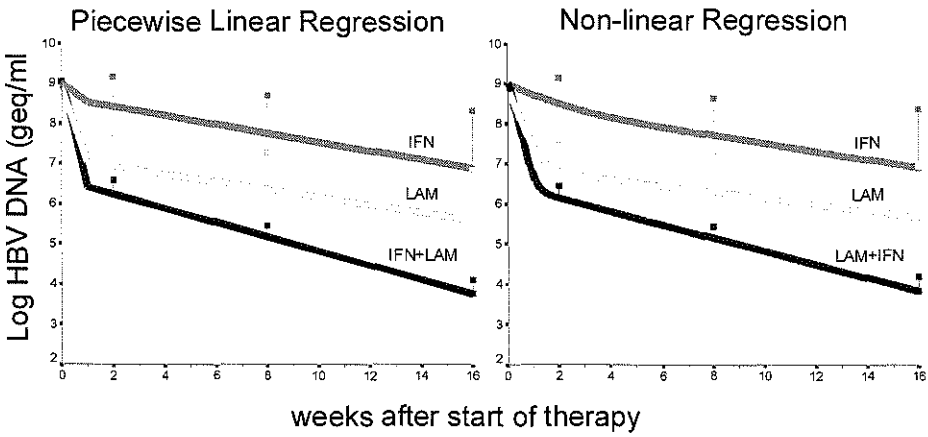


Figure 1 HBV DNA decline curves (population median curve and 75 percentile), calculated by piecewise linear regression and by non-linear regression. Note the marked decrease of HBV DNA of LAM (with or without IFN) in phase 1, and the stronger effect of IFN (with or without LAM) on phase 2. The range of HBV DNA values (75 percentiles) with combination therapy is smaller than with monotherapy with LAM or IFN, reflecting a more uniform pattern of response. These results appear to be independent of the regression analysis used.

Table 2

Viral kinetics: decrease in HBV DNA over time calculated by 2 methods.

		decrease in HBV DNA (log/week)		
model	Time period	LAM (x 95% CI)	IFN (x 95% CI)	LAM + IFN (x 95% CI)
1	Wk 0-1	1.85 (1.58-2.12)†	0.48 (0.18-0.78)†	2.71 (2.28-3.14)
	Wk 1-16	0.10 (0.05-0.14)††	0.11 (0.07-0.16)	0.18 (0.11-0.24)
2	Phase 1	1.53 (1.20-1.86)†	0.41 (0.00-0.87)†	2.65 (1.96-3.28)
	Phase 2	0.08 (0.04-0.12)††	0.13 (0.05-0.15)	0.17 (0.10-0.23)

Results are given as means and 95% confidence intervals.

Model 1: piecewise linear regression. Model fitting information: log likelihood = 1254.0 (AIC=-634.0).

Model 2: non-linear regression. Model fitting information: log likelihood = 1263.4 (AIC=-638.7).

†significantly different from LAM + IFN: $p<0.001$

††significantly different from LAM + IFN: $p<0.05$

Time to viral negativity by therapy group Assuming that the slope of the viral decline of the second phase can be extrapolated, the time to viral negativity was estimated using the results of model 2 (table 3). The mean estimated time for complete elimination of all viral particles from serum (till 1 copy/ml) is 274 days (39 weeks) for combination therapy, which is significantly shorter than that for monotherapy of either drug (Table 3). The probability to reach the detection limit of currently available HBV DNA assays over time using the results of model 1 are shown in figure 2. The combination of lamivudine and IFN markedly decreased the time necessary to reach HBV DNA negativity by either liquid hybridization or PCR (detection limit 400 geq/ml). Furthermore this figure shows that by combining both drugs, HBV DNA negativity by PCR is theoretically obtainable in nearly all patients, whereas for monotherapies the curve tends to level off around 70% of patients.

Kinetic results corrected for baseline ALT: Independently of the therapy given, there was an overall significant effect of baseline ALT on the decline rate of HBV DNA of the second phase ($p_{overall}$= 0.004): a higher ALT was associated with a faster decline in HBV DNA. After correction for baseline ALT the decline of HBV DNA during the first phase remained significantly faster for combination therapy compared to either lamivudine or IFN monotherapy. For the second phase the difference in decline of HBV DNA between combination therapy and lamivudine was 0.10 log/week (95% C.I. 0.03-0.17, p=0.008) and between combination therapy and interferon 0.11 log/week (95% C.I. 0.03-0.19, p=0.006). In contrast; the second phase of lamivudine and IFN therapy showed comparable decline: the overall difference was 0.01 log/week week (95% C.I. -0.04-0.07) after correction for baseline ALT (p=0.73).

Discussion

In this study combination therapy of lamivudine and IFN results in a significant more rapid decline of hepatitis B virus, both in the initial and in the second phase. According to theoretical extrapolation of the viral kinetic data the mean duration of therapy necessary to obtain viral clearance from serum was 39 weeks, whereas it is more than 85 weeks with monotherapy of IFN or lamivudine (table 3). Based on current data, nearly 100% of patients will reach the detection limit of the HBV DNA-PCR assay within 52 weeks, this result will occur in less than 50-60% of patients receiving monotherapy for this period.

In chronic HCV, viral clearance from serum is the generally accepted key outcome measure, in HBV viral negativity by PCR is not an endpoint of antiviral therapy. The two endpoints proposed at a recent NIH workshop (10) are HBV DNA less than 10^5 copies/ml reflecting inactive chronic hepatitis B, and HBsAg-negativity reflecting a sustained response and – in most instances - cure.

Table 3

Viral dynamics: dynamic parameters derived from the non-linear regression analysis for the 3 different therapy groups, LAM, IFN and LAM+IFN.

	phase 1			phase 2		
	Clearance of serum virus		Inhibition of virus production	Loss of Infected cells		Estimated time for viral negativity
	Clearance rate (1/day) c	Virus half-life (days) $T_{½}$	Efficacy (%) ε	Death rate (1/day) δ	Infected cell half-life (days) $T_{½,2}$	(days)
LAM	0.50	1.4	99.0	0.025	26.7	620
	(n.a.)	(n.a.)	(98.9-99.1)	(0.0004-0.062)	(11.2-149.8)	(190-3387)
IFN	0.13	5.6	82.7	0.043	16.1	594
	(n.a.)	(n.a.)	(68.3-97.9)	(0.0004-0.107)	(6.5-179.7)	(192-7549)
LAM+IFN	0.86	0.8	99.8	0.054	12.7	274
	(n.a.)	(n.a.)	(99.7-99.9)	(0.031-0.083)	(8.4-22.6)	(183-321)

Results are expressed as the mean population averages with the 5th – 95th percentile of individual patients between brackets. Note the small variation in δ for LAM + IFN combination therapy

n.a: not applicable since the random effect for C was fixed by necessity in the model.

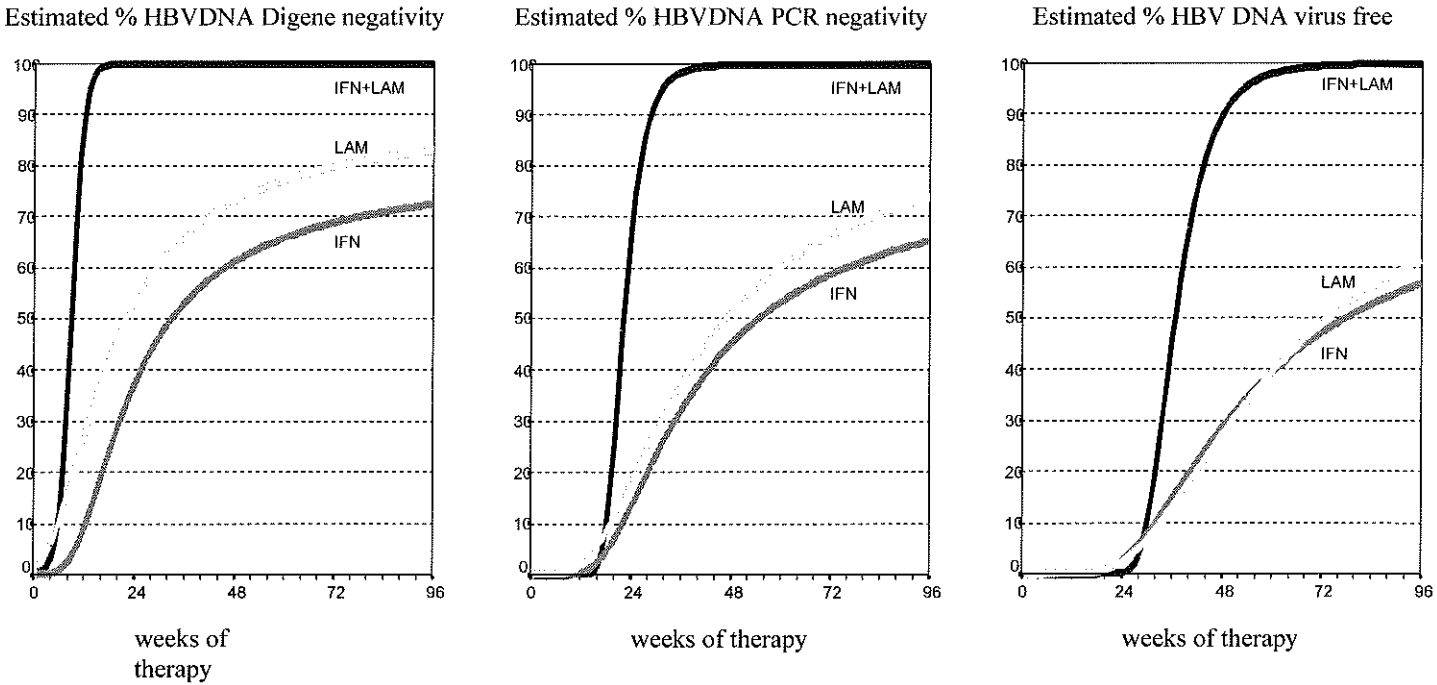


Figure 2 The probability to reach the detection limit of currently available HBV DNA assays as well as complete elimination of virus from serum (1 copy/ml), by therapy group over time using the results of model 1. These results, based on extrapolation of the week 0-16 data, suggest that 1 year of combination therapy produces maximal virus suppression as well as a clinically significant improvement over monotherapy.

Patients reaching a HBsAg negative state also are HBV DNA–PCR negative, but HBV DNA–PCR negativity does not equal HBsAg negativity. In HBV, inactivechronic hepatitis can be the result of treatment in maximally 40% of patients on IFN therapy (11, 12), whereas loss of HBsAg occurs in about 10% over a period of 5 years. We believe that the percentages of responses will increase when virus clearance from serum will also become a goal of treatment; we are aware that this concept needs further proof.

Data on viral kinetics and dynamics are often based on a limited number of patients (4, 5, 13-15). Also in this study the confidence in the results and conclusions is hampered by the small number of patients, especially in the combination therapy group. Selection bias was minimized by either randomly choosing patients from a large group (IFN), or including all patients treated in the trial (lamivudine) or all patients from 2 centers (combination therapy). This approach, however, resulted in significant differences for various baseline factors; we corrected our model calculations successfully for the differences in baseline ALT, the only different factor of proven significance. Although the data for combination therapy were only based on 8 patients, we believe the data are valid since the pattern of response to combination therapy was rather uniform (small range for ε, δ and estimated time for viral elimination, table 3) in contrast to the wide range of responses seen with IFN or lamivudine monotherapy. We had the choice to enlarge the combination group to 13 patients or to test the model in a new cohort of 5 patients. We attributed the largest confidence to confirmation of our findings in another group; in fact, the results for the 8 study patients were similar to those of the new cohort of 5 consecutive patients. Despite the small number of patients studied and the differences in baseline characteristics, we think the results of our analysis can be used as the best available rough estimates; however, further research is warranted to determine more precise values of viral kinetics in chronic hepatitis B.

The major conclusions about the kinetics of HBV DNA decay were independent of the model used. Dynamic parameters can only be calculated with the non-linear regression analysis (5). It has to be taken into account this model is designed for a 4-week treatment period in HCV patients during IFN. We used the model generally accepted for assessment of 4-week IFN treatment in HCV for an extended treatment period of 16 weeks in HBV patients on lamivudine, IFN or combination therapy. For the original model, it was assumed that the major effect of IFN is blocking viral replication rather than blocking *de novo* infection (5). We think this assumptions is also true for IFN and lamivudine in hepatitis B, although blocking de novo infection may play a role in IFN containing therapies. Further developments in mathematical modeling and antiviral therapy however are necessary before the biological effects of antiviral therapy can be described with confidence.

We were hampered by the lack of "week one" samples in the IFN treated group. By piecewise linear regression analysis we could not calculate a breakpoint because of the

paucity of early data points. We therefore choose to set the breakpoint at week one, the same as for the other therapies. Repeating the analysis without a breakpoint for IFN gave similar results (results not shown), and hence did not influence the conclusions. This and other studies (4) using only first samples at 1 week, suggest a transition of the first to second phase within the first week in HBV. Daily sampling during the first week of therapy will result in a higher calculated value of the clearance of the free virus from serum, a decrease of the corresponding half life of the virus, and a shorter duration of the first phase (6).

This study suggests that combination therapy increases the slope of the viral decline from serum, presumably by causing greater inhibition of virus production. Previous studies showed that higher dosing of antiviral therapy only increased the duration of the first phase but did not affect the slope of the viral decline from blood (5). The increased efficacy of combination therapy of famciclovir and lamivudine was also caused by the prolongation of the first phase, combination therapy of these 2 nucleoside analogues did not influence the half life of free virus (15). Future studies, including frequent early sampling, have to elucidate whether the increased effectiveness of lamivudine-IFN combination therapy is due to an increased viral decline curve, a prolonged first phase or a combination of both effects.

Baseline ALT has been identified as a key baseline factor influencing the HBeAg seroconversion rate (1, 11, 16). The importance of pre-treatment ALT as a marker of host immune reactivity also emerged from this study, since a significant positive effect of ALT on the slope of viral decay was observed, independently of the therapy given. It has been shown previously in HCV that the slope of the second phase is positively correlated to baseline ALT levels, reflecting pre-treatment cellular immune responsiveness (5). This supports the hypothesis that the second slope is dependent on the immune-mediated death rate of infected cells (δ), next to the blocking effect of drug on viral replication.

Therefore it is of major importance to correct for baseline ALT levels when comparing the effects of different treatments. After correction for baseline ALT the second phase of the viral decay curve became comparable for the two monotherapies, whereas combination therapy was associated with a significantly faster viral decline in both phases compared to monotherapy. To obtain sustained viral clearance, the death rate of the infected cells is probably the most important factor. The combination of lamivudine and FN reduced the half life of the infected cells compared to lamivudine monotherapy.

We used the kinetics of the second slope of viral decay to predict the estimated time to negativity, showing a strong reduction in time necessary to obtain viral negativity with combination therapy. Since biological mechanisms may change over time, the extrapolation of data for prediction of the duration of therapy has to be interpreted with caution.

Nevertheless, this study shows that the duration of combination therapy in HBV patients has to be prolonged to at least 26-46 weeks, assuming that complete virus suppression is a prerequisite for improved results of antiviral therapy in HBV.

References

1. Schalm SW, Heathcote J, Cianciara J, Farrell G, Sherman M, Willems B, Dhillon A, Moorat A, Barber J, Gray DF. Lamivudine and alpha interferon combination treatment of patients with chronic hepatitis B infection: a randomised trial. Gut. 2000 ;46:562-8.
2. Schiff ER, Karayalcin S, Grimm IS, Perrillo RP, Dienstag JL, Husa P, Man RA de, Goodman Z, Condreay LD, Crowther LM, Woessner MA, McPhilips PJ, Brown NA. Lamivudine and 24 weeks of lamivudine/interferon combination therapy in hepatitis B patients who previously failed interferon. J Infect Dis. Submitted.
3. Mutimer D, Dowling D, Cane P, Ratcliff D, Tang H, O'Donnell K, Shaw J, Elias E, Pillay D. Additive antiviral effects of lamivudine and α-interferon in chronic hepatitis B infection. Antiviral Therapy 2000;5:273-7.
4. Tsiang M, Rooney JF, Toole JJ, Gibbs CS. Biphasic clearance kinetics of hepatitis B virus from patients during adefovir dipivoxil therapy. Hepatology 1999;29:1863-9.
5. Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, Perelson AS. Hepatitis C viral dynamics in vivo and the Antiviral Efficacy Of Interferon-Alpha Therapy. Science 1998;282:103-7.
6. Wolters LMM, Hansen BE, Niesters HGM, Levi-Drummer RS, Neumann AU, Schalm SW, Man de RA. The influence of baseline characteristics on viral dynamic parameters in chronic hepatitis B patients treated with lamivudine. J Hepatol 2002, accepted for publication.
7. Janssen HLA, Gerken G, Carreño V, Marcellin P, Naoumov NV, Craxi A, Ring-Larsen H, Kitis G, Hattum J van, Vries RA de, Michielsen PP, Kate FJW ten, Hop WCJ, Heijtink RA, Honkoop P, Schalm SW. Interferon alfa for chronic hepatitis B infection: increased efficacy of prolonged treatment. Hepatology 1999;30:238-43.
8. Mutimer D, Naoumov N, Honkoop P, Marinos G, Ahmed M, Man RA de, McPhilips P, Johnson M, Williams R, Elias E, Schalm SW. Combination alpha-interferon and lamivudine therapy for alpha-interferon-resistant chronic hepatitis B infection: results of a pilot study. J Hepatol 1998;28:923-9.
9. Heermann KH, Gerlich WH, Chudy M, Schaefer S, Thomssen R. Quantitative detection of hepatitis B virus DNA in two international reference plasma preparations. Eurohep Pathobiology Group. J Clin Microbiol. 1999;37:68-73.
10. Lok A, Heathcote EJ, Hoofnagle JH. Management of hepatitis B: 2000- summary of a workshop Gastroenterology 2001;120:1828-1853.
11. Krogsgaard K, Bindslev N, Christensen E, Craxi A, Schlichting P, Schalm S, Carreno V, Trepo C, Gerken G, Thomas HC, et al. The treatment effect of alpha interferon in chronic hepatitis B is independent of pre-treatment variables. Results based on individual patient data from 10 clinical controlled trials. J Hepatol. 1994;21:646-55.
12. Wong DK, Cheung AM, O'Rourke K, Naylor CD, Detsky AS, Heathcote J. Effect of alpha-interferon treatment in patients with hepatitis B e antigen chronic hepatitis B. A meta-analysis. Ann Intern Med. 1993;119:312-23.
13. Nowak MA, Bonhoeffer S, Hill AM, Boehme R, Thomas HC, McDade H. Viral dynamics in hepatitis B virus infection. P Natl Acad of Sci USA 1996;93:4398-4402.
14. Bekkering FC, Brouwer JT, Hansen BE, Schalm SW. Hepatitis C viral kinetics in difficult to treat patients receiving high dose interferon and ribavirin. J Hepatol 2001;34:435-40.
15. Lau GK, Tsiang M, Hou J, Yuen S, Carman WF, Zhang L, Gibbs CS, Lam S. Combination therapy with lamivudine and famciclovir for chronic hepatitis B-infected Chinese patients: a viral dynamics study. Hepatology 2000;32:394-9.
16. Chien RN, Liaw YF, Atkins M. Pretherapy alanine transaminase level as a determinant for hepatitis B e antigen seroconversion during lamivudine therapy in patients with chronic hepatitis B. Hepatology 1999;30:770-4.

IV.3 DURABILITY OF HBeAg SEROCONVERSION FOLLOWING ANTIVIRAL THERAPY FOR CHRONIC HEPATITIS B: RELATION TO TYPE OF THERAPY AND PRETREATMENT SERUM HBV DNA AND ALT

A. B. van Nunen[1], B. E. Hansen[2], D. J. Suh[3], H. F. Löhr[4], L. Chemello[5], H. Fontaine[6], J. Heathcote[7], B. C. Song[3], H. L.A. Janssen[1], R. A. de Man[1], and S. W.Schalm[1]

Departments of [1]Gastroenterology & Hepatology and [2]Biostatistics, Erasmus University Hospital Rotterdam, The Netherlands;
[3]Department of Internal Medicine, Asan Medical Center, Seoul, Korea;
[4]1st Medical Clinic, Johannes Gutenberg Universität, Mainz, Germany;
[5]Instituto Medicina Clinica, Padova, Italy;
[6]Hepatology Unit, Necker Hospital, Paris, France;
[7]Toronto Western Hospital, Toronto, Canada.

Submitted for publication

Abstract

Background & Aims: Interferon (IFN) induced HBeAg-seroconversion is durable in 80-90% of chronic hepatitis B patients. Preliminary reports on the durability of HBeAg seroconversion following lamivudine are contradictory. We investigated the durability of response following IFN, lamivudine or IFN-lamivudine combination therapy in a meta-analysis of individual patient data.

Patients and Methods: Twenty-four centers included in total 130 patients with an HBeAg seroconversion (HBeAg negative, anti-HBe positive) at the end of antiviral therapy: 59 with lamivudine, 49 with interferon, and 22 with combination therapy. Relapse was defined as confirmed reappearance of HBeAg.

Results: The 3-year cumulative HBeAg relapse rate by the Kaplan-Meier method was 54% for lamivudine, 32% for IFN and 23% for combination therapy (p=0.01). Cox regression analysis identified pre-treatment HBV DNA, ALT, gender and therapy as independent predictive factors of post-treatment relapse; Asian race, previous therapy, center and type of study were not predictive of relapse. The relative HBeAg relapse risk of lamivudine compared to IFN therapy was 4.6 and that of combination therapy to IFN therapy 0.7 ($p_{overall}$=0.01).

Conclusions: The durability of HBeAg seroconversion following lamivudine treatment was significantly lower than that following IFN or IFN-lamivudine combination therapy. The risk of relapse after HBeAg seroconversion was also related to pre-treatment levels of serum ALT and HBV DNA, but independent of Asian race.

Introduction

Loss of HBeAg, either spontaneously or following alpha-interferon (IFN) therapy, significantly improves the clinical outcome and survival in chronic hepatitis B virus (HBV) patients (1-3). Therefore HBeAg loss with seroconversion to antiHBe has remained a major end-point of antiviral therapy in chronic HBV infection.

Monotherapy of 16-26 weeks of IFN is associated with loss of serum HBeAg in 20 to 40% of patients, depending on baseline ALT levels (4). Several long-term follow-up studies have been carried out in IFN responders showing that IFN induced HBeAg seroconversion is sustained in around 80-90% of patients (1-2, 5-10). IFN responders are more likely to clear HBsAg (23-86%) from serum than spontaneous seroconverters during a median follow up of 3.8 to 9 years (1-2, 5-6, 10).

HBeAg-seroconversion is observed in 15-30% of patients at 1-2 years of lamivudine therapy (11-14). The clinical implication of lamivudine induced HBeAg seroconversion is suggested to be comparable to that of seroconversion following IFN therapy (12,15-16). A relapse of HBeAg was observed in 19% (8/42) of patients with a response after lamivudine (16). On the contrary, a single-center cohort study showed a considerable higher percentage of relapse, increasing to 50% (16/32) at 2 years (17).

Combination therapy of 16-26 weeks results in HBeAg seroconversion in 29% of treatment - naive patients, and in 12% of previous IFN non-responders (13, 18). Reports on durability of seroconversion after IFN-lamivudine combination therapy reflect only the first 16 weeks after therapy; similar relapse rates are reported for responders after combination therapy (22%) compared to lamivudine (23%) and IFN (25%) (19).

Large comparative long-term post-treatment studies are lacking. To investigate the post-treatment durability of HBeAg seroconversion following lamivudine, IFN monotherapy or IFN-lamivudine combination therapy, we performed a multicenter meta-analysis of individual patient data. Since such a study does not include randomization*, we have taken strict precautions to minimize differences in outcome due to age, sex, race, previous therapy, baseline ALT and HBVDNA, and treatment center. Multivariate analysis stratified for center was performed to obtain the currently best estimate of the durability of HBeAg seroconversion following the 3 therapies; in addition factors predictive of relapse were identified.

---

* it is impossible to do a randomized study on the posttreatment durability of HBeAg seroconversion since the intervention is prior to reaching the inclusion criterium.

Patients and Methods

Long-term follow-up data of chronic HBV patients with a HBeAg seroconversion after antiviral therapy were collected. We contacted 53 centers that had patients with HBeAg seroconversion in randomized controlled trials, in which we participated ourselves (13, 18, 20-21). In addition 5 centers submitted data from patients treated in cohort studies between 1998-2000. Patients who were HBV DNA and HBeAg positive before start of antiviral therapy were eligible if they seroconverted after receiving lamivudine in a dose of at least 100 mg daily for 24 weeks or more, IFN monotherapy 30 million units per week for at least 16 weeks or IFN-lamivudine combination therapy for at least 16 weeks.

HBeAg seroconversion was defined as loss of HBeAg, appearance of anti-HBe and HBV DNA negativity (hybridization methodology), on 2 occasions at least 1 month apart, at the end of lamivudine monotherapy, or within 12 months of initiation of IFN therapy in case of IFN mono or combination therapy (9). Patients were excluded if at baseline there were co-infections (HCV, HDV or HIV), signs of decompensated cirrhosis (defined as jaundice, variceal bleeding, ascites or encephalopathy) or other causes of liver disease .

Virologic and biochemical measurements

We collected follow-up data on HBeAg, anti-HBe, HBVDNA, HBsAg and ALT, 1 month before the end of therapy, at the end of therapy, 3, 6 and 12 months after the end of therapy and yearly thereafter.

Serum HBeAg, anti-HBe and HBsAg were measured using routine commercially available immunoassays IMx (Abbott, Chicago, IL), Kodak Amerlite (Kodak Clinical & Diagnostics, Amersham, UK) or DiaSorin (Vercelli, Italy). Depending on the center and date of measurement, serum HBV DNA was measured using the solution hybridization assay by Abbott (Chicago, Ill, detection limit $2x10^7$ geq/ml), bDNA-assay (Chiron, Emeryville, CA, limit of detection $7x10^5$ geq/ml) or a hybrid capture tube assay (Digene, Murex, UK, detection limit $1,5x10^6$ geq/ml); all HBV DNA results were converted to Eurohep genome equivalents (22). ALT (IU/l) was expressed as times the upper limit of normal (ULN).

Definition of relapse

Relapse was defined as reappearance of HBeAg in serum, confirmed by HBV DNA positivity in the same sample or by HBeAg positivity in a consecutive sample. Relapse of HBV DNA was defined as reappearance of HBV DNA in serum above the cut-off for the method employed.

Statistics

Results are presented as mean ±standard deviation. The cumulative relapse rate for the total study population was calculated by the Kaplan-Meier method with the log-rank test for statistical comparison between groups. Multivariate analysis was performed by Cox

regression analysis with the likelihood ratio test for statistical significance. To account for a possible center effect the Cox regression was stratified by center; centers with less than 4 patients were clustered in one stratum. Furthermore, interactions between therapy, Asian race, baseline ALT-level, baseline HBV DNA, gender and previous therapy were investigated. Sensitivity analysis was performed excluding one center at a time. The level of significance was set to $p<0.05$.

To determine whether the duration of lamivudine therapy influences relapse rates, a Cox regression analysis stratified for center was performed on the subset of patients on lamivudine (n=59) with 3 categories (24-48 wks, 48-72 wks, and more than 72 wks).

Results

Worldwide 24 centers from 14 countries included 130 patients with chronic hepatitis B who had responded to antiviral therapy with HBeAg seroconversion (in 1 case no data on anti-HBe were available). Baseline characteristics are presented in table 1. Fifty-nine patients had received lamivudine, 49 IFN and 22 combination therapy; seventy-one patients received the treatment as participant in an international RCT. Follow-up till time of relapse or till the last observation, ranged from 4 weeks to 7 years for both HBeAg and HBV DNA. At start of follow-up, groups were comparable regarding sex, age and pre-treatment serum levels of HBV DNA and ALT. There were differences in the distribution of race, previous antiviral therapy, and participation in a RCT between the 3 groups.

Figure 1 shows the percentage of sustained HBeAg seroconversion and HBV DNA negativity for the total study population by Kaplan Meier analysis. A statistically significant higher relapse rate of HBeAg and/or HBVDNA positivity was observed after the end of lamivudine monotherapy compared to IFN monotherapy or combination therapy.

The results of the univariate analysis, shown in table 2, demonstrate that lamivudine therapy, male sex, high pre-treatment HBV DNA and low ALT are potential predictive factors for relapse of HBeAg and HBV DNA in serum. Age, race, previous antiviral therapy, center and study (RCT or cohort study) were not significantly related to the outcome.

Multivariate Cox analysis stratified by center showed that high pre-treatment HBV DNA, low ALT, lamivudine therapy and (male) sex were independent predictive factors of post-treatment relapse (Table 3). There were no significant interactions. Sensitivity analysis excluding each center one at a time gave similar relative risks as the outcome given in table 3. Subgroup analysis for patients with or without previous antiviral therapy (IFN and/or lamivudine) showed equal relapse risks in both groups. In further analysis, race remained included because of the unequal distribution across therapies. Patients with a lamivudine-induced HBeAg seroconversion show a significantly higher risk of relapse compared to compared to IFN therapy is 4.6 (95% CI:1.4-14.5) for lamivudine monotherapy and 0.7 (0.1-

Table 1

Baseline characteristics of chronic HBeAg-positive patients prior to succesfull antiviral therapy, by therapy group

	IFN	LAM	IFN+LAM
	n=49	n=59	n=22
sex, male	36 (73%)	42 (71%)	14 (64%)
age (years)*	41 (± 16)	37 (± 12)	43 (± 15)
ethnicity			
Caucasian, other	39 (80%)	19 (33%)	18 (82%)
Asian	10 (20%)	39 (66%)	3 (14%)
log HBV DNA*†	8.4 (±1.0)	8.7 (± 0.8)	8.5 (± 0.8)
ALT (xULN)*	4.3 (± 2.4)	4.5 (± 4.2)	3.3 (± 2.5)
previous therapy			
IFN	9 (18%)	26 (44%)	8 (36%)
LAM	0 (0%)	4 (7%)	1(5%)
type of study			
RCT‡	46 (94%)	10 (17%)	15 (68%)
cohort	3 (6%)	49 (83%)	7 (32%)

*means ± standard deviation

†corrected for the HBV DNA test used: Abbott or bDNA pg/ml are converted to Eurohep genome equivalents/ml (geq/ml)

‡RCT: randomized controlled trial

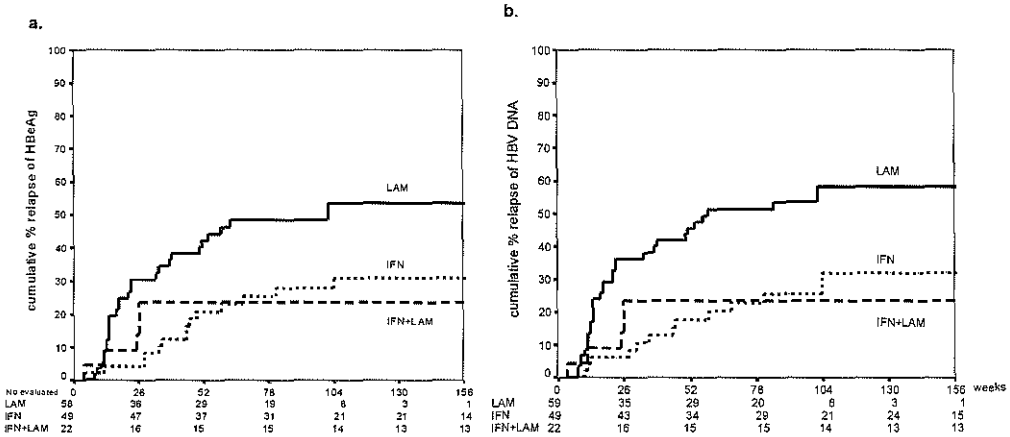


Figure 1 The cumulative percentage of sustained HBeAg seroconversion (a.) and HBV DNA negativity (b.) for the total study population. HBeAg-relapse at 1 year of follow up was observed in 42% of lamivudine, in 21% of IFN and in 23% of combination therapy treated patients, increasing to 54%, 32% and 23% respectively after 3 years ($p_{overall}$=0.01).

Table 2

Univariate analysis (Cox regression) stratified on center.

Variable	HBeAg relapse		HBV DNA relapse	
	relative risk (95% CI)	p-value (log likelihood)	relative risk (95% CI)	p-value (log likelihood)
Therapy		0.0008		0.0005
IFN	1		1	
LAM	7.4 (2.5-22.4)	0.0003	7.1 (2.4-20.1)	0.0002
COMBI	1.3 (0.8-4.5)	0.68	1.1 (0.3-3.8)	0.87
Age (yr)	1.00 (0.98-1.02)	0.87	1.00(0.98-1.03)	0.70
Sex				
Male	1			
Female	0.4 (0.2-0.9)	0.02	0.5 (0.2-1.1)	0.05
Race				
Caucasian	1		1	
Asian	0.8 (0.3-2.3)	0.75	1.0 (0.4-2.6)	0.97
Previous Therapy		0.24		0.05
None	1		1	
IFN	1.3 (0.6-2.6)	0.49	1.4 (0.7-2.7)	0.37
LAM	3.9 (0.8-19.3)	0.11	6.6 (1.4-31.3)	0.02
Study type				
RCT	1		1	
Cohort	1.3 (0.3-5.6)	0.75	1.85 (0.5-6.8)	0.38
HBV DNA (log)	1.51 (1.04-2.21)	0.03	1.50 (1.03-2.19)	0.03
HBV DNA				
HBV DNA $<10^8$	1		1	
HBV DNA $>10^8$	2.2 (1.2-4.2)	0.01	2.1 (1.1-3.8)	0.02
ALT		0.04		0.07
≤2	1		1	
2-5	0.4 (0.2-0.9)	0.03	0.5 (0.2-1.0)	0.04
≥5	0.4 (0.1-0.9)	0.02	0.4 (0.2-1.0)	0.04
Duration of lamivudine therapy		0.28		0.22
24-48 wks	1		1	
48-72 wks	0.4 (0.2-1.2)	0.11	0.4 (0.2-1.1)	0.09
> 72 wks	0.5 (0.1-2.8)	0.45	0.7 (0.2-2.8)	0.57

Table 3

Multivariate analysis (Cox regression stratified on center and correcting for sex, HBV DNA, ALT and race).

Variable	*HBeAg relapse* RR (95% CI)	p-value (log-likelihood)	*HBV DNA relapse* RR (95% CI)	p-value (log-likelihood)
therapy		0.01		0.01
IFN	1		1	
LAM	4.6 (1.4-14.5)	0.01	3.9 (1.3-12.1)	0.02
COMBI	0.7 (0.1-3.9)	0.72	0.6 (0.1-2.9)	0.50
sex				
male	1		1	
female	0.4 (0.2-0.9)	0.04	0.5 (0.2-1.2)	0.12
race				
Caucasian	1		1	
Asian	0.8 (0.3-2.4)	0.74	1.1 (0.4-3.0)	0.84
log HBV DNA	1.61 (1.03-2.52)	0.03	1.7 (1.1-2.6)	0.02
ALT		0.03		0.05
≤ 2	1		1	
2-5	0.4 (0.2-0.9)	0.02	0.4 (0.2-0.9)	0.03
≥ 5	0.3 (0.1-0.8)	0.01	0.4 (0.2-0.9)	0.03

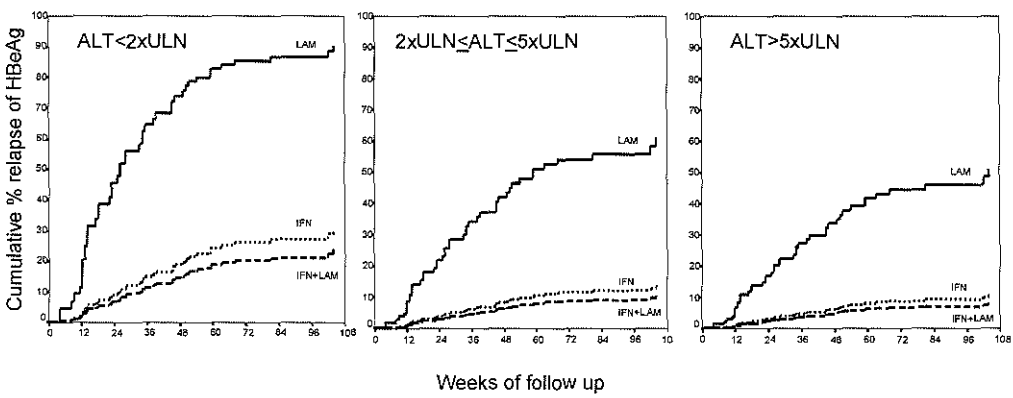


Figure 2 The cumulative percentage of relapse for the three therapies, corrected for the baseline factors pretreatment HBV DNA, ALT, gender and race can be calculated for a specific patient, using the results of the Cox regression (the estimated relative risks and the baseline hazard). In this figure the risk is illustrated for a male Caucasian patient with a mean pre-treatment HBV DNA level ($4x10^6$ geq/ml) after lamivudine, IFN and IFN-lamivudine combination therapy for three different ALT: (a) ALT < 2 x ULN, (b) ALT 2-5 x ULN, (c) ALT > 5 x ULN. Relapse rates are particularly high in patients with baseline ALT < 2 x ULN.

3.9) for combination therapy ($p_{overall}$=0.01).The relative risk of an HBV DNA relapse in serum compared to IFN therapy is 3.9 (95% CI:1.3-12) for lamivudine monotherapy and 0.6 (0.13-2.9) for combination therapy ($p_{overall}$=0.010). The risk of relapse over time for the three therapies corrected for pretreatment HBV DNA, gender and any possible race effect can be calculated for a given patient. Figure 2 illustrates the risk of relapse rate over time for a male Caucasian patient with a mean pre-treatment HBV DNA level ($4x10^8$ geq/ml) for 3 different categories of baseline ALT.

The relative risk of relapse after lamivudine therapy was not significantly influenced by the duration of therapy (p=0.28), but a trend towards a diminished relapse rate with longer treatment was found.

Discussion

The durability of HBeAg seroconversion after lamivudine therapy was reported to be 80-90% in the first studies, performed in Western countries (12,15-16, 19). Recently, Song et al. observed a relapse of viral activity in half of responding patients after withdrawal of lamivudine monotherapy in an Asian cohort study (17). Ethnicity and duration of therapy prior to seroconversion have been suggested to be predictive factors for post-treatment relapse. In this study comparing long-term post-treatment data in 130 responders after lamivudine, IFN and IFN-lamivudine combination therapy, lamivudine induced HBeAg seroconversion was significantly less durable than HBeAg seroconversion following IFN-containing therapies, independent of race and duration of therapy. However, the pretreatment factors high serum HBV DNA and low serum ALT were associated with a higher relapse rate

The US studies comprised a low number of patients with HBeAg seroconversion, 11 and 5 respectively with a follow-up of 4 –12 months. The studies reported by Schiff et al. in two abstracts only included patients who remained HBeAg negative 3 months after the end of therapy, thereby excluding early relapsers (16). We have tried to increase the accuracy of the estimate of the durability of HBeAg seroconversion by including a large number of responders in the study, by prolonging the duration of follow-up to 3 years, and by thorough statistical analysis. We corrected for differences in baseline characteristics by using multivariate analysis. The finding that our results were valid for each center separately should markedly increase the confidence in the results.

However, factors that were no part of the multivariate analysis may still be of relevance. It is possible that patients undergoing relapse are more likely to return to their physician than patients with a sustained response. We collected data from more than 90% of responder patients of the centers that participated, minimizing the likelihood of a selection bias. Furthermore, this potential pitfall would affect all three therapies and should therefore not influence the relative risk.

The HBeAg relapse rate following IFN therapy in this study population (32% in 3 years) may appear high. However, when corrected for mean HBV DNA levels and stratified for ALT category the post-treatment relapse rate following IFN (figure 2) was in accordance with the literature (1-2, 5-10).

Serum HBV DNA and ALT have been identified as predictors of response to antiviral therapy in chronic HBV infection (1, 23). More recently, the degree of ALT elevation was found to be the most powerful predictor for HBeAg seroconversion (13, 24). In this study pre-treatment HBV DNA levels were the major predictors for sustained response. In contrast to Song (17), we also found a significant predictive value of pre-treatment ALT levels for the durability of HBeAg seroconversion (higher baseline ALT – lower chance of relapse).

The differences in relapse following lamivudine and IFN therapy suggest a lack of an efficient immune control following HBeAg seroconversion in lamivudine treated patients. Resolution of acute hepatitis B is associated with a strong humoral and cellular immune response which is often maintained for years by persistence of minute amounts of HBV in liver or serum (25-27). In contrast to acute HBV infections, chronic HBV is generally associated with a weak and ineffective antiviral T-cell response (26, 28). Spontaneous exacerbations and antiviral therapy with IFN can elucidate a significant T-helper cell response preceding HBeAg seroconversion (29-31). Although Boni et al reported a restored HBV specific T-cell response following the strong decline in HBV DNA levels in lamivudine treated patients, HBcAg specific T-cell response remained undetectable during lamivudine therapy in another study (31-32). The differences in relapse after lamivudine and IFN-therapies in our study suggest induction of immune control as an essential element for long-term durability of HBeAg seroconversion.

Acknowledgments

We thank the following investigators who submitted data for this International Meta-Analysis of Individual Patient Data project: G.C. Farrell (Australia), M Adler, P Michielsen, F Nevens (Belgium), B. Willems (Canada), P Husa, J. Svejda (Czech Republic), H Ring-Larsen (Denmark), MJP Arthur, M. Bassendine, PR Mills, T Warnes, (England), P Marcellin (France), GE Kitis (Greece), J. Cianciara (Poland), M Buti, (Spain), G Norkrans (Sweden), BCM Vroom (The Netherlands).

We are grateful to Prof. dr.Th. Stijnen (The Netherlands) for his critical evaluation of the statistical methods used and his assessment of its validity for this study.

References

1. Niederau C, Heintges T, Lange S, *et al.* Long-term follow-up of HBeAg-positive patients treated with interferon alfa for chronic hepatitis B. N Engl J Med 1996;334:1422-7.
2. Lau DT, Everhart J, Kleiner DE, *et al.* Long-term follow-up of patients with chronic hepatitis B treated with interferon alfa. Gastroenterology 1997;113:1660-7.
3. Liaw YF, Tai DI, Chu CM, *et al.* The development of cirrhosis in patients with chronic type B hepatitis: a prospective study. Hepatology 1988;8:493-6.
4. Krogsgaard K, Bindslev N, Christensen E, *et al.* The treatment effect of alpha interferon in chronic hepatitis B is independent of pre-treatment variables. Results based on individual patient data from 10 clinical controlled trials. European Concerted Action on Viral Hepatitis (EUROHEP). J Hepatol 1994;21:646-55.
5. Krogsgaard K. The long-term effect of treatment with interferon-alpha 2a in chronic hepatitis B. The Long-Term Follow-up Investigator Group. The European Study Group on Viral Hepatitis (EUROHEP). Executive Team on Anti-Viral Treatment. J Viral Hepat 1998;5:389-97.
6. Carreno V, Castillo I, Molina J, *et al.* Long-term follow-up of hepatitis B chronic carriers who responded to interferon therapy. J Hepatol 1992;15:102-6.
7. Fattovich G, Giustina G, Realdi G, *et al.* Long-term outcome of hepatitis B e antigen-positive patients with compensated cirrhosis treated with interferon-alpha. European Concerted Action on Viral Hepatitis (EUROHEP). Hepatology 1997;26:1338-42.
8. Lin SM, Sheen IS, Chien RN, Chu CM, *et al.* Long-term beneficial effect of interferon therapy in patients with chronic hepatitis B virus infection therapy. Hepatology 1999;29:971-5.
9. Lok AS, Chung HT, Liu VW, *et al.* Long-term follow-up of chronic hepatitis B patients treated with interferon alfa. Gastroenterology 1993;105:1833-8.
10. Koorenman J, Baker B, Waggoner J, *et al.* Long-term remission of chronic hepatitis B after alpha-interferon therapy. Ann Intern Med 1991;114:629-34.
11. Lai CL, Chien RN, Leung NWY, *et al.* A one-year trial of lamivudine for chronic hepatitis B. N Engl J Med 1998;339:61-8.
12. Dienstag JL, Schiff ER, Wright TL, *et al.* Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med 1999;341:1256-63.
13. Schalm SW, Heathcote J, Cianciara J, *et al.* Lamivudine and alpha interferon combination treatment of patients with chronic hepatitis B infection: a randomised trial. Gut 2000;46:562-8.
14. Liaw YF, Leung NW, Chang TT, *et al.* Effects of extended lamivudine therapy in Asian patients with chronic hepatitis B. Gastroenterology 2000 Jul;119(1):172-80.
15. Dienstag JL, Schiff ER, Mitchell M, *et al.* Extended lamivudine retreatment for chronic hepatitis B: maintenance of viral suppression after discontinuation of therapy. Hepatology 1999;30:1082-7.
16. Schiff ER, Cianciara J, Karayalcin S, *et al* and the International Lamivudine Investigator Group. Durable HBeAg and HBsAg seroconversion after lamivudine for chronic hepatitis B (CHB). J Hepatol 2000(suppl);2:99(A).
17. Song BC, Suh DJ, Lee HC, *et al.* Hepatitis B e antigen seroconversion after lamivudine therapy is not durable in patients with chronic hepatitis B in Korea. Hepatology 2000;32:803-6.
18. Schiff ER, Karayalcin S, Grimm IS, *et al.* Lamivudine and lamivudine/interferon combination therapy in patients with hepatitis B who previously failed interferon: an international controlled trial. J Inf Dis, submitted.
19. Schiff E, Cianciara J, Willems B, *et al.* Durability of HBeAg responses to lamivudine and interferon in the early post-treatment period in adults with chronic hepatitis B. Hepatology 2000(suppl);32:377(A).
20. Janssen HL, Gerken G, Carreno V, *et al.* Interferon alfa for chronic hepatitis B infection: increased efficacy of prolonged treatment. The European Concerted Action on Viral Hepatitis (EUROHEP). Hepatology 1999;30:238-43.
21. Mutimer D, Naoumov N, Honkoop P, *et al.* Combination alpha-interferon and lamivudine therapy for alpha-interferon-resistant chronic hepatitis B infection: results of a pilot study. J Hepatol 1998;28:923-9.
22. Heermann KH, Gerlich WH, Chudy M, *et al.* Quantitative detection of hepatitis B virus DNA in two international reference plasma preparations. Eurohep Pathobiology Group. J Clin Microbiol 1999;37:68-73.
23. Brook MG, Karayiannis P, Thomas HC. Which patients with chronic hepatitis B virus infection will respond to alpha-interferon therapy? A statistical analysis of predictive factors. Hepatology 1989;10:761-3.

24. Chien RN, Liaw YF, Atkins M. Pretherapy alanine transaminase level as a determinant for hepatitis B e antigen seroconversion during lamivudine therapy in patients with chronic hepatitis B. Asian Hepatitis Lamivudine Trial Group. Hepatology 1999;30:770-4.
25. Penna A, Del Prete G, Cavalli A, *et al.* Predominant T-helper 1 cytokine profile of hepatitis B virus nucleocapside-specific T cells in acute self-limited hepatitis B. Hepatology 1997;25:1022-7.
26. Lohr HF, Krug S, Herr W, *et al.* Quantitative and functional analysis of core-specific T-helper cell and CTL activities in acute and chronic hepatitis B. Liver 1998;18:405-413.
27. Rehermann B, Ferrari C, Pasquinelli C, *et al.* The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response. Nat Med. 1996;2:1104-8.
28. Ferrari C, Penna A, Bertoletti A, *et al.* Cellular immune response to hepatitis B virus-encoded antigens in acute and chronic hepatitis B infection. J Immunol 1990;145:3442-9.
29. Tsai SL, Chen PJ, Lai PM, *et al.* Acute exacerbations of chronic type B hepatitis are accompanied by increased T cell responses to hepatitis B core and e antigens. J Clin Invest 1992;89:87-96.
30. Jung MC, Diepolder HM, Spengler U, *et al.* Activation of a heterogenous hepatitis B (HB) core and e antigen specific CD4+ T-cell population during seroconversion to anti-HBe and anti-HBs in hepatitis B virus infection. J Virol 1995;69:3358-68.
31. Marinos G, Torre F, Chokshi S, *et al.* Induction of T-helper cell response to hepatitis B core antigen in chronic hepatitis B: a major factor in activation of the host immune response to the hepatitis B virus. Hepatology 1995;22:1040-9.
32. Boni C, Bertoletti A, Penna A, *et al.* Lamivudine treatment can restore T cell responsiveness in chronic hepatitis B. J Clin Invest 1998;102:968-75.

IV.4 IS COMBINATION THERAPY WITH LAMIVUDINE AND INTERFERON-ALPHA SUPERIOR TO MONOTHERAPY WITH EITHER DRUG?

A.B. van Nunen[1], H.L.A. Janssen[1], L.M.M. Wolters[1], H.G.M. Niesters[2], R.A. de Man[1], S.W. Schalm[1]

Departments of [1]Hepatogastroenterology and [2]Virology, University Hospital Rotterdam, The Netherlands

Antiviral Research 2001;52:139-46.

Abstract

For the treatment of chronic hepatitis B (CHB) two drugs have been licensed world-wide: interferon-alpha (IFN) and lamivudine. Both drugs significantly increase the hepatitis B e-antigen (HBeAg) seroconversion rate, but a sustained response occurs in less than 40% of patients.

To explore whether there is an additional benefit of combining these two drugs, we reviewed the literature on lamivudine-IFN combination therapy in comparison to the two monotherapies in compensated, HBeAg positive, CHB patients. We focussed on two clinically relevant outcome measures: HBeAg seroconversion and change in liver histology. Candidates for lamivudine-IFN combination therapy were, previously untreated, patients with moderately elevated alanine aminotransferase (ALT). Such regimen should still be considered experimental.

Viral kinetics may provide insight into how long therapy should be continued; prolongation of therapy to 52 weeks currently appears a reasonable approach. According to principles of anti-viral therapy today, simultaneously dosing of both drugs is to be preferred, since rapid maximal virus suppression is thought to be essential to prevent drug resistance and enhance seroconversion. From an immunological point of view, pre-treatment with lamivudine or IFN may alter the virus-host balance and set the stage for the other drug to enhance the effect of treatment. Further clinical research on lamivudine-IFN combination therapy appears warranted.

Introduction

For the treatment of chronic hepatitis B (CHB) two drugs have been licensed world-wide: interferon-alpha (IFN) and lamivudine. Both drugs have been investigated extensively as monotherapy; they significantly increase the HBeAg seroconversion rate, but a sustained treatment response occurs in less than 40% of patients. The question arises whether there is an additional benefit of combining these two drugs. In this paper, we review the literature on lamivudine-IFN combination therapy in comparison to the two monotherapies in compensated, HBeAg-positive, CHB patients. We focussed on two clinically relevant outcome measures: HBeAg seroconversion, and change in liver histology.

1. Selection of clinical studies for review

Pre-treatment patient characteristics, especially levels of alanine aminotransferase (ALT), have a major impact on the treatment outcome (1-3). Therefore, different trials should only be compared after correction of baseline values. We aimed at including all published large* multicenter randomized trials in treatment-naive, HBeAg-positive, compensated, immunocompetent, CHB patients with standard lamivudine therapy (≥100 mg daily for 52 weeks), standard IFN (10 MU three times a week (tiw) or 5 MU daily for 16-26 weeks) monotherapy and combination therapy of both drugs. In addition to five eligible studies (1-6) we have included large international meta-analyses on IFN (7-9); meta-analyses on lamivudine or combination therapy are lacking. Furthermore, all other publications on lamivudine-IFN combination therapy were assessed.

2. Hepatitis B e-antigen seroconversion

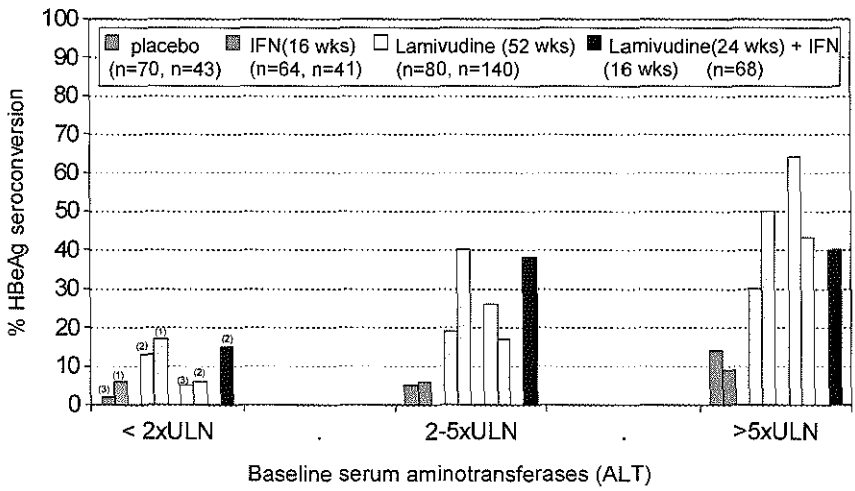
2.1 Standard Therapy

The HBeAg response in relation to baseline ALT is illustrated in figure 1.

A large Eurohep meta-analysis of individual patient data, published by Krogsgaard et al (7) in 1994, showed that the response to IFN therapy is dependent on baseline patient-characteristics. Often, IFN therapy is said to be associated with loss of HBeAg in 30-40% of patients. However, IFN increases the spontaneous response rate, which varies between 5 and 20%, with a factor 2 resulting in response in 15-40% of patients (1-3). Although the relative response is similar for all patients, those with elevated pre-treatment transaminases and low baseline HBV DNA levels have the largest absolute benefit from IFN therapy (7, 9-11).

* more than 40 patients in a treatment arm

Figure 1 Overview of treatment results in IFN naive, HBeAg positive chronic hepatitis B patients, by treatment modality; relation between baseline ALT and response.



After one year of lamivudine monotherapy, HBeAg seroconversion occurs in maximal 20% of patients (2, 4-5). Elevated baseline ALT is also the strongest predictor of lamivudine induced HBeAg seroconversion: multivariate analysis showed that both ALT and histologic activity index (HAI) were highly significant correlated with HBeAg seroconversion, but the contribution of HBV DNA to the predicted response was negligible (12). Lamivudine increases the response rate with a factor less than 2 in case of ALT < 2 times the upper limit of normal (ULN), but this factor rises to 4 in case of ALT > 5 x ULN (2-3).

The efficacy of lamivudine-IFN combination therapy given for 16 weeks after 8 weeks of lamivudine pre-treatment, was compared to standard IFN (10 MU tiw for 16 weeks) and lamivudine (100 mg qd for 52 weeks) monotherapy in 230 treatment-naive HBeAg-positive patients. Combination therapy resulted in a HBeAg seroconversion rate of 29% in the intention-to-treat analysis. This outcome was not significantly higher than after IFN (19%) or lamivudine (18%) monotherapy. However a per-protocol analysis, excluding all patients who did not meet the inclusion criteria at baseline or receive the assigned treatment, revealed a significant higher seroconversion rate in the combination group. Thirty-six percent of patients receiving combination therapy responded versus 19% and 22% in the lamivudine and IFN monotherapy groups, respectively (2).

In this study, a subgroup analysis based on pre-treatment ALT was performed, showing a 2-6-fold increase in the seroconversion rate in patients receiving combination therapy over that expected spontaneously. The absolute increase in the response was most pronounced in the group with moderately elevated ALT levels (2-5 x ULN). For patients with moderately elevated

pre-treatment serum transaminases, combination therapy may have a benefit over monotherapy.

2.2 Prolongation of therapy

Prolongation of both IFN and lamivudine leads to increased HBeAg seroconversion rates. Prolongation of IFN therapy from the standard 16 to 32 weeks enhanced clearance of HBeAg and HBV DNA in patients with HBV DNA levels below 10 pg/ml (Abbott assay) at 16 weeks of therapy (6).

Extension of lamivudine therapy from the standard 1 year to 2 years was associated with a modest increase in response to 21-27% (13-15). In an Asian study, continuation for 4 years in 58 patients showed an increased HBeAg seroconversion rate of 47% (16). However, the definition of HBeAg seroconversion was altered in this study concentrating on HBeAg negativity independently of HBV DNA negativity. In nearly half of these responders a YMDD variant was detectable (16). The emergence of lamivudine resistant strains is a major problem of long-term therapy; it has been reported in up to 60% of Caucasians at 2 years of therapy and 40-55% of Asian patients at 2-3 years (14-15, 17).

Prolonged combination therapy of IFN and lamivudine has not been investigated yet, with the exception of some cases. Figure 2 illustrates a case of a young woman receiving lamivudine 150 mg daily for 1 year simultaneously with IFN 10 MU tiw till week 32, followed by 5 MU tiw up till 1 year. This patient seroconverted and lost hepatitis B surface antigen (HBsAg). The response was sustained after withdrawal of therapy during the follow-up of 1 year.

2.3 Durability of response

HBeAg seroconversion, either spontaneously or following IFN therapy, significantly reduces morbidity and mortality (11, 18-19).

Several long-term follow-up studies have been performed to establish the durability of IFN induced HBeAg seroconversion and the effect of the durability of the response on disease progression and survival (8, 11, 18, 20-24). Responders to IFN therapy have a significantly lower risk to develop cirrhosis (22). In addition IFN has been identified as an independent factor related to a decreased risk for HCC development (22). Survival is found to be improved in 5 out of 6 studies assessing the impact of HBeAg seroconversion (8, 11, 18, 20-22). In cirrhotic patients IFN induced viral clearance improves survival, although Cox regression revealed ALT as the most important predictor of improved survival in these patients (21-22). IFN induced HBeAg seroconversion is sustained in around 90% of patients (Fig 2) (8, 11, 18, 20, 22-24).

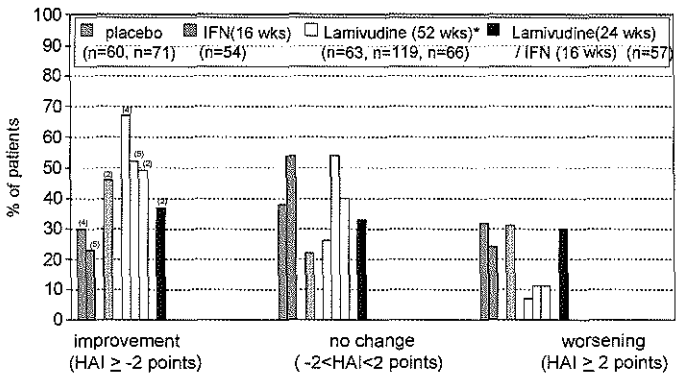
Lamivudine induced HBeAg seroconversion is suggested to be comparable to HBeAg seroconversion after IFN therapy (13, 16, 25). Forty-three lamivudine responders (defined as

HBeAg negative and detectable anti-HBe, or HBeAg and HBV DNA negative (bDNA) patients) were followed for a median of 21 months after cessation of therapy; only 19% (8/42) relapsed (25). This encouraging report, however, needs confirmation, since the patient population was highly selected from several lamivudine trials, by including only patients who remained responders for at least 3 months off lamivudine or who had seroconversion maintained on at least 2 occasions off therapy over time. In contrast to these reports, single-center cohort studies show a relapse percentage that increases with time of follow-up (26-27). One study comprising 34 responders (HBeAg negative and anti-HBe positive) to lamivudine therapy reported a cumulative relapse percentage up to 49% at 2 years of follow-up (26). Figure 3 shows the durability of HBeAg seroconversion during long-term follow-up after withdrawal of lamivudine or IFN therapy.

No long-term follow-up data after combination therapy induced HBeAg seroconversion are available.

3. Improvement of liver histology

Figure 4 illustrates effect of various therapies on the necro-inflammatory component of the Knodell scoring system (0-18 points). Histological improvement is defined as more than 2 points reduction of the necro-inflammatory score. An important factor, hampering the comparison of histological results of lamivudine with other therapies in several larger studies, is the fact that all biopsies were taken at the end of lamivudine monotherapy but 6 months after withdrawal of IFN or combination therapy.



* measured at the end of 52 weeks of therapy

Figure 4 Histologic changes after standard monotherapy with IFN (16 weeks) or lamivudine (12 months), or combination therapy with both drugs (lamivudine for 24 weeks combined with IFN for 16 weeks) evaluated by the Knodell necro-inflammatory scoring system excluding the fibrosis component (0-18 points). Improvement was defined as a reduction of at least 2 points, worsening as an increase of at least 2 points. Biopsies are taken at the end of lamivudine therapy in contrast to the end of follow up for the other therapy groups. Improvement during lamivudine therapy is clearly illustrated; sustained improvement can only be assessed for IFN and lamivudine-IFN and appears small at 6 months after therapy.

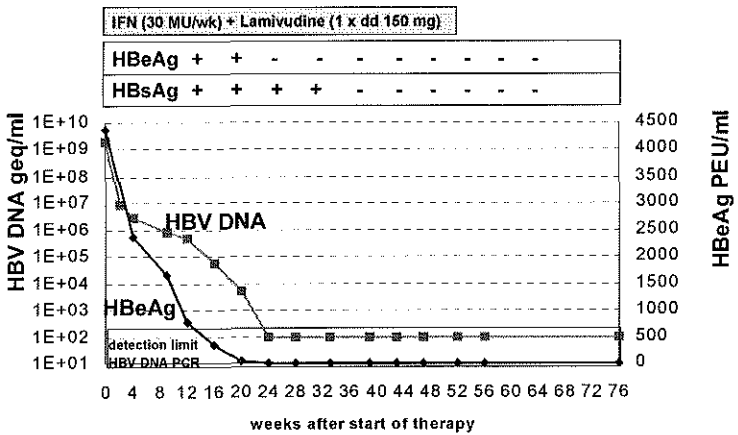


Figure 2 Prolonged combination therapy of lamivudine and IFN for 52 weeks in an individual Caucasian woman of 23 years. Note the complete response occurring during therapy.

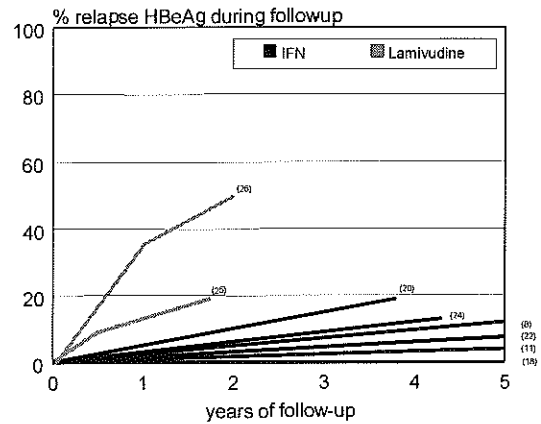


Figure 3 Durability of HBeAg seroconversion after withdrawal of monotherapy with IFN or lamivudine during long-term follow up. In case the follow up after withdrawal of IFN therapy was beyond 5 years, the line was cut off at 5 years.

Lamivudine significantly reduced the HAI score and prevented progression of fibrosis in HBeAg positive patients at 1 year of therapy (2, 4-5, 28-29). Results following withdrawal of lamivudine therapy are not available. Lamivudine shows histological benefit in about 50% of the patients, whereas IFN therapy showed improvement in 45% and combination therapy in 38% of patients. The difference between active treatments appears more clear when disease progression is taken as the outcome measure: the number of patients with worsening of histology during lamivudine is minimal (7-11%), whereas IFN, combination therapy and placebo were associated with worsening of histology in about one-third of patients.

The differences between lamivudine and IFN can be partially explained by the fact that these are overall results, including responders as well as non-responders to therapy. In IFN and combination therapy, histological improvement is dependent on a virological response (2, 4, 30).

4. Safety

Combination therapy with IFN and lamivudine up to 26 weeks is generally well tolerated (2, 31-33). Combining both drugs does not change the safety profile; side effects are mainly IFN related (2, 33). IFN has a wide range of dose-depending side effects, which requires dose reduction in 20-40% of the patients and discontinuation in about 5% of the patients (1-2, 6,9). No pharmacokinetic interactions, that might affect the efficacy of both drugs, were observed during co-administration (32). Stopping lamivudine, also as part of combination therapy, is associated with a slightly increased risk of post treatment flares in around 20% of patients (2, 33-34).

5. Other experiences with combination therapy in chronic hepatitis B

The enhanced seroconversion rate for combination therapy, as reported by Schalm et al (2), could not be confirmed in a study of 238 HBeAg positive, previous IFN non-responders (33). Compared to the study in treatment-naive patients a similar trial design was used; only the arm with IFN-monotherapy was replaced by placebo. At week 52, 12% of patients receiving combination therapy seroconverted for HBeAg, which was comparable to placebo (13%) but lower than lamivudine (18%) therapy.

The reason for the lack of response to lamivudine-IFN therapy most likely relates to patient characteristics. A subgroup analysis based on pre-treatment ALT is lacking, but median baseline ALT and HBV DNA levels were comparable in both studies. In general, host immunity was not overall low, in view of the response rate in the placebo and lamivudine groups. It is, however, conceivable that previous IFN non-responders have an immune status that makes it very difficult to respond to 16 weeks of IFN, even in the presence of lamivudine therapy.

Another study in 20 previous IFN non-responders, receiving combination therapy, showed a response in 20% of patients (32). This response was sustained in only 1 out of 4 patients. In a third study in previous non-responders, 6 months of lamivudine followed by 6 months of IFN led to a sustained response in 45% of patients, but this needs further investigation (35). Finally, a study of 26 weeks of combination therapy in HBeAg-negative patients resulted in HBV DNA PCR negativity in 95%; however, withdrawal of combination therapy for this time period often led to a relapse of viral activity (31).

Conclusions and implications for future research

The relatively low sustained response rates with either IFN or lamivudine monotherapy, the possibility of relapse of viral activity after cessation of lamivudine monotherapy and the time-dependent emergence of viral resistance to lamivudine emphasize the need for combination therapy.

In previously untreated patients, those with moderately elevated ALT are candidates for lamivudine-IFN combination therapy. Such regimen should still be considered experimental. Viral kinetics may provide insight how long therapy should be continued; prolongation of therapy to 52 weeks currently appears a reasonable approach. According to principles of antiviral therapy today, simultaneously dosing of both drugs is to be preferred, since rapid maximal virus suppression is thought to be essential to prevent drug resistance and enhance seroconversion. From an immunological point of view, pretreatment with lamivudine or IFN may alter the virus-host balance and set the stage for the other drug to enhance the effect of treatment. Further clinical research on lamivudine-interferon combination therapy appears warranted.

References

1. Perrillo RP, Schiff ER, Davis GL, Bodenheimer HC Jr, Lindsay K, Payne J, Dienstag JL, O'Brien C, Tamburro C, Jacobson IM, et al. A randomized, controlled trial of interferon alfa-2b alone and after prednisone withdrawal for the treatment of chronic hepatitis B. The Hepatitis Interventional Therapy Group. N Engl J Med 1990;323:295-301.
2. Schalm SW, Heathcote J, Cianciara J, Farrell G, Sherman M, Willems B, Dhillon A, Moorat A, Barber J, Gray DF. Lamivudine and alpha interferon combination treatment of patients with chronic hepatitis B infection: a randomised trial. Gut 2000;46:562-8.
3. Chien RN, Liaw YF, Atkins M. Pretherapy alanine transaminase level as a determinant for hepatitis B e antigen seroconversion during lamivudine therapy in patients with chronic hepatitis B. Asian Hepatitis Lamivudine Trial Group. Hepatology 1999;30:770-4.
4. Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray DF. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med 1998;339:61-8.
5. Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HWL, Goodman Z, Crowther L. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med 1999;341:1256-63.
6. Janssen HL, Gerken G, Carreno V, Marcellin P, Naoumov NV, Craxi A, Ring-Larsen H, Kitis G, van Hattum J, de Vries RA, Michielsen PP, Ten Kate FJW, Hop WCJ, Heijtink RA, Honkoop P, Schalm SW. Interferon alfa for chronic hepatitis B infection: increased efficacy of prolonged treatment. The European Concerted Action on Viral Hepatitis (EUROHEP). Hepatology 1999;30:238-43.
7. Krogsgaard K, Bindslev N, Christensen E, Craxi A, Schlichting P, Schalm S, Carreno V, Trepo C, Gerken G, Thomas HC, Kragh Andersen P, Ring-Larsen H. The treatment effect of alpha interferon in chronic hepatitis B is independent of pre-treatment variables. Results based on individual patient data from 10 clinical controlled trials. European Concerted Action on Viral Hepatitis (EUROHEP). J Hepatol 1994;21:646-55.
8. Krogsgaard K. The long-term effect of treatment with interferon-alpha 2a in chronic hepatitis B. The Long-Term Follow-up Investigator Group. The European Study Group on Viral Hepatitis (EUROHEP). Executive Team on Anti-Viral Treatment. J Viral Hepat 1998;5:389-97.
9. Wong DK, Cheung AM, O'Rourke K, Naylor CD, Detsky AS, Heathcote J. Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B. A meta-analysis. Ann Intern Med1993;119:312-23.
10. Brook MG, Karayiannis P, Thomas HC. Which patients with chronic hepatitis B virus infection will respond to alpha-interferon therapy? A statistical analysis of predictive factors. Hepatology 1989;10:761-3.
11. Lau DT, Everhart J, Kleiner DE, Park Y, Vergalla J, Schmid P, Hoofnagle JH. Long-term follow-up of patients with chronic hepatitis B treated with interferon alfa. Gastroenterology 1997;113:1660-7.
12. Perrillo RP, Schalm SW, Schiff ER, Brown NA, Woessner MA, Sullivan M. Predictors of HBeAg seroconversion in chronic hepatitis B patients treated with lamivudine. Hepatology 1999;30:317A.
13. Dienstag JL, Schiff ER, Mitchell M, Casey DE, Gitlin N, Lissoos T, Gelb LD, Condreay L, Crowther L, Rubin M, Brown N. Extended lamivudine retreatment for chronic hepatitis B: maintenance of viral suppression after discontinuation of therapy. Hepatology 1999;30:1082-7.
14. Buti M, Jardi R, Cotrina M, Rodriguez-Frias F, Cruz de Castro E, Costa X, Esteban R, Guardia J. Two years of lamivudine therapy in patients with chronic hepatitis B. Analysis of efficacy and emergence of HBV genomic variations. J Hepatol 2000;2:111A.
15. Liaw YF, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Chien RN, Dent J, Roman L, Edmundson S, Lai CL for the Asia Hepatitis Lamivudine Study Group. Effects of extended lamivudine therapy in Asian patients with chronic hepatitis B. Gastroenterology 2000;119:172-80.
16. Chang TT, Lai CL, Liaw YF, Guan R, Lim SG, Lee CM, Ng KY, Leung NWY, Nicholls GJ, Pearce MA. Incremental increases in HBeAg seroconversion and continued ALT normalization in Asian chronic HBV (CHB) patients treated with lamivudine for four years. International Symposium on Viral Hepatitis and Liver Disease, April 2000, Atlanta.
17. Leung NW, Lai CL, Chang TT, Guan R, Lee CM, Ng KY, Wu PC, Dent JC, Edmundson S, Liaw YF. Three year lamivudine therapy in chronic HBV. J Hepatol 1999;30:59A.
18. Niederau C, Heintges T, Lange S, Goldmann G, Niederau CM, Mohr L, Haussinger D. Long-term follow-up of HBeAg-positive patients treated with interferon alfa for chronic hepatitis B. N Engl J Med 1996;334:1422-7.
19. Liaw YF, Tai DI, Chu CM, Chen TJ. The development of cirrhosis in patients with chronic type B hepatitis: a prospective study. Hepatology 1988;8:493-6.

20. Carreno V, Castillo I, Molina J, Porres JC, Bartolome J. Long-term follow-up of hepatitis B chronic carriers who responded to interferon therapy. J Hepatol 1992;15:102-6.
21. Fattovich G, Giustina G, Realdi G, Corrocher R, Schalm SW. Long-term outcome of hepatitis B e antigen-positive patients with compensated cirrhosis treated with interferon-alpha. European Concerted Action on Viral Hepatitis (EUROHEP). Hepatology 1997;26:1338-42.
22. Lin SM, Sheen IS, Chien RN, Chu CM, Liaw YF. Long-term beneficial effect of interferon therapy in patients with chronic hepatitis B virus infection therapy. Hepatology 1999;29:971-5.
23. Lok AS, Chung HT, Liu VW, Ma OC. Long-term follow-up of chronic hepatitis B patients treated with interferon alfa. Gastroenterology 1993;105:1833-8.
24. Koorenman J, Baker B, Waggoner J, Everhart JE, Bisceglie AM, Hoofnagle JH. Long-term remission of chronic hepatitis B after alpha-interferon therapy. Ann Intern Med 1991;114:629-34.
25. Schiff E, Cianciara J, Karayalcin S, Kowdley K, Woessner M, McMullen S, Pearce M, Brown N. Durable HBeAg and HBsAg seroconversion after lamivudine for chronic hepatitis B (CHB). J Hepatol 2000;2:99A.
26. Song BC, Suh DJ, Lee HC, Chung YH, Lee YS. Seroconversion after lamivudine treatment is not durable in chronic hepatitis B. Hepatology 1999;30:348A.
27. Fontaine H, Driss F, Lagneau JL, Zylerberg H, Brechot C, Pol S, Necker CHU. Hepatitis B virus reactivation after lamivudine discontinuation. Hepatology 1999;30:349A.
28. Suzuki Y, Kumada H, Ikeda K, Chayama K, Arase Y, Saitoh S, Tsubota A, Kobayashi M, Koike M, Ogawa N, Tanikawa K. Histological changes in liver biopsies after one year of lamivudine treatment in patients with chronic hepatitis B infection. J Hepatol 1999;30:743-8.
29. Goodman Z, Dhillon AP, Wu PC, Gray F, Atkins M, Stevenson C, Barber J, Brown N, Crowther L, Woessner M. Lamivudine treatment reduces progression to cirrhosis in patients with chronic hepatitis B. J Hepatol 1999;30:59A.
30. Brook MG, Petrovic L, McDonald JA, Scheuer PJ, Thomas HC. Histological improvement after anti-viral treatment for chronic hepatitis B virus infection. J Hepatol 1989;8:218-25.
31. Alexopoulou A, Zafiropoulou R, Papakonstantinou A, Hadziyannis SJ. Randomised trial in HBeAg negative patients with replicating virus with ganciclovir vs lamivudine in combination with interferon. Evaluation of long-term efficacy. Hepatology 1998;28:490A.
32. Mutimer D, Naoumov N, Honkoop P, Marinos G, Ahmed M, de Man RA, McPhillips P, Johnson M, Williams R, Elias E, Schalm SW. Combination alpha-interferon and lamivudine therapy for alpha-interferon-resistant chronic hepatitis B infection: results of a pilot study. J Hepatol 1998;28:923-9.
33. Schiff ER, Karayalcin S, Grimm IS, Perillo RP, Dienstag JL, Husa P, de Man RA, Goodman Z, Condreay LD, Crowther LM, Woessner MA, McPhillips PJ, Brown NA and an International Lamivudine Investigator Group. Lamivudine and lamivudine/interferon combination therapy in patients with hepatitis B who previously failed interferon: an international controlled trial. Ann Int Med 2002, accepted for publication.
34. Honkoop P, de Man RA, Heijtink RA, Schalm SW. Hepatitis B reactivation after lamivudine. Lancet 1995;346:1156-7.
35. Thabut D, Serfaty L, Andreani T, Fontaine H, Chazouilleres O, Pol S, Poupon R. Sequential treatment with lamivudine and interferon in patients with chronic hepatitis B non responders to interferon alone. J Hepatol 2000;2:103A.

Chapter V

Discussion

V. DISCUSSION AND FUTURE IMMUNE MODULATORY STRATEGIES IN THE TREATMENT OF CHRONIC HEPATITIS B

Natural history of chronic hepatitis B

Worldwide over 350 million people are infected with the hepatitis B virus (HBV), which is the major cause of liver related deaths. Of all chronically infected HBV patients, between 10-30% have active chronic liver disease (1-2).

Inactive chronic HBV carriers (immune *control* phase, table 1) have an uneventful long-term prognosis; cirrhosis is not likely to develop, the yearly incidence of HCC is 0,1% and their 5-year survival rate is comparable to the normal population (97%) (3-6). Although low levels of viral replication may persist, there is no indication for antiviral therapy (7-8).

Active HBV on the other hand is often a progressive disease leading to liver cirrhosis and hepatocellular carcinoma (HCC) after 10 to 30 years. The progression rate of chronic liver disease to cirrhosis is 2-4% per year and eventually 20-35% will develop cirrhosis (5, 9-11). The annual HCC rate is 1%, increasing to 2-10% in cirrhotics (5, 11). The 5-year survival rate is decreased to 86%, which may decrease further to less than 35% after liver decompensation (4, 12-13).

The loss of active HBV replication, reflected by loss of HBeAg from serum and normalization of serum transaminases, is an important factor significantly improving morbidity and mortality in cirrhotic as well as non-cirrhotic patients (14-17). Moreover one study identifies biochemical disease remission as the best positive predictor of improved survival in compensated cirrhosis (18).

Obviously the primary goal of antiviral therapy in chronic HBV is sustained inactivation or elimination of HBV and biochemical disease activity. Therapy is targeted to those patients with active HBV, HBeAg positivity in combination with high HBV DNA and ALT levels (immune *elimination* phase) or in those infected with a precore mutant (immune *escape* phase), high HBV DNA and ALT levels. The benefit of antiviral therapy in immune tolerant patients is unclear.

Table 1

Selection of chronic HBV patients for antiviral therapy based on virological and biochemical parameters.

Phase of Immune activity:		*Tolerance*	*Elimination*	*Control or Residual*	*Escape*
Virological	HBeAg	+	+	–	–
	HBV DNA	10^9 geq/ml	10^7-10^9 geq/ml	<10^5 geq/ml	>10^5 geq/ml
Biochemical	ALT	<ULN	↑	<ULN	↑
	ALT>5xULN*	–	+	–	+
Prevalence		10%	10%	60%	10%
Advice on antiviral Rx		(+)	+	–	+

* Hepatitis flare followed by HBeAg seroconversion in 25%

Table 1 illustrates the selection of HBV patients for antiviral therapy based on virological and biochemical parameters. In this thesis we focussed on how to improve standard antiviral therapy. In addition we tried to identify patients who benefit most from therapy.

The basis of antiviral therapy in HBV

The first agent licensed for the treatment of HBV was interferon-alpha (IFN). IFN is a natural glycoprotein with direct antiviral and immunostimulating properties (see chapter 1).
Sustained IFN responders have a significantly improved morbidity and mortality (11, 14-15, 18, 19). However, IFN monotherapy induces HBeAg seroconversion in maximal 20% of patients with low baseline ALT levels to 40% of patients with activated baseline host immunity (20). The biochemical remission parallels the virological response in 94% of patients (21). Response is sustained in approximately 90% of patients (14-15, 18-19, 21-24).
Patients with high HBV DNA levels and low ALT levels before start of interferon therapy are unlikely to respond. Immune tolerance is probably induced by an abundance of viral particles and proteins creating an unfavorable position for interferon to restore the T-cell hyporesponsiveness characteristic for chronic HBV.

Since the end of 1999 lamivudine has been registered for the treatment of chronic HBV. Lamivudine is a cytidine dideoxynucleoside analogue that acts as a chain terminator after incorporation in the viral DNA and is a strong virus suppressive agent.
Hypothetically maximal virus suppression by lamivudine therapy could enable the host immune response to restore and eliminate the virus infected cells over time. However loss of HBeAg and gain of anti-HBe is described in only 20% of patients at one year of lamivudine monotherapy (25-26, 27). More important we showed that lamivudine induced HBeAg seroconversion is significantly less durable compared to HBeAg seroconversion following IFN-containing therapies and withdrawal is mostly followed by virus return. On the other hand prolongation is hindered by the development of resistant strains, documented in more than half of all patients after 2 to 3 years of therapy (28-30).
The limited effect of lamivudine monotherapy is probably due to the fact that lamivudine does not affect the ccc-DNA pool and is dependent of the host immune response for viral elimination. As for IFN therapy patients with higher baseline ALT levels are more likely to have a sustained response to lamivudine therapy. On the other hand those with low ALT levels are unlikely to respond and have higher relapse rates (27, this thesis). Strong suppression of HBV replication reduces the expression of epitopes of viral proteins on the cell surface, hampering the class I-mediated CD8+ CTL response. In patients with no baseline host immune reactivity, it is therefore very unlikely the T-cell hyporesponsiveness will be restored during lamivudine therapy as has been published earlier (31). This may

account for the high relapse rate reported by others and us and suggest additional immune modulating therapy is necessary to obtain viral elimination (32, this thesis).

How to improve antiviral therapy in chronic HBV

Combination therapy seems the way to go. Several approaches aiming to improve the efficacy of antiviral therapy have been investigated or suggested.

a. Combination of nucleoside analogues

Several other nucleoside analogues including famcyclovir, adefovir dipivoxil, emcitabine, entecavir, clevudine among others, are available. The combination of 2 or more nucleoside analogues, acting complementary with regard to inhibition of the viral polymerase and viral replication, is likely to obtain long-term control of HBV. Not only the degree of HBV suppression will be increased, this approach may also prevent viral resistance as has been successfully shown in HIV (33).

The combination of lamivudine with famciclovir, a relatively weak HBV suppressive drug, for 12 weeks showed to significantly increase the inhibition of viral replication (34). Both adefovir dipivoxil and entecavir appear to be effective in patients with lamivudine resistant HBV mutant strains (35).

Th goal of combined nucleoside combination therapy would be obtaining sustained long term virus suppression. In the absence of host immune activity, it is very unlikely a combination of nucleoside analogues will actively eliminate HBV infected cells and subsequently ccc-DNA, the template for transcription of pregenomic RNA. Only of one of the newer compounds, entecavir, it is claimed it does affect the ccc-DNA pool in woodchucks (30-37).

b. Combination therapy of lamivudine and anti-HBs

Anti-HBs is well known in the prevention of perinatal transmission, post-exposure prophylaxis and post-liver transplantation but relatively unexplored in the treatment of chronic hepatitis B. The major effect of anti-HBs is preventing transmission of HBV by binding and neutralizing circulating HBsAg. In addition suggested immunomodulatory effects of antibodies in chronic hepatitis include antibody-dependent cellular cytotoxicity (ADCC), modulation of antigen presentation leading to stimulation of production of pro-inflammatory cytokines, formation of antigen-antibody complexes preventing infection and promoting opsonization by macrophages, and interference with the assembly and secretion of HBsAg (38-45).

We showed the administration of anti-HBs in vivo, both of HBIg and MAb, can induce a temporary reduction and neutralization of HBsAg in lamivudine treated patients. Although in preclinical studies MAb showed a 200-times higher affinity for HBsAg compared to HBIg, our in vitro results showed nearly 100% inhibition of HBsAg by HBIg, the MAb however was only

partially effective. Galun et al showed comparable results in patients not pretreated with lamivudine using a mixture of two monoclonal antibodies (46). He also observed a reduction in HBV DNA. These two antibodies were tested previously in a trimera mouse model showing an inhibition of viral replication and reduction of viral load.

Galun et al suggested to add anti-HBs to lamivudine treated patients (46). In our trials however this appeared an unsuccessful strategy. This is probably because infection of hepatocytes may be interrupted by infusion of anti-HBs, but there will still be a reservoir of infected cells, which will not be removed. Furthermore despite lamivudine pretreatment there are still high amounts of anti-HBs necessary, increasing the risk of side effects and inconvenience due to the amount of infusions. Lamivudine therapy blocks viral replication and the amount of Dane-particles is reduced. Although it was less accurate investigated as for IFN, this is accompanied by a considerable decrease in HBsAg levels but still relatively high levels were detectable (47-48, this thesis).

The development of a monoclonal directed against the Dane particle preventing viral spread would possibly be more effective in combination with intermittent low dose lamivudine therapy.

c. Combination therapy of lamivudine and IFN

We showed combining lamivudine and IFN drugs may increase the initial response rate if given for a prolonged period of time. According to these data the duration of combination therapy given in the previous trials was way to short explaining in part the limited efficacy reported up till now (27, 49-50). One trial using 24 weeks of combination therapy showed to indeed increase the efficacy (51).

It has to be taken into account these data were obtained using a biphasic viral kinetic model designed for IFN therapy in HCV. However it was previously shown it fits data obtained during lamivudine therapy in chronic HBV very well (52). Secondly we did not have multiple measurements in the first phase, therefore we had to focus on the second phase. At last no long-term data were available and the estimated time to viral negativity was calculated by extrapolation of the second phase. It is however possible the model may be accurate for long-term therapy or there may be a third phase when extending therapy, this has not been investigated yet during antiviral therapy for chronic HBV. Future viral kinetic studies using multiple measurements during the first phase and extending of the model beyond 16 weeks is necessary.

Combining IFN and lamivudine significantly decreased the relatively high relapse rate observed after lamivudine monotherapy (this thesis). In general patients with high pre-treatment HBV DNA and low ALT levels pretreatment are at higher risk for post treatment relapse of viral activity. The positive correlation between higher ALT, response and HBV

DNA decline was shown already before (20, 27, this thesis). Targeting combination therapy to this group of patients will significantly increase efficacy and reduce relapse rates. The identification of other baseline factors such as genotype is important. The clinical significance of hepatitis B genotypes is still unclear although there is growing evidence for the correlation with HBeAg seroconversion, progression of liver disease, the occurrence of YMDD mutations and certain subtypes (53-56).

The optimal schedule of lamivudine-IFN therapy is unclear yet. Previously it was shown that pretreatment with IFN before initiation of lamivudine therapy only delayed the effect of combination therapy on HBV DNA levels (50). Lamivudine pretreatment has been suggested, although the partial overlapping treatment schedule described was unsuccessful. This was nevertheless in contrast to previously published results (57). Intermittent lamivudine therapy would probably face the same problems as partial overlapping schedules.

Based on the reports on combination therapy an international multi-center study was started. Because of the strong effect of the pretreatment ALT level on the initial response rate and its positive effect on prevention of post-treatment relapse, we focussed on those patients with elevated ALT levels. This study investigates the efficacy of 52 weeks of combination therapy in immune reactive patients reflected by elevated pre-treatment ALT levels; first results are expected within the coming year. Future studies will have to determine the optimal duration of combination lamivudine-IFN therapy and therapeutic schedule.

Since a new array of new nucleoside analogues with similar or even stronger antiviral effect compared to lamivudine, are in use or under development we may expect several combinations with IFN in the future. The combination of pretreatment with L-FMAU followed by surface antigen vaccination was able to disrupt immune tolerance in woodchucks (58). This may support the hypothesis sequential or intermittent nucleoside analogue treatment followed by immunomodulating therapy may be effective.

d. Other immune modulating drugs

Although no conclusive results on monotherapy with recombinant anti-HBV, specific T cell or DNA vaccines, thymosine α and specific cytokines are available so far, combination with these drugs and new nucleoside analogues in the future may increase the efficacy of antiviral therapy (see chapter 1) (59-64).

e. New developments

Recently the use of hammerhead ribozyme-mediated gene therapy has been introduced as a potential novel therapeutic agent. Specific ribozymes mediated cleavage of HBV RNA reduces HBV replication *in vitro* in HepG2 cells (65).

In conclusion

Future studies need to take into account baseline factors like ALT and HBV DNA and probably genotype to evaluate and target antiviral therapy. The use of viral kinetics may give more lucidity about the effect and duration of therapy needed.

Combination therapy of immunomodulating drugs and nucleoside analogues is necessary to improve effectivity, break immune tolerance and prevent relapse in chronic HBV. Lamivudine-IFN is a potent combination especially in patients with elevated baseline ALT, the optimal duration and the use of an overlapping or sequential schedule are still unclear. In patients with low immune reactivity a combination with several nucleoside analogues may obtain long-term virus suppression.

References

1. Hoofnagle JH, Shafritz DA, Popper H. Chronic type B hepatitis and the "healthy" HBsAg carrier state. Hepatology 1987;7:758-63.
2. Dragosics B, Ferenci P, Hitchman E, Denk H. Long-term follow-up study of asymptomatic HBsAg-positive voluntary blood donors in Austria: a clinical and histologic evaluation of 242 cases. Hepatology 1987;7:302-6.
3. de Franchis R, Meucci G, Vecchi M, Tatarella M, Colombo M, Del Ninno E, Rumi MG, Donato MF, Ronchi G. The natural history of asymptomatic hepatitis B surface antigen carriers. Ann Intern Med 1993;118:191-4.
4. Weissberg JI, Andres LL, Smith CI, Weick S, Nichols JE, Garcia G, Robinson WS, Merigan TC, Gregory PB. Survival in chronic hepatitis B. An analysis of 379 patients. Ann Intern Med 1984;101:613-6.
5. Chu CM. Natural history of chronic hepatitis B virus infection in adults with emphasis on the occurrence of cirrhosis and hepatocellular carcinoma. J Gastroenterol Hepatol 2000;15 Suppl:E25-30.
6. Sie MPS, de Man RA, Niesters HGM, Blankenstein M. Which chronic HBsAg positive HBeAg negative hepatitis B needs long-term follow-up? Hepatology 2000,32:455A.
7. Torbenson M, Thomas DL. Occult hepatitis B. Lancet Infect Dis 2002;2:479-86
8. Mason AL, Xu L, Guo L, Kuhns M, Perrillo RP. Molecular basis for persistent hepatitis B virus infection in the liver after clearance of serum hepatitis B surface antigen. Hepatology 1998;27:1736-42.
9. Hoofnagle JH. The clinical spectrum and course of chronic hepatitis B. workshop on management of chronic hepatitis B: 2000. September 8-10, 2000, National Institutes of Health,Bethesda, Maryland.
10. Maddrey WC. Hepatitis B--an important public health issue. Clin Lab 2001;47:51-5.
11. Fattovich G, Schalm SW. Effect of antiviral therapy for chronic viral hepatitis on patient survival. (submitted).
12. Fattovich G, Giustina G, Christensen E, Pantalena M, Zagni I, Realdi G, Schalm SW. Influence of hepatitis delta virus infection on morbidity and mortality in compensated cirrhosis type B. The European Concerted Action on Viral Hepatitis (EUROHEP). Gut 2000;46:420-6.
13. Fattovich G, Giustina G, Schalm SW, Hadziyannis S, Sanchez-Tapias J, Almasio P, Christensen E, Krogsgaard K, Degos F, Carneiro de Moura M, et al. Occurrence of hepatocellular carcinoma and decompensation in western European patients with cirrhosis type B. The EUROHEP Study Group on Hepatitis B Virus and Cirrhosis. Hepatology 1995;21:77-82.
14. Lin SM, Sheen IS, Chien RN, Chu CM, Liaw YF. Long-term beneficial effect of interferon therapy in patients with chronic hepatitis B virus infection therapy. Hepatology 1999;29:971-5.
15. Niederau C, Heintges T, Lange S, Goldmann G, Niederau CM, Mohr L, Haussinger D. Long-term follow-up of HBeAg-positive patients treated with interferon alfa for chronic hepatitis B. N Engl J Med 1996;334:1422-7.
16. Realdi G, Fattovich G, Hadziyannis S, Schalm SW, Almasio P, Sanchez-Tapias J, Christensen E, Giustina G, Noventa F. Survival and prognostic factors in 366 patients with compensated cirrhosis type B: a multicenter study. The Investigators of the European Concerted Action on Viral Hepatitis (EUROHEP). J Hepatol 1994;21:656-66.
17. Di Marco V, Lo Iacono O, Camma C, Vaccaro A, Giunta M, Martorana G, Fuschi P, AlmasioPL, Craxi A. The long-term course of chronic hepatitis B. Hepatology 1999;30:257-64.
18. Fattovich G, Giustina G, Realdi G, Corrocher R, Schalm SW. Long-term outcome of hepatitis B e antigen-positive patients with compensated cirrhosis treated with interferon-alpha. European Concerted Action on Viral Hepatitis (EUROHEP). Hepatology 1997;26:1338-42.
19. Lau DT, Everhart J, Kleiner DE, Park Y, Vergalla J, Schmid P, Hoofnagle JH. Long-term follow-up of patients with chronic hepatitis B treated with interferon alfa. Gastroenterology 1997;113:1660-7.
20. Krogsgaard K, Bindslev N, Christensen E, Craxi A, Schlichting P, Schalm S, Carreno V, Trepo C, Gerken G, Thomas HC, Kragh Andersen P, Ring-Larsen H. The treatment effect of alpha interferon in chronic hepatitis B is independent of pre-treatment variables. Results based on individual patient data from 10 clinical controlled trials. European Concerted Action on Viral Hepatitis (EUROHEP). J Hepatol 1994;21:646-55.
21. Krogsgaard K. The long-term effect of treatment with interferon-alpha 2a in chronic hepatitis B. The Long-Term Follow-up Investigator Group. The European Study Group on Viral Hepatitis (EUROHEP). Executive Team on Anti-Viral Treatment. J Viral Hepat 1998;5:389-97.
22. Carreno V, Castillo I, Molina J, Porres JC, Bartolome J. Long-term follow-up of hepatitis B chronic carriers who responded to interferon therapy. J Hepatol 1992;15:102-6.
23. Lok AS, Chung HT, Liu VW, Ma OC. Long-term follow-up of chronic hepatitis B patients treated with interferon alfa. Gastroenterology 1993;105:1833-8.

24. Koorenman J, Baker B, Waggoner J, Everhart JE, Bisceglie AM, Hoofnagle JH. Long-term remission of chronic hepatitis B after alpha-interferon therapy. Ann Intern Med 1991;114:629-34.
25. Lai CL, Chien RN, Leung NWY, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray F. A one-year trial of lamivudine for chronic hepatitis B. N Engl J Med 1998;339:61-8.
26. Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, Crowther L, Condreay LD, Woessner M, Rubin M, Brown NA. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med 1999;341:1256-63.
27. Schalm SW, Heathcote J, Cianciara J, Farrell G, Sherman M, Willems B, Dhillon A, Moorat A, Barber J, Gray F. Lamivudine and alpha interferon combination treatment of patients with chronic hepatitis B infection: a randomised trial. Gut 2000;46:562-8.
28. Buti M, Jardi R, Cotrina M, Rodriguez-Frias F, Cruz de Castro E, Costa X, Esteban R, Guardia J. Two years of lamivudine therapy in patients with chronic hepatitis B. Analysis of efficacy and emergence of HBV genomic variations. J Hepatol 2000;2:111A.
29. Liaw YF, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Chien RN, Dent J, Roman L, Edmundson S, Lai CL. Effects of extended lamivudine therapy in Asian patients with chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. Gastroenterology 2000;119:172-80.
30. Leung NW, Lai CL, Chang TT, Guan R, Lee CM, Ng KY, Lim SG, Wu PC, Dent JC, Edmundson S, Condreay LD, Chien RN. Extended lamivudine treatment in patients with chronic hepatitis B enhances hepatitis B e antigen seroconversion rates: results after 3 years of therapy. Hepatology 2001;33:1527-32.
31. Boni C, Bertoletti A, Penna A, Cavalli A, Pilli M, Urbani S, Scognamiglio P, Boehme R, Panebianco R, Fiaccadori F, Ferrari C. Lamivudine treatment can restore T cell responsiveness in chronic hepatitis B. J Clin Invest 1998;102:968-75.
32. Song BC, Suh DJ, Lee HC, Chung YH, Lee YS. Hepatitis B e antigen seroconversion after lamivudine therapy is not durable in patients with chronic hepatitis B in Korea. Hepatology 2000;32:803-6.
33. Balzarini J. Suppression of resistance to drugs targeted to human immunodeficiency virus reverse transcriptase by combination therapy. Biochem Pharmacol 1999;58:1-27.
34. Lau GK, Tsiang M, Hou J, Yuen S, Carman WF, Zhang L, Gibbs CS, Lam S. Combination therapy with lamivudine and famciclovir for chronic hepatitis B-infected Chinese patients: a viral dynamics study. Hepatology 2000;32:394-9.
35. Perrillo R, Schiff E, Yoshida E, Statler A, Hirsch K, Wright T, Gutfreund K, Lamy P, Murray A. Adefovir dipivoxil for the treatment of lamivudine-resistant hepatitis B mutants. Hepatology 2000;32:129-34.
36. Genovesi EV, Lamb L, Medina I, Taylor D, Seifer M, Innaimo S, Colonno RJ, Standring DN, Clark JM. Efficacy of the carbocyclic 2'-deoxyguanosine nucleoside BMS-200475 in the woodchuck model for hepatitis B infection. Antimicrob Agents Chem 1998;42:3209-17.
37. Colonno RJ, Genovesi EV, Median I, Lamb L, Durham S, Corey L, Locarnini S, Tennant BC, Clark JM. Long-term therapy with entecavir (BMS-200475) in the woodchuck model of chronic hepatitis infection. Abstract of the 40th ICAAC September 2000, 172.
38. Wen YM, Wu XH, Hu DC, Zhang QP, Guo SQ. Hepatitis B vaccine and anti-HBs complex as approach for vaccine therapy. Lancet 1995;345:1575-6.
39. McCluskie MJ, Wen YM, Di Q, Davis HL. Immunization against hepatitis B virus by mucosal administration of antigen-antibody complexes. Viral Immunol 1998;11:245-52.
40. Celis E, Abraham KG, Miller RW. Modulation of the immunological response to hepatitis B virus by antibodies. Hepatology 1987;7:563-8.
41. Wen YM, Xiong SD, Zhang W. Solid matrix-antibody-antigen complex can clear viraemia and antigenaemia in persistent duck hepatitis B virus infection. J Gen Virol 1994;75:335-9.
42. Wen YM, Qu D, Zhou SH. Antigen-antibody complex as therapeutic vaccine for viral hepatitis B. Int Rev Immunol. 1999;18:251-8.
43. Schilling R, Ljaz S, Williams R, Naoumov NV. Internalisation of monoclonal and polyclonal hepatitis B immunoglobulins in human hepatoma cells and their effect on the assembly and secretion of HBsAg. Hepatology 1999;30:246A.
44. Krawczynski K, Fattom A, Culver D, Kamili S, Spelbring J, Basham L, Sapan C, Naso R, Purdy M, Carson D, Experimental immune treatment of hepatitis C virus (HCV) infection: passive anti-HCV (HBCig) transfer in chronically infected chimpanzees. J Hepatol 2000;2:37A.
45. Schneiders AM, Schulz, M, Leifeld L, Duesberg U, Koopman J, Matz B, Inchauspe G, Sauerbruch T, Spengler U. E2 antibodies mediate antibody dependent cellular cytotoxicity in HCV. J Hepatol 2000;2:37A.

46. Galun E, Eren R, Safadi R, Ashour Y, Terrault N, Keeffe EB, Matot E, Mizrachi S, Terkieltaub D, Zohar M, Lubin I, Gopher J, Shouval D, Dagan S. Clinical evaluation (phase I) of a combination of two human monoclonal antibodies to HBV: safety and antiviral properties. Hepatology 2002;35:673-9.
47. Janssen HL, Kerhof-Los CJ, Heijtink RA, Schalm SW. Measurement of HBsAg to monitor hepatitis B viral replication in patients on alpha-interferon therapy. Antiviral Res 1994;23:251-7.
48. Nevens F, Main J, Honkoop P, Tyrrell DL, Barber J, Sullivan MT, Fevery J, De Man RA, Thomas HC. Lamivudine therapy for chronic hepatitis B: a six-month randomized dose-ranging study. Gastroenterology 1997;113:1258-63.
49. Schiff ER, Karayalcin S, Grimm IS, Perillo RP, Dienstag JL, Husa P, de Man RA, Goodman Z, Condreay LD, Crowther LM, Woessner MA, McPhillips PJ, Brown NA and an International Lamivudine Investigator Group. Lamivudine and lamivudine/interferon combination therapy in patients with hepatitis B who previously failed interferon: an international controlled trial. Ann Int Med 2002, accepted for publication.
50. Mutimer D, Naoumov N, Honkoop P, Marinos G, Ahmed M, de Man R, McPhillips P, Johnson M, Williams R, Elias E, Schalm S. Combination alpha-interferon and lamivudine therapy for alpha-interferon-resistant chronic hepatitis B infection: results of a pilot study. J Hepatol 1998;28:923-9.
51. Barbaro G, Zechini F, Pellicelli AM, Francavilla R, Scotto G, Bacca D, Bruno M, Babudieri S, Annese M, Matarazzo F, Di Stefano G, Barbarini G. Long-term efficacy of interferon alpha-2b and lamivudine in combination compared to lamivudine monotherapy in patients with chronic hepatitis B. An Italian multicenter, randomized trial. J Hepatol 2001;35:406-11.
52. Wolters LM, Hansen BE, Niesters HG, Zeuzem S, Schalm SW, de Man RA. Viral dynamics in chronic hepatitis B patients during lamivudine therapy. Liver 2002;22:121-6.
53. Kao JH, Chen PJ, Lai MY, Chen DS. Genotypes and clinical phenotypes of hepatitis B virus in patients with chronic hepatitis B virus infection. J Clin Microbiol 2002;40:1207-9.
54. Kobayashi M, Arase Y, Ikeda K, Tsubota A, Suzuki Y, Saitoh S, Kobayashi M, Suzuki F, Akuta N, Someya T, Matsuda M, Sato J, Kumada H. Clinical characteristics of patients infected with hepatitis B virus genotypes A, B, and C. J Gastroenterol 2002;37:35-9.
55. Zollner B, Petersen J, Schafer P, Schroter M, Laufs R, Sterneck M, Feucht HH. Subtype-dependent response of hepatitis B virus during the early phase of lamivudine treatment. Clin Infect Dis 2002;34:1273-7.
56. Chu CJ, Lok AS. Clinical significance of hepatitis B virus genotypes. Hepatology 2002;35:1274-6.
57. Serfaty L, Thabut D, Zoulim F, Andreani T, Chazouilleres O, Carbonell N, Loria A, Poupon R. Sequential treatment with lamivudine and interferon monotherapies in patients with chronic hepatitis B not responding to interferon alone: results of a pilot study. Hepatology 2001;34:573-7.
58. Menne S, Roneker CA, Korba BE, Gerin JL, Tennant BC, Cote PJ. Immunization with surface antigen vaccine alone and after treatment with 1-(2-fluoro-5-methyl-beta-L-arabinofuranosyl)-uracil (L-FMAU) breaks humoral and cell-mediated immune tolerance in chronic woodchuck hepatitis virus infection. J Virol 2002;76:5305-14.
59. Zeuzem S, Carreno V. Interleukin-12 in the treatment of chronic hepatitis B and C. Antiviral Res 2001;52:181-8.
60. Michel M, Pol S, Brechot C, Tiollais P. Immunotherapy of chronic hepatitis B by anti HBV vaccine: from present to future. Vaccine 2001;19:2395-9.
61. Heathcote J, McHutchison J, Lee S, Tong M, Benner K, Minuk G, Wright T, Fikes J, Livingston B, Sette A, Chestnut R. A pilot study of the CY-1899 T-cell vaccine in subjects chronically infected with hepatitis B virus. The CY1899 T Cell Vaccine Study Group. Hepatology 1999;30:531-6.
62. Vitiello A, Ishioka G, Grey HM, Rose R, Farness P, LaFond R, Yuan L, Chisari FV, Furze J, Bartholomeuz R, et al. Development of a lipopeptide-based therapeutic vaccine to treat chronic HBV infection. I. Induction of a primary cytotoxic T lymphocyte response in humans. J Clin Invest 1995;95:341-9.
63. van Hattum J, Rash Mc, Boland GJ, Leroux-Roels G, Vandepapeliere P, Early evaluation of two schedules of administration of a novel therapeutic vaccine for chronic hepatitis B. Hepatology 2000;32:458A.
64. Couillin I, Pol S, Mancini M, Driss F, Brechot C, Tiollais P, Michel ML. Specific vaccine therapy in chronic hepatitis B: induction of T cell proliferative responses specific for envelope antigens. J Infect Dis 1999;180:15-26.
65. Feng Y, Kong YY, Wang Y, Qi GR. Intracellular inhibition of the replication of hepatitis B virus by hammerhead ribozymes. J Gastroenterol and Hepatol 2001;16:1125-30.

Chapter VI

Summary

Samenvatting

Dankwoord

Curriculum vitae

Bibliography

List of Abbreviations

Summary

The search for antiviral agents in chronic hepatitis B virus (HBV) infected patients faces multiple problems. Monotherapy with either alpha-interferon (IFN) or lamivudine has limited efficacy; viral clearance -loss of HBeAg and gain of anti-HBe- is the result in only 20-40% of patients. Although nucleoside analogues are very potent inhibitors of viral replication, viral resistance complicates long-term therapy, whereas withdrawal is followed by a relapse of viral replication and inflammatory activity. This stresses the need for new therapeutic approaches.

The studies presented in this thesis focus on combination of immune-modifying drugs (anti-HBs and IFN) and the nucleoside analogue lamivudine in chronic compensated HBV patients. Theoretically this approach may increase the HBeAg seroconversion rate and enhance sustained viral clearance by combining strong initial virus suppression and immune-mediated clearance of remaining infected cells. The correlation between pre-treatment patient characteristics -especially ALT levels- and the degree of virus suppression, response and post-treatment relapse rate was investigated in more detail. These data may better tailor antiviral therapy to subgroups of HBV infected patients.

Although anti-HBs is used extensively in the post-transplantation period in HBV, in only 3 small trials it was used for the treatment of chronic HBV. In chapter II, three studies are described using polyclonal antibodies (HBIg, Hepatect®) or a human monoclonal anti-hepatitis B antibody preparation recognizing the stable 'a'-determinant of the HBsAg molecule (MAb) in chronic HBV patients. After infusion of anti-HBs, antigen-antibody complexes are formed rapidly, theoretically preventing the spread of the virus to non-infected cells. In addition these complexes have been described to promote opsonisation increasing the uptake by macrophages and enhance the HBsAg specific T-cell response. In theory monoclonal antibodies may also have specificity to certain determinants involved in binding or penetration of the virus.

The first study describes 10 chronic HBV patients, 4 received the MAb and 6 a combination of IFN plus the MAb (chapter II.1). Although a reduction in HBsAg levels of more than 50% was achieved in all patients, neutralization of circulating HBsAg was not obtainable. No long-term effects were observed despite in 3 patients treated with combination therapy. In these patients serum HBV DNA levels became undetectable, however this effect probably has to be attributed to IFN.

The last 2 studies describe the effects of HBIg respectively MAb in chronic HBV patients pre-treated with lamivudine to obtain maximal viral load reduction (HBV DNA levels undetectable by hybridization assay). Experimental enzyme-immunoassays (EIA´s) were developed to evaluate the neutralizing efficacy of the MAb in the circulation. With monoclonal antibody

conjugates HBsAg, MAb and HBsAg-MAb complexes were measurable in serum seperately. *In vitro* HBIg showed complete neutralization, whereas the Mab was only partially effective. *In vivo* the neutralizing effect of HBIg was limited to those patients with very low HBsAg levels. The MAb, which has a 200-fold binding capacity compared to HBIg, also only showed a temporary HBsAg neutralizing effect also related to pretreatment HBsAg levels. During MAb therapy the antibody could be detected in circulating HBsAg/anti-HBs complexes. No long-term effects were observed. Unfortunately passive immunization, even in HBV DNA PCR negative patients, was not a feasible option.

In chapter III a case is described of a chronic HBV patient on lamivudine therapy, superinfected with the hepatitis A virus (HAV). After 8 weeks of lamivudine monotherapy the initial fast drop in HBV DNA ceased and levels around 10^7-10^6 geq/ml remained detectable. A sudden further decline of HBV DNA below the limit of detection of the assay (400 geq/ml) and the loss of HBeAg from serum following a peak of serum transaminases, accompanied an acute HAV infection. The induction of a HAV mediated IFN-gamma peak just before the rise in serum transaminases, was pivotal in the additional suppression of HBV DNA replication. Several other cytokines -IFN-alpha, TNF-alpha and IL-12- remained undetectable; a small IL-10 peak was measurable which could have contributed to the self-limitation of the response. No HBV-specific T cell response was elucidated. This probably accounts for the relapse of viral activity after resolution of the HAV infection and withdrawal of lamivudine therapy, despite the temporary addition of IFN-alpha therapy.

The potential benefit of lamivudine-IFN combination therapy in chronic HBV was evaluated in four studies described in chapter IV. Several studies on combination therapy were published previously. Although in 2 studies an enhanced HBeAg seroconversion rate following combination therapy was reported compared to monotherapy with either lamivudine or IFN, the overall results remained disappointing. Combination therapy appears to be the most beneficial approach in patients with elevated baseline ALT levels (2-5 x ULN) (chapter IV.4). We tried to optimize the effect of combining both drugs by using a different treatment schedule, our initial hypothesis being that by viral load reduction IFN efficacy might be enhanced. IFN was added after at least 16 weeks of lamivudine monotherapy (chapter IV.1). Combination therapy was given for at least 8 weeks followed by withdrawal of lamivudine, IFN monotherapy was continued for at least 8 weeks. During lamivudine monotherapy HBV DNA levels decreased a median of 3.2 logs, 66% of final decrease was obtained in the initial 4 weeks. Thereafter the decline of HBV DNA was more gradual (0.2-0.3 logs per month). Adding IFN further increased the degree of virus suppression by a median of 1.4 log, 86% was obtained in the first 4 weeks of combination therapy. However withdrawal of lamivudine

and subsequently IFN was accompanied by a relapse of viral activity in most patients. HBeAg seroconversion was obtained in 4/24 patients, this was sustained in only 2/24 patients (8%). Although the degree of virus suppression was superior during lamivudine-IFN combination therapy, at the end of follow-up no additional benefit was observed using the schedule studied.

In the second study the effect of combination therapy was further evaluated using a biphasic mathematical model (chapter IV.2). It was shown that combining both drugs enhanced the decay rate of HBV DNA from serum during both phases of viral decline. Simultaneously dosing of IFN and lamivudine decreased the half-life of the virus and of the infected cell significantly. Independently of the therapy given, a significant correlation between baseline ALT levels and the decline rate of HBV DNA levels was found. More importantly extrapolation of the second phase of the bi-phasic model showed that, theoretically, combining both drugs will reduce the estimated time needed to obtain serum viral elimination and it will increase the percentage of patients that may reach this point to nearly 100%. Although short-term combination therapy (16 weeks) did not increase efficacy in previous studies these results suggest a strong benefit of combination therapy over monotherapy with either drug when given for a prolonged period of time.

There is a debate about the durability of lamivudine induced HBeAg-seroconversion. A large international multicenter meta-analysis of individual patient data following IFN, lamivudine or lamivudine-IFN therapy induced HBeAg-seroconversion was designed (chapter IV.3). At 3 years of follow-up a relapse of HBeAg in serum was observed in 54% of responders to lamivudine therapy compared to 32% following IFN and 23% following combination therapy. High ALT levels and low HBV DNA levels were positively related to lower relapse rates, independently of the therapy given. Correction for baseline factors, HBV DNA, ALT, race and sex, further increased the relapse risk following lamivudine therapy and decreased the risk of post-treatment relapse after IFN-containing therapies. The relative relapse risk of HBeAg after withdrawal of lamivudine was 4.6 compared to IFN monotherapy. So these results clearly show lamivudine induced HBeAg-seroconversion is significantly less durable compared to seroconversion following IFN-containing therapies.

Long-term lamivudine-IFN combination therapy will probably increase the initial response rate substantially, decrease the duration of therapy needed and minimize the risk of relapse of viral activity after withdrawal of therapy compared to monotherapy with either drug, especially when targeted to patients with elevated baseline serum transaminases. The effect of long-term combination therapy in different patient populations and the optimal treatment schedule, dose and duration of therapy will be subjects of future studies.

Samenvatting

In de zoektocht naar antivirale middelen voor de behandeling van patiënten chronisch geïnfecteerd met het hepatitis B virus (HBV) stuit men op multiple problemen. Monotherapie met interferon-alpha (IFN) of lamivudine heeft een beperkte effectiviteit; slechts in 20-40% van de patiënten wordt klaring van het virus –verlies van HBeAg en aanmaak van anti-HBe- bereikt. Ondanks dat nucleoside analoga een sterke virus onderdrukkende werking hebben, wordt behandeling op de langere duur beperkt door het ontstaan van virale resistentie, terwijl aan de andere kant het stoppen van behandeling gepaard gaat met de terugkeer van het virus in serum en opvlamming van de ontstekingsactiviteit. Bovenstaande benadrukt de noodzaak van een nieuwe therapeutische aanpak in chronische HBV.
De studies die in dit proefschrift gepresenteerd worden richten zich op het gebruik van een combinatie therapie van immuunmodulerende middelen (anti-HBs en IFN) en nucleoside analoga in chronische gecompenseerde HBV patiënten. Theoretisch kan de combinatie van sterke initiële virus onderdrukking met immuun gemedieerde opruiming van geïnfecteerde cellen leiden tot een verhoogd percentage HBeAg seroconversie en blijvende virale klaring. Patientenkenmerken voor start van therapie -met name ALT- zijn onderzocht in relatie tot de mate van virus suppressie, het percentage respons en terugkeer van het virus na stoppen van therapie. Op basis van deze data kan de therapie beter gericht worden op bepaalde subgroepen van patiënten.

Alhoewel het gebruik van anti-HBs uitgebreid onderzocht is in de periode na levertransplantatie, is het effect in chronische HBV slechts in drie kleine onderzoeken beschreven. In hoofdstuk II worden drie nieuwe studies beschreven die het gebruik van polyclonale antilichamen (HBIg, Hepatect®) of van een humaan monoclonaal anti- hepatitis B antilichaam (MAb) gericht tegen het stabiele deel van het HBsAg molecuul, "a-determinant", in chronische HBV bestuderen. Na infusie van anti-HBs vormen zich snel antigeen-antilichaam complexen welk theoretisch de verspreiding van het virus naar niet-geïnfecteerde cellen voorkomt. Daarbij is beschreven dat deze complexen de "opsonisatie" stimuleren en zo de uptake door macrophagen vergroot en de HBsAg specifieke T-cell response stimuleren. In theorie kunnen monoclonale antilichamen ook specifiek gericht zijn tegen bepaalde delen die betrokken zijn bij de binding en penetratie van het virus.
De eerste studie beschrijft tien chronische HBV patiënten, vier werden behandeld met het MAb en zes met IFN-MAb combinatie therapie (hoofdstuk II.1). Hoewel een reductie van HBsAg met meer dan 50% werd bereikt in alle patiënten, was neutralisatie van circulerend HBsAg niet haalbaar. Er werden geen lange termijn effecten waargenomen, behoudens in drie patiënten die behandeld werden met combinatie therapie. In deze groep werd serum

HBV DNA ondetecteerbaar. Het is echter mogelijk dat dit effect aan de werking van IFN alleen kan worden toegeschreven.
In de laatste twee studies wordt het effect van HBIg respectievelijk MAb beschreven in chronische HBV patiënten die zijn voorbehandeld met lamivudine om een maximale reductie van de virale load te verkrijgen (HBV DNA negativiteit gemeten met een hybridisatie test). Experimentele enzyme-immunoassays (EIA´s) werden ontwikkeld om de HBsAg-neutralizerende werking van het MAb in de circulatie te evalueren. Met monoclonale antilichaam conjugaten werd het mogelijk om HBsAg, MAb en HBsAg-MAb complexen apart te meten. *In vitro* was het mogelijk complete neutralisatie te verkrijgen met HBIg, echter MAb was slechts gedeeltelijk effectief. *In vivo* was het tijdelijke neutraliserend effect van HBIg beperkt tot die patiënten met zeer lage HBsAg waarden. Met het MAb, wat een 200 maal sterkere binding van HBsAg ten opzichte van HBIg vertoont, werd ook slechts tijdelijke HBsAg neutralisatie bereikt hetgeen ook gerelateerd was aan lage HBsAg waarden voor start van de behandeling. Gedurende de behandeling met MAb kon het MAb geïdentificeerd worden in circulerende HBsAg/anti-HBs complexen. Er zijn geen lange termijn effecten waargenomen en het bleek dat passieve immunizatie, zelfs in HBV DNA PCR negatieve patiënten geen haalbare optie was.

In hoofdstuk III word een casus beschreven van een chronische hepatitis B patiënt geïnfecteerd met het hepatitis A virus (HAV) tijdens behandeling met lamivudine. Na 8 weken behandeling met lamivudine buigt de initiële snelle daling van het HBV DNA zich af en blijft op ongeveer 10^7-10^6 geq/ml hangen. Volgend op een acute HAV infectie gepaard gaande met een piek in serum transaminasen treedt een plotselinge snelle daling van HBV DNA op tot beneden de detectielimiet van de test (400 geq/ml) alsmede het verlies van HBeAg. Het induceren van een HAV gemedieerde IFN-gamma piek net voor de snelle stijging van serum transaminasen, bleek een essentiële rol te spelen in de additionele suppressie van HBV DNA replicatie. Verschillende andere cytokines –IFN-alpha, TNF-alpha en IL-12- bleven negatief; een kleine piek in IL-10 was meetbaar hetgeen theoretisch kan hebben geleid tot een gelimiteerde respons. Er werd geen HBV specifieke T-cell response gegenereerd, hetgeen waarschijnlijk de terugkeer van het virus na beëindiging van de HAV infectie en het stoppen van lamivudine, verklaart, ondanks de tijdelijke toevoeging van IFN.

In hoofdstuk IV worden vier studies beschreven die het potentiële effect van lamivudine-IFN combinatie therapie in chronische HBV bestuderen. Voorheen zijn verschillende lamivudine-IFN studies verricht. Alhoewel in twee studies een toegenomen HBeAg seroconversie werd beschreven na combinatie therapie in vergelijking met IFN of lamivudine monotherapie, blijft de meerwaarde van combinatie therapie vooralsnog onduidelijk en zijn de resultaten in het

algemeen teleurstellend. Combinatie therapie heeft het grootste effect in patiënten met een middelmatig verhoogde ALT (2-5 keer de bovengrens van normaal) voor start van therapie (hoofdstuk IV.4).

Een aangepast behandelingsschema werd gebruikt om het effect van combinatie therapie te vergroten, ervan uitgaande dat het effect van IFN therapie wordt versterkt door reductie van de virale load voor start van behandeling. IFN werd toegevoegd aan voorbehandeling met tenminste 16 weken lamivudine therapie (hoofdstuk IV.1). Combinatie therapie werd gedurende ten minste acht weken gecontinueerd waarna lamivudine werd gestopt en IFN monotherapie nog ten minste acht weken werd gegeven. Tijdens lamivudine monotherapie daalde HBV DNA met een mediaan van 3.2 log, 66% van deze reductie in virale load werd in de eerste vier weken bereikt. Daarna vlakte de daling af (0.2-0.3 log per maand). Het toevoegen van IFN zorgde voor een verdere virus onderdrukking met een mediaan van 1.4 log, hiervan werd 86% bereikt in de eerste vier weken van combinatie therapie. Echter het achtereenvolgens stoppen van lamivudine en vervolgens IFN ging gepaard met een terugkeer van de virale load in vrijwel alle patiënten. HBeAg seroconversie werd bereikt in vier van de 24 patiënten, het was echter slecht in 2/24 (8%) blijvend. Alhoewel de mate van virus suppressie gedurende combinatie therapie met lamivudine en IFN superieur was, werd geen voordeel bereikt met het gebruikte behandelingsschema.

In de tweede studie is het effect van combinatietherapie geëvalueerd met behulp van een biphasisch mathematisch model (hoofdstuk IV.2). Er werd aangetoond dat combinatie van beide middelen de daling van het virus gedurende beide fases versterkt. Het gelijktijdig geven van IFN en lamivudine verminderde de halfwaardetijd van het virus en de geïnfecteerde cel aanzienlijk. Onafhankelijk van de therapie die werd gegeven werd er een correlatie gevonden tussen ALT voor start van behandeling en de mate van daling van HBV DNA. Belangrijker, extrapolatie van de gegevens van de tweede fase van het biphasische model toonde aan dat, theoretisch, combinatie van beide middelen de geschatte behandelingsduur, nodig om klaring van HBV DNA te verkrijgen, verkort en dat het percentage patiënten wat dit punt bereikt verhoogd wordt naar vrijwel 100%. Hoewel kortdurende combinatie therapie (16 weken) de effectiviteit in voorafgaande studies niet vergrootte, suggereren deze resultaten een sterk voordeel van combinatie therapie boven monotherapie met een van beide geneesmiddelen indien dit gedurende een langere periode wordt gecontinueerd.

Er is discussie over de duurzaamheid van lamivudine geïnduceerde HBeAg seroconversie. Een grote internationale meta-analyse werd opgezet die de individuele patiënten data over HBeAg seroconversie na IFN, lamivudine of lamivudine-IFN combinatie therapie bestudeerde (hoofdstuk IV.3). In 54% van de lamivudine responders werd na 3 jaar follow-up een terugkeer van HBeAg in serum geobserveerd, vergeleken met 32% na IFN therapie en

23% na combinatie therapie. Een hoge ALT en laag HBV DNA waren positief gerelateerd aan een lagere kans op terugkeer, onafhankelijk van de therapie die gegeven werd. Na correctie voor baseline factoren, HBV DNA, ALT, ras en geslacht werd een toename van de kans op terugkeer gezien na stoppen van lamivudine therapie, echter het risico daalde na stoppen van IFN-bevattende therapieën. Het relatieve risico op terugkeer na stoppen van lamivudine was 4.6 vergeleken met IFN monotherapie. Deze resultaten tonen dus duidelijk aan dat HBeAg-seroconversie na lamivudine minder duurzaam is vergeleken met seroconversie na IFN-bevattende therapie.

Langdurige behandeling met een combinatie van lamivudine en IFN zal waarschijnlijk de initiële kans op response vergroten, de duur van de behandeling verkorten en het risico op terugkeer van het virus na stoppen van de behandeling verkleinen, in vergelijking met monotherapie met een van beide middelen, met name als deze behandeling wordt gericht op patiënten met verhoogde serum transaminasen voor start van therapie. Het effect van langdurige combinatie therapie in verschillende patiënten populaties en het optimale behandelingsschema, dosis en duur van behandeling zijn onderwerp van vervolgstudies.

Dankwoord

Promoveren is naast een individuele ook een sociale aangelegenheid. Collegialiteit, begeleiding en coöperatie van velen zijn hierbij van belang. Een aantal mensen wil ik graag persoonlijk bedanken.

Als eerste bedank ik mijn promotor, Prof. dr. S.W. Schalm, voor zijn gedrevenheid en vakkundige visie waarbij zijn dynamisch alsmede kritisch blik op mij de meeste indruk heeft gemaakt.

Zeer veel dank ben ik Rob de Man verschuldigd voor zijn inhoudelijke kennis, strategisch denken, persoonlijke interesse vertrouwen en steun. Je bescheidenheid toont zich in je treffende en motiverende metafoor van de koekjesfabriek. Bij de ontwikkeling van het concept, de productie en verpakken van het product heb jij heel wat meer bijgedragen dan alleen de mooie strik om het pakje zoals je het initieel schilderde.

Ruud Heijtink, zonder jouw handwerk, alsmede dat van je medewerkers op het laboratorium, was de enorme bulk samples slechts in een vriezer beland. Bedankt voor alle energie en wetenschappelijke input die je in onze studies gestoken hebt.

Bert Niesters, virologisch bepalingen waar ik nu weken op moet wachten werden door jou in een handomdraai verricht hetgeen vele van mijn studies mogelijk heeft gemaakt. Ook wil ik iedereen op de afdeling diagnostiek en het secretariaat bedanken voor hun hulp bij de reeksen samples die verwerkt moesten worden.

Beste Betinna, je dinsdag draaide alleen maar om hepatitis B. Later werd ik zelfs op je vrije dag tussen je kinderen en het avondeten ingepland. Je computer heeft overuren gedraaid, maar je wist de meest mooie berekeningen te voorschijn te toveren. Bedankt voor je eindeloze geduld en tijd.

Harry Janssen, in mijn laatste periode heb ik samen met je hard gewerkt aan de opzet en uitvoering van een grote internationale trial. Ik hoop dat hij net zo succesvol als voorspeld mag eindigen.

Het secretariaat van de SLO was behalve het zakelijke centrum ook de bron van veel gezelligheid en het laatste nieuws. Ik weet niet hoe alles de deur uit was gegaan zonder jouw zorg, Sylvia. Wil en Mieke, mijn dank voor het regelen en bemiddelen in vele praktische zaken.

In de patientenzorg werd ik bijgestaan door de polidames op de aangename dinsdagmiddag HBV-spreekuren en de verpleging op de Clinical Research Unit bij het uitwerking van de vele trials en begeleiding van de geincludeerde patienten.

Mijn collega's op de dakpoli hebben ervoor gezorgd dat het leerzame ook met het aangename werd gecombineerd. Begonnen met Pieter Honkoop, Michael, Bart, Frank Bekkering, Frank Vleggaar en Thekla en geëindigd met Jan-Maarten, Thjon, Pieter ter

Borg en Marjolein. Ook de afdeling senioren op de dakpoli was onmisbaar, Henk van Buuren, Hans Brouwer en Sjoerd de Rave bedankt voor jullie hulp, met name bij de scholing van mijn stem.

Leonieke, samen hebben we het toch maar mooi gedaan. Alle ups en downs van ons onderzoek en het leven hebben we besproken. Ik denk niet dat veel mensen een dergelijke vriendschap uit hun werk halen. We zijn nu niet meer de hepatitis B meisjes en zien elkaar veel minder vaak, maar ik hoop dat het nog heel wat jaren mag duren.

Collega's uit het Maxima, bedankt voor de steun het afgelopen 1,5 jaar. Hierna hoeven jullie mijn gezeur nooit meer aan te horen, ook jullie zijn van mijn promotielast ontdaan.

Mart en Clemens, voor jullie betekende Rotterdam eindelijk samen zijn. Voor mij het tegendeel. Mede dankzij jullie ben ik me thuis gaan voelen en ik mis nu zelfs de dynamiek van deze indrukwekkende stad.

Ruth, je hebt altijd weinig woorden nodig. Al zat je in Groningen, telefonisch stond je me in het heetst van de strijd toch altijd met wijze raad bij. Gelukkig kom je nu eindelijk weer wat dichterbij wonen. Ik ben er trots op dat jij mijn andere paranimf wilt zijn.

Jos en Tineke, jullie zijn er altijd en overal. Nu heb ik eindelijk eens een kans om jullie te zeggen hoe veel dat voor me betekent. Door jullie ben ik geworden wie ik ben en heb ik kunnen doen wat ik heb gedaan.

Merel en Rach, heerlijk 3,5 jaar lang 2 zusjes in de buurt, ook al is afstand voor jullie nooit een belemmering.

Liefste Bas, mijn meest kritische noot en onbegrensde steun. Een dissertatie is niet alleen een proeve van wetenschappelijke bekwaamheid. Het is ook een ware test voor de relatie. Ik geloof dat we geslaagd zijn, jij in ieder geval. Ik heb weer tijd en heel veel toekomstplannen die ik samen met je hoop waar te maken.

Curriculum Vitae

De auteur van dit proefschrift werd op 29 mei 1971 in Eindhoven geboren. Na het V.W.O.-B eindexamen aan het Mgr. Zwijsen college te Veghel werd in 1989 gestart met de studie geneeskunde aan de Rijksuniversiteit Limburg. Tijdens haar co-schappen werkte zij vanaf 1995 tot 1997 mee aan een onderzoek naar osteoporose bij patiënten met de ziekte van Crohn op de afdeling maag, -darm -en leverziekten van het Academisch Ziekenhuis Maastricht (Prof. Dr. R.W. Stockbrűgger). Na het behalen van het artsexamen in 1997 was zij gedurende 3½ jaar als arts-onderzoeker werkzaam op de afdeling maag, -darm -en leverziekten van het Academisch Ziekenhuis Rotterdam. Onder begeleiding van Prof. Dr. S.W. Schalm en Dr. R.A. de Man werd onderzoek verricht naar antivirale therapie in chronische hepatitis B patiënten uiteindelijk leidend tot de totstandkoming van dit proefschrift. Met de opleiding tot internist werd in januari 2001 begonnen in het Sint Joseph Ziekenhuis in Veldhoven (opleider: Dr. A.W.L. van den Wall Bake). Samen met haar partner Bas van Alphen woont zij sindsdien in Eindhoven.

Bibliography

van Nunen AB, Janssen HL, Wolters LM, Niesters HG, de Man RA, Schalm SW. Is combination therapy with lamivudine and interferon-alpha superior to monotherapy with either drug? Antiviral Res 2001;52:139-46.

Heijtink RA, van Nunen AB, van Bergen P, Ostberg L, Osterhaus AD, de Man RA. Administration of a human monoclonal antibody (TUVIRUMAB) to chronic hepatitis B patients pre-treated with lamivudine: monitoring of serum TUVIRUMAB in immune complexes. J Med Virol 2001;64:427-34.

van Nunen AB, Baumann M, Manns MP, Reichen J, Spengler U, Marschner JP, de Man RA. Efficacy and safety of an intravenous monoclonal anti-HBs in chronic hepatitis B patients. Liver 2001;21:207-12.

Wolters LM, van Nunen AB, Niesters HG, de Man RA. Contrasting patterns of response to lamivudine monotherapy in chronic hepatitis B patients. Scand J Gastroenterol 2000;232:74-8.

Schoon EJ, van Nunen AB, Wouters RS, Stockbrugger RW, Russel MG. Osteopenia and osteoporosis in Crohn's disease: prevalence in a Dutch population-based cohort. Scand J Gastroenterol 2000;232:43-7.

van Nunen AB, Pontesilli O, Uytdehaag F, Osterhaus AD, de Man RA. Suppression of hepatitis B virus replication mediated by hepatitis A-induced cytokine production. Liver;2001;21:45-9.

Wolters LM, van Nunen AB, Honkoop P, Vossen AC, Niesters HG, Zondervan PE, de Man RA. Lamivudine-high dose interferon combination therapy for chronic hepatitis B patients co-infected with the hepatitis D virus. J Viral Hepat 2000;7:428-34.

Schalm SW, Wolters LM, van Nunen AB, Niesters HG, de Man RA. New nucleoside analogues for chronic hepatitis B. Acta Gastroenterol Belg 2000;63:191-3.

van Nunen AB, de Man RA, Heijtink RA, Niesters HG, Schalm SW. Lamivudine in the last 4 weeks of pregnancy to prevent perinatal transmission in highly viremic chronic hepatitis B patients. J Hepatol 2000;32:1040-1.

Van Nunen AB, de Man RA, Heijtink RA, Vossen ACTM, Schalm SW. Passive immunization of chronic hepatitis B patients on lamivudine therapy: a feasible issue? J Viral Hepat 2002;9:221-8.

Abbreviations

AL(A)T	alanine aminotransferase
anti-HBe	antibody against HBeAg
anti-HBs	antibody against HBsAg
AS(A)T	aspartate aminotransferase
CCC-DNA	covalently closed circular DNA
CHB	chronic hepatitis B
CI	confidence interval
CMV	cytomegalo virus
DNA	deoxyribonucleic acid
EBV	Ebstein Barr virus
EIA	enzyme immune assay
ELISA	enzyme labeled immuno solid phase assay
geq	genome equivalents
HAI	histology activity index
HAV	hepatitis A virus
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B envelop antigen
HBIg	hepatitis B immune globulin
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDV	hepatitis D virus
HIV	human immunodeficiency virus
IFN (-α), (-γ)	interferon (-alpha), (-gamma)
IL (-2), (-10), (-12)	interleukin (-2), (-10), (-12)
IU	international units
LAM	lamivudine
LPR	lympho-proliferative response
LSI	lymphocyte stimulation index
LTx	liver transplantation
MAb	monoclonal antibody
MU	mega units
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PCRQ	quantitative polymerase chain reaction
RCT	randomised controlled trial
PEI	Paul Ehrlich Institute
pg	pico gram
RNA	ribonucleic acid
RR	relative risk
SD	standard deviation
SE(M)	standard error (of the mean)
Th	T helper cell
TNF-α	tumor necrosis factor alpha
ULN	upper limit of normal