IMMUNE RESPONSE AND IMMUNOMODULATION IN CHRONIC HEPATITIS B VIRUS INFECTION

D. SPRENGERS

Immune response and immunomodulation in chronic hepatitis B virus infection

Immuunrespons en immunomodulatie in chronische
Hepatitis B virus infectie

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof.dr. S.W.J. Lamberts
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 1 november 2006 om 13.45 uur

door

Dave Sprengers

geboren te Goirle

Promotiecommissie

Promotor:	Prof.dr. H.L.A. Janssen
Copromotor:	Dr. R.G. van der Molen
Overige leden:	Prof.dr. J.H.P. Wilson Prof.dr. A.D.M.E. Osterhaus Prof.dr. S.W. Schalm

This study was supported by a grant from The Netherlands Organisation for Health Research and Development (ZonMw, Agiko-stipendium dossiernummer 920-03-244).

Publication of this thesis was financially supported by: ZonMw, Zambon Nederland BV, Novartis Pharma BV,

Aan mijn ouders

TU REMANEBIS AMICUS MAXIMUS PERPETUO

Contents

Page

Chapter 1: introduction 1

Chapter 2: Functional impairment of myeloid and plasmacytoid dendritic cells of chronic hepatitis B patients 17

Chapter 3: Flow cytometry of fine-needle-aspiration-biopsies: a new method to monitor intrahepatic immunological environment in chronic viral hepatitis 33

Chapter 4: Different composition of intrahepatic lymphocytes in the immune-tolerance and immune-clearance phase of chronic hepatitis B 45

Chapter 5: Analysis of intrahepatic HBV-specific cytotoxic T-cells during and after acute HBV infection in humans 59

Chapter 6: *In vivo immunization* following virus suppression: a novel approach for inducing immune control in chronic hepatitis B 73

Chapter 7: *In vivo immunization* in combination with peg-interferon for chronic hepatitis B virus infection 79

Chapter 8: Induction of CD4+ CD25+ regulatory T-cells associated with non-response to pegylated Interferon-α therapy for chronic hepatitis B virus infection 93

Chapter 9: Discussion 109

Summary 123
Samenvatting
Dankwoord
Curriculum Vitae

Abbreviations

ALT	alanine ominotransferase
anti-HBc	antibodies against hepatitis B core antigen
anti-HBe	antibodies against hepatitis B e antigen
anti-HBs	antibodies against hepatitis B surface antigen
CHB	chronic hepatitis B virus
CTL	cytotoxic T-cells
DC	dendritic cells
DNA	deoxyribonucleic acid
FNAB	fine-needle aspiration biopsy
GM-CSF	granulocyte/macrophage colony-stimulating factor
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCV	hepatitis C virus
HDV	hepatitis D virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IFN	interferon
IL	interleukin
IVI	in vivo immunization
LIL	liver infiltrating lymphocytes
mDC	myeloid dendritic cells
NK cell	Natural Killer cell
PB	peripheral blood
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cells
Peg-IFN	pegylated interferon
poly (I:C)	polyriboinosinic polyribocytidylic acid
Pre-S1 Ag	hepatitis B pre-S1 antigen
Pre-S2 Ag	hepatitis B pre-S2 antigen
SAC	*Staphylococcus aureus* Cowen strain I
Th1/Th2	type-1/type-2 T helper cell
Th-cell	T-helper cell
TNF	tumor necrosis factor
Treg	regulatory T-cells

CHAPTER 1

Introduction

Modified from:
Immunomodulatory therapy for chronic hepatitis B virus infection
D. Sprengers, H.L.A. Janssen, Fundam Clin Pharmacol 2005; 19: 17-26

Chronic hepatitis B virus infection

Hepatitis B virus (HBV) is one of the most prevalent viral pathogens of man with almost a third of the world population having evidence of infection and around 350 million chronically infected patients [1]. Chronic hepatitis B is characterized by inflammatory liver disease of variable severity and is associated with a significantly increased risk of developing cirrhosis, liver failure and hepatocellular carcinoma [1, 2].

HBV is a non-cytopathic virus and liver injury is mainly mediated by the host immune response against virus infected liver cells and by the production of inflammatory cytokines. A vigorous, polyclonal and multispecific cytotoxic (CTL) and helper T (Th) cell response to HBV is readily detectable in the peripheral blood of patients with acute self-limited hepatitis B, but is weak, antigenically restricted or undetectable in patients with chronic HBV infection [3]. This T cell response is believed to be responsible for the elimination of the hepatitis B virus.

Two major classes of antiviral therapeutics have been adopted to treat the infection: drugs that directly interfere with virus replication and drugs that modulate anti-viral immune response. In most countries lamivudine, adefovir dipivoxil and entecavir are the only approved inhibitors of viral replication (HBV-DNA polymerase inhibitors). Although lamivudine leads to rapid and almost absolute discontinuation of HBV replication [4], breakthrough of resistant HBV variants limits long-term use [5, 6]. These limitations underline the need for specific and non-specific immunotherapeutic strategies in order to enhance or broaden the defective T cell response in chronically infected patients.

In this chapter we will focus on the immune response to HBV and we will review reported data on immunotherapeutic strategies like immunomodulatory drugs (cytokines and Thymic derivates) and vaccine therapies using currently available recombinant anti-HBV vaccines, lipopeptide-based T cell vaccine and newly developed genetic vaccines.

The hepatitis B virus

HBV is a partly double-stranded DNA virus belonging to the group of hepadna viridae [7](fig 1). The replication of HBV is believed to occur preferentially in the hepatocyte. Analysis of the nucleotide sequence revealed 4 open reading frames, regions of the genome witch may code for viral antigens [7]. Although the HBV genome contains only 3200 nucleotides, its compactness and circular composition, employing overlapping genes for production of several viral antigens, make the virus highly efficient for replication. After entry into the hepatocyte's nucleus the virus is uncoated and the genomic DNA is converted to a supercoiled form of covalently closed fully double-stranded DNA, which is transcribed to pregenomic messenger RNA (mRNA). This viral mRNA is transported to the cytoplasm where it codes for the production of viral proteins. mRNA is reverse transcribed into a minus strand of DNA which is utilized as a template for completion of the plus strand of the newly synthesized viral DNA. The HBV-DNA-polymerase exerts both a DNA polymerase activity and a reverse transcriptase activity. Out of these viral proteins and viral DNA in the cytoplasm of the

hepatocyte new HBV-particles are assembled, that subsequently leave the cell into the circulation.

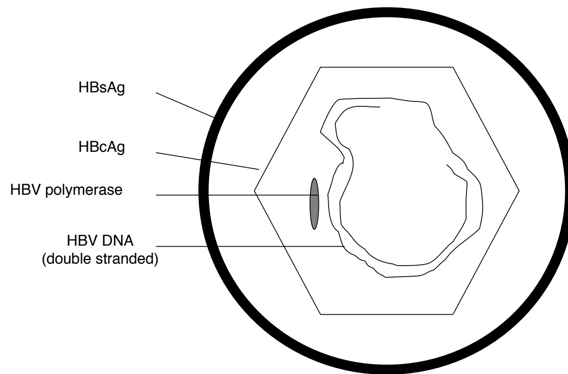


Figure 1. Schematic overview of an HBV-particle, also known as the Dane particle. The HBV-genome encodes for several proteins that are used to assemble the Dane particle. The outer surface of the virus consists of three types of hepatitis B surface antigen (HBsAg). Hepatitis B core antigen (HBcAg) forms the inner core of the Dane particle. The viral DNA and the HBV-polymerase reside inside the inner core of the virus.

The immune response to the hepatitis B virus in humans

Patients infected with HBV may develop one of two types of anti-HBV immune responses. The first is an effective anti-viral response that suppresses viral growth as the result of both non-specific (innate) as well as specific (adaptive) immunity. After early actions of natural killer (NK) cells, NK T cells and antiviral cytokines, individuals with acute self-limited HBV infection mount a vigorous polyclonal and multispecific Th and CTL response to epitopes within the HBV-envelope (HBe), nucleocapsid and polymerase proteins. The peptides are presented to the T cells by professional antigen presenting cells (APC) and on the surface of infected hepatocytes in the context of a MHC class I molecule (fig 2). The response is readily detectable in peripheral blood [8-11]. It often coincides with an elevation of serum alanine-aminotransferase levels and precedes clearance of HBe and HBs (surface) antigens and the development of neutralizing antibodies. Upon recognition of viral peptides CTL acquire the capacity to either cure HBV-infected cells via a non-cytopathic, cytokine-mediated inhibition of HBV replication, or to kill them via perforin-Fas ligand and TNFα-mediated death pathways [12-15]. Both effector functions have been observed during resolution of acute hepatitis B and this type 1 T cell (Th1) response persists even after clinical recovery [16]. In contrast, the second type of anti-HBV immune response is an ineffective one. The HBV-specific immune response is weak, antigenically restricted or undetectable in the blood of chronically infected patients, although individual HBV-specific T cell clones have been isolated and expanded from liver biopsies [17, 18]. Since HBV is considered a non-cytopathic virus and the degree of intrahepatic inflammatory leukocytic infiltrate is regarded as the histological hallmark of the severity of chronic hepatitis B, it has been

postulated that the HBV-specific immune response is too weak to eliminate HBV from all infected hepatocytes, but sufficiently strong to continuously destroy HBV-infected hepatocytes and to induce chronic inflammatory liver disease in persistently infected individuals. The reason for this inefficient, yet harmful nature of the cellular immune response in chronic hepatitis B is currently not known (table 1), but it emphasizes the need for immunomodulatory drugs capable of inducing an effective T cell response that enables eradication of chronic HBV infection.

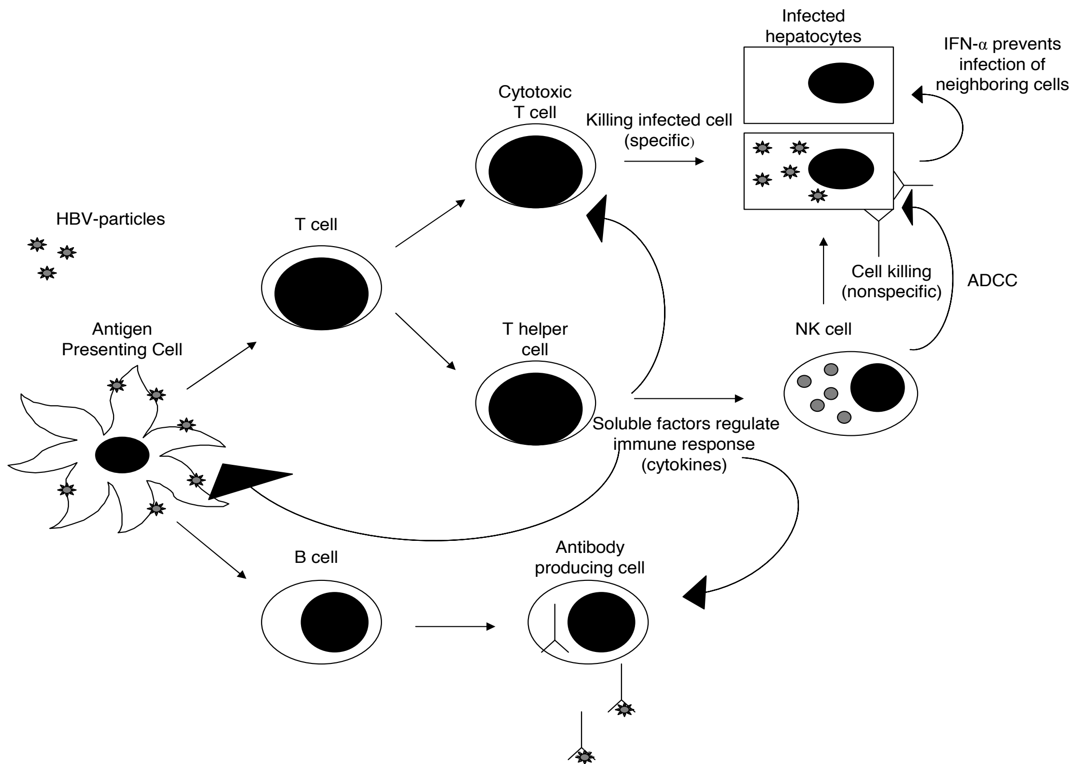


Figure 2. Schematic overview of cellular immune response to the hepatitis B virus. Viral antigens are processed and presented to T cells (on the infected cell surface) and B cells (free antigenic pieces or viruses) by Antigen Presenting Cells. Complexing of antibody produced by B cells, with virus (opsonization) increases the engulfment of the virus by phagocytes and can neutralise virus. Binding of antibody to infected target cells activates antibody-dependent cell-mediated cytotoxicity (ADCC). T cells (helper as well as cytotoxic) produce cytokines with numerous immunoregulatory actions. Products of T cells activate macrophages for killing of ingested virus and natural killer cells for nonspecific cytotoxicity against virus-infected cells. Interferons (IFN-α/β) are produced by infected hepatocytes for protection of surrounding cells against virus infection.

Treatment of chronic hepatitis B

The aim of treatment of chronic viral hepatitis B is to suppress HBV replication before significant irreversible liver damage occurs. Ideally, the primary goals of therapy are to suppress viral replication and to induce remission of liver disease. The long-term goals of treatment are to eliminate the virus, prevent disease progression to cirrhosis or liver cancer and to improve patient survival. Definitions of responses to antiviral therapy can be classified as virologic, biochemical and histological [19]. In patients who were initially HBeAg positive effective treatment of chronic HBV is defined as sustained clearance of circulating HBeAg with production of antibodies to HBe and decrease of HBV-DNA to < 10^5 geq/ml (virologic response); improvement in liver disease as determined by normalisation of serum alanine transaminase levels (biochemical response) and reduction of necroinflammation on liver biopsies (histological response). In active HBeAg negative chronic hepatitis, characterized by detection of anti-HBe antibodies without HBeAg in serum, detectable HBV DNA using non-PCR-based methods, elevated alanine transaminase levels and hepatic necro-inflammation [20], virologic response is defined as decrease of HBV-DNA to < 10^5 geq/ml [19].

Reports on immunomodulatory antiviral agents that are under review (table 2) will be analysed for these response parameters.

Table 1. Possible causes of persistent HBV infection

Factor	
Viral factors	- High viral load - High replication rate - Viral inhibition of antigen presentation - Viral mutations that abrogate, anergize or antagonize antigen recognition by specific T-cells - Immunosuppressive effects of virus
Host factors	- Immunological tolerance - Exhaustion of T-cell response - Insufficient co-stimulation of virus-specific T-cells - Infection of immunologically privileged sites - Host HLA background - Inefficient viral antigen processing by antigen presenting cells or hepatocytes - Inefficient responsiveness of B- or T-cells; alteration of the Th1-Th2 balance

Immunotherapeutic strategies to control chronic hepatitis B virus infection

cytokines

Interferon-α therapy

Interferons are cytokines with immunomodulatory, antiproliferative and antiviral properties. Interferon-α (IFN-α) has marked immunoregulatory but less pronounced antiviral effects [21, 22]. IFN-α was licensed in 1992 and after being extensively studied, it is the first approved treatment for chronic HBV infection in most countries. IFN-α has been shown to inhibit viral RNA pregenomic packaging into core particles and also to

enhance expression of HBsAg on hepatocytes [23].
Multiple studies of IFN-α therapy in chronic HBV have demonstrated beneficial effects. A sustained response improves survival, but frequency of response is low [24-26]. According to a meta-analysis which included 15 randomised controlled studies involving 837 patients IFN-α achieves HBeAg-seroconversion in 33% of treated patients using 16 weeks of therapy compared to 12% of controls. Importantly, loss of HBsAg was recorded in 7.8% of IFN-treated patients compared with only 1.8% of controls. Loss of detectable HBV DNA, as determined by hybridisation assay, and normalisation of ALT level were also more common in treated than control patients [27]. However, results were obtained in selected patient cohorts with marked disease activity at baseline. Recent large studies showed response rates of 20% [28]. There was a strong correlation between baseline ALT and HBeAg seroconversion rate: approximately 5-10% for serum ALT two-fold elevated, 20% for baseline ALT of two-fivefold the upper limit of normal and 30-40% for ALT five-fold elevated. Post hoc analysis also revealed other major pre-treatment factors correlated with response to IFN-α: low HBV DNA levels and increased scores of activity and fibrosis on liver biopsy [19].
The optimal duration of interferon therapy for HBV is not well established. In a large multicenter trial it was demonstrated that continuing therapy for 32 weeks was beneficial in patients who did not clear HBeAg after 16 weeks, but who had at that time low levels of HBV DNA (<10 pg/ml) [29]. The durability of responses and continued benefit of IFN-α therapy were recently assessed in several long-term follow-up studies. Studies from North America and Europe reported that 95-100% of responders remained HBeAg negative during 5-10 years of follow-up and ultimately 30-86% of responders lost HBsAg. In contrast, Asian studies show only low rate of durable response with no obvious difference between controls and treated patients [30-33].
Patients who were initially HBeAg negative appear to relapse more often after IFN-α therapy than HBeAg positive patients. In HBeAg-negative chronic viral hepatitis response rates are highly variable with 6-24% of patients maintaining a sustained response 12-18 months after cessation of therapy [34, 35]. More recently a study showed a beneficial effect of 24 months of IFN-α therapy, with 30% sustained responders in a selected group of HBeAg negative patients [30].

Recently, Pegylated IFN-α (Peg-IFN) was developed through the process of pegylation, in which a polyethylene glycol (PEG) polymer molecule is attached to the base IFN-α molecule to produce a drug with a prolonged half-life [36]. After showing considerable improvement in efficacy over conventional IFN-α in chronic hepatitis C infection, preliminary results showed that after a 24-weeks follow-up twice as many HBeAg-positive patients receiving Peg-IFNα-2a for 6 months achieved both virologic and biochemical response compared with patients receiving conventional IFN-α; 24% vs, 12% respectively [37]. In a large multicentre, double-blind controlled trial Peg-IFNα-2b monotherapy for 52 weeks induced loss of HBeAg in 36% of patients after 26

weeks of follow-up [38]. These results indicate that Peg-IFN treatment is at least equal but probably superior in efficacy to conventional IFN-α for HBeAg-positive CHB. Additionally, in this multicentre trial it was investigated whether a combination of Peg-IFNα-2b and the nucleoside analogue lamivudine has beneficial effects. The combination Peg-IFNα-2b – lamivudine resulted in a higher HBeAg seroconversion rate at end of treatment compared to patients treated with Peg-IFNα-2b monotherapy, but equal response rates at the end of follow up. Results of a large study (n = 537) comparing 48 weeks of Peg-IFNα-2a with and without lamivudine to lamivudine alone, in the treatment of HBeAg-negative patients, showed that the combination of Peg-IFNα-2a with lamivudine did not improve response rates over Peg-IFNα-2a monotherapy. At the end of 24 weeks follow-up virologic response was achieved in 43% Peg-IFNα-2a-treated patients vs. 44% of patients treated with the combination therapy [39].

Table 2. Immunological functions of components used as immunomodulatory drugs to improve the immune response to HBV

Component	
IFN-α	- Inhibits viral replication; degrades viral components - Induces production of IL-15 and consequently enhances T-cell growth - Augments lytic activity of NK cells and CTL - Enhances expression of cell-surface antigens by the major histocompatibility complex (MHC) - Modulates production of proinflammatory cytokines like IL-1, TNF-α and IL-8
IL-2	- Activates T cells and induces differentiation of B cells; growth factor of T- and B-cells - Stimulates production of antiviral cytokines TNF-α and IFN-γ
IL-12	- Proliferation of pre-activated T-cells and NK cells - Enhances generation of CTL - Augments cytotoxic activity of CTL and NK cells - Induces production of antiviral cytokines TNF-α and particularly IFN-γ
GM-CSF	- Stimulates differentiation of hematopoietic precursor cells - Major stimulatory cytokine for viability, differentiation and function of DC, the most important cell type for stimulation of primary T-cell mediated immune responses. - Increases expression of MHC class II molecules on antigen presenting cells.
Thymosine-α	- Stimulates activity of NK cell-mediated cytotoxicity - Stimulates production of IL-2 and IFN-γ - Increases maturation of T-cells
Vitamin E	- Increases lymphocyte proliferation and IL-2 production - Raises activity of NK cells

Interleukine-2

Interleukine-2 (IL-2) has been known for many years as a T cell growth promoting factor. It has been demonstrated in a pilot study that IL-2 enhances CD4+ T cells and NK cells in chronic hepatitis B [40]. Despite the report of clearance of HBsAg and the appearance of anti-HBs antibodies in an HIV-infected patient after recombinant IL-2 therapy [41], a pilot study of natural human IL-2 in patients with chronic HBV did not

result in either a biochemical or immunological response [42]. A randomised controlled trial of IL-2 versus placebo in HBV patients co-infected with HIV did show a significant increase in CD4+ T cell count but IL-2 treatment did not modify HBV replication [43].
In a double-blind randomised controlled trial 31 patients with chronic HBV infection received different doses of IL-2 subcutaneously [44]. No differences in the frequency of loss of HBeAg were observed between treated and placebo groups and decrease in serum HBV DNA concentration was similar in both populations. Studies investigating IL-2 in combination with other antiviral therapies have also been performed. A randomised controlled trial showed no additional effect of a combination of IL-2 with IFN-α, with respect to HBV DNA decrease, HBeAg seroconversion or ALT normalisation [45]. Combining HBsAg vaccination with lamivudine only or with lamivudine and IL-2 did not show any additional efficacy of IL-2 [46].

Interleukine-12
Interleukine-12 (IL-12) is a product of activated inflammatory cells like monocytes, macrophages and dendritic cells [47]. IL-12 has a direct proliferative effect on pre-activated T cells and NK cells, and it induces these cells to produce cytokines, such as granulocyte-macrophage colony- stimulatory factor, TNF-α and particularly IFN-γ. Therefore, IL-12 favours the differentiation of Th1 cells.
It was shown that amongst patients with chronic HBV undergoing IFN-α treatment, only those who clear HBV show a substantial increase in the production of biologically active IL-12 [48]. The peak of serum IL-12 occurred after the hepatitis flare but preceded or coincided with HBeAg seroconversion. Recently a multicentre open label phase I/II trial was conducted to test the efficacy of once weekly injections of 0.03, 0.25 and 0.50 μg/kg rHuIL-12 over a 12 week course [49]. rHuIL-12 was generally well tolerated, although associated with transient decreases of neutrophils and lymphocyte counts, and with elevations in serum transaminases and bilirubin. ALT normalisation together with sustained clearance of HBV DNA and HBeAg was observed at the end of follow-up in 3/46 patients, one in every dose group. Two patients seroconverted to anti-HBe. Thus, the antiviral activity of rHuIL-12 in patients with chronic HBV does not appear to be advantageous compared to other currently available treatments. Alternative dosing strategies may increase the antiviral efficacy of IL-12.

Granulocyte-macrophage colony-stimulating factor
Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) is the major stimulatory cytokine for the viability, differentiation and function of dendritic cells [50]. It augments the expression of T cell co-stimulatory molecules and increases the expression of MHC class II molecules, which are crucial for antigen recognition and initiation of an immune response by CD4+ T cells [51-53]. The consequences of the immunostimulatory activity of rhGM-CSF have been enhanced T cell proliferative responses to suboptimal antigen concentrations and the augmentation of primary antibody responses *in vitro* and *in vivo* [54].
A pilot study including 9 patients with chronic HBV infection showed that rhGM-CSF

therapy for 6 weeks significantly enhanced TNF-α and IL-1 production in cultured mononuclear cells. A significant reduction of serum HBV DNA levels was also observed [55]. In a preliminary study for previous IFN non-responders rhGM-CSF plus IFN-α for 4 months led to HBV DNA negativity by a hybridization assay in 5 of 8 patients [56]. These data suggest that the combination of rhGM-CSF and IFN-α may be more effective at clearing HBV DNA than IFN-α alone.

Vaccine therapy for chronic hepatitis B virus infection

Immunotherapy using recombinant anti-HBV vaccine.

Pilot clinical studies established that specific vaccine therapy by standard anti-HBV vaccination (GenHevac B) could cancel or reduce HBV replication in of chronic carrier subjects [57, 58]. These results were confirmed in a recent controlled study [59] showing both the efficacy and limitations of (standard) vaccine therapy in chronic infection. The 118 included patients were 'naive' subjects who had never received any previous HBV therapy. They were given either five intramuscular injections of 20μg of a preS2/S vaccine (GenHevac B, Pasteur-Merieux; n = 46), an S vaccine (Recombivax Merck & Co.; n = 34) or placebo as a control (n = 37). After 12 months follow-up and five vaccine injections, there was no difference in the rate of serum HBV DNA reduction as determined by a hybridisation assay, between vaccinated and unvaccinated subjects. However, in the first 6 months following vaccination patients treated with either vaccine were significantly more likely to clear serum HBV DNA and seroconvert to anti-HBe than untreated controls. At 12 months these differences lost significance due to increased seroconversion in the placebo group. Disappearance of serum HBsAg was not observed. In another randomized placebo controlled therapeutic vaccination trial including 22 chronically infected patients, a vaccine containing pre-S1, pre-S2 and S antigenic components, did not induce an HBsAg-specific induction of Th1-lymphokines or HBV-specific CD8+ T cells [60]. This result might explain why a therapeutic surface vaccine, although safe and well tolerated is mostly not able to break tolerance leading to the clearance of the hepatitis B virus.

T cell vaccine for chronic hepatitis B virus infection.

The goal of an alternative vaccination strategy is to use peptide epitopes that are recognized by cytotoxic T lymphocytes (CTL) as immunogens for the development of prophylactic and therapeutic vaccines. Recently, a lipopeptide-vaccine (CY-1899) was designed consisting of HBV core antigen peptide 18-27 as the CTL epitope, tetanus toxoid peptide 830-843 as the T helper peptide, and two palmitic acid molecules as the lipids. A dose escalation trial (5, 50, and 500 μg) carried out in 26 normal subjects showed that this vaccine was safe and able to induce a primary HBV-specific CTL response [61]. The aim of a following study was to determine whether repeated doses of CY-1899 given subcutaneously to 19 patients with chronic hepatitis B could initiate in vivo CTL activity and viral clearance [62]. Patients received up to 4 doses (ranging from 0.05 mg to 15 mg) 6 weeks apart. Administration of the single-epitope vaccine, CY-1899, initiated CTL activity, but of a magnitude lower than that observed during

spontaneous HBV clearance. This low-level CTL activity was not associated with viral decline and no significant changes in liver biochemistry or viral serology were observed during follow-up.

DNA vaccines for chronic hepatitis B virus infections
New approaches of vaccination for HBV are now based on intramuscular injection of plasmid DNA encoding HBV antigen [63]. The so-called DNA vaccines induce immune responses against antigens synthesized *in vivo* after introduction of DNA's encoding antigen sequences. In the context of class I and class II MHC molecules, the endogenous synthesis of antigen leads to appropriate antigen-presentation which will result in strong humoral and cellular immune responses. This novel approach to immunization may overcome failures of the traditional antigen-based approach and may provide effective therapeutic vaccines.
After being extensively studied in animal models [63, 64], recently in a phase I clinical trial three groups of healthy volunteers (n = 4 each group) received three administrations of DNA encoding the surface antigen of HBV at a dose of 1, 2, or 4 μg [65]. The vaccine was safe and well tolerated. All the volunteers developed protective antibody responses. In volunteers who were positive for the HLA class I A2 allele, the vaccine also induced antigen-specific CD8+ T cells that bound HLA-A2/$HBsAg_{335–343}$ tetramers, secreted IFN-γ and lysed target cells presenting an HBsAg CTL epitope. Enumeration of HBsAg-specific T cells producing cytokines indicated preferential induction of a Type 1 T helper cell response. These results provide the first demonstration of a DNA vaccine inducing protective antibody titres and both humoral and cell-mediated immune responses in humans. Further studies are needed to evaluate therapeutic efficacy of DNA vaccines in the setting of chronic HBV infection.

Other immunomodulators
Thymosine alpha1
Thymosine alpha-1 is a bovine thymus extract that accelerates the replenishment and maturation of thymocytes, stimulates differentiation into active T cells and restores T cell function by T cell-mediated antibody production [66]. Thymosine alpha-1 (T-α1) concentrations are low in patients with chronic HBV [67]. Trials of T-α1 suggest that it may be at least as effective as IFN-α in patients with raised serum ALT who are HBeAg and HBV DNA positive [68, 69]. A recent meta-analysis containing 353 patients evaluated the efficacy of T-α1 [68, 70-72]. Tolerance to T-α1 was good in all trials. Compared to placebo there was no significant biochemical response but odds ratio for virologic response to T-α1, measured by HBeAg seroconversion rate and HBV DNA clearance in a hybridization assay, at the end of therapy, at 6 and at 12 months post-treatment were 0.56, 1.67 and 2.67 respectively. Finally there was a significant increase in virologic response overtime after discontinuation of T-α1 treatment. This suggests that viral clearance may be non-cytolitic and may be mediated by IFN-γ and TNF-α secreted by activated CTL [73]. It was shown that IFN-γ producing CD4+ T cells are increased in peripheral blood of T-α1 treated patients and that NK T cells and CTL

appear to be augmented in the liver [74].
Few studies have concentrated on combination therapy. A recent trial analysed the efficacy of T-α1 plus famciclovir for 26 weeks vs. famciclovir alone vs. placebo [75]. The combination group showed significantly higher HBV DNA reduction, and at 52 weeks 5 patients (15.6%) experienced HBeAg seroconversion vs. none in the other groups. Sustained serological clearance of HBeAg was associated with activation of CD4+ HBV-specific T-cell reactivity.

Vtamin E

Vitamin E (α-tocopherol) is an essential vitamin with anti-oxidant properties [76] that is also able to enhance the cell-mediated immunity [76, 77]. Recent studies showed its possible beneficial role in the treatment of chronic hepatitis C virus infection [78, 79]. In a randomised controlled pilot study 15 – of which 12 HBeAg negative- patients with chronic HBV infection were treated for three months with 300 mg twice daily [80]. Baseline ALT and HBV DNA were comparable to 17 non-treated patients. At the end of a follow-up of 15 months after therapy, ALT and HBV DNA serum levels were significantly lower in patients receiving vitamin E, with 7 subjects exhibiting a complete response (ALT normal and HBV DNA negative in hybridisation assay) vs. none of the controls.
Vitamin E may probably be used as a supplementary treatment rather than monotherapy for chronic HBV infection. Larger studies are needed to confirm these interesting results and to evaluate different dosing regimes and combinations with other antiviral drugs.

Conclusions

The primary aim in the treatment of chronic hepatitis B is to induce sustained disease remission and prevent liver failure and/or hepatocellular carcinoma. The recent emergence of drug-resistant HBV mutants and post-treatment relapse as a consequence of nucleoside analogue monotherapy emphasizes that the principal goal should be to stimulate a successful immune response.
At present the only evident effective immunomodulatory therapy is IFN-α. Compared to conventional IFN-α Peg-IFN appears to achieve higher response rates in HBeAg positive patients. Both in HBeAg-positive and HBeAg-negative patients combination of Peg-IFN and lamivudine was not superior to Peg-IFN alone. Important antiviral and immunomodulatory effects of other cytokines in combination with pegylated interferon-α, may be more successful than monotherapy with either component.
Future immunotherapy approaches should aim to induce a co-ordinate activation of all components of the adaptive immune response to restore a multispecific anti-HBV immune response. Vaccines able to elicit both HBV-specific T and B cell responses in humans, against multiple HBV antigens, present a logical and promising immunomodulatory strategy. However, many factors, like antigen selection, frequency and timing of vaccination and safety issues, need to be evaluated before therapeutic vaccination can be applied to induce durable disease remission in patients with chronic HBV infection.

References

1. Kane M. Global programme for control of hepatitis B infection. Vaccine 1995; 13 Suppl 1:S47-9.
2. Wright TL, Lau JY. Clinical aspects of hepatitis B virus infection. Lancet 1993; 342:1340-4.
3. Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. Annu Rev Immunol 1995; 13:29-60.
4. Benhamou Y, Dohin E, Lunel-Fabiani F, et al. Efficacy of lamivudine on replication of hepatitis B virus in HIV-infected patients. Lancet 1995; 345:396-7.
5. Lai CL, Chien RN, Leung NW, et al. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med 1998; 339:61-8.
6. Angus P, Vaughan R, Xiong S, et al. Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. Gastroenterology 2003; 125:292-7.
7. Tiollais P, Pourcel C, Dejean A. The hepatitis B virus. Nature 1985; 317:489-95.
8. Maini MK, Boni C, Lee CK, et al. The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. J Exp Med 2000; 191:1269-80.
9. Rehermann B, Lau D, Hoofnagle JH, Chisari FV. Cytotoxic T lymphocyte responsiveness after resolution of chronic hepatitis B virus infection. J Clin Invest 1996; 97:1655-65.
10. Livingston BD, Crimi C, Grey H, et al. The hepatitis B virus-specific CTL responses induced in humans by lipopeptide vaccination are comparable to those elicited by acute viral infection. J Immunol 1997; 159:1383-92.
11. Guidotti LG, Ando K, Hobbs MV, et al. Cytotoxic T lymphocytes inhibit hepatitis B virus gene expression by a noncytolytic mechanism in transgenic mice. Proc Natl Acad Sci U S A 1994; 91:3764-8.
12. Rehermann B. Intrahepatic T cells in hepatitis B: viral control versus liver cell injury. J Exp Med 2000; 191: 1263-8.
13. Ganem D. Persistent infection of humans with hepatitis B virus: mechanisms and consequences. Rev Infect Dis 1982; 4:1026-47.
14. 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.
15. Heise T, Guidotti LG, Cavanaugh VJ, Chisari FV. Hepatitis B virus RNA-binding proteins associated with cytokine-induced clearance of viral RNA from the liver of transgenic mice. J Virol 1999; 73:474-81.
16. Guidotti LG, Chisari FV. Cytokine-mediated control of viral infections. Virology 2000; 273:221-7.
17. Barnaba V, Franco A, Alberti A, Balsano C, Benvenuto R, Balsano F. Recognition of hepatitis B virus envelope proteins by liver-infiltrating T lymphocytes in chronic HBV infection. J Immunol 1989; 143:2650-5.
18. Ferrari C, Penna A, Giuberti T, et al. Intrahepatic, nucleocapsid antigen-specific T cells in chronic active hepatitis B. J Immunol 1987; 139:2050-8.
19. Lok AS, Heathcote EJ, Hoofnagle JH. Management of hepatitis B: 2000--summary of a workshop. Gastroenterology 2001; 120:1828-53.
20. Hadziyannis SJ, Vassilopoulos D. Hepatitis B e antigen-negative chronic hepatitis B. Hepatology 2001; 34: 617-24.
21. Oritani K, Kincade PW, Zhang C, Tomiyama Y, Matsuzawa Y. Type I interferons and limitin: a comparison of structures, receptors, and functions. Cytokine Growth Factor Rev 2001; 12:337-48.
22. Levy DE. Whence interferon? Variety in the production of interferon in response to viral infection. J Exp Med 2002; 195:F15-8.
23. Lau JY, Bain VG, Naoumov NV, Smith HM, Alexander GJ, Williams R. Effect of interferon-gamma on hepatitis B viral antigen expression in primary hepatocyte culture. Hepatology 1991; 14:975-9.
24. 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.
25. Villeneuve JP, Condreay LD, Willems B, et al. Lamivudine treatment for decompensated cirrhosis resulting from chronic hepatitis B. Hepatology 2000; 31:207-10.
26. van Zonneveld M, Honkoop P, Hansen BE, et al. Long-term follow-up of alpha-interferon treatment of patients with chronic hepatitis B. Hepatology 2004; 39:804-10.
27. 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.
28. 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.
29. 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.
30. Lampertico P, Del Ninno E, Vigano M, et al. Long-term suppression of hepatitis B e antigen-negative

chronic hepatitis B by 24-month interferon therapy. Hepatology 2003; 37:756-63.
31. 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.
32. 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.
33. Ikeda K, Saitoh S, Suzuki Y, et al. Interferon decreases hepatocellular carcinogenesis in patients with cirrhosis caused by the hepatitis B virus: a pilot study. Cancer 1998; 82:827-35.
34. Manesis EK, Hadziyannis SJ. Interferon alpha treatment and retreatment of hepatitis B e antigen-negative chronic hepatitis B. Gastroenterology 2001; 121:101-9.
35. Oliveri F, Santantonio T, Bellati G, et al. Long term response to therapy of chronic anti-HBe-positive hepatitis B is poor independent of type and schedule of interferon. Am J Gastroenterol 1999; 94:1366-72.
36. Kozlowski A, Harris JM. Improvements in protein PEGylation: pegylated interferons for treatment of hepatitis C. J Control Release 2001; 72:217-24.
37. Cooksley WG, Piratvisuth T, Lee SD, et al. Peginterferon alpha-2a (40 kDa): an advance in the treatment of hepatitis B e antigen-positive chronic hepatitis B. J Viral Hepat 2003; 10:298-305.
38. Janssen HLA, Senturk H, Zeuzem S, et al. Peginterferon alfa-2B and lamivudine combination therapy compared with peginterferon alfa-2B for chronic HBeAg positive hepatitis B: a randomized controlled trial in 307 patients. Hepatology 2003; 38:1323.
39. Marcellin P, Lau GKK, Bonino F, et al. Peginterferon alfa-2a (40KD) (pegasys) monotherapy is more effective than lamivudine monotherapy in the treatment of HBeAg-negative chronic hepatitis B: 73-week results from a phase III, partially double-blind study of pegasys alone vs. pegasys plus lamivudine vs. lamivudine. J Hepatol 2004; 40 suppl 1:95A.
40. Onji M, Kondoh H, Horiike N, et al. Effect of recombinant interleukin 2 on hepatitis B e antigen positive chronic hepatitis. Gut 1987; 28:1648-52.
41. Gianotti N, Uberti-Foppa C, Boeri E, et al. Hepatitis B surface antigen clearance and appearance of antibodies against hepatitis B surface antigen after treatment with recombinant interleukin 2 in a human immunodeficiency virus-infected patient. Hepatology 2000; 32:1409-10.
42. Tilg H, Vogel W, Tratkiewicz J, et al. Pilot study of natural human interleukin-2 in patients with chronic hepatitis B. Immunomodulatory and antiviral effects. J Hepatol 1993; 19:259-67.
43. Thibault V, Delaugerre C, Calvez V, Costagliola D, Tubiana R, Katlama C. Interleukin 2 treatment does not modify hepatitis B or C replication in human immunodeficiency virus-infected patients: results from a randomized control trial. Hepatology 2002; 35:238-9.
44. Artillo S, Pastore G, Alberti A, et al. Double-blind, randomized controlled trial of interleukin-2 treatment of chronic hepatitis B. J Med Virol 1998; 54:167-72.
45. Bruch HR, Korn A, Klein H, et al. Treatment of chronic hepatitis B with interferon alpha-2b and interleukin-2. J Hepatol 1993; 17 Suppl 3:S52-5.
46. Dahmen A, Herzog-Hauff S, Bocher WO, Galle PR, Lohr HF. Clinical and immunological efficacy of intradermal vaccine plus lamivudine with or without interleukin-2 in patients with chronic hepatitis B. J Med Virol 2002; 66:452-60.
47. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol 2003; 3:133-46.
48. Rossol S, Marinos G, Carucci 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.
49. Zeuzem S, Hopf U, Carreno V, et al. A phase I/II study of recombinant human interleukin-12 in patients with chronic hepatitis C. Hepatology 1999; 29:1280-7.
50. Markowicz S, Engleman EG. Granulocyte-macrophage colony-stimulating factor promotes differentiation and survival of human peripheral blood dendritic cells in vitro. J Clin Invest 1990; 85:955-61.
51. Fischer HG, Frosch S, Reske K, Reske-Kunz AB. Granulocyte-macrophage colony-stimulating factor activates macrophages derived from bone marrow cultures to synthesis of MHC class II molecules and to augmented antigen presentation function. J Immunol 1988; 141:3882-8.
52. Morrissey PJ, Bressler L, Park LS, Alpert A, Gillis S. Granulocyte-macrophage colony-stimulating factor augments the primary antibody response by enhancing the function of antigen-presenting cells. J Immunol 1987; 139:1113-9.
53. Germain RN. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. Cell 1994; 76:287-99.
54. Liehl E, Hildebrandt J, Lam C, Mayer P. Prediction of the role of granulocyte-macrophage colony-stimulating factor in animals and man from in vitro results. Eur J Clin Microbiol Infect Dis 1994; 13 Suppl 2:S9-17.
55. Martin J, Quiroga JA, Bosch O, Carreno V. Changes in cytokine production during therapy with granulocyte-macrophage colony-stimulating factor in patients with chronic hepatitis B. Hepatology 1994; 20:1156-61.

56. Van Thiel DH, Friedlander L, Kania RJ, Molloy PJ, Hassanein T, Faruki H. A preliminary experience with GM-CSF plus interferon in patients with HBV and HCV resistant to interferon therapy. J Viral Hepat 1997; 4 Suppl 1:101-6.
57. 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.
58. Pol S, Couillin I, Michel ML, et al. Immunotherapy of chronic hepatitis B by anti HBV vaccine. Acta Gastroenterol Belg 1998; 61:228-33.
59. Pol S, Nalpas B, Driss F, et al. Efficacy and limitations of a specific immunotherapy in chronic hepatitis B. J Hepatol 2001; 34:917-21.
60. Jung MC, Gruner N, Zachoval R, et al. Immunological monitoring during therapeutic vaccination as a prerequisite for the design of new effective therapies: induction of a vaccine-specific CD4+ T-cell proliferative response in chronic hepatitis B carriers. Vaccine 2002; 20:3598-612.
61. Vitiello A, Ishioka G, Grey HM, 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.
62. Heathcote J, McHutchison J, Lee S, et al. 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.
63. Thermet A, Rollier C, Zoulim F, Trepo C, Cova L. Progress in DNA vaccine for prophylaxis and therapy of hepatitis B. Vaccine 2003; 21:659-62.
64. Davis HL, McCluskie MJ, Gerin JL, Purcell RH. DNA vaccine for hepatitis B: evidence for immunogenicity in chimpanzees and comparison with other vaccines. Proc Natl Acad Sci U S A 1996; 93:7213-8.
65. Roy MJ, Wu MS, Barr LJ, et al. Induction of antigen-specific CD8+ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. Vaccine 2000; 19:764-78.
66. Li CL, Zhang T, Saibara T, et al. Thymosin alpha1 accelerates restoration of T cell-mediated neutralizing antibody response in immunocompromised hosts. Int Immunopharmacol 2002; 2:39-46.
67. Sherman KE, Jones CC, Goldstein AL, Naylor PH. Low thymosin alpha-1 concentrations in patients chronically infected with the hepatitis B virus. Viral Immunol 1991; 4:195-9.
68. 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.
69. Andreone P, Cursaro C, Gramenzi A, et al. A randomized controlled trial of thymosin-alpha1 versus interferon alfa treatment in patients with hepatitis B e antigen antibody--and hepatitis B virus DNA--positive chronic hepatitis B. Hepatology 1996; 24:774-7.
70. Chan HL, Tang JL, Tam W, Sung JJ. The efficacy of thymosin in the treatment of chronic hepatitis B virus infection: a meta-analysis. Aliment Pharmacol Ther 2001; 15:1899-905.
71. Mutchnick MG, Lindsay KL, Schiff ER, et al. 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.
72. Zavaglia C, Severini R, Tinelli C, et al. A randomized, controlled study of thymosin-alpha1 therapy in patients with anti-HBe, HBV-DNA-positive chronic hepatitis B. Dig Dis Sci 2000; 45:690-6.
73. Guidotti LG, Ishikawa T, Hobbs MV, Matzke B, Schreiber R, Chisari FV. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. Immunity 1996; 4:25-36.
74. Sugahara S, Ichida T, Yamagiwa S, et al. Thymosin-alpha1 increases intrahepatic NKT cells and CTLs in patients with chronic hepatitis B. Hepatol Res 2002; 24:346-354.
75. Lau GK, Nanji A, Hou J, et al. Thymosin-alpha1 and famciclovir combination therapy activates T-cell response in patients with chronic hepatitis B virus infection in immune-tolerant phase. J Viral Hepat 2002; 9:280-7.
76. Meydani M. Vitamin E. Lancet 1995; 345:170-5.
77. Gogu SR, Blumberg JB. Vitamin E increases interleukin-2 dependent cellular growth and glycoprotein glycosylation in murine cytotoxic T-cell line. Biochem Biophys Res Commun 1993; 193:872-7.
78. von Herbay A, Stahl W, Niederau C, Sies H. Vitamin E improves the aminotransferase status of patients suffering from viral hepatitis C: a randomized, double-blind, placebo-controlled study. Free Radic Res 1997; 27:599-605.
79. Ota Y, Sasagawa T, Suzuki K, et al. Vitamin E supplementation increases polyunsaturated fatty acids of RBC membrane in HCV-infected patients. Nutrition 2004; 20:358-63.
80. Andreone P, Fiorino S, Cursaro C, et al. Vitamin E as treatment for chronic hepatitis B: results of a randomized controlled pilot trial. Antiviral Res 2001; 49:75-81.

CHAPTER 2

Functional impairment of myeloid and plasmacytoid dendritic cells of chronic hepatitis B patients

R.G. van der Molen,[1] D. Sprengers,[1] R.S. Binda,[1] E.C. de Jong,[2] H.G.M. Niesters[3] J.G. Kusters,[1] J. Kwekkeboom,[1] and H.L. A. Janssen[1]

[1] Dept. of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands
[2] Dept. of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
[3] Dept. of Virology, Erasmus MC University Medical Center, Rotterdam, The Netherlands

Hepatology 2004; 40: 738-746

Abstract

Dendritic cells (DC) play an important role in the induction of T cell responses. We hypothesize that the hampered anti-viral T cell response in chronic hepatitis B patients is a result of impaired DC function. In this study, we compared the number, phenotype and functionality of two important blood precursor DC, myeloid DC (mDC) and plasmacytoid DC (pDC), of chronic hepatitis B patients with healthy volunteers. No differences in percentages of mDC and pDC in peripheral blood mononuclear cells were observed between chronic hepatitis B patients and healthy controls. The allostimulatory capacity of isolated and *in vitro* matured mDC, but not of pDC, was significantly decreased in patients compared to controls. Accordingly, a decreased percentage of mDC expressing CD80 and CD86 was observed after maturation, compared to controls. In addition, mDC of patients showed a reduced capacity to produce tumor necrosis factor-α after a stimulus with synthetic double stranded RNA and interferon-γ. Purified pDC from patients produced less interferon-α, an important antiviral cytokine, in response to stimulation with *Staphylococcus aureus* Cowan strain I than pDC isolated from controls. In conclusion, we show that mDC and pDC are functionally impaired in patients with chronic hepatitis B. This might be an important way by which HBV evades an adequate immune response leading to viral persistence and disease chronicity.

Introduction

Hepatitis B virus (HBV) infection represents an enormous health problem world-wide. Today more than 350 million people are chronically infected with HBV and are at risk to develop liver cirrhosis or hepatocellular carcinoma. Up till now it is unclear why an individual develops a chronic carrier state. However, an inadequate immune response of the host is thought to play a critical role in the chronicity of the infection [1]. To recover from an acute HBV infection both a strong humoral and cellular immune response is required. During an acute infection patients exhibit a multispecific and polyclonal cytotoxic T cell (CTL) response and a strong type-1 T helper cell (Th1) response [2, 3]. Such HBV specific T cell responses are generally undetectable in chronic patients [4, 5]. Dendritic cells (DC) represent the most potent antigen presenting cells, and thus play an important role in the induction of specific T cell responses [6]. Functional defects in DC could therefore be an important mechanism of virus to evade host immune responses. In several chronic viral infections, such as human immune deficiency virus-1 and hepatitis C, impaired function of DC has been demonstrated [7-11]. For HBV evidence exists as well, that shows impaired DC function in chronic patients [12, 13]. Data on the functionality of DC in chronic HBV patients was obtained with in vitro generated monocyte derived DC (moDC). Recently, Osugi et al showed several differences between moDC and the in vivo present myeloid DC [14]. Therefore it would be more accurate to isolate and study the functionality of DC precursors present in vivo. Two major DC precursors are the myeloid (mDC) and plasmacytoid (pDC) dendritic cells [15, 16]. MDC are characterized by the absence of surface expression of lineage markers, the presence of markers such as CD1c (blood dendritic cells antigen-1 (BDCA1)) and CD11c. In response to bacterial compounds or CD40 ligand, mDC can produce large amounts of IL-12 and they require the presence of granulocyt/macrophage colony-stimulating factor (GM-CSF) for survival [15].
PDC, on the other hand, are characterized by the negativity of lineage markers and the presence of BDCA2, BDCA4 and CD123 (IL-3 receptor α-chain), they require the presence of IL-3 for survival and can produce high amounts of type I interferon (IFN), IFN-α and IFN-β, upon exposure to viruses as well as bacterial components such as CpG oligonucleotides [17-19]. The release of IFN-αβ initiates a cascade of event that eventually leads to the elimination of the virus. It can act on several cells and turn on biochemical pathways that restrict viral replication and render host cells resistant to further viral infection [20]. Moreover, type I IFN is frequently used as therapy for chronic HBV patients and leads to disease remission in approximately 20% to 35% of cases [21, 22]. Therefore, the role of type I IFN producing pDC could be of great interest in the pathogenesis of chronic HBV infection.
The aim of this study was to determine the number, phenotype and functionality of mDC and pDC precursor subsets in peripheral blood of chronic HBV patients and to compare these characteristics with those of healthy volunteers.

Material and Methods

Patients and controls

Peripheral heparinized blood samples were obtained from 30 patients with chronic hepatitis B (table 1). All patients were negative for antibodies against human immune deficiency virus, hepatitis C and hepatitis D. None of the patients was treated for chronic HBV infection or received any other medication 6 months previous to blood sampling. All patients had biopsy proven chronic hepatitis with minimal to moderate fibrosis. The median serum HBV-DNA load was 1.1×10^8 geq/ml (range 1.0×10^3 – 1.2×10^{10}) and median alanine transaminase level (ALT) was 48 U/l (range 9 – 243). Fifteen patients were HBV envelope antigen (HBeAg) positive and 15 patients were HBeAg negative and had antibodies to HBeAg. A control group, matched for age, gender and race, comprised of 19 healthy subjects who had no evidence of exposure to HBV (HBV surface antigen (HBsAg) negative). The study was approved by the local ethics committee and all patients and controls in the study gave informed consent before blood donation.

Table 1. Patient characteristics

	All patients (n=30)	Controls (n=19)
Sex (male/female)	19 / 11	13 / 6
Age (years)*	39 (19-69)	29 (22-43)
ALT (units/L)*	48 (9-243)	n.t.
HBV-DNA (geq/mL)*	1.1×10^8 (1.0×10^3-1.2×10^{10})	-
HBeAg (+)/anti-HBeAg (+)	15 / 15	-

n.t. not tested
* median (range)

Virological assessments

Serum HBsAg, HBeAg, and anti-HBe were determined quantitatively using the IMX system (Abbot Laboratories, North Chicago, IL) according to the manufacturers instructions. Serum HBV-DNA was determined using a HBV monitor assay (Roche Applied Science, Penzberg, Germany; detection limit 1×10^3 geq/ml). When the serum HBV-DNA was below 1×10^3 geq/ml HBV-DNA the assay was repeated using an in-house developed TaqMan PCR (detection limit 373 geq/ml) [23]. TaqMan PCR was also

used for determination of HBV-DNA in the DC subtypes. DNA was extracted from a pellet of $2x10^4$ DC. The pellet was resuspended in 190 µL double distilled water, to which 10 µl of a known amount of internal control, consisting of a seal herpes virus, was added. This material was extracted on a MagnaPure LC isolation system (Roche Applied Science) using the total nucleic acid isolation kit. Twenty µl of the isolated nucleic acid was used to detect HBV-DNA. Values for the internal control had to be within a range previously determined within the laboratory.

Analysis of myeloid and plasmacytoid dendritic cells subsets in peripheral blood
Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Isopaque gradient centrifugation. PBMC were incubated for 5 minutes with human immunoglobulins (Octagam, 1.3 mg/ml; Octapharma, Lachen, Switzerland) in PBS containing 1% w/v bovine serum albumin (BSA) before adding specific antibodies to determine the percentage of mDC and pDC. For mDC, PBMC were incubated with a cocktail of FITC-conjugated monoclonal antibodies to the lineage markers CD3, CD14, CD16, CD19, CD20 and CD56 (Becton Dickinson, San Jose, CA), anti-BDCA1 (CD1c)-PE (Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-CD11c-APC (Becton Dickinson). To determine the percentage of pDC, PBMC were incubated with the FITC conjugated lineage marker cocktail, anti-BDCA4-PE and anti-CD123-biotin (Becton Dickinson), and subsequently a secondary step with strepavidin-PerCP (Becton Dickinson). As controls, cells were stained with corresponding isotype-matched control monoclonal antibodies. Stained cells were analyzed using a four-color flow cytometer (FACScalibur, Becton Dickinson) and CellQuest software.

Isolation of myeloid and plasmacytoid dendritic cells
MDC ($BDCA1^+$) and pDC ($BDCA4^+$) were isolated from PBMC by positive immunomagnetic selection using the mini-MACS system (Miltenyi Biotec) according to the manufacturers instructions. Briefly, $BDCA1^+$ mDC were isolated by incubating CD19 depleted cells with a PE-conjugated monoclonal antibody (mAb) to BDCA1 followed by anti-PE magnetic beads and separation over a MS-column. In addition, $BDCA4^+$ pDC were isolated by after incubation with a PE-conjugated mAb to BDCA4 followed by anti-PE magnetic beads. Cells were resuspended in culture medium consisting of RPMI 1640 (Bio Whittaker, Verviers, Belgium) containing 10% fetal calf serum (Hyclone, Logan, UT). The isolated mDC and pDC were analyzed for purity by flow cytometry as mentioned in the previous section (mDC; $BDCA1^+$, lineage $markers^-$, $CD11c^+$ and pDC; $BDCA4^+$, lineage $markers^-$, $CD123^{high}$) and only used if >90% pure.

Expression of cell surface molecules on the surface of myeloid and plasmacytoid dendritic cells by flowcytometry
Freshly isolated mDC and pDC ($1x10^4$ cells) were incubated with CD80-FITC (Immunotech, Marseille, France), human leucocyte antigen (HLA)-DR-PerCP (Becton Dickinson) and CD86-APC (Becton Dickinson). As a control cells stained with

corresponding isotype-matched control monoclonal antibodies were used. Cells were analyzed on the flow cytometer and the mean fluorescence intensity and percent positive stained cells was determined.

Analysis of T cell stimulatory capacity of myeloid and plasmacytoid dendritic cells in a mixed lymphocyte reaction

Purified mDC and pDC were matured for 24 hours in 96-well flat bottom culture plates at different concentrations (1.25, 2.5, 5 and $10x10^3$ cells/200 µl) in culture medium containing IL-1β (50 ng/ml; Strathmann Biotech, Hannover, Germany) and TNF-α (25 ng/ml; Strathmann). For mDC GM-CSF (500 U/ml; Leucomax, Novartis Pharma, Arnhem, The Netherlands) and for pDC IL-3 (10 ng/ml; Strathmann) was added as a growth supplement. The next day culture supernatant was removed and nylon-wool purified T cells from a normal healthy volunteer ($1.5x10^5$ cells/200 µl) were added to the DC. After 5 days, cell proliferation was assessed by the incorporation of [^{3}H]thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK), 0.5 µCi/well was added and cultures were harvested 18 hours later. Phytohemagglutin 5 µg/ml (Murex, Paris, France) was added to T cells as a positive control.

Cytokine production of stimulated myeloid and plasmacytoid dendritic cells

Purified mDC were stimulated at a concentration of $4x10^4$ cells/200 µl in 96 flat bottom Costar plates (Costar, Cambridge, MA) in culture medium with synthetic double-stranded (ds)RNA, polyriboinosinic-polyribocytidylic acid (poly (I:C), 20 µg/ml; Sigma-Aldrich, St. Louis, MA) in combination with recombinant human IFN-γ (1000 U/ml; Strathmann) and in the presence of GM-CSF (500 U/ml). Purified pDC were stimulated at a concentration of $2x10^4$ cells/200 µl with *Staphylococcus aureus* Cowan strain I (SAC; 75 µg/ml; Calbiochem, San Diego, CA) and in the presence of IL-3 (10 ng/ml). Cells were cultured at 37°C and 5% CO_2 in a humified incubator. Supernatants were harvested after 24 hours. The level of IL-12p70 (Diaclone, Besançon, France) and IFN-α (Biosource International) were determined by standard ELISA according to the manufactures instructions. The levels of TNF-α, IL-6 and IL-10 were determined by specific solid-phase sandwich ELISA as previously described, using pairs of monoclonal antibodies and recombinant cytokine standards from Biosource International [24, 25].

Statistical analysis

Data are expressed as mean ± SEM, unless indicated otherwise. Data were analyzed with SPSS 11.5 for Windows (SPSS, Chicago, IL) using the Mann-Whitney test to compare variables between two independent groups. In all analyzes a p-value of <0.05 was considered statistically significant. Correlations were determined using the Spearman's correlation test. HBV patients were compared to healthy controls. To analyze the results on the basis of serum viral load and ALT, we categorized all HBV patients according to: 1) HBV-DNA > 10^8 geq/ml (high, n=16), HBV-DNA 10^5 to 10^8 geq/ml (intermediate, n=5), HBV-DNA < 10^5 geq/ml (low, n=9), and 2) ALT normal

(upper limit of normal (ULN) is 35 U/L, n=11), ALT normal to 2 x ULN (intermediate, n=9) and ALT > 2 x ULN (high, n=10).

Results

Myeloid and plasmacytoid dendritic cells percentages in peripheral blood are similar in chronic HBV patients and healthy controls

To determine whether a chronic HBV infection affects the frequencies of mDC and pDC in peripheral blood, the percentages of both cell types in PBMC were determined by flow cytometry. MDC were recognized in peripheral blood by staining with a cocktail of lineage markers (CD3, CD14, CD16, CD19, CD20 and CD56) and anti-BDCA1 (Fig 1A). PDC were characterized by staining with anti-BDCA4 and anti-CD123 (Fig 1B). No significant differences in the frequencies of both mDC and pDC were observed between chronic HBV patients and healthy controls (Fig 1C).

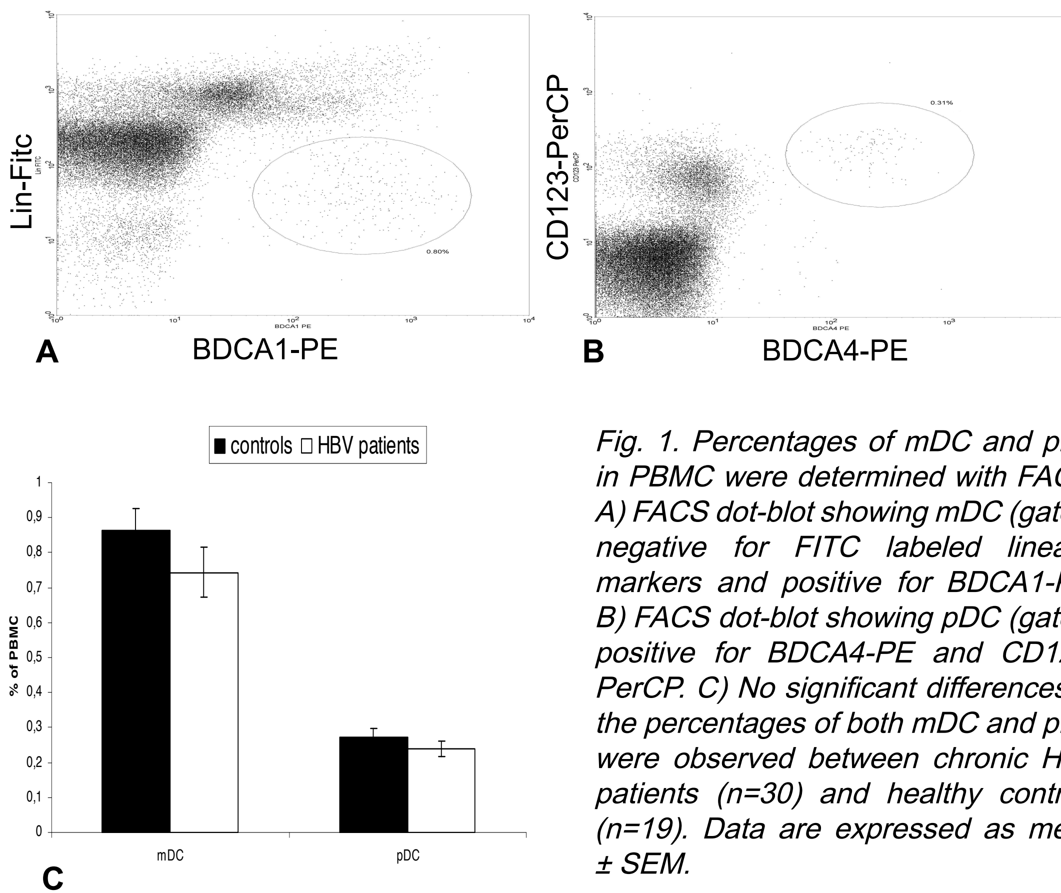


Fig. 1. Percentages of mDC and pDC in PBMC were determined with FACS. A) FACS dot-blot showing mDC (gated) negative for FITC labeled lineage markers and positive for BDCA1-PE. B) FACS dot-blot showing pDC (gated) positive for BDCA4-PE and CD123-PerCP. C) No significant differences in the percentages of both mDC and pDC were observed between chronic HBV patients (n=30) and healthy controls (n=19). Data are expressed as mean ± SEM.

Myeloid dendritic cells of chronic HBV patients are inhibited in their capacity to express costimulatory molecules upon in vitro maturation

Freshly isolated DC precursor populations from peripheral blood of patients and controls express only low amounts of the costimulatory molecules CD80 and CD86. After stimulation for 24 hours in the presence of IL-1β and TNF-α, the percentages of mDC and pDC expressing CD80 and CD86 as well as the mean fluorescence intensities of the positive cells were significantly increased. Maturation of mDC was significantly more increased compared to pDC as is shown in FACS histograms of one representative healthy control ($p<0.001$, Fig 2). No differences were observed in the mean fluorescence intensities of CD80 and CD86 on mDC from patients and controls. However, the percentages of mature mDC expressing CD80 and CD86 after stimulation were significantly reduced in the patient group ($p<0.05$, Table 2). The percentages of pDC expressing CD80 or CD86 of patients and controls were similar both before and after stimulation. A significant increase in mean fluorescence intensity of CD86 on pDC was found in the HBV patient group after stimulation ($p<0.05$). The HLA-DR mean fluorescence intensities were significantly increased on mDC and pDC after stimulation ($p<0.001$; Fig 2 and Table 2). No difference in HLA-DR mean fluorescence intensity was found when comparing patients to controls.

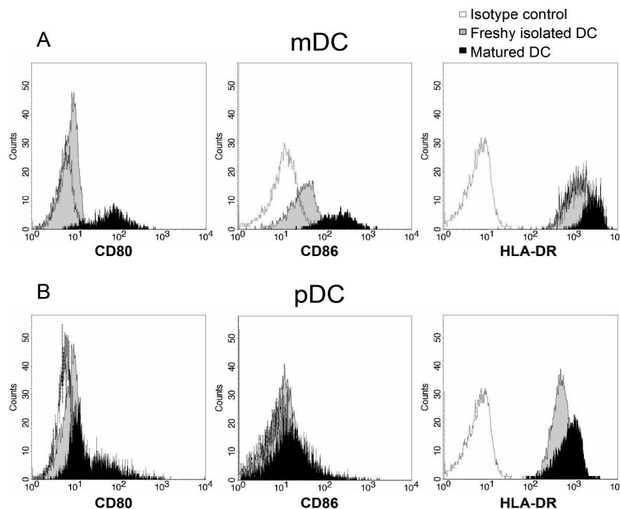


Fig 2. FACS histograms showing the mean fluorescence intensities of CD80, CD86 and HLA-DR of freshly isolated DC (gray histogram) and DC that were matured for 24 h. with IL-1β and TNF-α (black histogram) and isotype control (dotted line). Results from one representative healthy control are shown. The mean fluorescence intensities of CD80, CD86 and HLA-DR expression were increased after 24 h. maturation especially on mDC (A) and to a lesser extent on pDC (B).

Table 2. Phenotypic characteristics of mDC and pDC

DC	Markers		Freshly isolated DC		Matured DC	
			Patients	Controls	Patients	Controls
MDC	HLA-DR	%	98 ± 2.2	99 ± 0.6	96 ± 12	99 ± 3.1
		MFI	1374 ± 78	1476 ± 80	3669 ± 352	4016 ± 479
	CD80	%	2.6 ± 0.8	1.6 ± 0.6	74 ± 4.1*	84 ± 2.9
		MFI	8.8 ± 1.2	7.2 ± 0.3	64 ± 6.3	66 ± 4.1
	CD86	%	26 ± 3.9	27 ± 5.6	81 ± 4.8*	92 ± 1.2
		MFI	48 ± 5.1	46 ± 4.1	320 ± 41	311 ± 44
PDC	HLA-DR	%	95 ± 4.3	97 ± 4.0	96 ± 7.6	98 ± 3.5
		MFI	537 ± 39	587 ± 21	1644 ± 164	1579 ± 175
	CD80	%	1.8 ± 0.5	1.6 ± 0.7	54 ± 3.4	51 ± 4.0
		MFI	6.8 ± 0.5	5.9 ± 0.2	61 ± 5.7	52 ± 3.9
	CD86	%	2.8 ± 0.9	3.0 ± 1.4	31 ± 5.1	30 ± 7.4
		MFI	41 ± 25	14 ± 1.7	78 ± 9.3*	51 ± 6.2

* p<0.05 versus controls

The allostimulatory capacity of myeloid dendritic cells of chronic HBV patients is impaired

To asses whether the T cell stimulating capacity of the two DC subsets is affected in chronic HBV patients, we studied the ability of DC to stimulate T cells in the allogeneic mixed lymphocyte reaction. Mature mDC and pDC (obtained as described above) were added at different concentrations to T cells of a healthy third party. Fig 3A shows that mDC of patients were less efficient in inducing T cell proliferation than mDC isolated from controls at all ratios tested (p<0.05). PDC were capable of inducing T cell proliferation, but were less potent than mDC. This was also reflected by their lower expression of HLA-DR and costimulatory molecules, CD80 and CD86. No difference was found in the T cell stimulatory function of pDC from patients and controls (Fig 3B). Background proliferation of T cells only was <500 cpm. The proliferation of PHA stimulated T cells was similar in both groups (data not shown).

Impaired TNF-α production by myeloid dendritic cells and IFN-α production by plasmacytoid dendritic cells of chronic HBV patients

MDC are main producers of IL-12 and pDC of IFN-α. Chronicity of HBV infection is thought to be mainly caused by a reduced Th1 response, possibly caused by a reduced IL-12 and/or IFN-α production by DC. To investigate the capacity of the two subtypes of DC to produce cytokines, mDC were stimulated with poly I:C and IFN-γ to induce high IL-12p70 production and pDC were stimulated with SAC for high IFN-α production. Twenty four hours later culture supernatants were harvested and analyzed for the production of several cytokines using specific ELISAs. The production of IL-12p70, IL-10 and IL-6 by mDC was not significantly different when comparing patients with controls (Fig 4A-C). However, the secretion of TNF-α in the supernatant of stimulated mDC isolated from the patients was significantly reduced compared with healthy controls (Fig 4D). IFN-α was not detectable in the culture supernatant of stimulated mDC (data not shown).

SAC stimulated pDC of patients showed a significantly impaired production of IFN-α

compared to controls ($p<0.05$; Fig 5A). IL-10 production by pDC was increased in the patients although not significantly ($p=0.1$, Fig 5B). There was no significant difference in IL-6 and TNF-α production of SAC-stimulated pDC of controls and patients (Fig 5C-D). Only low amounts of IL-12p70 were detectable in SAC-stimulated pDC (data not shown).

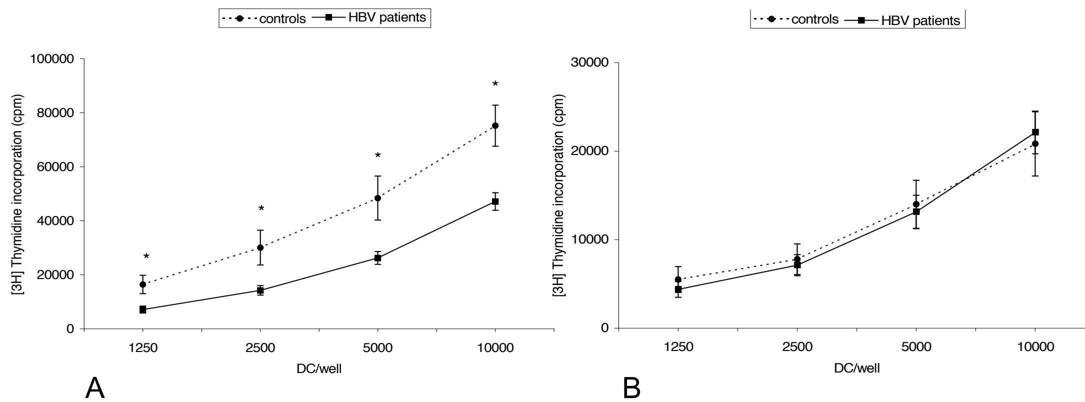


*Fig 3. Allostimulatory capacity of peripheral DC subtypes. Precursor mDC and pDC were matured for 24 h. with IL-1β and TNF-α. Subsequently DC were cultured at different numbers with T cells from a third party. After 5 days, the cells were pulsed for another 18 h. with [^{3}H]thymidine. A) mDC of chronic HBV patients (n=27) showed a significantly reduced capacity to stimulate allogeneic T cells at all ratios tested (*p<0.05) compared to healthy controls (n=15). B) No difference was found in allostimulatory capacity of pDC of patients (n=29) and healthy controls (n=15). Data are expressed as mean ± SEM counts per minute (CPM).*

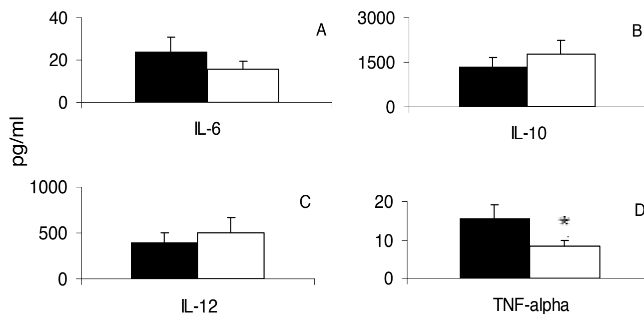


*Fig 4. Cytokine production by isolated peripheral precursor mDC of chronic HBV patients (n=25) and healthy controls (n=14) after stimulation with poly (I:C). After 24 h. stimulation cytokine production was determined in the culture supernatant by specific ELISAs. No difference was detected in the production of A) IL-6, B) IL-10 and C) IL-12p70 production between patients and healthy controls. D) Purified mDC of patients showed a significantly reduced capacity to produce TNF-α compared to healthy controls. Data are expressed in mean ± SEM, *p<0.05.*

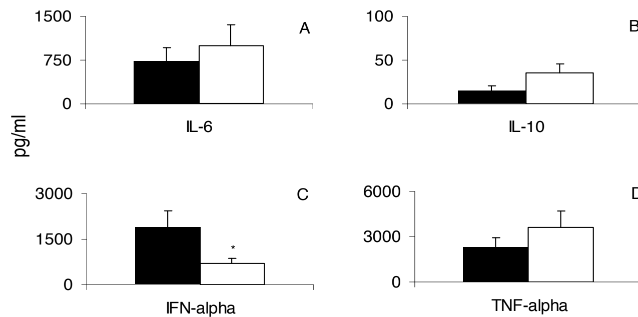


*Fig 5. Cytokine production by isolated peripheral precursor pDC of chronic HBV patients (n=23) and healthy controls (n=15) after stimulation with SAC. After 24 h stimulation cytokine production was determined in the culture supernatant by specific ELISAs. No difference was detected in the production of A) IL-6, B) IL-10 and D) TNF-α production between patients and healthy controls. C) PDC of patients were significantly impaired to produce IFN-α compared to healthy controls. Data are expressed in mean ± SEM, *p<0.05.*

Dendritic cell function in relation to viral load and alanine transaminase levels

When analyzing the obtained results of the HBV patients in relation to the viral load and ALT, we found that the capacity of mDC to produce TNF-α was significantly reduced in patients with low viral load as compared to patients with high viral load (p<0.05, Fig 6A). No relation was observed between TNF-α production and ALT levels.

The IFN-α production of pDC was significantly decreased in patients with high ALT as compared to patients with normal ALT (p<0.05, Fig 6B). Although all patients with high ALT exhibited HBV-DNA levels above 10^8 geq/ml, no relation was found between serum viral load and IFN-α production.

The expression of costimulatory molecules before and after maturation, the allostimulatory capacity and the capacity to produce cytokines by mDC and pDC were not related to viral load and ALT.

To exclude that the observed effects are the result of liver inflammation rather than a specific effect of the virus, we studied a separate group of patients with chronic inflammatory liver disease of non-viral origin (primary biliary cirrhosis n=2; hemochromatosis n=4) and a ALT comparable to chronic HBV patients (median 40 U/l, range 24-110 U/l). The mDC isolated from these patients showed similar expression of costimulatory molecules and also displayed similar allostimulatory capacity as healthy controls. Furthermore, the production of IFN-α by plasmacytoid dendritic cells was not reduced in those patients as compared to healthy controls (data not shown).

HBV-DNA was detectable in DC precursor subpopulations of chronic HBV patients

A functional impairment of the DC subsets could be caused by the presence of virus in the DC. To investigate this, HBV-DNA was determined in the DC by taqman PCR.

HBV-DNA was detected in mDC of 15 out of 30 chronic HBV patients. In 12 patients HBV-DNA was present in pDC. The level of HBV-DNA was significantly higher in pDC than mDC, $1.2x10^4 \pm 2.9x10^3$ geq/ml versus $6.7x10^3 \pm 2.2x10^3$ geq/ml respectively (mean ± SEM; $p<0.01$). The presence of HBV-DNA in the mDC and pDC was correlated with the serum HBV-DNA values (Spearman's correlations $r=0.64$, $p<0.05$ and $r=0.60$, $p<0.05$ respectively). HBV-DNA was not detected in any of the control DC tested. No correlation was observed between HBV-DNA presence in mDC and suppressed allostimulatory capacity and decreased CD80 and CD86 expression of matured mDC (data not shown).

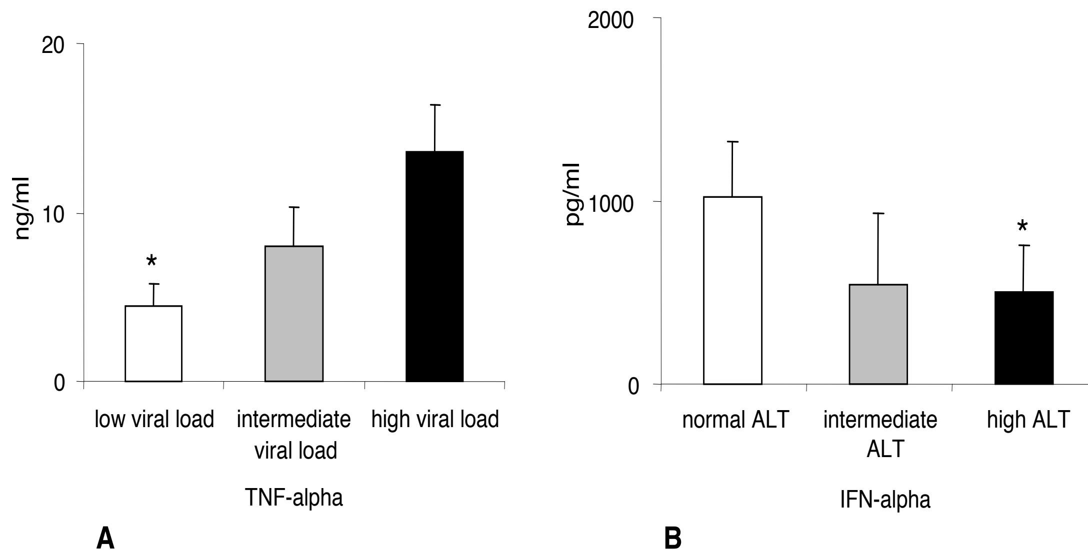


*Fig 6. A) The capacity of mDC to produce TNF-α was significantly reduced in patients with low viral load (all HBeAg negative) compared to patients with high viral load (94% HBeAg positive) B) The IFN-α production of pDC was decreased significantly in patients with high ALT as compared to the patients with a normal ALT. Data are expressed in mean ± SEM, *p<0.05.*

Discussion

DC are professional antigen presenting cells that are extremely potent in initiating a primary immune response. The weak or absent T cell responses found in chronic HBV patients could be the result of a defect in the DC compartment. Numbers and/or functionality of DC subsets in the blood may be affected by the presence of the virus. The present study showed that percentages of mDC and pDC in peripheral blood of chronic HBV patients and controls were similar. On the other hand, the functionality of the two DC precursor subsets was affected in HBV infected patients compared to healthy controls.

In vitro matured mDC isolated from chronic HBV patients exhibit an impaired

allostimulatory capacity compared with mDC from healthy controls. It is unlikely that this difference is caused by the difference in the HLA class II alleles mismatches, since the allostimulatory capacity between pDC of patients and healthy controls was similar. The reduced T cell stimulatory capacity may be due to the decreased upregulation of the co-stimulatory molecules, CD80 and CD86, after *in vitro* maturation of mDC from HBV patients. However, pDC of patients showed an increased expression of CD86 after *in vitro* maturation, while there was no difference in T cell stimulatory capacity of pDC of patients and controls. This suggests that other factors, such as co-stimulatory or inhibitory molecules, play a role in the decreased allostimulatory capacity. For example CD40 has been shown to serve as a co-stimulus for T cell activation and also CD40 ligation is a critical step in the final maturation of DC into fully competent APC [26].

Besides the expression of costimulatory molecules or inhibitory molecules also the capacity of mDC to produce certain cytokines is important for the T cell stimulatory capacity. MDC of HBV patients were impaired to produce TNF-α. The capacity of mDC to produce TNF-α was decreased significantly in patients with low viral load. Sheron et al showed that serum TNF-α levels and the *in vitro* production of TNF-α production by lipopolysaccharide-stimulated PBMC was lower in HBeAg negative patients with low viral load [27]. TNF-α has been shown to contribute to allostimulation, it can act as an autocrine growth factor for DC induced T cell proliferation [28]. Furthermore, TNF-α plays an essential role in the maturation of DC [29]. We demonstrated that addition of exogenous TNF-α induced maturation of DC. This was manifested by the increased expression of costimulatory molecules after maturation with IL-β and TNF-α. However, the maturation of mDC was reduced in the HBV patients compared to healthy controls. The capacity of mDC to produce TNF-α was most prominent in patients with low viral load, while the allostimulatory capacity of mDC was comparable for all HBV patients. This indicates that there may be an additional factor yet to be determined that also plays a role in the reduced allostimulatory capacity of mDC of HBV patients.

We showed that IL-12 production by mDC was not different between HBV patients and controls in contrast to previous studies using moDC. In previous studies a reduced allostimulation corresponded with a reduced IL-12 production in chronic HBV patients [12, 13]. In a study by Lohr *et al*, the reduced T cell response induced by moDC from chronic HBV patients could be restored by exogenous IL-12 [30]. Taken together the results on IL-12 production show that mDC are clearly different from moDC, as also previously shown by Osugi *et al* [14].

In addition to the impaired function of mDC in chronic HBV patients we found a strongly reduced IFN-α production by pDC from chronic HBV patients. Furthermore, a trend towards upregulation of the cytokines IL-6, TNF-α and IL-10 was observed in these patients, indicating an imbalance in cytokine production by pDC of HBV patients. IFNα is a pleiotropic modulator of host resistance and plays a critical role in the induction of an antiviral state [19, 31]. In other viral infections such as human immune deficiency virus-1, also a reduced capacity of pDC to produce IFN-α has been shown [32, 33]. Furthermore, it has been proposed in human immune deficiency virus infection

that pDC control viral replication through production of IFN-α [34]. This anti-viral effect of IFN-α may also play a role in chronic HBV infection, since we found a more reduced IFN-α production in patients with active disease (high ALT and high viral load). Recently, IFN-α has also been shown to play a role in plasma cell differentiation and subsequent antibody production [35]. Therefore, reduced IFN-α production may influence the production of HBV specific antibodies. Exogenous IFN-α therapy may restore the disturbed cytokine environment and could be successful in especially those patients with impaired endogenous IFN-α production. This issue is subject of further studies.

Our results indicate that both circulating DC subsets in peripheral blood of patients with chronic HBV infection are affected in their function. This functional impairment of DC was not observed in patients with chronic inflammatory liver disease of non-viral origin. Therefore, our findings appear indeed related to HBV and are not solely the consequence of inflammation of the liver. The DC dysfunction in chronic HBV patients may be caused directly by viral infection of DC, interfering with signaling pathways involved in maturation and cytokine production. We showed the presence of HBV-DNA in both DC subsets. Others have shown that PMBC and purified cell subsets contain HBV-DNA and also viral intermediates, suggestive of viral replication [36, 37]. Moreover, viral particles and HBV replication intermediates were found in moDC of chronic HBV patients [12, 13] and direct infection of DC has been described for other viruses [38-40]. HBV may also interact with DC through binding to the cell surface receptors, as previously shown for human immunodeficiency virus and hepatitis C infection [41]. From our results we cannot exclude that the presence of HBV-DNA in both DC subsets reflects attachment of the virus to the cell surface or uptake of the virus as a biological function of DC. The virus may also indirectly affect the DC subsets, for instance by infection of other cells which in turn produce cytokines causing a change in cytokine environment in the liver or lymphoid organs. Further research should determine the mechanism by which HBV interferes with the DC functions and whether the HBV-DNA we found in the DC represents replicating virus.

The question remains whether the chronic HBV infection in patients is the cause of a preexisting dysfunction of the DC subtypes or that the infection itself causes the dysfunction of DC. Recently a study on hepatitis C in chimpanzees suggested that the DC impairment is rather a consequence of persistent and active hepatitis C virus infection associated with disease progression [42]. Research studying the functionality of DC before and after successful therapy for HBV may provide answers for this issue.

In conclusion, our results show that both mDC and pDC are functionally impaired in chronic HBV patients. This could be one of the reasons for the absent or weak T cell responses and the subsequent continued HBV replication in these patients.

References

1. Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. Annu.Rev.Immunol. 1995; 13:29-60.
2. Maini MK, Boni C, Ogg GS, et al. Direct ex vivo analysis of hepatitis B virus-specific CD8(+) T cells associated with the control of infection. Gastroenterology 1999; 117:1386-96.
3. Webster GJ, Reignat S, Maini MK, et al. Incubation phase of acute hepatitis B in man: dynamic of cellular immune mechanisms. Hepatology 2000; 32:1117-24.
4. 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.
5. Maini MK, Boni C, Lee CK, et al. The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. J.Exp.Med. 2000; 191:1269-1280.
6. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998; 392:245-52.
7. Donaghy H, Gazzard B, Gotch F, Patterson S. Dysfunction and infection of freshly isolated blood myeloid and plasmacytoid dendritic cells in patients infected with HIV-1. Blood 2003; 101:4505-11.
8. Auffermann-Gretzinger S, Keeffe EB, Levy S. Impaired dendritic cell maturation in patients with chronic, but not resolved, hepatitis C virus infection. Blood 2001; 97:3171-3176.
9. Bain C, Fatmi A, Zoulim F, Zarski JP, Trepo C, Inchauspe G. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. Gastroenterology 2001; 120:512-524.
10. Kanto T, Hayashi N, Takehara T, et al. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. J Immunol 1999; 162:5584-91.
11. Steinman RM, Granelli-Piperno A, Pope M, et al. The interaction of immunodeficiency viruses with dendritic cells. Curr Top Microbiol Immunol 2003; 276:1-30.
12. Beckebaum S, Cicinnati V, Dworacki G, et al. Reduction in the Circulating pDC1/pDC2 Ratio and Impaired Function of Ex Vivo-Generated DC1 In Chronic Hepatitis B Infection. Clin.Immunol. 2002; 104:138.
13. Arima S, Akbar SM, Michitaka K, et al. Impaired function of antigen-presenting dendritic cells in patients with chronic hepatitis B: localization of HBV DNA and HBV RNA in blood DC by in situ hybridization. Int J Mol Med 2003; 11:169-74.
14. Osugi Y, Vuckovic S, Hart DN. Myeloid blood CD11c(+) dendritic cells and monocyte-derived dendritic cells differ in their ability to stimulate T lymphocytes. Blood 2002; 100:2858-66.
15. Liu YJ, Kanzler H, Soumelis V, Gilliet M. Dendritic cell lineage, plasticity and cross-regulation. Nat Immunol 2001; 2:585-9.
16. O'Doherty U, Peng M, Gezelter S, et al. Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. Immunology 1994; 82:487-93.
17. Bauer M, Redecke V, Ellwart JW, et al. Bacterial CpG-DNA triggers activation and maturation of human CD11c-, CD123+ dendritic cells. J Immunol 2001; 166:5000-7.
18. Siegal FP, Kadowaki N, Shodell M, et al. The nature of the principal type 1 interferon-producing cells in human blood. Science 1999; 284:1835-1837.
19. Cella M, Facchetti F, Lanzavecchia A, Colonna M. Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization. Nat Immunol 2000; 1:305-10.
20. Katze MG, He Y, Gale M, Jr. Viruses and interferon: a fight for supremacy. Nat Rev Immunol 2002; 2:675-87.
21. 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.
22. 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; 46:562-568.
23. Pas SD, Fries E, De Man RA, Osterhaus AD, Niesters HG. Development of a quantitative real-time detection assay for hepatitis B virus DNA and comparison with two commercial assays. J Clin Microbiol 2000; 38:2897-901.
24. Kalinski P, Schuitemaker JH, Hilkens CM, Kapsenberg ML. Prostaglandin E2 induces the final maturation of IL-12-deficient CD1a+CD83+ dendritic cells: the levels of IL-12 are determined during the final dendritic cell maturation and are resistant to further modulation. J Immunol 1998; 161:2804-9.
25. Snijders A, Hilkens CM, van der Pouw Kraan TC, Engel M, Aarden LA, Kapsenberg ML. Regulation of bioactive IL-12 production in lipopolysaccharide-stimulated human monocytes is determined by the expression of the p35 subunit. J Immunol 1996; 156:1207-12.
26. McLellan AD, Sorg RV, Williams LA, Hart DN. Human dendritic cells activate T lymphocytes via a CD40: CD40 ligand-dependent pathway. Eur J Immunol 1996; 26:1204-10.
27. Sheron N, Lau J, Daniels H, et al. Increased production of tumour necrosis factor alpha in chronic hepatitis

B virus infection. J Hepatol 1991; 12:241-5.
28. McKenzie JL, Calder VL, Starling GC, Hart DN. Role of tumour necrosis factor-alpha in dendritic cell-mediated primary mixed leucocyte reactions. Bone Marrow Transplant 1995; 15:163-71.
29. Steinman RM. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol 1991; 9:271-96.
30. Lohr HF, Pingel S, Bocher WO, et al. Reduced virus specific T helper cell induction by autologous dendritic cells in patients with chronic hepatitis B - restoration by exogenous interleukin-12. Clin Exp Immunol 2002; 130:107-14.
31. Kadowaki N, Antonenko S, Lau JY, Liu YJ. Natural interferon alpha/beta-producing cells link innate and adaptive immunity. J Exp Med 2000; 192:219-26.
32. Feldman S, Stein D, Amrute S, et al. Decreased interferon-alpha production in HIV-infected patients correlates with numerical and functional deficiencies in circulating type 2 dendritic cell precursors. Clin Immunol 2001; 101:201-10.
33. Chehimi J, Campbell DE, Azzoni L, et al. Persistent decreases in blood plasmacytoid dendritic cell number and function despite effective highly active antiretroviral therapy and increased blood myeloid dendritic cells in HIV-infected individuals. J Immunol 2002; 168:4796-801.
34. Soumelis V, Scott I, Gheyas F, et al. Depletion of circulating natural type 1 interferon-producing cells in HIV-infected AIDS patients. Blood 2001; 98:906-12.
35. Jego G, Palucka AK, Blanck JP, Chalouni C, Pascual V, Banchereau J. Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. Immunity 2003; 19:225-34.
36. Yoffe B, Noonan CA, Melnick JL, Hollinger FB. Hepatitis B virus DNA in mononuclear cells and analysis of cell subsets for the presence of replicative intermediates of viral DNA. J Infect Dis 1986; 153:471-477.
37. Gu JR, Chen YC, Jiang HQ, et al. State of hepatitis B virus DNA in leucocytes of hepatitis B patients. J Med Virol 1985; 17:73-81.
38. Blauvelt A, Asada H, Saville MW, et al. Productive infection of dendritic cells by HIV-1 and their ability to capture virus are mediated through separate pathways. J Clin Invest 1997; 100:2043-53.
39. Fugier-Vivier I, Servet-Delprat C, Rivailler P, Rissoan MC, Liu YJ, Rabourdin-Combe C. Measles virus suppresses cell-mediated immunity by interfering with the survival and functions of dendritic and T cells. J Exp Med 1997; 186:813-23.
40. Riegler S, Hebart H, Einsele H, Brossart P, Jahn G, Sinzger C. Monocyte-derived dendritic cells are permissive to the complete replicative cycle of human cytomegalovirus. J Gen Virol 2000; 81:393-9.
41. Geijtenbeek TB, van Kooyk Y. DC-SIGN: a novel HIV receptor on DCs that mediates HIV-1 transmission. Curr Top Microbiol Immunol 2003; 276:31-54.
42. Rollier C, Drexhage JA, Verstrepen BE, et al. Chronic hepatitis C virus infection established and maintained in chimpanzees independent of dendritic cell impairment. Hepatology 2003; 38:851-8.

CHAPTER 3

Flow cytometry of fine-needle aspiration biopsies: a new method to monitor intrahepatic immunological environment in chronic viral hepatitis

Modified from:

Flow cytometry of fine-needle-aspiration-biopsies: a new method to monitor intrahepatic immunological environment in chronic viral hepatitis
D. Sprengers[1], R.G. van der Molen[1], J.G. Kusters[1], J. Kwekkeboom[1], L.J.W. van der Laan[1], H.M.G. Niesters[2], E.J. Kuipers[1], R.A. De Man[1], S.W. Schalm[1], H.L.A. Janssen[1].
J. Viral Hepat 2005; 12: 507-512

Coarse vs. Fine-Needle aspiration biopsy
D. Sprengers[1], R.G. van der Molen[1], J.G. Kusters[1], H.L.A. Janssen[1]
J Hepatol 2004; 41: 503-504

[1] Dept. of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands
[2] Dept. of Virology, Erasmus MC University Medical Center, Rotterdam, The Netherlands

Abstract

Information about character and grade of the intrahepatic immune response in viral hepatitis is important for evaluation of disease stage and effect of therapy. Complications like haemorrhage provide a limitation to frequently performing tissue needle biopsies (TB), and have made many use peripheral blood as surrogate marker instead. The fine-needle aspiration biopsy (FNAB) of the liver represents a safe and atraumatic method that allows frequent cytological sampling. Our aim was to investigate whether flow cytometry of FNAB-specimens allows co-analysis of phenotype, function and specificity of key populations of liver infiltrating lymphocytes (LIL). In 20 consecutive patients with chronic viral hepatitis (10 HBV, 10 HCV) flow cytometry was performed on FNAB-cytology, simultaneously obtained lymphocytes isolated from a TB and peripheral blood mononuclear cells (PBMC). The ratio CD8+/CD4+ lymphocytes in FNAB correlated well with LIL from TB ($r = 0.78$, $p<0.05$) but differed from PBMC (mean ratio: 2.6; 2.1; 0.7 respectively). Similarly, a correlation was observed for percentage CD56+ NK-cells (mean %: 29.9; 32. 3; 14.5 respectively; $r = 0.69$, $p<0.05$). The percentage IFN-γ producing CD3+ lymphocytes in both FNAB and TB was higher than in PBMC (mean %: 41; 44; 22 respectively; $p<0.05$). Furthermore, tetrameric complexes allowed analysis of HBV-specific T-cells in FNAB-specimens. In conclusion, flow cytometry of FNAB allows easy, atraumatic and reliable analysis of lymphocytes obtained from the intrahepatic compartment. Therefore, the FNAB is a valuable tool in the study of immunopathology of viral hepatitis, and it may contribute to improved clinical evaluation of chronic viral liver disease.

Introduction

Many liver disorders, such as hepatitis B and C virus infection, are characterized by chronic inflammation induced by a host immune response. Information about character and grade of the intrahepatic immune response is important for evaluation of disease stage and effect of therapy. The most widely used liver sampling method, and the accepted standard, is the 14 Gauge core tissue needle biopsy. Even with current biopsy technology serious complications like pain, haemorrhage and bile leakage provide a limitation, especially for repeated use. The mortality rate after TB is approximately 1 in 10,000 patients [1]. Therefore, most human studies investigating viral hepatitis have focussed on immune effector cells derived from peripheral blood (PB). Although this provided valuable information, circulating and intrahepatic hepatitis B virus (HBV) specific T cells displayed different phenotypes with regard to their activation status and thus may differ in effector functions such as cytotoxicity and interferon gamma (IFN-γ) production [2]. Moreover, it was shown that resident intrahepatic populations of lymphocytes differ significantly from populations in the circulation, suggesting a unique immunological environment in the liver [3].
This emphasises the need for a safe technique to obtain sufficient ex vivo intrahepatic immune effector cells to monitor the immune response directed against viral hepatitis. Fine-needle-aspiration-biopsy (FNAB) has proved to be an easy, atraumatic and reliable method for the diagnosis of acute rejection in kidney-grafted patients [4] and more recently in liver-transplant recipients and patients with focal liver lesions [5-7]. Up to now flow cytometry on liver FNABs has concentrated on evaluating DNA content and ploidy in the context of intraabdominal masses [8, 9]. However, FNAB may also be a valuable tool in immunopathological research and clinical practice in chronic viral hepatitis. CD56+ Natural Killer cells (NK cells), CD8+ cytotoxic T cells (CTL) and CD4+ helper T cells (Th) have all been shown to be essential in the immune response against viral hepatitis [2, 10, 11]. The aim of this study was to assess whether flow cytometric analysis of phenotype and function of these key populations of immune effector cells obtained by FNAB and TB, allow equally good analysis of liver infiltrating lymphocytes in patients with chronic HBV and HCV infection. To determine potential "contamination" of the FNAB-aspirate with peripheral blood, lymphocytes from FNAB-cytology, TB and PB are analysed simultaneously.

Methods

Fine-Needle Aspiration Biopsy.
Twenty consecutive patients (10 HBV,10 HCV), undergoing TB for chronic HBV or HCV infection, were studied. TB was performed as part of the diagnostic evaluation and all patients signed informed consent to perform a FNAB just before the conventional tissue biopsy was taken. Details of the FNAB-procedure are described elsewhere [12]. Briefly, a mandarin containing 25-gauge (diameter 0.5 mm) needle (Braun AG, Melsungen, Germany) is punctured in the 8th or 9th right intercostal space. After

removal of the mandarin a 10 mL syringe filled with 2 mL RPMI supplemented with 0.1% human serum albumin (Sanquin CLB, Amsterdam, the Netherlands) and 25 IU/mL heparin (Leo Pharma, Breda, the Netherlands) is attached. Liver cells are aspirated by negative syringe pressure. Next, the needle is flushed with 3 mL culture medium to obtain the liver cells from inside the needle and the specimen is transferred into a 15 mL tube and placed on ice immediately. Per patient two FNABs are performed and the aspirates are pooled before further analysis. If at macroscopic evaluation an aspirate contained predominantly blood and only few tissue fragments, an additional specimen was taken immediately. Only representative FNAB-specimens [12], i.e. containing 7 hepatocytes/100 leukocytes in a May-Grünwald-Giemsa stain of a cytospin preparation made from a fraction of the aspirate, were analysed with flow cytometry.

Immunophenotyping and intracellular cytokine staining

PBMC were isolated from heparinized blood samples by Ficoll-Hypaque density gradient centrifugation. Erythrocytes were removed from FNAB-specimens by NH_4CL. Liver infiltrating lympocytes (LIL) from TB were isolated after digestion with collagenase type IV (0,5 mg/ml; Sigma Chemical Co., St. Louise, USA (Sigma)) and DNAse (25µg/ml; Gibco, Paisley Scotland) for 30 minutes at 37 ^{o}C. The cell suspension was washed twice before antibody staining and four-color flow cytometric evaluation using FACS Caliber (Becton and Dickinson Biosciences; San Jose, USA (BD)) and CELLQuest software (BD). Immunofluorescence staining was performed as described previously [2]. To determine IFN-γ production cells were stimulated with phorbol myristate acetate (PMA;50ng/ml; Sigma) and ionomycin (1µM; Sigma) in the presence of Brefeldin (10µg/ml; Sigma) or with Brefeldin only (negative control). The following antibodies were divided in 2 panels for FACS analysis: anti-CD4 PerCP Cy5, -hIFNγ FITC, -CD8 PerCP (BD); anti-CD8 APC (Dako, Glostrup, Denmark (Dako)) anti-CD56 PE, -CD56 APC and –CD3 PE (Immunotech, Marseille, France). Matched isotype antibodies were used for control staining. In a separate experiment it was determined that LIL obtained by FNAB display a similar degree of autofluorescence as lymphocytes from PBMC. Since FNABs contain limited cell numbers the isotype controls were performed with PBMC. A minimum of 30.000 lymphocytes isolated from PB and liver tissue was analysed for each labelling together with all lymphocytes collected with the FNAB.

HLA typing, HLA-A2 tetramer staining

Screening for HLA-A2 haplotype positive HBV patients was performed by staining PBMC with an anti-HLA-A2 antibody derived from a hybridoma (clone BB7.2, ATCC HB-82). Staining was followed by an FITC-conjugated rabbit anti-mouse anti-IgG (Dako) secondary antibody and flow cytometric analysis. In HLA-A2+ HBV patients HBV-specific T cells were stained with the soluble HLA-A2 peptide tetramer: HBc 18-27 (FLPSDFFPSV) APC-conjugated (ProImmune, Oxford, UK) together with anti-CD8 FITC (Dako). Staining was performed as reported before [2]. In control experiments with

4 HLA-A2 positive healthy controls and HLA-A2 negative HBV patients the tetramer bound to 0.019 ± 0.002 and 0.013 ± 0.004% of total CD8+ T cells respectively. Therefore background staining level was determined 0.025% (mean +2SD).

Statistical analysis
The paired Pearson correlation test was used to correlate results obtained by FNAB to LIL isolated from TB and lymphocytes from PB. The agreement between FNAB and TB was determined as described by Bland and Altman [13] and reported as B&A p-values.

Results

FNAB evaluation
In cytospin evaluation the FNAB-aspirates of 20 consecutive patients with viral hepatitis were scored as representative, i.e. contained 7 hepatocytes/100 leukocytes and were thus unlikely to be “contaminated” with large amounts of PBMC. Consequently all FNAB-specimens were suitable for analysis by flow cytometry. The FNAB sample contained a median of 10,042 (2,620 - 40,346) lymphocytes that were analysed by flow cytometry. Using trypane blue exclusion criteria the viability was >95%. All samples were collected without any complications to the patients.

Ratio CD8 / CD4
Cytotoxic T cells (CTL) and helper T cells have both been shown to be essential in the immune response to viral hepatitis [2, 14]. Lymphocytes isolated from PB and LIL from FNAB and TB of all 20 patients were evaluated for expression of CD8, a marker for CTL, and for expression of CD4, a marker for helper T cells. Fig 1 shows that LIL in both FNAB and TB contained significantly more CD8+ - than CD4+ lymphocytes as opposed to PB. There was a significant correlation between the two liver sampling methods. The mean ratio CD8+ / CD4+ lymphocytes was 0.7 (± 0.1) in PB, 2.6 (± 0.34) in LIL from TB and 2.1 (± 0.3) in the FNAB-aspirate (FNAB vs. TB: $r = 0.78$, $p < 0.0001$; B&A $p = 0.38$) (table 1).

Table 1. Comparison of key populations of lymphocytes present in FNAB, tissue biopsy and peripheral blood

	FNAB	Tissue Biopsy	PB	TB vs. FNAB
Ratio CD8+/CD4+ lymphocytes	2.1 [± 0.3]	2.6 [± 0.34]	0.7 [± 0.1]	r = 0.78, p < 0.0001 B&A p = 0.38
% CD56+ lymphocytes	32.3% [± 2.9]	29.9% [± 3.3]	14.5% [± 2.4]	r = 0.69, p = 0.0002 B&A p = 0.44
% IFNγ+ CD3+ lymphocytes	42% [± 4.6]	39% [± 6.1]	21% [± 3.5]	r = 0.79, p = 0.001 B&A p = 0.13

Results represent mean [± SEM] of ratio CD8+/CD4+ lymphocytes, percentage CD56+ lymphocytes and percentage IFNγ+ CD3+ lymphocytes in FNAB-cytology, LIL isolated from tissue biopsy (TB) and lymphocytes from peripheral blood of 20 patients with viral hepatitis.

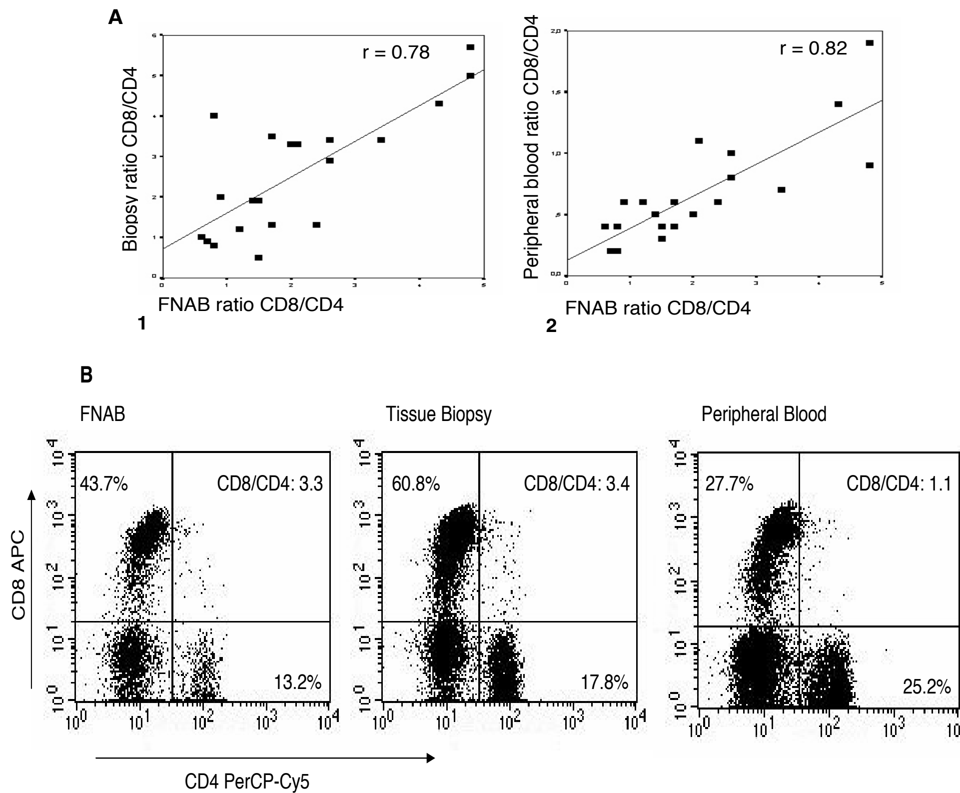


Fig 1.

A) Ratio CD8+ / CD4+ lymphocytes in tissue biopsy versus FNAB (1) and peripheral blood versus FNAB (2). Results of Pearson correlation test. With the method for assessing agreement between two methods of clinical measurement, as described by Bland and Altman, we find a non-significant p (p = 0.38) in A and a significant p (p < 0.0001) in B. This indicates a non-significant (A) and significant (B) difference between the two sampling techniques.

B) FACS dotblots of CD8+ / CD4+ lymphocytes in FNAB, tissue biopsy and peripheral blood. The number in the upper left quadrant indicates the percentage CD8+ lymphocytes. The number in the bottom right quadrant indicates the percentage CD4+ lymphocytes. The ratio CD8+ / CD4+ lymphocytes is depicted in the upper right quadrant. Data shown of a representative patient (patient 8).

Fraction CD56+ lymphocytes

A large proportion of intrahepatic lymphocytes consists of CD3- CD56+ Natural Killer cells (NK cells) [15]. Considering the potential importance of these cells in hepatic immunity, the fraction of CD56+ lymphocytes was determined in FNAB-specimens and TBs. Results show a significant correlation in percentage CD56+ cells in LIL from

FNABs and TBs. The mean percentage of CD56+ lymphocytes was 14.5 (± 2.4%) in PB, 29.9 (± 3.3%) in LIL isolated from the TBs and 32.3 (± 2.9%) in the FNAB-specimens (FNAB vs. TB: r = 0.69, p = 0.006; B&A p = 0.44) (Table 1).

IFN-γ production
Recent studies have shown that IFN-γ secretion by T cells plays an important role in the anti-viral immune response to HBV and HCV [10]. In 17 patients (20 minus 3 HLA A2+ HBV patients) the percentage IFN-γ producing CD3+ T cells was determined after 5-hour stimulation with PMA and ionomycine. In PB a mean of 21% of total CD3+ T cells produced IFN-γ versus 39% of the T cells isolated from the TBs and 42% of T cells in the FNAB-specimens (FNAB vs. TB: r = 0.79, p = 0.001; B&A p = 0.13) (Table 1). Fig 2 shows data of a representative patient. No significant difference was found between the number of IFN-γ producing NK cells in PB, TB and FNAB; the means were 50%, 54% and 63%, respectively (FNAB vs. TB: r = 0.62, p = 0.02; B&A p = 0.88).

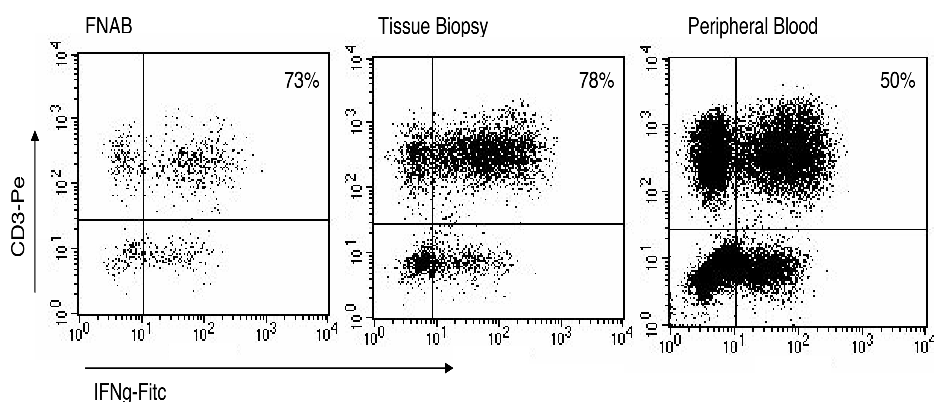


Fig 2.
IFN-γ production by CD3+ lymphocytes. The number in the upper right quadrant indicates the percentage IFNγ producing CD3+ lymphocytes of total CD3+ lymphocytes. Data shown of a representative patient (patient 2). In all cases background IFN-γ production was negligible.

HBc 18-27-tetramer binding of CD8+ lymphocytes
To investigate whether the FNAB provides sufficient material to adequately analyse cells that are present in the liver in very low frequencies, in HLA A2+ HBV patients virus-specific T cells were stained with tetrameric complexes able to visualise CD8+ T cells targeted at the HBV core epitope 18-27, [2]. Three out of 10 chronic HBV patients were HLA A2+. In 1 of 3 patients tetramer staining exceeded the background level of 0.025% of total CD8+ T cell population. In PB of this patient 0.19% of total CD8+ T cells showed tetramer binding, whereas this was 1.5% in LIL isolated from the TB and 1.3% in the FNAB-aspirate (fig 3).

Multiple sampling

Since material of multiple FNABs is pooled, sampling error may provide a limitation. In an additional experiment including 5 patients with diffuse liver disease, we compared the percentages CD4+, CD8+ and CD56+ lymphocytes present in two FNABs that were performed at the same time point but analysed separately. The median measurement error was 1.2% for CD4, 2.2% for CD8 and 5% for CD56, with high correlation in a paired Pearson test (r >/= 0.89, p </= 0.04).

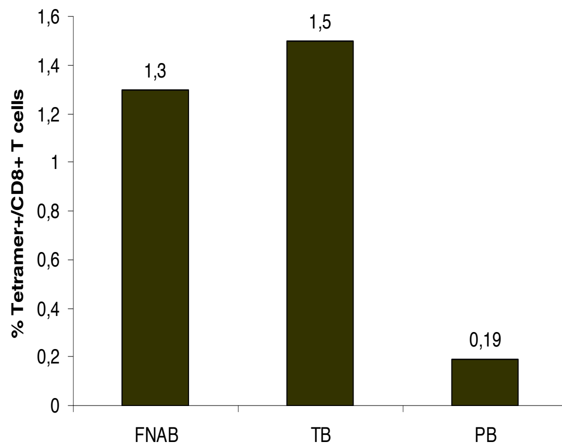


Fig 3.
In 3 HLA-A2+ HBV patients HBV-specific T cells were stained with the soluble HLA-A2 peptide tetramer HBc 18-27. In patient 1 tetramer positive CD8 + T cells were detectable in FNAB, TB and PB. HBc 18-27 tetramer binding in material from patient 4 and 6 did not exceed background level (data not shown).

Discussion

This is the first report on flow cytometric immunophenotyping of the intrahepatic compartment using Fine-Needle-Aspiration-Biopsies (FNAB) of the liver. We show that the FNAB-aspirate contains sufficient material for flow cytometry immunophenotyping experiments and functional assays; even when the cell population of interest is specific and present in small numbers, like HBV specific CD8+ T cells.

For clinical practice in hepatology the advantages of the liver FNAB are becoming increasingly apparent. The FNAB is an easy procedure that is much better tolerated than a standard liver tissue biopsy. Large series in which several thousand FNABs were evaluated describe an excellent safety profile [12]. Up to now in our clinic we have collected >500 FNAB-samples of the liver without any serious complications to the patient. Invasiveness is comparable to a venapunction and the FNAB can be performed at any patient visit without anaesthesia or other preparations. Therefore the FNAB, in contrast to a TB, is suitable for repetitive use as bedside procedure or

in the outpatient clinic, enabling frequent monitoring and improved clinical analysis of the intrahepatic immune response, for instance to evaluate the impact of anti-viral therapy on hepatic inflammatory infiltration.

In this study lymphocyte populations present in FNAB-specimens were compared to lymphocytes isolated from TB and PB. Theoretically, representativeness of FNAB-samples can be affected by "contamination" with PB, in which case the obtained results would correlate with PB instead of the TBs. Visual inspection for blood contamination during the FNAB-procedure as well as cytospin evaluation of the FNAB-aspirate are important in this respect. Representative FNAB-specimens, i.e. containing 7 hepatocytes/100 leukocytes in a May-Grünwald-Giemsa stain of a fraction of the aspirate, are analysed with flow cytometry. This procedure is based upon a comparison between the inflammatory cells of liver and blood samples as described for large numbers of liver FNABs in the setting of viral hepatitis and transplantation [16-18]. We show that in experienced hands these criteria are almost never met, since in none of the 20 consecutive patients the FNAB-sample had to be excluded from further analysis. Although a May-Grünwald-Giemsa stain of the aspirate is always analysed in parallel to prevent analysis of non-representative FNABs, it cannot fully exclude mixture with leukocytes from PB. Previous studies have reported that intrahepatic lymphocyte populations contain more CD8+ than CD4+ lymphocytes as opposed to PB [3, 15]. Therefore, the ratio CD8+/CD4+ lymphocytes can be used as a sensitive means to validate flow cytometry of FNAB in evaluating intrahepatic immune response. In this study the ratio CD8/CD4 in FNABs correlated well with that observed in TBs, and was significantly higher than that observed in PB.

As flow cytometric analysis does not require fixation of living cells, FNAB-cytology may be used to investigate the possible role of cytokines and virus specific T cells in the pathogenesis of chronic viral hepatitis. To illustrate this application of FNAB we show that the percentages intrahepatic CD3+ lymphocytes producing IFN-γ in FNAB and TB correlate well. In order to investigate whether FNAB provides sufficient material to analyse the virus-specific CD8+ T cell response, we used soluble HLA-A2 tetrameric complexes able to visualise T cells specific for the HBV core epitope 18-27. Compared to PB, there is sequestering of tetramer+ CD8+ T cells in LIL isolated from TB as well as in FNAB-cytology of a patient with chronic HBV, confirming previous reports [2]. It therefore appears that a median of 10,000 lymphocytes in the FNAB-aspirate allows analysis of HBV-specific CTL.

Not only does the FNAB-procedure allow for flow cytometric analysis of infrequent cell populations. In this genomic era other technologies like PCR and microarray allow reproducible analysis of extremely low cell numbers that can easily be obtained by FNAB. Therefore, diagnostic procedures like the FNAB, may also be increasingly important for understanding potential intracellular interactions between the host cell and the virus, that contribute to persistence of viral hepatitis.

Although results of TB and FNAB correlate well, sampling error may provide a limitation in flow cytometric analysis of FNABs since material of multiple biopsies is pooled. However, we showed that in this protocol the sampling error involved in

evaluation of FNAB-cytology is minimal. In fact, the FNAB-procedure could be used to perform multiple passes in different areas of the liver, thus reducing the sampling error that is one of the limitations of the standard tissue needle biopsies.
In conclusion, flow cytometry of fine-needle-aspiration-biopsy of the liver allows easy, atraumatic and reliable analysis of lymphocytes obtained from the intrahepatic compartment. Therefore, the FNAB is a valuable tool in the study of immunopathology of viral hepatitis that may contribute to improved clinical evaluation of chronic viral liver disease.

Acknowledgements

The authors wish to thank A Bertoletti from the Institute of Hepatology, University College London, for his valuable help and discussion. We would also like to thank T.J. Tang for advice regarding the FNAB procedure and B. Hansen for her assistance with the statistical analysis.

References

1. Bravo AA, Sheth SG, Chopra S. Liver biopsy. N Engl J Med 2001; 344:495-500.
2. Maini MK, Boni C, Lee CK, et al. The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. J Exp Med 2000; 191:1269-80.
3. Norris S, Collins C, Doherty DG, et al. Resident human hepatic lymphocytes are phenotypically different from circulating lymphocytes. J Hepatol 1998; 28:84-90.
4. von Willebrand E, Hayry P. Reproducibility of the fine-needle aspiration biopsy. Analysis of 93 double biopsies. Transplantation 1984; 38:314-6.
5. Schlitt HJ, Nashan B, Ringe B, et al. Differentiation of liver graft dysfunction by transplant aspiration cytology. Transplantation 1991; 51:786-93.
6. Franca AV, Valerio HM, Trevisan M, et al. Fine needle aspiration biopsy for improving the diagnostic accuracy of cut needle biopsy of focal liver lesions. Acta Cytol 2003; 47:332-6.
7. Greene CL, Fehrman I, Tillery GW, Husberg BS, Klintmalm GB. A clear distinction between “immune activation of rejection” and “no immune activation” in liver transplant aspiration cytology. Transplant Proc 1988; 20:661-2.
8. Fuhr JE, Kattine AA, Nelson HS, Jr. Flow cytometry of liver fine needle aspirates. J Surg Oncol 1994; 56: 153-8.
9. Granados R, Garcia-Vela JA, Fenandez-Segoviano P, et al. Flow cytometric DNA analysis on fine needle aspiration biopsies of liver lesions. Cytopathology 2002; 13:273-83.
10. Frese M, Schwarzle V, Barth K, et al. Interferon-gamma inhibits replication of subgenomic and genomic hepatitis C virus RNAs. Hepatology 2002; 35:694-703.
11. Lau GK, Suri D, Liang R, et al. Resolution of chronic hepatitis B and anti-HBs seroconversion in humans by adoptive transfer of immunity to hepatitis B core antigen. Gastroenterology 2002; 122:614-24.
12. Lautenschlager I, Hockerstedt K, Hayry P. Fine-needle aspiration biopsy in the monitoring of liver allografts. Transpl Int 1991; 4:54-61.
13. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986; 1:307-10.
14. Day CL, Seth NP, Lucas M, et al. Ex vivo analysis of human memory CD4 T cells specific for hepatitis C virus using MHC class II tetramers. J Clin Invest 2003; 112:831-42.
15. Hata K, Van Thiel DH, Herberman RB, Whiteside TL. Phenotypic and functional characteristics of lymphocytes isolated from liver biopsy specimens from patients with active liver disease. Hepatology 1992; 15:816-23.
16. Hayry P, von Willebrand E. Practical guidelines for fine needle aspiration biopsy of human renal allografts. Ann Clin Res 1981; 13:288-306.
17. Franceschini N, Goncalves LF, Prompt CA, Barros SG, Cerski CT, Costa CA. Fine needle aspirative biopsy of the liver in HBsAG-positive patients with end-stage renal failure. Ren Fail 1994; 16:491-9.
18. Kuijf ML, Kwekkeboom J, Kuijpers MA, et al. Granzyme expression in fine-needle aspirates from liver allografts is increased during acute rejection. Liver Transpl 2002; 8:952-6.

CHAPTER 4

Different composition of intrahepatic lymphocytes in the immune-tolerance and immune-clearance phase of chronic hepatitis B

D. Sprengers[1], R.G. van der Molen[1], J.G. Kusters[1], B. Hansen[1], H.G.M. Niesters[2], S.W. Schalm[1], H.L.A. Janssen[1].

[1] Dept. of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands
[2] Dept. of Virology, Erasmus MC University Medical Center, Rotterdam, The Netherlands

J Med Virol 2006; 78: 561-568

Abstract

Based on virological and biochemical parameters chronic HBV patients are divided into distinct clinical phases: the immune-tolerance phase, the immune-clearance phase and the inactive carrier state. Unclear is whether these phases have characteristic intrahepatic immune responses. We aimed to characterise the composition of liver-derived lymphocytes in patients with chronic HBV infection. In 47 patients the composition of liver-derived lymphocytes was analysed by flow cytometry of Fine-Needle-Aspiration-Biopsies of the liver. The proportion NK cells in the liver was significantly higher in immune-tolerant than in immune-clearance patients and inactive carriers. No differences were found in proportion CD4+ T-cells and CD8+ T-cells, in these phases. However, when patients in the immune-clearance phase, with similar ALT, were grouped according to viral load, the proportion CD8+ T-cells was higher in those with high viral load. In contrast, the proportion CD4+ T-cells was increased in patients with low HBV-DNA. These differences were absent in peripheral blood. Intrahepatic HBV-specific CD8+ T-cells were mainly found in immune-clearance-patients with low viral load.
In conclusion, clear differences in the intrahepatic cellular infiltrate were found between the various clinical phases of chronic HBV infection. These findings are relevant to the design of new, individualised anti-viral strategies.

Introduction

Hepatitis B virus (HBV) is a noncytopathic, hepatotropic DNA virus that can cause chronic hepatitis, cirrhosis and hepatocellular carcinoma [1, 2]. Viral clearance during HBV infection is tightly associated with the appearance of a vigorous virus-specific T-cell response [3, 4]. In contrast, viral persistence and chronic hepatitis are associated with a markedly diminished HBV-specific T-cell response [3, 4]. However, the difference between immune responses present in resolved versus chronic HBV infection has obscured the diversity present within chronic hepatitis B. Chronic HBV infection is a highly heterogeneous disease, and the levels of virus replication, liver disease activity and humoral responses can differ considerably [5, 6]. Based on biochemical and virological parameters several potentially successive phases of chronic HBV have been well described [7, 8]. In the immune-tolerance phase serum hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) are detectable; serum HBV-DNA levels are high and serum aminotransferases are normal or minimally elevated. In the immune-clearance phase, serum HBV-DNA levels decrease and serum aminotransferase levels increase. In some patients flares in this phase are followed by HBeAg seroconversion. A third phase, termed the inactive carrier state is characterized by low levels of HBV-DNA and normal concentrations of aminotransferases. Treatment is indicated for patients with active inflammation and viral replication. Low initial HBV-DNA levels, high ALT elevations and HBV genotype have been associated with improved response to therapy [9-11]. Treatment is not indicated for those in the inactive carrier state. Interestingly, the different profiles of chronic hepatitis B have also been associated with different magnitudes of HBV-specific immune responses. Episodes of acute flares during chronic HBV are associated with a recovery of HBV-specific CD4+ T cell responses [12, 13]. Increased levels of HBV-specific CD8+ T cell responses have only been demonstrated in patients with a low level of HBV replication, irrespective of degree of inflammation, [14-16]. These data have led to the hypotheses that in each of the stages of chronic HBV a distinct intrahepatic immunological milieu exists, that determines the response to treatment. Thus characterization of this intrahepatic immunological environment is relevant to both improved understanding of chronic HBV infection as well as the design of antiviral therapy. The aim of this study was to characterize the different phases of chronic HBV infection by analysing the composition of key populations of immune effector cells, i.e. (HBV-specific) CD8+ T cells, CD4+ T cells and CD56+ Natural Killer (NK) cells in the liver.

Methods

Patients

Fine-Needle Aspiration Biopsies (FNAB) were collected from fifty non-treated consecutive patients with chronic HBV infection and stable biochemical and virological parameters when monitored for a minimum of three months prior to the biopsy [17, 18].

All patients were HBsAg positive >6 months, HBV-DNA positive (> 100 geq/ml), anti-HBV core antigen (HBcAg) antibody positive, and HBeAg or anti-HBe positive. In all patients antibodies to HCV, HIV-1 HIV–2 and hepatitis Delta were absent. None of the patients had significant co morbidity. Patients were stratified to the different phases of chronic HBV according to predefined biochemical and virological parameters [19, 20]; immune-tolerant: alanine transaminase (ALT) normal (< 45 IU/l) and HBV-DNA >10^7 geq/ml; immune-clearance: ALT >45 IU/l and all levels of HBV-DNA; inactive state: ALT normal and HBV-DNA ≤ 10^6 geq/ml. All patients signed informed consent before the samples were collected.

Virological assessment

Blood samples for reported virological and biochemical data were obtained at the time of fine-needle aspiration biopsy. Serum HBeAg, HBsAg, anti-HBs, anti-HBc, anti-delta, anti-HCV and anti-HIV1/2 were determined using commercially available immunoassays (Abbott Laboratories, North Chicago, IL, USA). Serum HBV-DNA was measured with TaqMan real-time PCR (detection limit 100 geq/ml) validated on the Eurohep Standard [21].

PCR and HBV-DNA sequencing

HBV-DNA was extracted from serum samples taken at the time of fine-needle aspiration biopsy by using a MagnaPure LC station (Roche Applied Science, Penzberg, Germany). Both extraction and amplification efficiency was monitored by spiking the samples with a known amount of control virus [22]. For sequencing of the core gene, primers were used as described previously [23]. The amplifications were purified and the core region HBc 18-27 was sequenced directly by using an ABI 3100 automated sequencer (Applied Biosystems, Nieuwekerk aan den IJssel, the Netherlands). The genotype of the predominant HBV population was determined by analysis of sequenced portions of the core and surface antigen encoding DNA isolated from the serum [24].

Fine-Needle Aspiration Biopsy

Details of the fine-needle aspiration biopsy-procedure are described elsewhere [25]. Briefly, a mandarin containing 25-gauge (diameter 0.5 mm) needle (Braun AG, Melsungen, Germany) is punctured in the 8th or 9th right intercostal space. After removal of the mandarin a 10 mL syringe filled with 2 mL RPMI supplemented with 0.1% human serum albumin (Sanquin CLB, Amsterdam, the Netherlands) and 25 IU/mL heparin (Leo Pharma, Breda, the Netherlands) is attached. Liver cells are aspirated by negative syringe pressure. Next, the specimen is transferred into a 15 mL tube and placed on ice immediately before further analysis. Validation for immunological assessment of the liver using the fine-needle aspiration biopsy-aspirate, has been described previously [17, 18].

Immunophenotyping

Erythrocytes were removed from peripheral blood and fine-needle aspiration

biopsy-specimens by NH_4CL-lysis. The cell suspension was washed twice before antibody staining and four-color flow cytometric evaluation using FACS Caliber (Becton and Dickinson Biosciences; San Jose, USA (BD)) and CELLQuest software (BD). Immunofluorescence staining was performed as described previously [16]. The following antibodies were divided in 2 panels for FACS analysis of all lymphocytes collected with the fine-needle aspiration biopsy: anti-CD4 PerCP Cy5 (BD), anti-CD8 FITC (Dako, Glostrup, Denmark (Dako)); anti-CD8 APC (Dako); anti-CD56 Fitc (BD) and anti-CD3 PE (Immunotech, Marseille, France). Matched isotype antibodies were used for control staining. NK cells were defined as CD8- CD56+ lymphocytes.

HLA typing, HLA-A2 tetramer staining

Screening for HLA-A2 haplotype positive HBV patients was performed by staining PBMCs with an anti-HLA-A2 antibody derived from a hybridoma (clone BB7.2, ATCC HB-82). Staining was followed by an FITC-conjugated rabbit anti-mouse anti-IgG (Dako) secondary antibody and flow cytometric analysis. In HLA-A2+ HBV patients HBV-specific T-cells were stained with the soluble HLA-A2 peptide tetramer: HBc 18-27 (FLPSDFFPSV) APC-conjugated (ProImmune, Oxford, UK) together with anti-CD8 FITC (Dako). Staining was performed as reported before [16]. In control experiments with PBMCs of HLA-A2 positive healthy controls (n=4) and HLA-A2 negative HBV patients (n=4) the tetramer bound to 0.019 ± 0.002 and 0.013 ± 0.004% of total CD8+ T cells respectively. Therefore background staining level was determined 0.025% (mean +2SD).

Statistical analysis

Data are expressed as mean ± SEM, unless indicated otherwise. Oneway and multivariable analysis of variance was performed with SPSS 11.0.1 for Windows (SPSS, Chicago, IL). For pair wise comparison Bonferroni correction was applied. Statistical analyses were performed using χ^2 for categorical variables. Correlations were determined using the Spearman's correlation test. In all analyzes a p-value of <0.05 was considered statistically significant.

Results

Patients

Of 50 consecutive chronic HBV patients sampled, 47 were eligible. Three patients were excluded from further analysis: one patient due to co existing alcohol abuse, one for receiving lamivudine therapy, and one as a consequence of non-representative fine-needle aspiration biopsy in cytospin evaluation. The 47 chronic HBV patients, 36 (72%) male and with a median age of 35 (14 - 69), were stratified to the different phases of chronic HBV infection based on predefined biochemical and virological status (table I). Seven patients were assigned to the immune-tolerant phase with a median ALT of 33 IU/l (16-43), median viral load of $2.5x10^9$ geq/ml ($1.1x10^7$-$1.6x10^{10}$) and all HBeAg positive. Twenty-four patients were assigned to the immune-clearance

phase with a median ALT of 91.5 IU/l (48-863), median viral load of $9x10^7$ geq/ml ($1.6x10^3$-$1.1x10^{10}$) and 10 HBeAg positive. Sixteen patients were inactive carriers with median ALT of 25.5 IU/l (16-45), median HBV-DNA of $3.4x10^3$ geq/ml (162-$1.2x10^6$), all HBeAg negative. Except for race, there was even distribution of age, sex and genotype. The mean number of cells obtained by fine-needle aspiration biopsy and analyzed by flow cytometry was 7316 (± 1463) for patients in the immune-tolerant phase, 8159 (± 232) for patients in the immune-clearance phase, and 7349 (± 459) for inactive carriers. The differences in these cell counts were not significant. All samples were collected without any complications to the patients.

Table I. Patient characteristics at the time of aspiration biopsy.

Patients n=47	Immune tolerance (n=7)	Immune clearance (n=24)	Inactive Carriers (n=16)
Age*	41 (19-69)	31.5 (16-62)	35.5 (17-51)
Sex	3 M, 4 F	20 M, 4 F	10 M, 6 F
Race+	4 Asian, 1 Cauc., 2 Mixed Race	5 Asian, 17 Cauc., 2 Mixed Race	7 Asian, 5 Cauc., 4 Mixed Race
ALT* (U/l)	33 (16-43)	91.5 (48-863)	25.5 (16-45)
HBV-DNA* (geq/ml)	$2.5x10^9$ ($1.1x10^7$-$1.6x10^{10}$)	$9x10^7$ ($1.6x10^3$-$1.1x10^{10}$)	$3.4x10^3$ (162-$1.2x10^6$)
HBeAg positive	7	10	0
Genotype A-B-C-D-E	2-0-4-0-1	4-4-2-11-3	3-2-5-2-3 1ND
Cirrhosis	-	3x	1x

* Median values (range).
ND: Not Determined
+ Uneven distribution; p = 0.037 in χ^2 test.

The composition of liver derived lymphocytes in patients with chronic HBV infection

CD3- CD56+ NK cells, CD4+ T-cells and CD8+ T cells have all been shown to be involved in the anti-HBV immune response [13, 14, 26]. The proportion of these immune effector cells in liver derived lymphocytes and PBMCs of patients with chronic HBV is summarized in figure 1. In immune-tolerant patients the fraction NK cells in the liver was higher than in patients in the immune-clearance- and inactive-phase; mean percentage 41.1 ± 2.8, 29.8 ± 1.7 and 35.0 ± 2.0 respectively (immune-tolerance vs. immune-clearance p = 0.006, immune-tolerant vs. inactive carriers p = 0.052). Also in PBMCs the fraction NK cells was higher in immune-tolerant patients than in patients in the immune-clearance- and inactive phase of chronic HBV; mean % 28.2 (± 4.5), 17.1 (± 1.8) and 18.7 (± 2.9) respectively (immune-tolerance vs. immune-clearance p

= 0.015, immune-tolerant vs. inactive carriers p = 0.047).

No differences were found with respect to the proportion CD4+ T cells or CD8+ T-cells in the different phases of chronic HBV, either in the liver or peripheral blood. In patients in the immune-tolerant phase, the immune-clearance phase and the inactive carrier state the mean percentage CD4+ T cells in the liver was 25.2 ± 2.8, 25.8 ± 1.7 and 22.8 ± 2.0, respectively, in peripheral blood 37.7 (± 2.5) 41.6 (± 2.0) and 39.7 (± 2.3) respectively. In all three phases the liver contained more CD8+ T-cells than CD4+ T cells, whereas this was the opposite in peripheral blood. The mean percentage CD8+ T-cells in the liver was 34.5 ± 2.7 in patients in the immune-tolerant phase, 36.7 ± 2.0 in patients in the immune-clearance phase and 37.9 ± 2.2 in inactive carriers. In peripheral blood these percentages were considerably lower; 29.7 (± 3.1), 29.3 and (± 1.9) 31 (± 1.9) respectively. Results were independent of age, sex, race and HBV genotype.

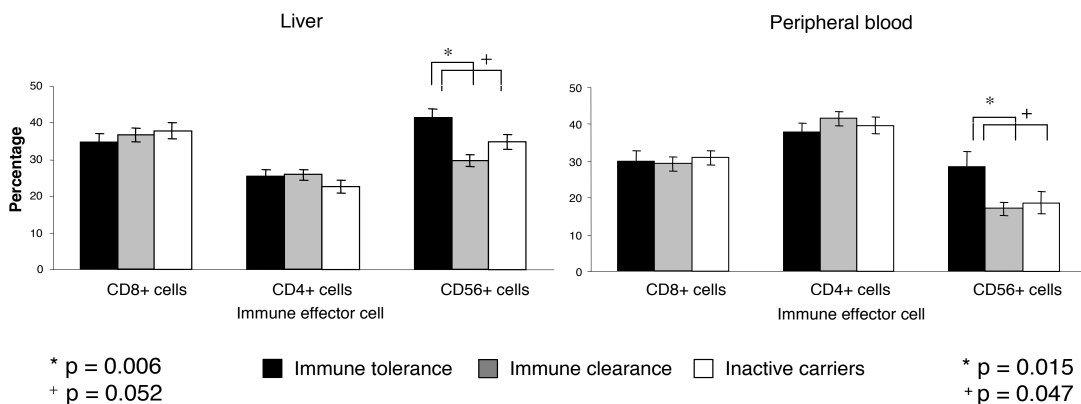


Figure 1.
Mean percentage CD8+ T-cells, CD4+ T-cells and NK cells in liver derived lymphocytes and peripheral blood of patients in the immune-tolerant phase (■), patients in the immune-clearance phase (▩) and inactive carriers (□).
In the liver and peripheral blood of immune-tolerant patients NK cells are the dominant lymphocyte population. In the liver of all three patient groups the frequencies of CD8+ T-cells are dominant over the frequencies of CD4+ T-cells, whereas the opposite is the case in peripheral blood.

The composition of peripheral blood and liver derived lymphocytes in patients in the immune-clearance phase of chronic HBV

In the immune-clearance phase serum HBV-DNA levels varied from high to low and some individuals had seroconverted from HBeAg positive to anti–HBeAg positive. Recently, it was suggested that a serum HBV-DNA of 10^7 geq/ml appears a dividing threshold below which circulating HBV-specific T-cells can consistently be detected *ex vivo* [14], suggesting that the different virological stages may be the result of dynamics in the immune response. Therefore, the composition of liver derived lymphocytes in

patients with high viral load (HBV-DNA>10^7 geq/ml; HBV-high) was compared to liver derived lymphocytes in patients with low viral load (HBV-DNA <10^7 geq/ml; HBV-low). Table II summarizes the characteristics of the 2 patient groups. Of 24 patients in the immune-clearance phase 14 had high viral load, with a median HBV-DNA of 5.7×10^8 geq/ml (2×10^7-1.1×10^{10}) and a median ALT of 95 IU/L (48-863). Ten patients had a low virus titer with a median HBV-DNA of 1.9×10^4 geq/ml (1.6×10^3-3.5×10^6) and a median serum ALT of 89 IU/L (63-120). The difference in ALT levels between the 2 groups was not significant. Of 14 HBV-high patients 10 were HBeAg positive, whereas all HBV-low patients had seroconverted to anti-HBe positive.

Table II: Patients in the immune-clearance phase

Immune-clearance Patients (n=24)	**HBV-DNA >1×10^7 geq/ml** (n=14)	**HBV-DNA <1×10^7 geq/ml** (n=10)
Age*	30 (20-62)	36.5 (16-59)
Sex	10 M, 4 F	10 M
Race+	2 Asian, 12 Cauc.	3 Asian, 5 Cauc., 2 Mixed Race
ALT* (U/l)	95.5 (48-863)	89 (63-120)
HBV-DNA* (geq/ml)	5.7×10^8 (2×10^7-1.1×10^{10})	1.9×10^4 (1.6×10^3-3.5×10^6)
HBeAg positive	10	0
Genotype A-B-C-D-E	2-2-1-9-0	2-2-1-2-3

*Median values (range).

+Uneven distribution; $p < 0.05$ in χ^2 test.

The difference in serum ALT levels between IC-hi and IC-lo was not significant.

The proportion of CD4+ T cells, CD8+ T cells and NK cells in the liver of patients in the immune-clearance phase is summarized in figure 2. Compared to patients with high HBV-DNA, in the liver of those with low HBV-DNA the mean percentage CD4+ T cells was higher; 22.5% (± 1.8) vs. 30.4% (± 1.4) respectively (p = 0.02). This difference was not found in PBMCs; the mean percentage CD4+ T cells was 41.2 (± 3.1) and 42.1 (± 2.1) respectively. Interestingly, the liver of patients in the immune-clearance phase and low viral load contained a significantly higher proportion CD4+ T cells than inactive carriers (30.4% ± 1.4 vs. 22.8 ± 2.0, respectively, p = 0.02). In

contrast, in immune-clearance patients with high viral load the fraction CD8+ T cells was higher than in patients with low viral load (mean percentage 40.3 ± 2.4 and 31.7 ± 2.8 respectively ($p = 0.02$)). Again, this difference was absent in peripheral blood; 31.4 (± 2.8) vs. 26.3 (± 2.2), respectively.
No difference was observed between the two immune-clearance-groups regarding the proportion of NK cells in the liver and peripheral blood. In the liver of patients with high viral load 29.5% ± 2.5 of total lymphocytes were NK cells, as compared to 30.2% ± 2.2 in patients with low viral load. In peripheral blood the means were 15.2% (± 2.1) and 19.6% (± 3.0) respectively.
To exclude association between proportions of intrahepatic lymphocyte populations a spearman correlation test was performed. Only weak negative correlations were found between CD8+ T cells and CD4+ T cells ($r = -0.3$), between CD4+ T cells and NK cells ($r = -0.2$) and between CD8+ T-cells and NK cells ($r = -0.2$). The intrahepatic distribution of all investigated lymphocyte subsets was independent of age, sex, genotype and race.

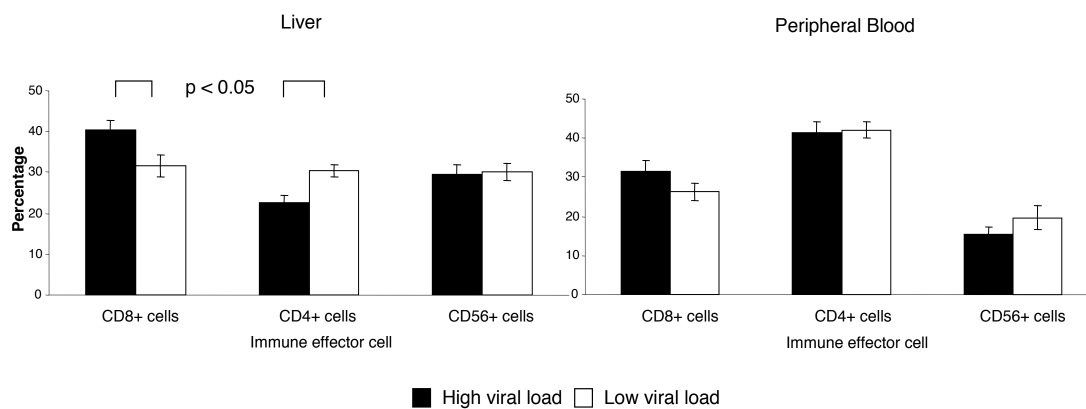


Figure 2.
Mean percentage CD8+ T-cells, CD4+ T-cells and NK cells in liver derived lymphocytes and PBMCs of patients in the immune-clearance phase. Patients were divided based upon HBV-DNA titers; $>10^7$ geq/ml (■) and $<10^7$ geq/ml (□).
In the liver of immune-clearance-patients with high viral load the proportion CD8+ T cells was significantly higher than in patients with low viral load. In the liver immune-clearance-patients with low viral load the percentage CD4+ T cells was significantly higher than in patients with high viral load. These differences were absent in peripheral blood.

Ex Vivo Frequency of HBV-specific CD8+ T-cells in liver
Fifteen of 47 patients (32%) were HLA-A2 positive; 3 patients in the immune-tolerant phase, 9 in the immune-clearance phase and 3 inactive carriers (table III). In these cases staining of HBV-specific T-cells was performed with soluble HLA-A2 tetrameric complexes able to visualize T-cells specific for the core epitope 18-27. In 3 of 15 patients tetramer staining exceeded the background level of 0.025% of total CD8+

T-cell population. All 3 were in the immune-clearance phase; 1 patient with high viral load (case 4) and 2 with low viral load (cases 10 and 12) (fig 3). Interestingly, in the only patient with high HBV-DNA and detectable HBV specific CD8+ T-cells (case 4), serum ALT of 187 IU/L normalized and HBV-DNA decreased from $2x10^7$ geq/ml to $1x10^4$ geq/ml within a month after sampling. The absence of core 18-27-specific CD8+ T-cells may be caused by viral mutations resulting in specific epitope inactivation [14]. Therefore, in all 15 HLA A2+ patients this core epitope was sequenced. Six patients were infected with an HBV strain carrying a valine-to isoleucine mutation at position 27. However, this amino acid mutation is not associated with core 18-27 unresponsiveness, since it was also detected in a patient who showed a CD8+ T-cell response against this epitope. One inactive carrier in whom no tetramer positive cells were detected (case 15), displayed an Aspartic acid – to – Asparagine substitution at position 22, which is associated with reduced CD8+ T-cell activation [27].

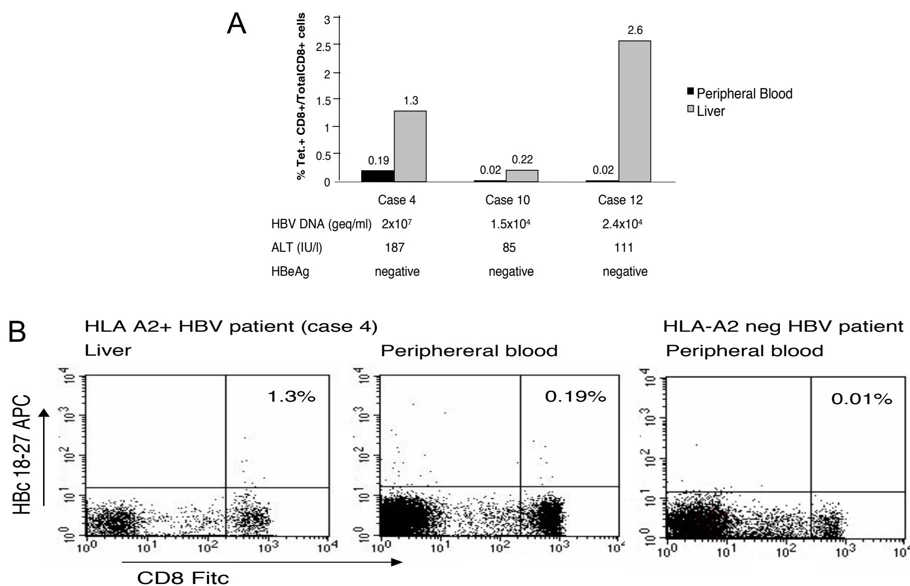


Figure 3. HBc 18-27-tetramer binding to CD8+ T cells.
3a) Percentage HBc 18-27-tetramer positive CD8 positive T cells in the liver and in peripheral blood of the 3 patients in which these cells were detected (Liver ◻, Peripheral blood ◼). In all 3 patients there is sequestering of tetramer positive cells in the liver. In the only patient with high HBV-DNA and detectable HBV-specific CD8+ T-cells, serum ALT normalized and HBV-DNA decreased to $1x10^4$ geq/ml within a month after sampling.
3b) Percentages HBc18-27 tetramer+ CD8+ T cells in a representative HLA-A2+ patient (case 4). The number in the upper right quadrant indicates the percentage HBc 18-27 tetramer+ CD8+ T cells of total CD8+ T cells. The right panel shows data of a representative HLA-A2- chronic HBV patient, serving as control for aspecific tetramer binding.

Discussion

It is now widely accepted that chronic HBV patients can be grouped into different well defined phases: an initial replicative, immune-tolerance phase, followed by gradual decrease of viral replication in the immune-clearance phase and a third asymptomatic inactive carrier state [7, 8].

We found that in immune-tolerant patients but not in immune-clearance patients and inactive carriers, lymphocytes that bear the classical NK phenotype are the dominant immune effector cell population in the liver. In fact, the distribution of NK cells, CD4+ and CD8+ T cells in immune-tolerant patients resembles the situation observed previously in the normal human liver [28] which may support the assumption that immune-tolerant patients, characterized by high viral load and normal ALT levels have a low endogenous immune response to HBV [9, 29].

In contrast to immunohistochemical staining of a standard liver biopsy, the fine-needle aspiration biopsy does not allow the necessary equal sample size in each patient in order to compare absolute cell numbers. It is known that inactive carriers have less cellular infiltrate than patients with active hepatitis [16] and therefore the absolute number of NK cells present in the liver of immune-tolerant patients may be lower than in the immune-clearance phase.

However, similar to previous immuno histochemistry studies we found a relative deficiency of CD4+ T cells compared to CD8+ T cells in the liver of patients with chronic HBV [30, 31]. Interestingly, in these previous studies it was noticed that in the HBeAg negative subpopulation of the individuals with active hepatitis, the fraction CD4+ T-cells had increased compared to HBeAg positive patients with active hepatitis [30]. Indeed, we found that in the immune-clearance phase, the patients with high viral load -of which a large majority is HBeAg positive-, had a lower proportion of intrahepatic CD4+ T cells, than patients with low viral load of which none were HBeAg positive. The immune-clearance phase comprises a heterogenic patient population, and the various virological stages may be the result of dynamics in the intrahepatic immune response.

Alternatively, the increased fraction of intrahepatic CD4+ T cells in patients with low HBV-DNA and hepatitis might be a consequence and not the cause of low HBV-DNA. Nevertheless, a previous study has shown that CD4+ T cells are required to sustain CD8+ T cell responses during chronic viral infection [32]. Therefore, an increment in the fraction CD4+ T cells in the liver of patients in the immune-clearance phase, may contribute to an improvement of the HBV-specific CD8+ T cell response which may result in a decreasing viral load. Our observation and that by others [14-16], that HBc 18-27–specific CD8+ T cells are primarily found in the liver of patients with active hepatitis and low HBV-DNA, may support this concept. Unexpectedly, in one patient in the immune-clearance phase with a high viral load, we found high frequencies of intrahepatic HBc 18-27 specific CD8+ cells. Shortly after obtaining the liver-sample, HBV-DNA levels dropped from $2x10^7$ geq/ml to$1x10^4$ geq/ml. Although this may have been the time point at which the antiviral immune response was developing, additional

samples were lacking for sequential analysis and follow-up.
In chronic HBV and chronic hepatitis C infection the efficacy of interferon-alpha in inducing sustained virologic response has been attributed to the efficacy in induction and maintenance of significant CD4+ T cell responses [33 34]. The improved response to anti-viral therapy of chronic HBV patients with hepatic inflammation and low viral load compared to patients with inflammation and high viral load [9, 10] may be a direct consequence of an increment in intrahepatic CD4+ T cells.
The differences in proportion CD8+ T cells and CD4+ T cells were absent in peripheral blood, which underlines the value of studying intrahepatic immunology as compared to immune effector cells derived from the circulation, in investigating immune pathology in chronic HBV infection.
In conclusion, analysis of the phenotype of intrahepatic immune response suggests that the clinical phases of chronic HBV infection, separated on biochemical and virological parameters, have characteristic intrahepatic immune responses. In immune-tolerant patients NK cells are the dominant immune effector cell population in the liver, and in the immune-clearance phase the patients with low viral load had a higher proportion of intrahepatic CD4+ T-cells, than patients with high viral load. These findings are relevant to the design of new, individualised anti-viral strategies.

References

1. Kane M. Global programme for control of hepatitis B infection. Vaccine 1995; 13 Suppl 1:S47-9.
2. Wright TL, Lau JY. Clinical aspects of hepatitis B virus infection. Lancet 1993; 342:1340-4.
3. Ferrari C, Penna A, Bertoletti A, et al. Cellular immune response to hepatitis B virus-encoded antigens in acute and chronic hepatitis B virus infection. J Immunol 1990; 145:3442-9.
4. Jung MC, Spengler U, Schraut W, et al. Hepatitis B virus antigen-specific T-cell activation in patients with acute and chronic hepatitis B. J Hepatol 1991; 13:310-7.
5. Lok AS, McMahon BJ. Chronic hepatitis B. Hepatology 2001; 34:1225-41.
6. Maruyama T, McLachlan A, Iino S, Koike K, Kurokawa K, Milich DR. The serology of chronic hepatitis B infection revisited. J Clin Invest 1993; 91:2586-95.
7. de Franchis R, Hadengue A, Lau G, et al. EASL International Consensus Conference on Hepatitis B. 13-14 September, 2002 Geneva, Switzerland. Consensus statement (long version). J Hepatol 2003; 39 Suppl 1: S3-25.
8. Lok AS. Chronic hepatitis B. N Engl J Med 2002; 346:1682-3.
9. Lok AS, Heathcote EJ, Hoofnagle JH. Management of hepatitis B: 2000--summary of a workshop. Gastroenterology 2001; 120:1828-53.
10. 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.
11. Janssen HL, van Zonneveld M, Senturk H, et al. Pegylated interferon alfa-2b alone or in combination with lamivudine for HBeAg-positive chronic hepatitis B: a randomised trial. Lancet 2005; 365:123-9.
12. Tsai SL, Chen PJ, Lai MY, et al. Acute exacerbations of chronic type B hepatitis are accompanied by increased T cell responses to hepatitis B core and e antigens. Implications for hepatitis B e antigen seroconversion. J Clin Invest 1992; 89:87-96.
13. Rossol S, Marinos G, Carucci 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.
14. Webster GJ, Reignat S, Brown D, et al. Longitudinal analysis of CD8+ T cells specific for structural and nonstructural hepatitis B virus proteins in patients with chronic hepatitis B: implications for immunotherapy. J Virol 2004; 78:5707-19.
15. Sobao Y, Tomiyama H, Sugi K, et al. The role of hepatitis B virus-specific memory CD8 T cells in the control of viral replication. J Hepatol 2002; 36:105-15.
16. Maini MK, Boni C, Lee CK, et al. The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. J Exp Med 2000; 191:1269-80.
17. Sprengers D, van der Molen RG, Kusters JG, et al. Flow cytometry of fine-needle-aspiration biopsies: a new method to monitor the intrahepatic immunological environment in chronic viral hepatitis. J Viral Hepat 2005; 12:507-12.
18. Sprengers D, van der Molen RG, Kusters JG, Janssen HL. Coarse vs. fine needle aspiration biopsy. J Hepatol 2004; 41:503-4; author reply 504.
19. Chu CJ, Hussain M, Lok AS. Quantitative serum HBV DNA levels during different stages of chronic hepatitis B infection. Hepatology 2002; 36:1408-15.
20. Hadziyannis SJ, Vassilopoulos D. Hepatitis B e antigen-negative chronic hepatitis B. Hepatology 2001; 34: 617-24.
21. Pas SD, Fries E, De Man RA, Osterhaus AD, Niesters HG. Development of a quantitative real-time detection assay for hepatitis B virus DNA and comparison with two commercial assays. J Clin Microbiol 2000; 38:2897-901.
22. Niesters HG. Clinical virology in real time. J Clin Virol 2002; 25 Suppl 3:S3-12.
23. Stuyver L, De Gendt S, Van Geyt C, et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol 2000; 81:67-74.
24. Norder H, Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology 1994; 198:489-503.
25. Lautenschlager I, Hockerstedt K, Hayry P. Fine-needle aspiration biopsy in the monitoring of liver allografts. Transpl Int 1991; 4:54-61.
26. Kakimi K, Guidotti LG, Koezuka Y, Chisari FV. Natural killer T cell activation inhibits hepatitis B virus replication in vivo. J Exp Med 2000; 192:921-30.
27. Bertoletti A, Southwood S, Chesnut R, et al. Molecular features of the hepatitis B virus nucleocapsid T-cell epitope 18-27: interaction with HLA and T-cell receptor. Hepatology 1997; 26:1027-34.
28. Norris S, Collins C, Doherty DG, et al. Resident human hepatic lymphocytes are phenotypically different from circulating lymphocytes. J Hepatol 1998; 28:84-90.
29. Lau GK, Nanji A, Hou J, et al. Thymosin-alpha1 and famciclovir combination therapy activates T-cell

response in patients with chronic hepatitis B virus infection in immune-tolerant phase. J Viral Hepat 2002; 9:280-7.

30. Montano L, Aranguibel F, Boffill M, Goodall AH, Janossy G, Thomas HC. An analysis of the composition of the inflammatory infiltrate in autoimmune and hepatitis B virus-induced chronic liver disease. Hepatology 1983; 3:292-6.
31. Yang PM, Su IJ, Lai MY, et al. Immunohistochemical studies on intrahepatic lymphocyte infiltrates in chronic type B hepatitis, with special emphasis on the activation status of the lymphocytes. Am J Gastroenterol 1988; 83:948-53.
32. Matloubian M, Concepcion RJ, Ahmed R. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. J Virol 1994; 68:8056-63.
33. 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.
34. Kamal SM, Fehr J, Roesler B, Peters T, Rasenack JW. Peginterferon alone or with ribavirin enhances HCV-specific CD4 T-helper 1 responses in patients with chronic hepatitis C. Gastroenterology 2002; 123: 1070-83.

CHAPTER 5

Analysis of intrahepatic HBV-specific cytotoxic T-cells during and after acute HBV infection in humans

D. Sprengers[1], R.G. van der Molen[1], J.G. Kusters[1], R.A. De Man [1], H.G.M. Niesters[2], S.W. Schalm[1], H.L.A. Janssen[1].

[1] Dept. of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands
[2] Dept. of Virology, Erasmus MC University Medical Center, Rotterdam, The Netherlands

J Hepatol 2006; 45: 182-189

Abstract

Characteristics of the intrahepatic virus-specific T-cell response in patients with acute hepatitis B virus (HBV) infection have not been studied due to the risk of complications associated with standard liver biopsies. In this study we aimed to characterize the virus-specific CD8+ T-cell response in the liver of patients with acute HBV infection using the fine-needle aspiration-biopsy (FNAB).

In HLA-A2 positive patients with acute HBV infection a FNAB was done at first presentation, at the time of HBsAg-seroconversion and three months after HBsAg-seroconversion. HLA-A2 tetramers were used to identify HBV-specific CD8+ T-cells in FNAB-cytology and peripheral blood (PB).

At first presentation there was a correlation between the frequency of intrahepatic CD8+ T-cells and the degree of liver damage. At all time points there was sequestering of HBV-specific CD8+ T-cells in the liver, and the percentage of intrahepatic HLA-DR expressing HBV-specific CD8+ T-cells was higher than in PB. Three months after HBsAg-seroconversion the frequency of intrahepatic HBV-specific CD8+ T-cells remained high.

In conclusion, HBV-specific CD8+ T-cells are compartmentalized in the liver during acute HBV infection. Their presence in the liver may suggest a role in the resolution of the infection. Intrahepatic HBV-specific CD8+ T cells remain detectable at high frequencies after HBsAg-seroconversion.

Introduction

Cytotoxic T lymphocytes (CTL) contribute to control of hepatitis B virus (HBV) infection by inhibiting viral replication in infected liver cells [1-3]. Patients with acute hepatitis who ultimately control the virus, possess a strong CTL response in peripheral blood (PB) that is able to recognize different epitopes within HBV proteins and persists without liver damage [2-4]. In contrast, this CTL response is barely detectable in the circulation of chronic patients despite biochemical and histological signs of liver damage [5 6]. In human HBV infection, studies have been hampered by the difficulty of studying the intrahepatic compartment. The few studies that have investigated HBV-specific immune responses in the liver have focussed on chronic infection and showed important differences between circulating and intrahepatic HBV-specific CD8+ T cells with respect to phenotype, activation status and frequency [7 8]. The virus-specific T cell response at the site of viral replication has not been studied yet in acute HBV patients. In this setting, sampling of the liver is rarely justifiable due to the serious complications associated with standard liver tissue biopsy techniques [9]. The fine-needle aspiration biopsy (FNAB) of the liver with an invasiveness comparable to a venapunction is much better tolerated, and can therefore be repeated numerous times[10, 11]. We have recently shown that flow cytometric analysis of liver derived lymphocytes in the FNAB-aspirate of patients with chronic viral hepatitis, allows reliable analysis of the intrahepatic compartment [10, 12]. This observation has facilitated the present study in which we aimed to characterize the virus-specific CD8+ T cell response in the liver of patients with acute HBV infection. HLA-A2 tetrameric complexes with 3 different peptides representing HBV core, envelope and polymerase epitopes were used to identify HBV-specific CD8+ T cells in FNAB-cytology. These antigen-specific populations were followed over the course of infection and their phenotype and frequencies were correlated with biochemical parameters.

Methods

Patients

Between January and July 2004 nine patients with acute HBV infection were referred to our clinic by local Community Health Centers (GGD). Five HLA-A2 positive adult-subjects with self-limited acute HBV-infection participated in the present study on the basis of the expression of the HLA-A2 allele and in all five patients the virus-specific CD8+ T cell response in the liver and peripheral blood was monitored at the time of first presentation (day 0) and soon after HBsAg had disappeared from the circulation (HBsAg-seroconversion). In four patients (patient 1, 2, 3 and 5) an additional sample was collected at day 30 and/or 90 days post HBs-seroconversion.

Diagnosis of acute hepatitis was based on increased serum alanine aminotransferase (ALT) activity (at least 10 times the upper limit of normal), first detection of hepatitis B surface (HBsAg) antigen and immunoglobulin (Ig) M anti-hepatitis B core antigen (anti-HBc) antibodies in the serum, along with the recent onset of jaundice and

associated symptoms. In all patients antibodies to hepatitis C virus (HCV), HIV-1 HIV–2 and hepatitis Delta were absent. None of the patients had significant co morbidity. All patients recovered clinically; the transaminase levels normalized, and HBsAg and HBeAg antigens became negative in the serum. All patients gave written informed consent before the samples were collected. This study was performed in agreement with the local Medical Ethics Committee.

FNAB-procedure
Intrahepatic immune cells were obtained by fine-needle aspiration biopsy (FNAB). Details of the FNAB-procedure are described elsewhere [10-12]. Briefly, a mandarin containing 25-gauge (diameter 0.5 mm) needle (Braun AG, Melsungen, Germany) is punctured in the 8th or 9th right intercostal space. After removal of the mandarin a 10 mL syringe filled with 2 mL RPMI supplemented with 0.1% human serum albumin (Sanquin CLB, Amsterdam, the Netherlands) and 25 IU/mL heparin (Leo Pharma, Breda, the Netherlands) is attached. Liver cells are aspirated by negative syringe pressure. Next, the needle is flushed with 3 mL culture medium to obtain the liver cells from inside the needle and the specimen is transferred into a 15 mL tube and placed on ice immediately.

Virological assessment
Blood samples for virological and biochemical data were obtained at the time of FNAB. Serum HBsAg, anti-HBsAg, HBeAg, anti-HBcAg, anti-delta, anti-HCV and anti-HIV1/2 were determined using commercially available immunoassays (Abbott Laboratories, North Chicago, IL, USA). Serum HBV DNA was measured with TaqMan real-time PCR (detection limit 362 geq/ml) validated on the Eurohep Standard [13].

PCR and HBV DNA sequencing
HBV DNA was extracted from serum samples taken at the time of FNAB by using a MagnaPure LC station (Roche Applied Science, Penzberg, Germany). Both extraction and amplification efficiency was monitored by spiking the samples with a known amount of control virus [14]. For sequencing primers were used as described previously [15]. The amplifications were purified and the core region 18-27, envelope 183-191 and polymerase 816-824 were sequenced directly by using an ABI 3100 automated sequencer (Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands). The genotype of the HBV population was determined by analysis of sequenced portions of the core and surface antigen encoding DNA isolated from the serum [16].

HLA typing
PBMC were isolated from heparinized blood samples by density gradient centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Screening for HLA-A2 haplotype positivity was performed by staining PBMC with an anti-HLA-A2 antibody derived from a hybridoma (clone BB7.2, ATCC HB-82). Staining was followed by an FITC-conjugated rabbit anti-mouse anti-IgG (Dako, Glostrup, Denmark

(Dako) secondary antibody and flow cytometric analysis.

Immunophenotyping and HLA-A2 tetramer staining

Erythrocytes were removed from FNAB-specimens by NH_4CL-lysis. This cell suspension and isolated PBMC were washed twice before antibody staining and four-color flow cytometric evaluation using FACS Caliber (Becton and Dickinson Biosciences; San Jose, USA (BD)) and CELLQuest software (BD). Immunofluorescence staining was performed as described previously [4]. HBV-specific T cells were stained with soluble HLA-A2/peptide tetramers. Sequences of HBV (genotype D) peptides used were: FLPSDFFPSV (core 18-27) PE-conjugated, FLLTRILTI (envelope 183-191) APC-conjugated, and SLYADSPSV (polymerase 816-824) APC-conjugated (all manufactured by ProImmune, Oxford, UK). These soluble HLA-A2/peptide tetramers in combination with anti-CD4 PE (BD), anti-CD8 FITC (Dako) and anti-HLA-DR PerCP (BD) monoclonal antibodies (MAbs) were divided in 2 panels for FACS analysis of all lymphocytes collected by FNAB, and at least $2x10^5$ PBMC per panel. Matched isotype antibodies were used for control staining. In control experiments with PBMC and FNAB-cytology of HLA-A2 positive patients with chronic non-HBV related liver disease (n=4) and HLA-A2 negative chronic HBV patients (n=4) the level of aspecific tetramer binding in PBMC was determined as 0.03% of total CD8+ T cells (mean +2SD). In FNAB-cytology this percentage was always lower. In analysis of tetramer binding in PBMC and liver derived lymphocytes background-staining level was set on 0.03% of total CD8+ T cells.

Results

Patients

Figure 1 shows the patient characteristics and the dynamics of HBV replication and liver inflammation, as evidenced by serum ALT levels, at the time points of sampling. All patients showed a similar pattern of serum HBV and ALT reduction. At first presentation, high levels of serum ALT and HBV DNA co-existed. Both parameters rapidly declined and serum ALT had reached normal levels by the time that HBsAg was cleared from the circulation. All patients presented at our out-patient clinic after the onset of symptoms and proceeded to clinical recovery without therapeutic interventions.

Ratio of CD8+ and CD4+ cells during acute HBV infection

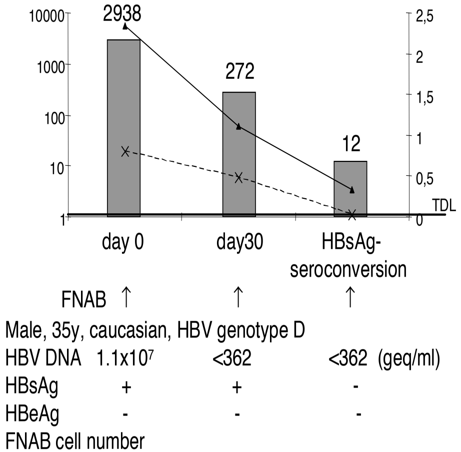
To investigate the dynamics of both lymphocyte subsets in human acute HBV infection, at day 0 and at the time of HBsAg-seroconversion we determined the percentages of circulating and intrahepatic CD8+ and CD4+ cells within the total lymphocyte population (mean ± SEM). At both time points the percentage of CD8+ T cells was higher in the liver (47.6 ± 1.6 and 36.9 ± 2.4, respectively) than in PB (30.5 ± 2.0 and 23.1 ± 1.5, respectively), and the percentage of CD4+ T cells was higher in the circulation (38.2 ± 5.1 and 50.5 ± 1.5, respectively) than in the liver (21.1 ± 3.1

1A

ALT (IU/L)

Tetr+ CD8+ cells / total CD8+ cells (%)

Patient 1

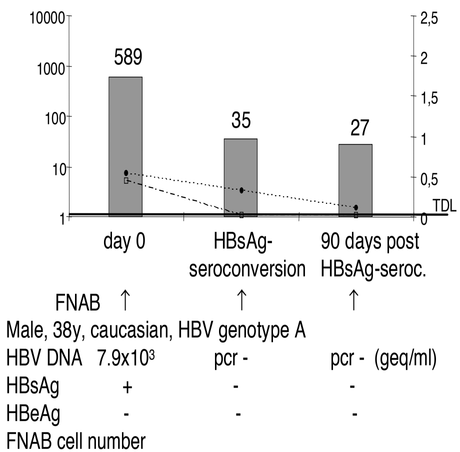


FNAB ↑ ↑ ↑

Male, 35y, caucasian, HBV genotype D

	day 0	day30	HBsAg-seroconversion
HBV DNA	$1.1x10^7$	<362	<362 (geq/ml)
HBsAg	+	+	-
HBeAg	-	-	-
FNAB cell number	22,770	16,125	7,875

Patient 2

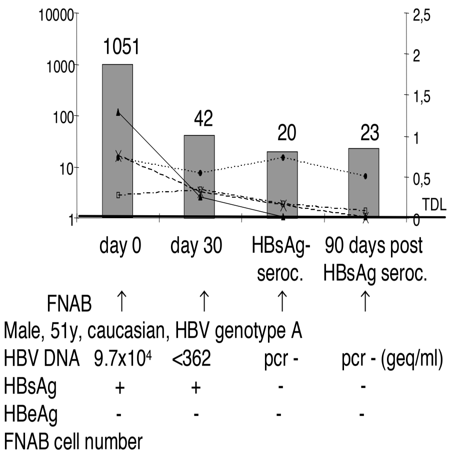


FNAB ↑ ↑ ↑

Male, 38y, caucasian, HBV genotype A

	day 0	HBsAg-seroconversion	90 days post HBsAg-seroc.
HBV DNA	$7.9x10^3$	pcr -	pcr - (geq/ml)
HBsAg	+	-	-
HBeAg	-	-	-
FNAB cell number	18,955	16,875	28,845

Patient 3

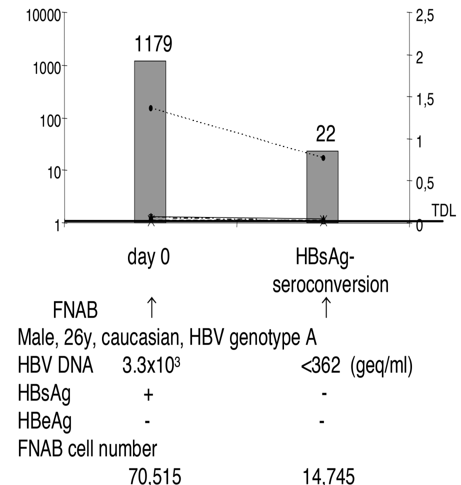


FNAB ↑ ↑ ↑ ↑

Male, 51y, caucasian, HBV genotype A

	day 0	day 30	HBsAg-seroc.	90 days post HBsAg seroc.
HBV DNA	$9.7x10^4$	<362	pcr -	pcr - (geq/ml)
HBsAg	+	+	-	-
HBeAg	-	-	-	-
FNAB cell number	20,355	12650	9,440	15,820

Patient 4

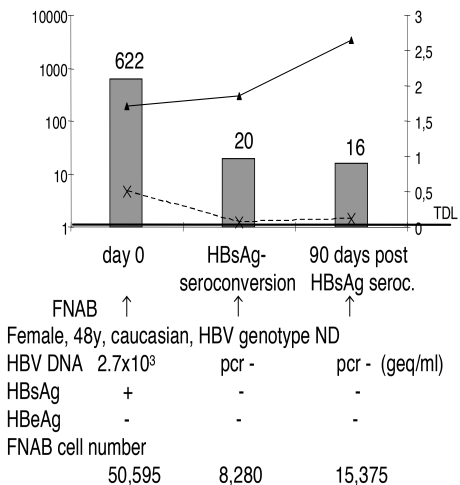


FNAB ↑ ↑

Male, 26y, caucasian, HBV genotype A

	day 0	HBsAg-seroconversion
HBV DNA	$3.3x10^3$	<362 (geq/ml)
HBsAg	+	-
HBeAg	-	-
FNAB cell number	70,515	14,745

Patient 5



FNAB ↑ ↑ ↑

Female, 48y, caucasian, HBV genotype ND

	day 0	HBsAg-seroconversion	90 days post HBsAg seroc.
HBV DNA	$2.7x10^3$	pcr -	pcr - (geq/ml)
HBsAg	+	-	-
HBeAg	-	-	-
FNAB cell number	50,595	8,280	15,375

ALT (IU/L)

Liver

% HBc 18-27+ CD8+ / total CD8+

% HBenv 183-191+ CD8+ / total CD8+

Peripheral Blood

% HBc 18-27+ CD8+ / total CD8+

% HBenv 183-191+ CD8+ / total CD8+

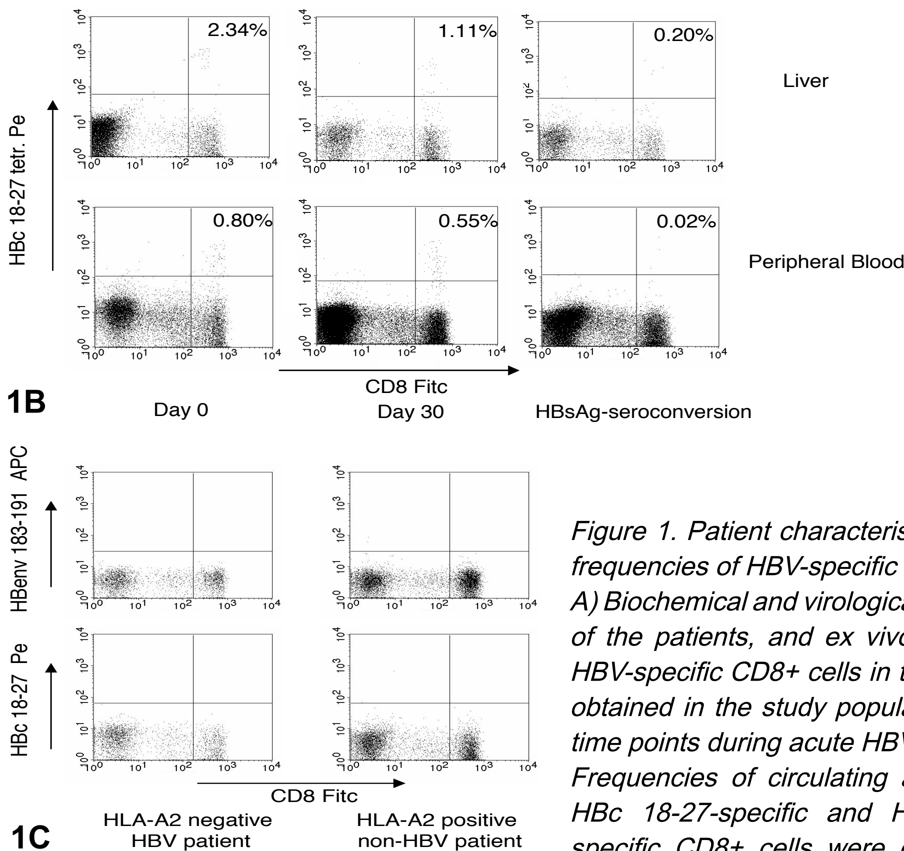


Figure 1. Patient characteristics and ex vivo frequencies of HBV-specific CD8+ cells.

A) Biochemical and virological characteristics of the patients, and ex vivo frequencies of HBV-specific CD8+ cells in the liver and PB, obtained in the study population at different time points during acute HBV infection.

Frequencies of circulating and intrahepatic HBc 18-27-specific and HBenv 183-191-specific CD8+ cells were obtained at first presentation (day 0), 30 days after first presentation (patients 1 and 3), at the time of HBsAg-seroconversion and 90 days post HBsAg-seroconversion (patients 2, 3 and 5). The frequencies were calculated as percentage HBc or HBenv-tetramer+ CD8+ cells of the total population CD8+ cells in the liver and PB. The frequencies of HBpol 816-824-specific CD8+ cells were always below the level of background staining and are not shown in the figures. The "FNAB cell number" indicates the cell numbers that were obtained by FNAB at each time point and that were analysed by flow cytometry.

The PCR-detection limit was 362 geq/ml. The arrows (↑) indicate the time points of sampling of the liver and PB in the individual patients. ND = Not Determined, TDL = Tetramer Detection Limit; 0.03% of total CD8+ cells.

B) Frequencies of intrahepatic and circulating HBV-sepcific CD8+ cells obtained in a representative patient (patient 1). The number in the upper right quadrant indicates the frequency HBc 18-27-specific CD8+ cells measured at the corresponding time point depicted at the bottom of the figure.

C) Results obtained in representative control patients. The upper half of the figure represents the background staining for the HBenv 183-191-tetramer observed in liver derived lymphocytes from a representative HLA-A2 negative patient with chronic HBV infection, and a representative HLA-A2 positive liver disease patient with no HBV infection. The bottom half of the figure shows the same for the HBc 18-27 tetramer.

and 30.1 ± 1.0, respectively). At all time points, the ratio of CD8+ / CD4+ cells in the liver was higher than in PB, indicating compartmentalization of CD8+ T cells in this organ (fig 2a). In three patients, the peak ratio of CD8+ / CD4+ cells was observed at first presentation, and when patients were divided on the basis of serum ALT at this time point, there was a correlation between degree of liver damage and the ratio of intrahepatic CD8+ / CD4+ cells ($r = 1.0$, $p < 0.01$) (fig 2b). These data suggest that CD8+ T cells are involved in disease pathogenesis through cytolytic liver damage.

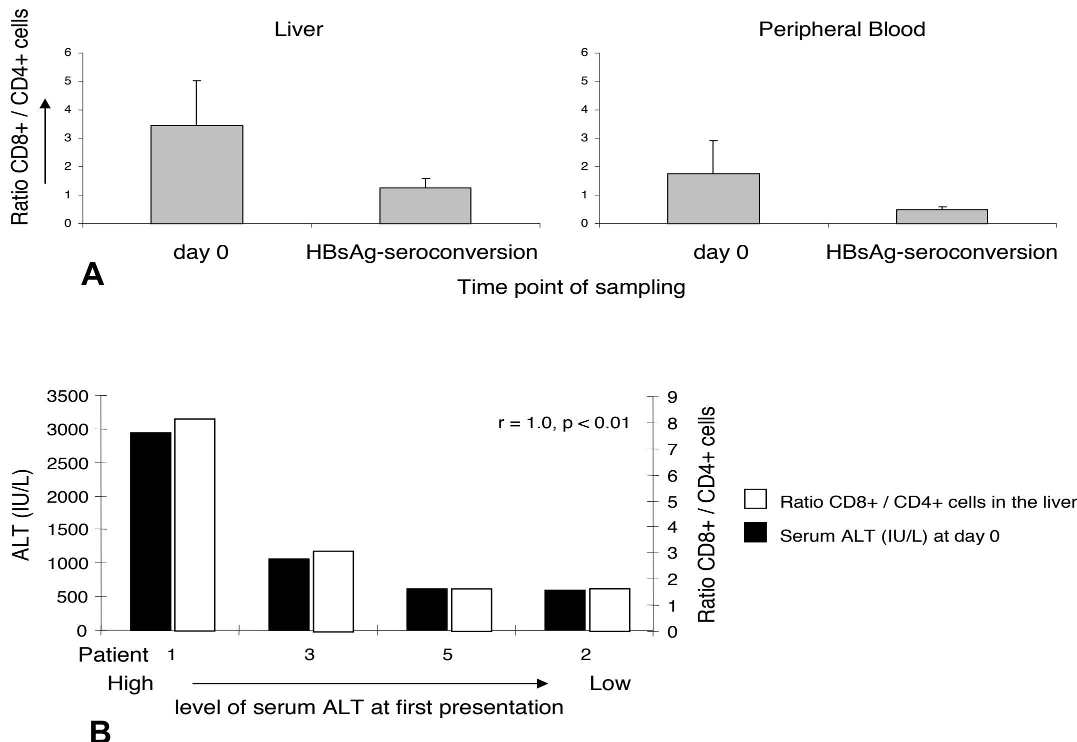


Figure 2. Ratio of CD8+ / CD4+ cells in the liver and PB.

A) Ratio of CD8+ / CD4+ cells in the liver and PB at first presentation (day 0) and at the time of HBsAg-seroconversion. The ratio of CD8+ / CD4+ cells was not determined at 90 days after HBsAg-seroconversion.

B) Ratio of CD8+ / CD4+ cells in the liver in relation to serum ALT level at first presentation. On the horizontal axis in the graph the patients are stratified on the basis of serum ALT at day 0, with decreasing value from left to right. On the vertical axes the corresponding ratio of CD8+ / CD4+ cells in the liver at day 0 is depicted, and the serum ALT levels measured at this time point (paired Spearman's correlation test r = 1,0 (p < 0.01). In patient 4 the ratio of CD8+ / CD4+ cells was not determined at first presentation and the data are therefore not shown.

Longitudinal analysis of the frequency of intrahepatic HBV-specific CD8 cells

To analyze the dynamics of intrahepatic HBV-specific CD8+ T cell responses, at several time points during acute HBV infection liver derived lymphocytes and PBMC from the patients were double stained directly *ex vivo*, with anti-CD8 MAbs and with HLA-A2/peptide tetrameric complexes able to visualize CD8+ T cells specific for core 18-27 (HBc 18-27), envelope 183-191 (HBenv 183-191) and polymerase 816-824 (HBpol 816-824). Figure 1a shows the frequencies of HBV-specific CD8+ T cells in the liver and PB at the time points of sampling. Figure 1b shows representative FACS results. During the course of infection, in the liver of four patients (patient 1, 3-5) HBc 18-27+ CD8+ T cells were detected, and in three patients (patient 2-4) HBenv 183-191+ CD8+ T cells were detected. In none of the patients HBpol 816-824+ CD8+ T cells were detectable at any time during infection, either in the liver or PB. Longitudinal analysis of the frequency of HBV-specific CD8+ T cells during recovery from the infection, as shown by progressive reduction and normalization of serum ALT and HBsAg-seroconversion, showed considerable variation between the intrahepatic compartment and PB. At all time points during infection there was sequestering of HBV-specific CD8+ T cells in the intrahepatic compartment, with a 1.2 to 21.7 fold concentration of HBc 18-27- or HBenv 183-191 specific cells in the liver compared to PB. The peak frequency of circulating HBV-specific CD8+ T cells occurred at first presentation and declined in correlation with biochemical and virological parameters. By the time that HBsAg was undetectable in the serum, in three patients (patients 1, 2 and 4) the percentage of circulating HBc 18-27- and HBenv 183-191-specific CD8+ T cells did not exceed the background level of 0.03%, as observed in controls (fig 1c). In contrast, at this time point high frequencies of tetramer positive cells (0.32 – 1.86% of total CD8+ T cells) were still detectable in the liver of all patients. Interestingly, in all patients sampled 90 days post HBsAg-seroconversion the frequency of intrahepatic HBenv 183-191+ CD8+ T cells and HBc18-27+ CD8+ T cells remained high (0.11 - 2.65%), suggesting an important role of intrahepatic virus-specific cells at this stage of the disease.

The absence of detectable intrahepatic HBpol 816-824-specific CD8+ cells may be caused by viral mutations resulting in specific epitope inactivation [3, 17]. Therefore, in all five patients this epitope was sequenced. Three patients were infected with an HBV strain carrying an aspartic acid-to valine mutation at position 820 (patient 2, 3 and 4) which may be associated with reduced CD8+ T cell activation.

HLA-DR expression on intrahepatic HBV-specific T cells during resolution of acute HBV infection

Figure 3 shows the percentage of HLA-DR expressing HBV-specific CD8+ T cells in the liver and PB. During the acute phase of HBV infection, HBenv 183-191 and HBc 18-27-specific CD8+ T cells had an activated phenotype because a large fraction of these cells expressed the activation marker HLA-DR. At this stage of disease, in all patients the percentage of HLA-DR expressing HBV-specific CD8+ T cells was higher in the liver than in PB, suggesting their increased reactivity at the site of virus

replication. During recovery from the infection, with progressive reduction of serum ALT levels, expression of HLA-DR in the liver and PB was down-regulated. However, at three months post HBsAg-seroconversion HLA-DR expression on HBc 18-27-specific cells in the liver of patient 5 had exceeded the level observed during the acute phase of infection (24% vs. 10%, respectively), in parallel with the increment in HBc 18-27-tetramer binding also observed in this patient, suggesting reactivation of these virus-specific cells.

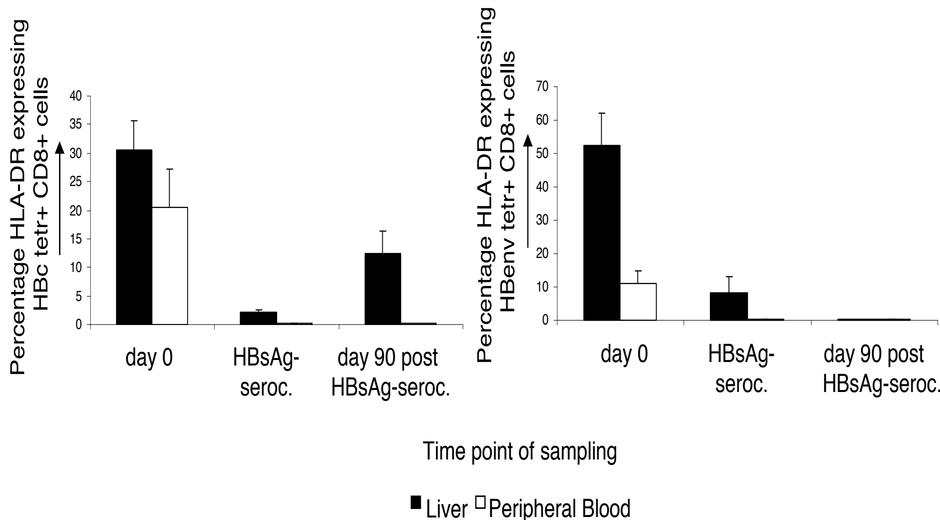


Figure 3. HLA-DR expression on HBV-specific CD8+ cells.
The left figure shows the percentage of HLA-DR expressing HBc18-27-specific CD8+ cells measured in the liver and PB of the study population (mean + SEM). The right figure shows the same for the HBenv 183-191-specific CD8+ cells. At 90 days post HBsAg-seroconversion only patients 2, 3 and 5 were sampled.

Discussion

Although several studies have previously shown important differences between circulating and intrahepatic HBV-specific CD8+ T cells in chronic HBV infection [7 8], this is the first report that describes differences in the characteristics of the virus-specific CD8+ T cell response in the liver and PB during human self-limiting acute HBV infection.

Interestingly, our results show a correlation between the distribution of CD8+ T cells in the liver and the degree of liver damage at first presentation. At the time of liver inflammation only a fraction of the intrahepatic CD8+ T cells displayed tetramer binding, and therefore our data may subscribe the relevance of nonspecific CD8+ T cell recruitment in liver damage in acute HBV infection. Moreover, an important finding of this study is that we did not observe a clear correlation between the degree

of liver damage and the profile of *ex vivo* intrahepatic HBV-specific CD8+ T cells. Based upon studies in animal models [18-20] and the finding that in the preclinical incubation phase of acute HBV infection, there is already a large fraction of circulating HBV-specific T cells preceding maximal liver damage, it has been suggested that HBV-specific T cells may infiltrate the liver where they can contribute to viral suppression, and subsequently trigger a cascade of events leading to liver damage [21]. This cascade may include recruitment of activated CD8+ T cells, not accounted for by tetramer staining. However, a limitation of the tetramer technology is that it can only be applied to study defined epitopes. Although the epitopes we used in this study have been chosen because they are among the most frequent CD8+ T cell epitopes found in HBV infection after screening with multiple peptides[3, 5, 22, 23], it has already been described that in acute HBV infection the T cell response is directed to multiple epitopes, including those not covered by our tetramers. Therefore, we may underestimate the HBV-specific CD8+ T cell response [3, 4, 21]. A more detailed longitudinal analysis of the intrahepatic events, which includes the incubation phase and a wide spectrum of T cell epitopes, is needed to further clarify the mechanism of liver damage in acute HBV infection.

In all patients high frequencies of intrahepatic HBV-specific cells remained detectable after normalization of serum ALT whereas the frequencies of circulating HBV-specific cells declined in parallel with serum ALT. Since at all time points of sampling the frequency of HBV-specific CD8+ T cells was higher in the liver than in PB, their disappearance from the circulation may indeed be explained by sequestering at the site of viral replication. Similar enrichment of virus-specific cells in the liver, has previously been found in patients with chronic HBV infection [7]. Although, it has been shown recently that in non-hepatotropic viruses activated primary or memory CD8+ T-cells migrate to the liver [24], and that in the normal human liver CD8+ T-cells are present at frequencies which are enriched compared to those in the blood [25], our finding may suggest that HBV-specific CD8+ T-cells contribute to clearance of the virus. Our data do not establish whether HBV-specific CD8+ T cells control virus replication through the secretion of cytokines alone, or in combination with direct lysis of infected cells. However, other groups have already shown that CD8+ cells are capable of these HBV-specific functional responses [26-28].

It has previously been shown that in acute HBV infection virus-specific CD8+ T cells are still present in the circulation for several months after complete clinical and serological recovery [3, 26]. Here we show that these cells can also be detected in the liver at three months post HBsAg-seroconversion, but at higher frequencies than in PB. Interestingly, at this time point in one patient both the frequency of intrahepatic HBc18-27-specific CD8+ cells as well as the level of HLA-DR expression was increased, suggesting reactivation of virus specific, presumably memory T cells. A CD8+ T cell-response may be maintained by continued antigenic stimulation by virus that has not yet been eradicated from the liver. This concept is supported by the finding in a previous study, that in a majority of patients with long-lasting T cell responses in peripheral blood, minute amounts of HBV-DNA could be detected years

after seroconversion [29].
In conclusion, our measurements of circulating and intrahepatic HBV-specific CD8+ cells in patients with self-limiting acute HBV infection have demonstrated that virus-specific cells are compartmentalized in the liver. Their sequestering in the liver may suggest a role in resolution of the acute infection. For several months after complete HBsAg seroconversion intrahepatic HBV-specific CD8+ T cells remain detectable at high frequencies.

Acknowledgements

The authors wish to thank Rehka Binda and Raymond Pot for their assistance in obtaining and processing the FNAB-samples.

References

1. Guidotti LG, Chisari FV. To kill or to cure: options in host defense against viral infection. Curr Opin Immunol 1996; 8:478-83.
2. Nayersina R, Fowler P, Guilhot S, et al. HLA A2 restricted cytotoxic T lymphocyte responses to multiple hepatitis B surface antigen epitopes during hepatitis B virus infection. J Immunol 1993; 150:4659-71.
3. Rehermann B, Fowler P, Sidney J, et al. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. J Exp Med 1995; 181:1047-58.
4. Maini MK, Boni C, Ogg GS, et al. Direct ex vivo analysis of hepatitis B virus-specific CD8(+) T cells associated with the control of infection. Gastroenterology 1999; 117:1386-96.
5. Bertoletti A, Costanzo A, Chisari FV, et al. Cytotoxic T lymphocyte response to a wild type hepatitis B virus epitope in patients chronically infected by variant viruses carrying substitutions within the epitope. J Exp Med 1994; 180:933-43.
6. Rehermann B, Chang KM, McHutchinson J, et al. Differential cytotoxic T-lymphocyte responsiveness to the hepatitis B and C viruses in chronically infected patients. J Virol 1996; 70:7092-102.
7. Maini MK, Boni C, Lee CK, et al. The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. J Exp Med 2000; 191:1269-80.
8. Bertoletti A, D'Elios MM, Boni C, et al. Different cytokine profiles of intraphepatic T cells in chronic hepatitis B and hepatitis C virus infections. Gastroenterology 1997; 112:193-9.
9. Bravo AA, Sheth SG, Chopra S. Liver biopsy. N Engl J Med 2001; 344:495-500.
10. Sprengers D, van der Molen RG, Kusters JG, Janssen HL. Coarse vs. fine needle aspiration biopsy. J Hepatol 2004; 41:503-4; author reply 504.
11. Lautenschlager I, Hockerstedt K, Hayry P. Fine-needle aspiration biopsy in the monitoring of liver allografts. Transpl Int 1991; 4:54-61.
12. Sprengers D, van der Molen RG, Kusters JG, et al. Flow cytometry of fine-needle-aspiration-biopsies: a new method to monitor intrahepatic immunological environment in chronic viral hepatitis. J Viral Hepat 2005; 12:507-12.
13. Pas SD, Fries E, De Man RA, Osterhaus AD, Niesters HG. Development of a quantitative real-time detection assay for hepatitis B virus DNA and comparison with two commercial assays. J Clin Microbiol 2000; 38:2897-901.
14. Niesters HG. Clinical virology in real time. J Clin Virol 2002; 25 Suppl 3:S3-12.
15. Stuyver L, De Gendt S, Van Geyt C, et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol 2000; 81:67-74.
16. Norder H, Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology 1994; 198:489-503.
17. Maini MK, Reignat S, Boni C, et al. T cell receptor usage of virus-specific CD8 cells and recognition of viral mutations during acute and persistent hepatitis B virus infection. Eur J Immunol 2000; 30:3067-78.
18. Moriyama T, Guilhot S, Klopchin K, et al. Immunobiology and pathogenesis of hepatocellular injury in hepatitis B virus transgenic mice. Science 1990; 248:361-4.
19. Nakamura I, Nupp JT, Cowlen M, et al. Pathogenesis of experimental neonatal woodchuck hepatitis virus infection: chronicity as an outcome of infection is associated with a diminished acute hepatitis that is temporally deficient for the expression of interferon gamma and tumor necrosis factor-alpha messenger RNAs. Hepatology 2001; 33:439-47.
20. Ando K, Moriyama T, Guidotti LG, et al. Mechanisms of class I restricted immunopathology. A transgenic mouse model of fulminant hepatitis. J Exp Med 1993; 178:1541-54.
21. Webster GJ, Reignat S, Maini MK, et al. Incubation phase of acute hepatitis B in man: dynamic of cellular immune mechanisms. Hepatology 2000; 32:1117-24.
22. Rehermann B, Ferrari C, Pasquinelli C, Chisari FV. 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.
23. Penna A, Chisari FV, Bertoletti A, et al. Cytotoxic T lymphocytes recognize an HLA-A2-restricted epitope within the hepatitis B virus nucleocapsid antigen. J Exp Med 1991; 174:1565-70.
24. Masopust D, Vezys V, Usherwood EJ, et al. Activated primary and memory CD8 T cells migrate to nonlymphoid tissues regardless of site of activation or tissue of origin. J Immunol 2004; 172:4875-82.
25. Ward SM, Jonsson JR, Sierro S, et al. Virus-specific CD8+ T lymphocytes within the normal human liver. Eur J Immunol 2004; 34:1526-31.
26. Urbani S, Boni C, Missale G, et al. Virus-specific CD8+ lymphocytes share the same effector-memory phenotype but exhibit functional differences in acute hepatitis B and C. J Virol 2002; 76:12423-34.
27. Thimme R, Wieland S, Steiger C, et al. CD8(+) T cells mediate viral clearance and disease pathogenesis

during acute hepatitis B virus infection. J Virol 2003; 77:68-76.
28. Menne S, Roneker CA, Roggendorf M, Gerin JL, Cote PJ, Tennant BC. Deficiencies in the acute-phase cell-mediated immune response to viral antigens are associated with development of chronic woodchuck hepatitis virus infection following neonatal inoculation. J Virol 2002; 76:1769-80.
29. Penna A, Artini M, Cavalli A, et al. Long-lasting memory T cell responses following self-limited acute hepatitis B. J Clin Invest 1996; 98:1185-94.

CHAPTER 6

In vivo immunization following virus suppression: a novel approach for inducing immune control in chronic hepatitis B

D. Sprengers[1], H.L.A.Janssen[1], J.Kwekkeboom[1], H.G.M.Niesters[2], R.A. de Man[1], S.W.Schalm[1]

[1] Dept. of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands
[2] Dept. of Virology, Erasmus MC University Medical Center, Rotterdam, The Netherlands

J Viral Hepat 2003: 10: 7-9

Despite the presence of an effective vaccine since 1982, Hepatitis B still ranks among the highest causes of mortality from infectious diseases worldwide. There is growing evidence that an active antiviral treatment strategy in those with progressive disease associated with active viral replication may lead to significant reduction in morbidity and mortality [1]. One approach similar to the strategy so successful in HIV is to suppress viral replication with (a combination of) nucleoside analogues; according to current experience such therapy needs to be given indefinitely. Another strategy aims at the induction of immune control by a treatment course of limited duration; this approach, if equally effective, is to be preferred for obvious reasons such as compliance, side effects and costs.
The two currently licensed drugs: lamivudine and interferon-alpha are each examples of the two different approaches. Their effectiveness is limited either by the high incidence of resistance (lamivudine) or low incidence of inducing immune control (interferon). Combination of a standard course of interferon therapy with lamivudine may increase the rate of inducing immune control [2, 3], but a sustained therapeutic response (HBeAg negativity, HBV DNA $<10^5$ geq/ml and ALT normality 6-12 months after stopping treatment) is observed in only a minority of patients.
We observed that combining lamivudine with interferon therapy induces a more rapid decline of the viral load than monotherapy [4]. We assume that profound virus suppression may allow recovery of the non-responsiveness of the effector arm of the immune response [5]; however, at the same time it may weaken the antigen expression on infected cells that need to be removed by the immune response.
In vivo immunization following virus suppression aims to optimize conditions for an effective immune response: following rapid and profound virus suppression by interferon-lamivudine combination therapy, lamivudine is withdrawn intermittently for 4 weeks during continued interferon therapy. Withdrawal of lamivudine will lead to increased viral replication in infected cells with stronger antigen expression, and to a rapid rise in viral load in the blood with subsequent immune stimulation. We assume that the continuation of interferon therapy will help the development of an immune response, but most importantly will prevent re-infection of hepatocytes during renewed active viral replication.

We have treated two chronic hepatitis B patients (♂ 21 years, ♂ 36 years, both HBeAg negative and HBV-DNA positive) with in vivo immunization following suppression of viral replication with interferon-alpha and lamivudine. After 6-12 months of combination therapy lamivudine therapy was interrupted initially for 2 weeks and subsequently for 4-6 weeks while interferon therapy was continued. In both patients with profound virus suppression (HBV DNA $< 10^3$ copies per ml) we observed a rapid rebound of viral replication upon withdrawal of lamivudine; the peak HBVDNA was smaller after the second period of withdrawal. Rises in hepatitis activity followed each episode of renewed viral replication. After re-introduction of lamivudine HBVDNA became undetectable by PCR followed by normalization of serum ALT (figure 1).

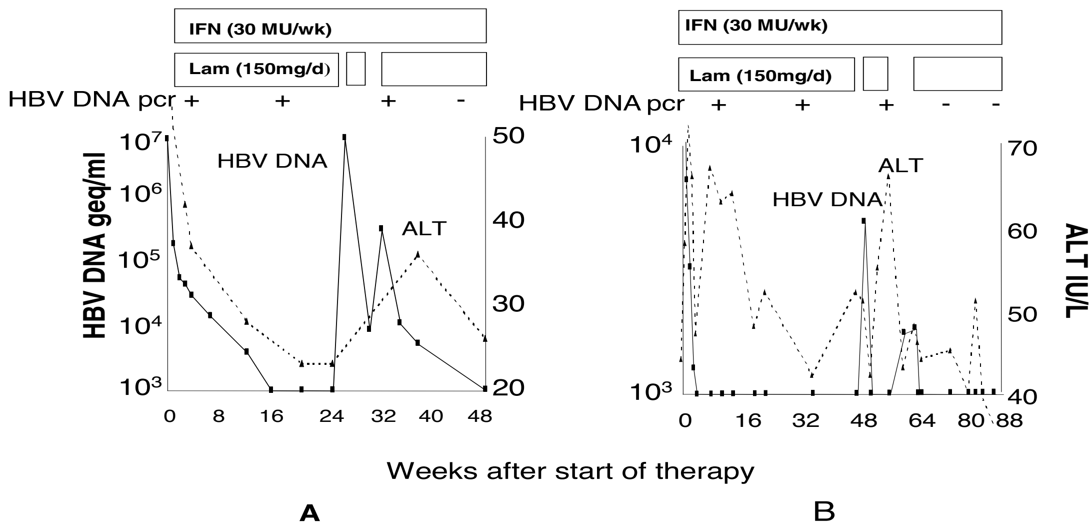


Fig. 1.
Assessment of viral load (HBV DNA) and hepatitis activity (ALT) in two patients with chronic HBV infection undergoing in vivo immunization *following viral suppression. HBV DNA was quantified using HBV monitor assay (Roche Diagnostics; detection limit $1x10^3$ geq/ml). Below 10^3 geq/ml HBV DNA was measured by an kinetic PCR (TaqMan based assay; detection limit 373 geq/ml). Baseline HBV DNA: A: ♂ 21y $9,9x10^6$ geq/ml, B: ♂ 36y $1,6x10^5$ geq/ml.*
Patiënt A shows a characteristic bi-phasic decline of HBV DNA, and marked rebound upon stopping lamivudine. The second rebound was lower than the first and associated with some hepatitis activity.
Patiënt B, with a HBV DNA above 10^5 geq/ml at start, showed a rapid decline and HBV DNA was undetectable by the Roche assay for a year. Upon stopping lamivudine HBV DNA rebounded twice; the second peak was lower than the first. Hepatitis activity was associated with both flares. Finally HBV DNA became negative by PCR.

These observations are in our opinion a strong stimulus to further explore the concept of *in vivo immunization.* Many factors need to be optimized like the duration of virus suppression prior to withdrawal, the duration of lamivudine withdrawal, the number of withdrawals, the dose of interferon required to prevent infection of hepatocytes without interfering with the immune response. Monitoring of HBV specific CD8 cytotoxic and CD4 helper cells in addition to sensitive measurements of HBVDNA appears essential for answering these questions.
Conceptually *in vivo immunization* differs from structured treatment interruptions, as described in HIV infection [6]. Treatment is not interrupted but for only one component of therapy. We assume that the continuation of interferon therapy will keep uninfected hepatocytes in their antiviral state' and that the pool of infected hepatocytes continues to decrease; in case of treatment interruption one must assume a rapid spread of the

infection and an increase in the number of infected cells.
In vivo immunization is also different from currently designed therapeutic vaccines. The immune system in patients with chronic hepatitis B is only slightly impaired; the impairment in immune responsiveness is hepatitis B specific rather than general. There is evidence that the immune responsiveness actually is hepatitis B strain specific, since patients with chronic hepatitis B apparently can clear a superinfection with another hepatitis B strain [7]. Therapeutic vaccines usually restrict the antigenic profile to a few epitopes thought to be essential [8]; presently there is limited proof about their effectiveness in generating a cellular immune response [9]. Since immune responses in resolved hepatitis B infections are often directed against several but variable epitopes [10], we believe that the best immune stimulus is the whole virus with a very broad antigenic profile. Furthermore, live attenuated vaccines permit efficient MHC class I presentation of antigen to stimulate CTL's [11].

Immune stimulation by the current approach of withdrawal of lamivudine during continued interferon therapy may suffice in some patients; it is conceivable that in many others additional adjuvant therapy is needed, for instance to shift the CD4 response to a Th1 profile or to maintain the HBV specific T-helper response of sufficient duration. We believe this concept of in vivo immunization as a novel therapeutic approach for chronic hepatitis B merits to be investigated further in a cooperative effort between academic institutions and pharmaceutical industry.

References

1. Niederau C, Heintges T, Lange S, Goldmann G, Niederau CM, Mohr L, et al. Long-term follow-up of HBeAg-positive patients treated with interferon alfa for chronic hepatitis B. N Engl J Med. 1996, 30; 334: 1422-7.
2. Schalm SW, Heathcote J, Cianciara J, Farrell G, Sherman M, Willems B, et al. Lamivudine and alpha interferon combination treatment of patients with chronic hepatitis B infection: a randomised trial. Gut. 2000; 46: 562-8.
3. Barbaro G, Zechini F, Pellicelli AM, Francavilla R, Scotto G, Bacca D, et al. 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.
4. van Nunen AB, Hansen BE, Mutimer DJ, Niesters HG, de Man RA, Schalm SW. Viral kinetics during 16 weeks of interferon and lamivudine monotherapy versus interferon-lamivudine combination therapy in chronic hepatitis B patients. Hepatology 2000; 32: 379A.
5. Boni C, Penna A, Ogg G, Bertoletti A, Pilli M, Cavallo C et al. Lamivudine treatment can overcome cytotoxic T-cell hyporesponsiveness in chronic hepatitis B: New perspectives for immune therapy. Hepatology 2001; 33: 963-971.
6. Carcelain G, Tubiana R, Samri A, Calvez V, Delaugerre C, Agut H et al. Transient mobilization of Human immunodeficiency Virus (HIV)-specific CD4 T-helper cells fails to control virus rebound during intermittent antiretroviral therapy in chronic HIV type 1 infection. J Virol 2001; 75: 234-241.
7. Kao JH, Chen PJ, Lai MY, Chen DS. Acute exacerbations of chronic hepatitis B are rarely associated with superinfection of hepatitis B virus. Hepatology. 2001; 34: 817-23.
8. Michel M-L, Pol S, Brechot C, Tiollais P. Immunotherapy of chronic hepatitis B by anti HBV vaccine: from present to future. Vaccine 2001; 19: 2395-2399.
9. Heathcote J, McHutchison J, Lee S, Tong M, Benner K, Minuk G, et al. 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.
10. Rehermann B, Fowler P, Sidney J, Person J, Redeker A, Brown M, et al. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. J Exp Med. 1995; 181: 1047-58.
11. Seder RA, Hill AVS. Vaccines against intracellular infections requiring cellular immunity. Nature 2000; 406: 793-798.

CHAPTER 7

In vivo immunization in combination with peg-interferon for chronic hepatitis B virus infection

D. Sprengers[1], R.G. van der Molen[1], R. Binda[1], J.G. Kusters[1], R.A.de Man[1], H.G.M. Niesters[2], S.W. Schalm[1], H.L.A. Janssen[1].

[1] Dept. of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands
[2] Dept. of Virology, Erasmus MC University Medical Center, Rotterdam, The Netherlands

Submitted

Abstract

Only in a minority of patients with chronic HBV (CHB) infection will treatment with IFNα or nucleoside analogues lead to sustained virological response. *In vivo immunization (IVI)* following virus suppression aims to optimize conditions for an effective immune response: following rapid and profound virus suppression by interferon-lamivudine combination therapy, lamivudine is withdrawn intermittently during continued interferon therapy. It is thought that withdrawal of lamivudine will lead to increased viral replication and increased antigen expression with subsequent immune stimulation.

The aim of this prospective pilot study was to evaluate *IVI* as a therapeutic approach for CHB. Fourteen HBeAg positive CHB patients were treated for 42 weeks with a combination of pegylated interferon-alpha 2b and lamivudine. After 12 weeks of combination therapy lamivudine was withdrawn intermittently for 3 consecutive periods of 4 weeks until it was permanently stopped in week 36.

At the end of follow-up (week 52) all patients had remained HBeAg positive and the median viral load was similar to baseline. During the initial 12 weeks of treatment, there was a reduction of both the HBV-specific proliferation capacity of Th-cells and the frequencies IFNγ-producing cells. During the lamivudine interruption-cycle there was an inverse relation between the increase of HBV-DNA, and the decrease in proliferation capacity and frequency of IFNγ-producing cells. The intrahepatic fraction of CD8+ T-cells increased during lamivudine withdrawal.

In conclusion, *IVI* was able to transiently stimulate the HBV-specific immune responsiveness of T-cells, but the magnitude of the response was insufficient to cause a beneficial virological effect.

Introduction

Chronic hepatitis B (CHB) is characterized by inflammatory liver disease of variable severity and is associated with a significantly increased risk of developing cirrhosis, liver failure and hepatocellular carcinoma [1, 2]. Active antiviral treatment of patients with progressive disease and active viral replication may lead to significant reduction in morbidity and mortality [3]. One approach similar to the strategy so successful in HIV is to suppress viral replication with (a combination of) nucleoside analogues such as lamivudine, adefovir dipivoxil or entecavir. Since these agents usually do not induce immune control, in most patients they need to be given indefinitely. However, lomg-term effect may be limited by the incidence of resistance [4, 5]. Another strategy aims at the induction of immune control by a treatment course of limited duration. This approach, of which interferon alpha (IFNα) therapy is the generally used example, is to be preferred. However, in only a minority of patients conventional or pegylated IFNα (Peg-IFNα) therapy leads to sustained virological response [6, 7]. Although several other components have been investigated to achieve immune control over the virus in the past [8], recently most attention has been focused on vaccine therapy for CHB. Therapeutic vaccination strategies aim at inducing or increasing the HBV-specific CD8+ and CD4+ T-cell responses that are weak, antigenically restricted or undetectable in the blood of chronically infected patients. However, until now standard anti-HBV vaccination as well as DNA based vaccines have mostly not been able to induce the clearance of the hepatitis B virus in humans [9, 10]. Therapeutic vaccines usually restrict the antigenic profile to a few epitopes thought to be essential [11]. Since immune responses in resolved hepatitis B infections are often directed against several but variable epitopes [12], the best immune stimulus may be the whole virus with a very broad antigenic profile.
In vivo immunization (IVI) following virus suppression aims to optimize conditions for an effective immune response: following rapid and profound virus suppression by interferon-lamivudine combination therapy, lamivudine is withdrawn intermittently for 3 consecutive periods of 4 weeks during continued interferon therapy. Withdrawal of lamivudine will lead to increased viral replication in infected cells with export of complete virus particles, a rapid rise in viral load and increased antigen expression on the hepatocyte membrane, with subsequent immune stimulation. It is assumed that the continuation of interferon therapy will help the development of an immune response, and will prevent re-infection of hepatocytes during renewed active viral replication [13-15]. It has recently been reported that *IVI* was able to transiently suppress viral replication in two patients with CHB [16]. These observations were a strong stimulus to further explore the concept of *IVI* as a therapeutic approach for chronic HBV infection in a prospective pilot study.

Patients and Methods

Patients and study design

Seventeen patients with CHB and alanine transaminase (ALT) levels greater than normal (>45 IU/l) were included in the study. All patients were HBsAg positive >6 months, HBV-DNA positive (>10^5 geq/ml), anti-HBV core antigen (HBcAg) antibody positive, and HBeAg positive. Patients had not received anti-HBV therapy within 6 months prior to enrollment. None of the patients had significant co-morbidity or cirrhosis. Antibodies to HCV, HIV-1, HIV–2 and hepatitis Delta were absent. Patients were treated for 42 weeks with a combination of 100μg pegylated interferon-alpha 2b (Peg-IFNα) weekly and 100mg lamivudine once daily. After 12 weeks of combination therapy lamivudine was withdrawn intermittently for 3 consecutive periods of 4 weeks until week 36, after which it was permanently stopped. Continuous Peg-IFNα therapy was given until week 42. There was a post-treatment follow-up period of 10 weeks, until week 52. Figure 1 shows the treatment schedule. Patients exhibiting HBeAg loss within the first 12 weeks were excluded from *in vivo* immunization and offered continuous PEG-IFNα and lamivudine therapy up to 12 months. The primary outcome measure was loss of HBeAg from serum. Secondary outcome measures were HBV-DNA levels below the level of detection in PCR assay, emergence of of mutations in the YMDD motif of HBV polymerase at week 42 and induction of an HBV-specific type 1 immune response. All patients signed informed consent before enrolment. This study was performed in agreement with the local Medical Ethics Committee.

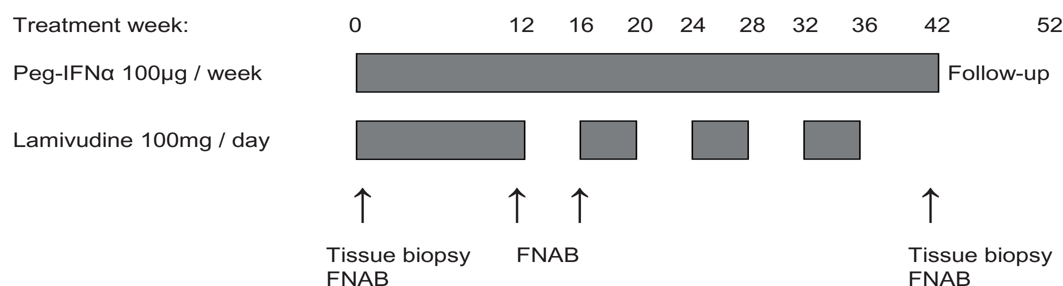


Fig 1. Study design.
Patients were treated for 42 weeks with a combination of 100μg pegylated interferon-alpha 2b weekly and 100mg lamivudine daily. After 12 weeks of combination therapy lamivudine was withdrawn intermittently for 4 weeks until week 36, after which it was permanently stopped. Continuous Peg-IFNα therapy was given until week 42. The follow up period lasted until week 52.

Virological assessment

Blood samples for reported virological and biochemical data were obtained every 4 weeks. Serum HBeAg, HBsAg, anti-HBs, anti-HBc, anti-delta, anti-HCV and anti-HIV1/2 were determined using commercially available immunoassays (Abbott Laboratories, North Chicago, IL, USA). Serum HBV-DNA was measured with TaqMan

real-time PCR (detection limit 100 geq/ml) validated on the Eurohep Standard [17]. HBV-DNA was extracted from serum samples by using a MagnaPure LC station (Roche Applied Science, Penzberg, Germany). For sequencing, primers were used as described previously [18]. The genotype of the predominant HBV population was determined by analysis of sequenced portions of the core and surface antigen encoding DNA isolated from the serum [19]. At week 0 and 42 HBV polymerase mutant analysis was performed on HBV-DNA using a Line Probe assay (INNO-LiPA HBV DR; Innogenetics N.V., Gent, Belgium) [18].

Standard tissue needle biopsy and fine-needle aspiration biopsy of the liver

Liver histology was assessed at baseline and at the end of Peg-IFNα treatment. The biopsies were scored centrally by an experienced pathologist who was unaware of the chronological order of the biopsy. Histological scoring was performed according to the histological activity index, as described by Ishak [20]. Improvement of histology was defined as a reduction of at least two points for the necroinflammatory score (range 0-18) and one point for the fibrosis score (range 0-6). Optionally, a fine-needle aspiration biopsy (FNAB) was combined with a standard tissue biopsy at baseline and at the end of treatment (42 weeks), and additionally at 12 and 16 weeks (fig 1). Details of the FNAB-procedure are described elsewhere [21]. Briefly, a mandarin containing 25-gauge (diameter 0.5 mm) needle (Braun AG, Melsungen, Germany) is punctured in the 8th or 9th right intercostal space. After removal of the mandarin a 10 mL syringe filled with 2 mL RPMI supplemented with 0.1% human serum albumin (Sanquin CLB, Amsterdam, the Netherlands) and 25 IU/mL heparin (Leo Pharma, Breda, the Netherlands) is attached. Liver cells are aspirated by negative syringe pressure. Next, the specimen is transferred into a 15 mL tube and placed on ice immediately before further analysis. Validation for immunological assessment of the liver using the fine-needle aspiration biopsy-aspirate, has been described previously [22].

Isolation of the PBMCs and flow cytometric Analysis

At the start of therapy (0 weeks), at 12 weeks and at every 4 weeks thereafter until the end of the study (t=52wks) PBMCs from individual patients were obtained by ficoll separation (Ficoll-Paque™ plus, Amersham Biosciences, Buckinghamshire, UK). The PBMCs were immediately frozen in medium containing 10% DMSO and stored at –135°C until further use. Flow cytometric analysis was performed on the stored samples and freshly obtained FNAB-cytology using fluorochrome conjugated antibodies specific for the surface markers CD4 and CD8 (Dako, Glostrup, Denmark). After staining the cells were analyzed using a four-color cytometer (FACScalibur™, CELLQuest Pro™ software, Beckton Dickinson Biosciences; San Jose, USA).

Proliferation assay

The proliferative capacity of Th cells was determined as reported previously [23]. Isolated PBMCs of all time points investigated were cultured in triplicate in a concentration of $1X10^5$ cells per well in 100 μl RPMI 1640 (Bio Whittaker, Verviers,

Belgium) containing 5% pooled human serum (Dept. of Immunohematology and Bloodbank, Leiden University Medical Center, Leiden, The Netherlands) and penicillin/streptomycin (Gibco, Paisley, UK). The cells were stimulated with 1 µg/ml HBV core antigen (HBcAg) (Biomerieux, Boxtel, The Netherlands), 5 µg/ml Phytohemagglutin (Murex, Paris, France), or not stimulated and cultured for 6 days. After 5 days of incubation the cells were pulsed with 0.25 µCi/well of [^{3}H]thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK). The cells were harvested 16 hours later. Proliferation, was determined by liquid scintillation counting of the harvested cells and expressed as stimulation index (SI). The SI was calculated from the counts per minute found with antigen divided by that found without antigen.

Elispot assay

Detection of IFNγ (U-CyTech, Utrecht, The Netherlands) was performed using an ELISPOT kit according to the manufacturer's instructions. Of all time points investigated, cultures of PBMCs were established in triplicate on round bottom 96-wells plates (Corning Inc., New york, USA), in a concentration of $1X10^5$ cells per well in 100 µl RPMI 1640 (Bio Whittaker) containing 5% pooled human serum (Leiden University Medical Center) and penicillin/streptomycin (Gibco). The cells were stimulated with 1 µg/ml HBV core antigen (HBcAg) (Biomerieux), 5 µg/ml Phytohemagglutin (Murex), or medium only (negative control) for 24 hours at 37 degrees Celsius, after which they were transferred into flat bottom, anti- IFNγ-coated 96-wells plates (Nunc A/S, Roskilde, Denmark) for another 24-hours incubation period. Cytokine spots were visualised by biotin-labelled antibodies and their numbers were established by using a Bioreader 3000 from BioSys (Karben, Germany).

Statistical analysis

Flow cytometry data, cytokine profile and proliferation capacity were compared using the Wilcoxon matched pairs signed rank sum test. The paired Pearson correlation test was used to correlate reported data. For these analyses SPSS 11.5 for Windows (SPSS, Chicago, IL) was used. Where applicable data are reported as mean ± standard error of the mean (SEM).

Results

Patients and side effects

Seventeen HBeAg positive patients were enrolled in the study (the intention to treat population). After 12 weeks of treatment with continuous lamivudine and Peg-IFNα combination therapy, 3 patients (18%) had lost HBeAg and these patients were excluded from *IVI* and offered continuous PEG-IFNα and lamivudine therapy up to 12 months. Fourteen patients (10 male) with a median age of 26 (22 – 61) were evaluated for the effect of *IVI* (the per protocol population) (table 1). The side effect profile in the 14 *IVI* patients and the 3 patients that received continuous PEG-IFNα and lamivudine therapy was similar, and it resembled data reported in previous clinical studies using

Peg-IFNα [7]. Common side effects included flu-like symptoms, headache, fatigue and local reaction at the injection site, and all were reversible.

Table I. Per protocol population characteristics at inclusion.

Patient	Sex	Age (years)	ALT (IU/L)	HBV DNA (cp/ml)	Genotype
1	M	47	136	$4.7x10^8$	D
2	M	30	48	$5.1x10^9$	D
3	F	31	74	$1.4x10^9$	B
4	F	22	301	$7.8x10^8$	B
5	M	23	82	$9.8x10^8$	C
6	M	26	56	$3.4x10^{10}$	E
7	M	26	174	$7.7x10^8$	D
8	M	61	57	$7.0x10^9$	A
9	M	23	69	$8.3x10^7$	C
10	M	24	57	$1.9x10^9$	B
11	M	26	46	$1.4\ x10^{10}$	D
12	M	22	250	$1.3\ x10^{10}$	D
13	F	28	47	$3.3\ x10^6$	D
14	F	26	46	$1.7\ x10^9$	D

median age: 26y (22 – 61)
median HBV DNA: $1.3x10^9$ cp/ml ($3.3x10^6$ - $3.4x10^{10}$)
median ALT: 63 IU/l (46 – 301)

ALT, alanine aminotransferase.

Viral kinetics during the study

At the end of follow-up (52 wks) all 14 patients had remained HBeAg and HBV DNA positive, and there were thus no virological responders. At baseline the median viral load was $1.34x10^9$ cp/ml ($3.3x10^6$ - $3.4x10^9$) and at 52 weeks it was $2.0x10^8$ cp/ml ($5.0x10^4$ - $1.1x10^{10}$) (Fig 2a). After 12 weeks of continuous Peg-IFNα – lamivudine combination therapy HBV-DNA had decreased by a median of 4.4 log_{10} (5.9 – 2.7) to $5.5x10^4$ cp/ml ($1.0x10^3$ – $1.2x10^6$). When lamivudine was withdrawn during the first interruption of 4 weeks, median HBV DNA levels increased by 3.3 log_{10} (1.8 – 4.7) to $1.3x10^8$ cp/ml ($4.0x10^3$ - $3.5x10^9$). Reintroduction of lamivudine at 16 wks reduced the viral load by 2.7 log_{10} (1.1 – 3.7) to $2.4x10^5$ cp/ml ($1.0x10^3$ – $1.2x10^7$) within 4 weeks. A similar pattern of response was observed during and after the second and third interruption of lamivudine therapy without a significant further reduction of the median HBV DNA levels. Permanent cessation of lamivudine at week 36 resulted in an increase in median HBV DNA levels from $8.5x10^5$ ($1.0x10^3$ – $1.2x10^7$) to $4.8x10^7$ cp/ml ($1.0x10^3$ – $1.5x10^{10}$) at week 42, and a further rise of 0.64 log_{10} (-3.8 – 3.0) occurred after discontinuation of Peg-IFNα at week 42. In 2 patients (patient 12 and 14; 14%) at the end of the study the viral load had stabilized at a significantly reduced level as compared to baseline ($1.3x10^{10}$ and $1.7x10^9$ vs. $2.6x10^5$ and $6.1x10^5$ cp/ml, respectively) (fig2b).

ALT response

At the end of treatment the median ALT level had reduced from 63 IU/L (46 – 301) at baseline to 38 IU/L (19-436) at 42 wks (fig 2a). There was no clear correlation between median ALT and HBV DNA levels during the course of the study (r = 0.33, p = 0.35). Theoretically, an HBV-specific immune response induced by the treatment regimen, may result in hepatitis. In 2 of 14 patients (patient 12 and 14; 14%) there was a clear hepatitis flare (ALT ≥100 IU/L) during *IVI.* In both patients the hepatitis activity appeared to be related to withdrawing lamivudine therapy, with temporal ALT rises and in patient 14 transient viral suppression following the treatment interruptions (fig 2b). In 4 patients (patient 1,6, 11 and 12; 29%) a reversible hepatitis flare occurred after stopping both lamivudine and Peg-IFNα therapy.

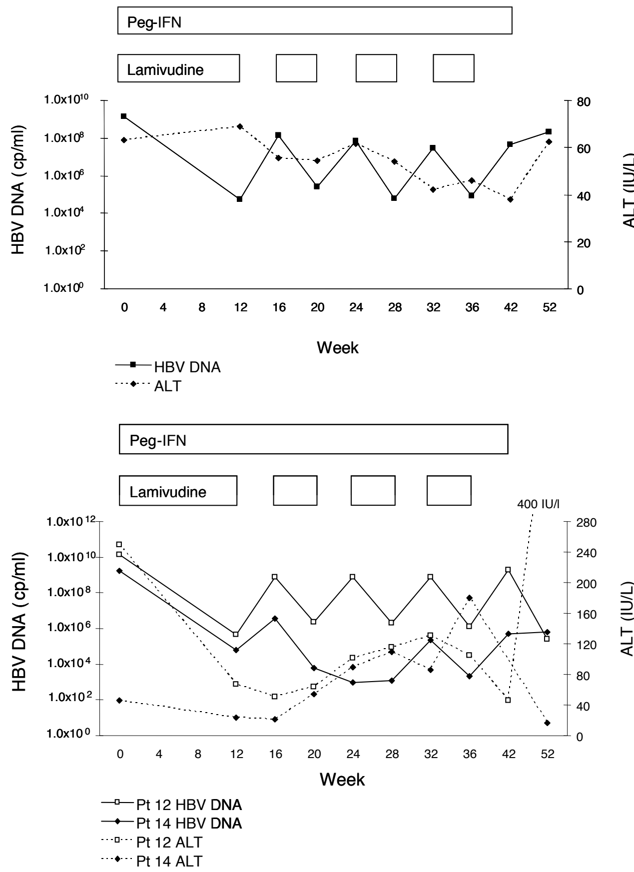


Fig 2. Assessment of viral load (HBV DNA) and hepatitis activity (ALT).
2a) Median HBV DNA and ALT levels of the patient population during the study.
2b) HBV DNA and ALT levels of patients 12 and 14. In both patients there was marked hepatitis activity during IVI, and in patient 14 transient viral suppression during the second lamivudine interruption. At the end of the study, in both patients the viral load had stabilized at a significantly reduced level as compared to baseline.

Longitudinal analysis of HBV-specific Thelper-cell proliferation and IFNγ production
A proliferation assay was performed to measure the immune responsiveness of Th-cells to HBcAg longitudinally during *IVI*. In the first 12 weeks of treatment, there was a progressive reduction of the mean proliferation capacity of Th-cells from a SI of 5.7 (± 2.5) to 3.3 (± 0.9) (fig 3a). During the first lamivudine interruption in all patients there was a further decrease in proliferation capacity to a mean SI of 0.6 (± 0.2). When lamivudine was reintroduced at week 16 in all patients the HBV-specific proliferation capacity partly restored to a mean SI of 3.5 (± 1.6) at week 20. In fact, also during and after the second and third lamivudine interruption an increase in proliferation capacity coincided with decreasing HBV DNA levels and vice versa. To analyze the kinetics of antiviral Th1-activity the frequency of IFNγ-producing cells after *in vitro* stimulation with HBcAg, was measured longitudinally during therapy (fig 3b). In the first 12 weeks of continuous lamivudine – Peg-IFNα combination therapy the mean frequency of IFNγ producing cells decreased in all patients (85 ±36 and 3 ±1.5 IFNγ producing cells /1x10^5 cells, respectively). During the lamivudine interruptions the course of the frequency of IFNγ-producing cells was similar to the Th-cell proliferative response (r = 0.7, p = 0.038); there was an inverse relation between the level of HBV DNA and the frequency of IFNγ-producing cells in the second and third lamivudine interruption. Therefore, *IVI* appeared to stimulate the HBV-specific immune responsiveness of Th-cells, but the magnitude of the response was low, and compared to baseline at the end of therapy in none of the patients the virus-specific proliferation capacity of Th-cells, or their capacity to produce IFNγ had improved.

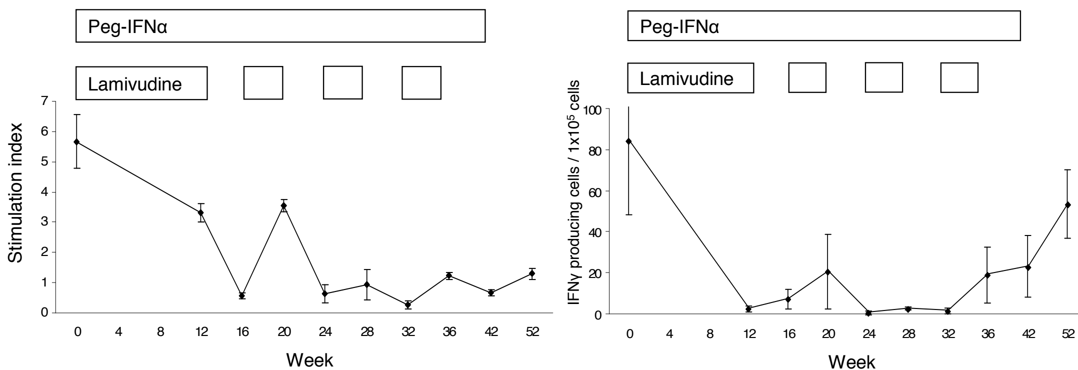


Fig 3. Logitudinal analysis of HBV-specific Thelper-cell proliferation and IFNγ production
3a) Mean proliferation capacity (± sem) of Th-cells obtained during the study, after stimulation with HBcAg.
3b) Mean frequency (± sem) of IFNγ producing Th-cells during the study, after stimulation with HBcAg.

Histology and intrahepatic immune response

Data on the change in histology of the liver between baseline and end of therapy were available for 13 of 14 patients. Fibrosis scores improved in 1 patient (8%) and worsened in 5 patients (38%). Improvements in inflammatory scores were seen in 4 patients (31%) and inflammation had increased in 3 patients (23%). In 9 patients the intrahepatic immune response was monitored by FNAB at week 0 and 12, after the first lamivudine interruption (week 16) and at the end of therapy (week 42). Figure 4 shows that compared to week 12 , at week 16 the ratio CD8+/CD4+ cells in the liver had increased by 26% (1.5 ± 0.08 and 1.9 ± 0.11, respectively, $p>0.05$). This rise in the fraction of CD8+ T-cells occurred in parallel with increased HBV DNA levels and presumably stronger antigen expression by hepatocytes due to lamivudine interruption. Interestingly, in patient 12 and 14 there was a relatively strong rise of the fraction of intrahepatic CD8+ T-cells (2x the baseline value; fig 4).

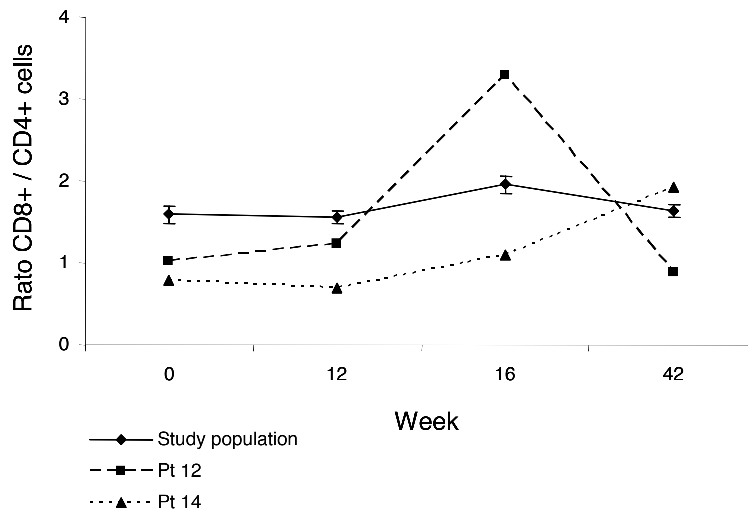


Fig4. Ratio CD8+ / CD4+ cells in liver derived lymphocytes obtained at baseline (week 0), after 12 weeks of continuous lamivudine – Peg-IFNα therapy (week 12), after the first lamivudine interruption (week 16), and at the end of therapy (week 42), in the study population (mean ± sem) and in patients 12 and 14.

Lamivudine resistance

One patient (patient 11) had previously been treated with lamivudine and was known to harbor a YMDD-mutant that was also identified after *IVI*. All other patients were treatment naïve and did not harbor a YMDD-mutant prior to inclusion. Of this group, after *IVI* in one patient (patient 3) a YMDD-mutant was detected.

Discussion

Although it was previously reported that *IVI* was able to transiently suppress viral replication in two patients with CHB [16], in the current pilot study we were unable to show a beneficial effect on the response rate, compared to continuous Peg-IFNα – lamivudine combination therapy or Peg-IFNα monotherapy for 52 weeks [7]. One explanation for the absence of a sustained virological response in the current study, may be that in most patients we were unable to reach sufficiently low levels of HBV DNA prior to the lamivudine interruptions, for a significant HBV-specific immune response to develop. In animal models high titers of viral antigens have been shown to negatively affect the anti-viral immune response [24]. Previous studies conducted in patients with chronic HBV infection treated with IFNα monotherapy [25], lamivudine [26], or a combination therapy of IFNα and lamivudine [27], suggest that T-cell responsiveness may be enhanced after significant reductions in viremia levels to $<10^5$ cp/ml. Whereas in the previous report on *IVI* [16] both patients had undetectable HBV DNA levels before lamivudine interruption, in the current study after 12 weeks of lamivudine - Peg-IFNα combination therapy in 9 patients the viral load was above this threshold. Therefore, future attempts to achieve immune control over the virus by *IVI* may be more successful when, prior to the nucleoside analogue interruptions, the HBV DNA levels are more profoundly reduced, and when the periods of interruption are shortened to limit the viral rebound.

Recently, it has been suggested that chronic patients with high viral load and those who harbor the HBV genotypes C or D display lower response rates to Peg-IFNα therapy than individuals with HBV genotypes A or B [7]. In our study, the median level of HBV-DNA was 1.3×10^9 cp/ml and only 4 of 14 patients harbored an HBV-strain with genotype A or B. Although the mechanism by which the genotype affects the immunomodulatory properties of IFNα is yet unknown, both the high viral load and the large fraction of patients with genotypes C and D may have negatively influenced the outcome of this study.

After the lamivudine interruptions a transient increase in proliferation capacity and IFNγ production by Th-cells was observed, which indicates up-regulation of a Th1-response. Furthermore, in parallel with increased viral replication and presumably stronger antigen expression by hepatocytes, during the first lamivudine interruption the intrahepatic fraction of CD8+ T-cells increased. An increment of CD8+ T-cells in the liver has been correlated to response to IFNα-therapy [28]. Thus although *IVI* transiently stimulates the HBV-specific immune responsiveness of T-cells, the magnitude of the response is apparently insufficient to cause a sustained virological effect in the majority of patients.

In 2 patients at the end of the study the viral load had stabilized at a significantly reduced level as compared to baseline. Compared to the rest of the study population, in these patients *IVI* did not induce more explicit proliferation of Th-cells or a higher frequency of IFNγ-producing cells after stimulation with HBcAg *in vitro*. However, in both patients a relatively strong rise of the fraction intrahepatic CD8+ T-cells was

detected, as well as hepatitis activity that appeared to be related to withdrawing lamivudine therapy. In one of them (patient 14) the flare coincided with transient HBV DNA suppression. This may suggest that in these patients *IVI* was able to induce an intrahepatic anti-viral immune response that contributed to partial immune control over the virus infection.

With respect to safety and tolerability, there was no difference with previous studies using lamivudine in combination with Peg-IFNα [7]. No dangerous flares (ALT >10x upper limit of normal) or flares leading to liver failure were encountered after lamivudine interruption. Also, despite the potential risk that lamivudine interruptions may induce viral resistance, the emergence of YMDD-mutants during *IVI*, did not exceed the frequency observed after lamivudine monotherapy for 12 months [4, 29].

In conclusion, in this prospective pilot study *IVI* was able to transiently stimulate the HBV-specific immune responsiveness of T-cells, but the magnitude of the response was insufficient to cause a beneficial virological effect, compared to currently used treatment strategies. Prior to initiating a larger, controlled study, factors such as the potency of virus suppression before withdrawal, the duration of nucleoside analogue withdrawal or the number of withdrawals, may have to be reconsidered to optimize the study design.

References

1. Kane M. Global programme for control of hepatitis B infection. Vaccine 1995; 13 Suppl 1:S47-9.
2. Wright TL, Lau JY. Clinical aspects of hepatitis B virus infection. Lancet 1993; 342:1340-4.
3. 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.
4. Lai CL, Chien RN, Leung NW, et al. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med 1998; 339:61-8.
5. Angus P, Vaughan R, Xiong S, et al. Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. Gastroenterology 2003; 125:292-7.
6. van Zonneveld M, Honkoop P, Hansen BE, et al. Long-term follow-up of alpha-interferon treatment of patients with chronic hepatitis B. Hepatology 2004; 39:804-10.
7. Janssen HL, van Zonneveld M, Senturk H, et al. Pegylated interferon alfa-2b alone or in combination with lamivudine for HBeAg-positive chronic hepatitis B: a randomised trial. Lancet 2005; 365:123-9.
8. Sprengers D, Janssen HL. Immunomodulatory therapy for chronic hepatitis B virus infection. Fundam Clin Pharmacol 2005; 19:17-26.
9. Pol S, Nalpas B, Driss F, et al. Efficacy and limitations of a specific immunotherapy in chronic hepatitis B. J Hepatol 2001; 34:917-21.
10. Mancini-Bourgine M, Fontaine H, Brechot C, Pol S, Michel ML. Immunogenicity of a hepatitis B DNA vaccine administered to chronic HBV carriers. Vaccine 2005.
11. Michel ML, Pol S, Brechot C, Tiollais P. Immunotherapy of chronic hepatitis B by anti HBV vaccine: from present to future. Vaccine 2001; 19:2395-9.
12. Rehermann B, Fowler P, Sidney J, et al. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. J Exp Med 1995; 181:1047-58.
13. Oritani K, Kincade PW, Zhang C, Tomiyama Y, Matsuzawa Y. Type I interferons and limitin: a comparison of structures, receptors, and functions. Cytokine Growth Factor Rev 2001; 12:337-48.
14. Sen GC. Viruses and interferons. Annu Rev Microbiol 2001; 55:255-81.
15. Bonvin M, Achermann F, Greeve I, et al. Interferon-inducible expression of APOBEC3 editing enzymes in human hepatocytes and inhibition of hepatitis B virus replication. Hepatology 2006; 43:1364-1374.
16. Sprengers D, Janssen HL, Kwekkeboom J, Niesters HG, de Man RA, Schalm SW. In vivo immunization following virus suppression: a novel approach for inducing immune control in chronic hepatitis B. J Viral Hepat 2003; 10:7-9.
17. Pas SD, Fries E, De Man RA, Osterhaus AD, Niesters HG. Development of a quantitative real-time detection assay for hepatitis B virus DNA and comparison with two commercial assays. J Clin Microbiol 2000; 38:2897-901.
18. Stuyver L, De Gendt S, Van Geyt C, et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol 2000; 81:67-74.
19. Norder H, Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology 1994; 198:489-503.
20. Ishak K, Baptista A, Bianchi L, et al. Histological grading and staging of chronic hepatitis. J Hepatol 1995; 22:696-9.
21. Lautenschlager I, Hockerstedt K, Hayry P. Fine-needle aspiration biopsy in the monitoring of liver allografts. Transpl Int 1991; 4:54-61.
22. Sprengers D, van der Molen RG, Kusters JG, et al. Flow cytometry of fine-needle-aspiration biopsies: a new method to monitor the intrahepatic immunological environment in chronic viral hepatitis. J Viral Hepat 2005; 12:507-12.
23. Lau GK, Suri D, Liang R, et al. Resolution of chronic hepatitis B and anti-HBs seroconversion in humans by adoptive transfer of immunity to hepatitis B core antigen. Gastroenterology 2002; 122:614-24.
24. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. J Virol 2003; 77:4911-27.
25. 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; 18:405-13.
26. 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.
27. Marinos G, Naoumov NV, Williams R. Impact of complete inhibition of viral replication on the cellular immune response in chronic hepatitis B virus infection. Hepatology 1996; 24:991-5.
28. Tang TJ, Kwekkeboom J, Mancham S, et al. Intrahepatic CD8+ T-lymphocyte response is important for therapy-induced viral clearance in chronic hepatitis B infection. J Hepatol 2005; 43:45-52.

29. Santantonio T, Mazzola M, Iacovazzi T, Miglietta A, Guastadisegni A, Pastore G. Long-term follow-up of patients with anti-HBe/HBV DNA-positive chronic hepatitis B treated for 12 months with lamivudine. J Hepatol 2000; 32:300-6.

CHAPTER 8

Induction of CD4+ CD25+ regulatory T-cells associated with non-response to pegylated Interferon-α therapy for chronic hepatitis B virus infection

D. Sprengers[1], J.N. Stoop[1], R.S. Binda[1], J.G. Kusters[1], B.Haagmans[2], P. Carotenuto[2], A. Artsen[2], A.D.M.E. Osterhaus[2], R.G. van der Molen[1*] H.L.A. Janssen[1*].
* Equal contribution

[1] Dept. of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands
[2] Dept. of Virology, Erasmus MC University Medical Center, Rotterdam, The Netherlands

Submitted

Abstract

Little is known about why treatment with interferon alpha (IFNα) leads to a response in only a minority of patients with chronic HBV. It was recently shown that in these patients CD4+ CD25+ regulatory T-cells (Treg) can suppress the HBV-specific immune response. We aimed to investigate whether in non-responders to IFNα therapy Treg contribute to treatment failure by down-regulating the HBV-specific T-cell responses. Fourteen patients received pegylated IFNα monotherapy for 52 weeks and were followed-up for 26 weeks. Compared to non-responders responders displayed an increased HBV-specific Th-cell proliferation. Whereas there was no difference in the frequencies of CD4+ CD25+ Treg between responders and non-responders at the start of treatment, during and after therapy in responders these frequencies decreased, and in non-responders there was a significant increase in the frequency of CD4+ CD25+ Treg during treatment. In contrast to the responders, the non-responders showed a significant increase in frequency of IL-10 producing cells. Treg depletion resulted in increased proliferation capacity and increased frequencies of HBV-specific INFγ-producing cells, but did not affect the frequency of IL-10 producing cells measured during the course of the treatment In conclusion, this study indicates that there may be an important role for regulatory T-cells, in HBV-persistence during and after Peg-IFNα therapy.

Introduction

Chronic infection with hepatitis B virus (HBV) is characterized by inflammatory liver disease of variable severity and is a major cause of liver failure and hepatocellular cancer world-wide [1, 2]. HBV is a non-cytopathic virus, and liver injury is mainly mediated by the host immune response against virus infected liver cells [3]. Therefore, in chronic hepatitis B infection (CHB) effective therapy is needed to stop viral replication and progression of liver damage.
Currently, interferon alpha (IFNα), a drug that modulates the anti-viral immune response, represents the first treatment of choice in CHB [4]. However, in only 20-30% of CHB patients conventional IFNα therapy leads to sustained virological response [5, 6] and the recent introduction of pegylated-IFNα, a drug with a prolonged half-life, has not changed the picture that the majority of treated patients does not respond to therapy [7].
Previous studies have defined two T helper cell subsets, Th1 and Th2, which are characterized by distinct and mutually exclusive patterns of cytokine production and different functions [8]. Th1 cells produce IFNγ, IL-2 and promote cellular immune reactions, while Th2 cells produce IL-4, -5, -and IL-13, and enhance humoral immune response [8]. Resolution of acute HBV infection has been associated with a vigorous polyclonal and multispecific Th1 response [9], whereas in the blood of chronically infected patients the HBV-specific Th1-response is weak, antigenically restricted or undetectable [10, 11]. Peripheral T cells contain an immunoregulatory sub-population which expresses CD4, CD25 (the IL-2 receptor α-chain) and CD45RO, and have a continuous intracellular expression of the cytotoxic T lymphocyte-associated antigen-4 (CTLA-4). These regulatory T cells (Treg) are capable of inhibiting the effector functions of $CD4^+$, $CD8^+$ and natural killer T cells [12-15]. In a recent study it was shown that patients with a chronic HBV infection have a higher percentage of Treg in their peripheral blood compared to healthy controls and individuals with a resolved HBV infection [16]. These Treg were able to suppress the HBV-specific immune response in a dose dependent manner. Although the mechanism of this suppressive effect is not clear, studies in other chronic viral infections have shown that upon stimulation Treg may contribute to persistent viral infection by modulation of the virus-specific Th1 response [17].
Little is known about why treatment with pegylated IFN leads to a response in only a minority of patients. Elucidating the mechanism responsible for treatment failure may result in improved control of the virus infection and better treatment strategies. Therefore, in this study, we aimed to analyze longitudinally the virus-specific Th-cell responses and cytokine patterns during and after treatment of patients with CHB with Peg-IFNα-2b monotherapy. We hypothesized that in non-responders to IFNα therapy Treg contribute to treatment failure by down-regulating the HBV-specific Th1 responses.

Patients and methods

Patients

Patient material used for this study was derived from individuals that participated in a multicenter, randomized, double-blind study carried out to compare the efficacy of pegylated interferon-alpha 2b (Peg-IFN) monotherapy to a combination regimen with lamivudine for treatment of patients with HBeAg positive chronic hepatitis B [7]. All patients were HBeAg positive, had alanine amino transferase (ALT) levels twice the upper limit of normal, had not received antiviral therapy within 6 months prior to enrollment and displayed no serious co morbidity. Patients were treated for 32 weeks with 100µg Peg-IFN weekly and with 50µg Peg-IFN weekly for another 20 weeks (total 52 weeks) after which they were followed-up for 26 weeks. Response to therapy was defined as HBeAg-loss at the end of follow-up. For the current study we used material from all locally enrolled patients that retrospectively had received Peg-IFN monotherapy, and of whom material was obtained after signing informed consent (n=14).

Isolation of the PBMCs and Flow Cytometric Analysis

At the start of therapy (t=0), 8 weeks later (t=8), at the end of therapy (t=52) and at the end of follow-up (t=78) PBMCs from patients were obtained by ficoll separation (Ficoll-Paque™ plus, Amersham Biosciences, Buckinghamshire, UK). The PBMCs were immediately frozen in medium containing 10% DMSO and stored at –135°C until further use. PBMC from different timepoints were tested simultaneously to avoid interassay variations. Flow cytometric analysis was performed on the stored samples using fluorochrome conjugated antibodies specific for the surface markers CD4, CD45RO and CD25 diluted in PBS/ 0.3% bovine serum albumin. The cells were fixed by incubation with intraprep reagent 1 and permeabilized by incubation with intraprep reagent 2 (Beckman-Coulter, Marseille, France). Anti-CTLA-4 antibody was added during permeabilization. The following antibodies were used: anti-CD4-PerCP-Cy5.5 (SK3) (Pharmingen, San Diego, CA), anti-CD45RO-APC (UCHL1) (Becton Dickinson, San Jose, CA), anti-CD25-FITC (2A3) (Becton Dickinson), anti-CTLA-4-PE (BNI3) (Immunotech, Marseille, France). For the CD45RO, CD25 and CTLA-4 antibodies, isotype matched control antibodies were used to determine the level of background staining. After staining the cells were analyzed using a four-color cytometer (FACScalibur™, CELLQuest Pro™ software, Beckton Dickinson). The FoxP3 antibody (clone PCH101, eBiosciences, San Diego, Ca) staining was performed according the manufacturers instructions. Briefly, cells surface markers were stained with anti CD25-PE (M-A251) (Pharmingen) anti-CD4-PerCP-Cy5.5 (SK3) (Pharmingen). The cells were fixed and permeabilized with Fix/perm buffer and permeabilization buffer (eBiosciences) anti-FoxP3-APC was added during permeabilization. To determine the level of background staining, for anti-CD25-PE and anti-FoxP3-APC isotype matched control antibodies were used.

$CD4^+CD25^+$ T cell isolation

Thawed PBMCs obtained at t=0, t=8, t=52 and t=78 were used for $CD4^+CD25^+$ T cell isolation. $CD4^+$ T cells were isolated from PBMCs by negative selection using the untouched $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+CD25^+$ T cells were isolated from $CD4^+$ T cells using anti CD25-microbeads (Miltenyi biotec). The isolations were performed according to manufacturer's instructions. The $CD4^-$ and the $CD4^+CD25^-$ fraction were pooled and were used as CD25 (Treg)-depleted responder cells. Purity of the cell fractions was determined by flow cytometry analysis with antibodies against CD3, CD4 and CD25. The following antibodies were used: anti-CD3-FITC (clone UCHT1, Immunotech), anti-CD4-PerCP-Cy5.5 (clone SK3, Becton Dickinson) and anti-CD25-PE (clone M-A251, Pharmingen). An isotype matched control antibody was used to determine the level of background staining for the anti-CD25-PE antibody. The $CD4^+CD25^+$ Treg purification method resulted in a Treg fraction containing more than 90% pure $CD4^+CD25^+$ Treg and a Treg-depleted cell fraction containing all other cell types present in PBMCs.

Proliferation assay

Th-cell proliferation was determined as reported previously [18]. Isolated PBMCs and the pooled CD25-depleted cells of all time points investigated were cultured in triplicate in a concentration of $1X10^5$ cells per well in 100 µl RPMI 1640 (Bio Whittaker, Verviers, Belgium) containing 5% pooled human serum (Dept. of Immunohematology and Bloodbank, Leiden University Medical Center, Leiden, The Netherlands) and penicillin/streptomycin (Gibco, Paisley, UK). The cells were stimulated with 1 µg/ml HBV core antigen (HBcAg) (a kind gift by M van Roosmalen, Biomerieux, Boxtel, The Netherlands), 5 µg/ml Phytohemagglutin (Murex, Paris, France), or not stimulated and cultured for 6 days. After 5 days of incubation the cells were pulsed with 0.25 µCi/well of $[^3H]$thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK). The cells were harvested 16 hours later. Proliferation, was determined by liquid scintillation counting of the harvested cells and expressed as stimulation index (SI). The SI was calculated from the counts per minute found with antigen divided by that found without antigen.

Elispot assay

Detection of IFNγ (U-CyTech, Utrecht, The Netherlands) and IL-10 (Sanquin, Amsterdam, The Netherlands) was performed using ELISPOT kits according to the manufacturer's instructions. Of all time points investigated, cultures of PBMCs and the pooled CD25-depleted cells of were established in triplicate on round bottom 96-wells plates (Corning Inc., New york, USA), in a concentration of $1X10^5$ cells per well in 100 µl RPMI 1640 (Bio Whittaker) containing 5% pooled human serum (Leiden University Medical Center) and penicillin/streptomycin (Gibco). The cells were stimulated with 1 µg/ml HBV core antigen (HBcAg) (M. van Roosmalen, Biomerieux), 5 µg/ml Phytohemagglutin (Murex) (positive control) or medium only (negative control) for 24 hours at 37 degrees Celsius, after which they were transferred into flat bottom,

anti- IFNγ or anti-IL-10-coated 96-wells plates (Nunc A/S, Roskilde, Denmark and Millipore, Molsheim, France, respectively) for another 24-hours incubation period. Cytokine spots were visualised by biotin-labelled antibodies and their numbers were established by using a Bioreader 3000 from BioSys (Karben, Germany). A response was considered positive if the number of spots was equal to or greater than 2.5 times the background, and the reported HBcAg-specific data is the number of spots counted minus the background level.

Statistics
Treg flow cytometry data, cytokine profiles and proliferation capacity of PBMC obtained from non-responders and responders to antiviral therapy were compared using the Mann Whitney U test. Longitudinal analysis of frequencies of Treg, cytokine profiles and proliferation capacity during the study period, was performed with the Wilcoxon matched pairs signed rank sum test. For these analyses SPSS 11.5 for Windows (SPSS, Chicago, IL) was used. Where applicable data are reported as mean ± standard error of the mean (SEM).

Results

Patients
Table I shows the patient characteristics. At the end of follow-up (t=78) 8 patients had remained HBeAg positive (non-responders), and 6 patients had sustained loss of HBeAg (responders; 43%). At the start of treatment (t=0), the 8 non-responders had a median ALT of 102 IU/l (range 47-394) versus 168 IU/l (range 72-236) in responders, and the median viral load measured $2.3x10^9$ cp/ml (range $1.6x10^8$-$7.1x10^9$) versus $1.1x10^9$ cp/ml (range $1.7x10^8$-$5.7x10^9$), respectively. These differences were not significant. At the end of therapy and at the end of follow-up HBV DNA levels were $1.8x10^8$ cp/ml ($1.4x10^3$-$4.7x10^8$) and $5.3x10^8$ cp/ml ($3.4x10^5$-$4.9x10^9$) in non-responders, vs. $1.0x10^3$ cp/ml (480-$2.9x10^4$) and 473 cp/ml (<373-970) in responders, respectively.

HBV-specific Thelper-cell proliferation
A proliferation assay was performed to measure the immune responsiveness of Th-cells to HBcAg longitudinally during the course of the treatment. Whereas there was considerable variation between the responders, the non-responders showed a similar pattern of response characterized by a decreasing proliferation capacity of Th-cells during Peg-IFNα therapy, which appeared to restore in the follow-up period (fig 1). At the start of therapy there was no significant difference between responders and non-responders (mean SI = 4.83 ± 0.63 and 4.43 ± 0.38, respectively). However, in non-responders at 8 and 52 weeks the mean proliferation capacity of Th-cells had decreased by 35% and 76%, respectively compared to t=0 ($p < 0.05$), whereas 5 of 6 responders showed a profound increase in the Th-cell proliferative response to HBcAg (SI > 2 x baseline level) at one or more time points analyzed.

Table I. Patient characteristics

Patients (case)	Sex	Age	Race	ALT (U/l) t=0	HBV-DNA (geq/ml) t=0	Response to therapy
1	Male	58	Cauc.	78	1.6×10^9	-
2	Male	47	Asian	125	1.9×10^8	-
3	Male	32	Asian	47	1.6×10^8	-
4	Male	27	Cauc.	70	3.0×10^9	-
5	Male	28	Cauc.	325	7.1×10^9	-
6	Male	49	Cauc.	394	5.7×10^9	-
7	Male	38	Cauc.	79	7.9×10^9	-
8	Female	21	Cauc.	160	2.2×10^8	-
9	Male	35	Cauc.	151	5.2×10^9	+
10	Male	41	Asian	131	1.2×10^9	+
11	Male	43	Cauc.	185	5.7×10^9	+
12	Male	41	Asian	236	4.4×10^8	+
13	Male	29	Cauc.	203	9.9×10^8	+
14	Female	29	Asian	72	1.7×10^8	+

All 14 patients were HBeAg positive at inclusion and were treated with PEG-IFNα for 52 weeks. At the end of therapy 6 patients (case 9 – 14) had serocoverted to HBeAg-negative and this seroconversion was sustained at the end of a follow-up period of 26 weeks. There were 8 non-responders (HBeAg positive, case 1-8) at end of therapy. t = 0 is start of therapy

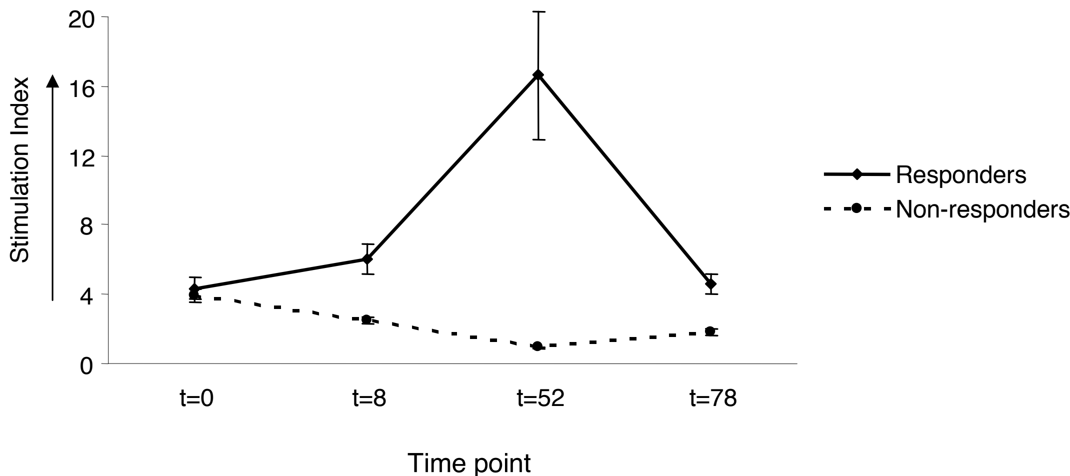


Fig 1. Mean proliferation capacity (± sem) of Th-cells of patients with chronic HBV, obtained at different time points during PEG-IFNα therapy, after stimulation with HBcAg. The time points analyzed were: t=0, t=8, t=52 and t=78 weeks. In non-responders, compared to t=0 at t=8 and t=52 the reduction in stimulation index was significant (p=0.016 at both time points). At 8 and 52 weeks of treatment the differences between responders and non-responders showed a trend towards significance (p = 0.07 and p = 0.10, respectively).

Longitudinal analysis of IFNγ producing cells in response to HBcAg

To analyze the kinetics of antiviral Th1-activity, the frequency of IFNγ-producing cells after *in vitro* stimulation with HBcAg was determined longitudinally during therapy. There was a wide variation between individual patients, and not at any time point analyzed there was a significant difference in frequencies of IFNγ producing Th-cells between responders and non-responders (fig 2). At the end of PEG-IFNα therapy in 6 of 8 non-responders and in 4 of 6 responders the mean number of IFNγ producing cells after stimulation with HBcAg, had decreased. Compared to t=0, at this time point in the non-responders and responders there was a mean reduction of 39% and 72% (p = 0.18 and p = 0.43), respectively.

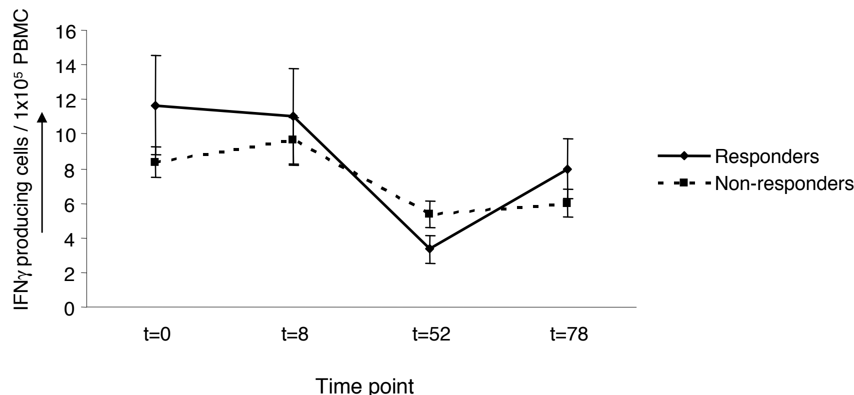


Fig 2. Mean frequencies (± sem) of IFNγ producing Th-cells at treatment week 0, 8, 52 and 78, according to treatment response.

Frequencies of CD4+ CD25+ regulatory T cells during PEG-IFNα therapy

At all time points investigated we determined the frequencies of CD4+ CD25+ Treg. Based upon several recent studies which showed that the $CD4^{+}CD25^{+}$ cells with high expression of CD25 are Treg [12, 16, 19], CD45RO and CTLA-4 were used as additional markers in our flow cytometry experiments to identify this cell population. Typical data as obtained by flow cytometry of PBMCs from a representative responder and non-responder, are shown in figure 3a. Figure 3b shows the mean frequencies of CD4+ CD25+ Treg in responders and non-responders during treatment. At baseline responders and non-responders had a comparable frequency of CD4+ CD25+ Treg (2.21 ± 0.10 vs 2.23 ± 0.19 respectively). At the end of therapy in non-responders the frequency had increased by 74% to a mean of 3.6% ± 0.18 (p = 0.008), whereas in responders it had decreased by 20% to a mean of 1.75% ± 0.09 (p = 0.031;responders vs. non-responders p = 0.008). At the end of follow-up in non-responders the frequency CD4+ CD25+ Treg returned to the baseline level, whereas in responders it further decreased to below baseline (responders vs. non-responders p = 0.074).

CD4+ CD25+ Treg express the *forkhead*/winged helix family protein Foxp3, which is a key regulator for the development and function of Treg, and currently the most specific marker for this cell population [20-22]. The recent availability of an adequate Foxp3

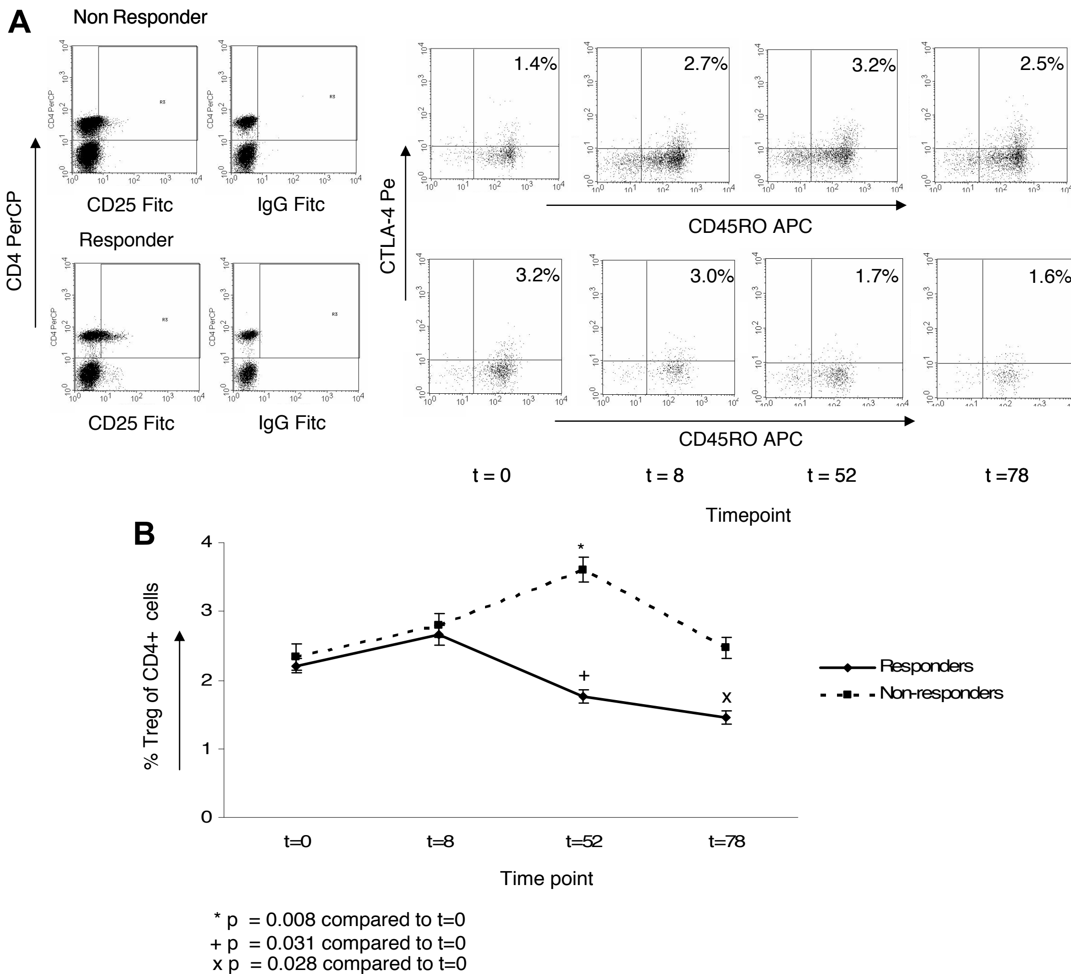


Fig 3. Percentage of Treg (CD4, CD25, CD45RO and CTLA-4 positive cells) within the CD4⁺ cell fraction, determined by FACS staining on PBMC of 8 non-responders and 6 responders to PEG-IFNα therapy. The time points analyzed were treatment week 0, 8, 52 and 78.
3a) Flow cytometry staining experiment from a representative responder (case 10) and non-responder (case 4) to Peg- IFNα therapy, during the course of the study. Antibodies were used against CD4, CD25, CD45RO and CTLA-4. The gates were positioned using isotype matched control antibodies as shown in the two dot-blots on the left. $CD4^+CD25^+$ cells were analyzed for CTLA-4 and CD45RO expression, and the number in the the upper right quadrant of de four dot blots on the right indicates the percentage CTLA-4+ CD45RO+ CD4+ CD25+ cells of total CD4+ cells (Treg).
3b) Mean frequencies of Treg (± sem) during the study. Compared to the baseline level, in non-responders there was a significant increase in the frequency of Treg at the end of therapy, whereas in responders there was a significant decrease. At the end of therapy the frequency of Treg was higher in non-responders than responders (p = 0.008). At t=78 in non-responders the frequency had returned to baseline level, whereas in responders it continued to decrease (responders vs. non-responders p = 0.074)

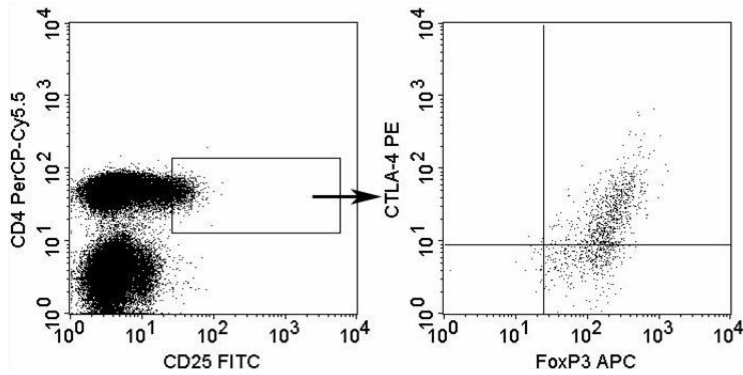


Fig 3c) Foxp3 expression by CD4+ CD25+ CTLA-4+ cells in a representative untreated chronic HBV patient. The number in the right upper quadrant of the right dot-blot indicates the percentage Foxp3+ cells of total CD4+ CD25+ CTLA-4+ cells.

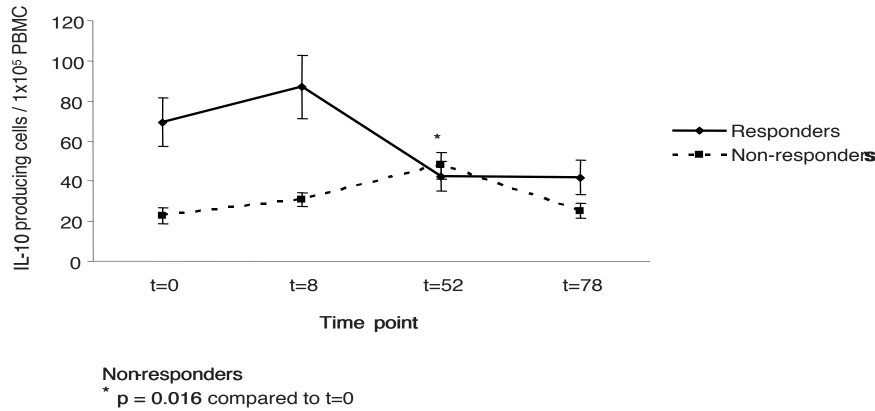
antibody enabled us to validate our Treg staining, by determining in a separate group of 5 untreated chronic HBV patients the Foxp3 expression by CD4+ CD25+ CTLA4+ cells. Almost all CD4+ CD25+ CTLA-4+ cells expressed the Foxp3 protein (mean percentage Foxp3+ / CD4+ CD25+ CTLA-4+ cells: 98% ± 0.46), which indicates that the combination of cell surface markers used to determine the Treg-frequencies in the study population, indeed most likely identifies a suppressive CD4+ CD25+ T-cell population. Figure 3c shows the flow cytometry data of a representative patient.

Longitudinal analysis of IL-10 producing cells in response to HBcAg

Previously, it has been suggested that in chronic HBV patients treated with the combination of ribavirin and IFNα, IL-10 cytokine production is associated with response to therapy [23]. Therefore, in our patient population we longitudinally studied the frequency of IL-10 producing Th-cells after stimulation with HBcAg *in vitro*. There was a wide variation between individual patients of both patient groups. At no time point analyzed there was a significant difference between responders and non-responders (fig 4). However, in contrast to the responders, during treatment the non-responders showed a gradual increase in frequency of IL-10 producing cells from a mean of 22.7 ± 4.0 / $1x10^5$ PBMC at t=0 to a mean of 48 ± 6.7 / $1x10^5$ PBMC at t=52 weeks ($p = 0.016$). This number returned to the baseline level after cessation of the therapy. In several studies IL-10 has been implicated as a potential mediator of suppression of the immune response by CD4+ CD25+ Treg [17, 24]. Although, in responders and non-responders the trends of IL-10 producing cells and of CD4+ CD25+ Treg were similar, there was no significant correlation between these two parameters (data not shown).

HBV-specific immune response after CD4+ CD25+ regulatory T-cell depletion

In four patients (2 responders, 2 non-responders) sufficient cells were obtained to



Non-responders
* p = 0.016 compared to t=0

Fig 4. Mean frequencies of IL-10 producing Th-cells (± sem) at treatment week 0, 8, 52 and 78, according to treatment response.

analyse the effects of CD4+ CD25+ Treg depletion on IFNγ and IL-10 production and proliferation capacity after stimulation of PBMC with HBcAg *in vitro.* When Treg were depleted, both the number of cells that produces IFNγ, as well as the proliferation capacity of Th-cells increased (fig 5). This suggests that the CD4+ CD25+ T cell population studied is indeed able to suppress the antiviral immune response. Interestingly, Treg depletion did not influence the number of IL-10 producing cells.

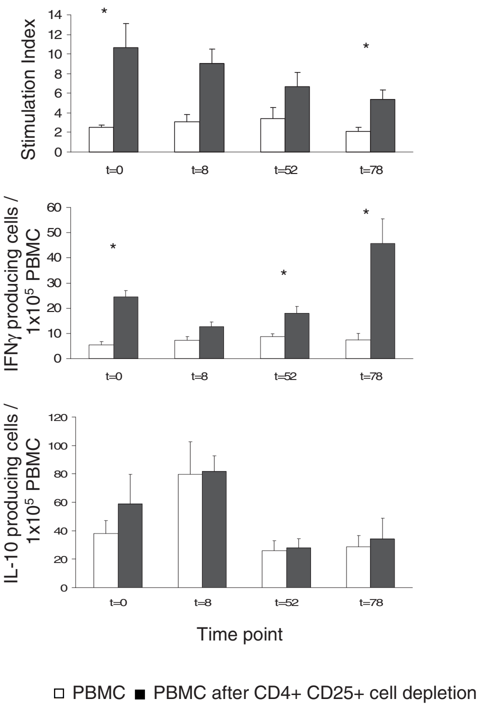


Fig 5.
CD4+ CD25+ regulatory T-cell depletion.
*The effects of CD4+ CD25+ Treg depletion on the mean proliferation capacity (top), and the mean frequency of IFNγ (middle) and IL-10 (bottom) producing cells in four patients (2 responders, 2 non-responders) after stimulation of PBMC with HBcAg in vitro. PBMC were depleted of CD4+ CD25+ cells at all time points during the study (week 0, 8, 52 and t=78). Despite the low patient number, at most time points there was a trend towards significance with respect to increased proliferation capacity and increased frequency of IFNγ producing cells after Treg depletion (* p ≤ 0.1).*

Discussion

Peg-IFNα therapy leads to sustained virological response in a minority of chronic HBV infected patients [7]. Elucidating the mechanism responsible for treatment failure may result in improved control of the virus infection and better treatment strategies. In this study we have investigated the HBV-specific Th-cell responses in peripheral blood of HBeAg-positive CHB patients treated with PEG-IFNα. Since it was recently shown that patients with chronic HBV infection have a higher percentage of CD4+ CD25+ Treg in their peripheral blood than individuals with a resolved infection, and that these Treg were capable of inhibiting the HBV-specific immune response [16], we have investigated the role of this T-cell population in the outcome of therapy. The results show that during therapy, in the majority of responders there is increased proliferation of Th-cells after stimulation with HBcAg, whereas in non-responders there is a clear decrease. Previously, it has been described that responders to conventional IFNα display a similar increased Th-cell proliferation capacity [25] which may reflect increased Th-cell reactivity to the virus. In contrast to several reports describing increased IFNγ production in responders to IFNα therapy only [25, 26], we found a decrease in the frequency of IFNγ producing cells during therapy in both responders and non-responders, to a similar extend. This discrepancy may be explained by differences in time points investigated or by methodological differences, since previously IFNγ was measured in supernatants [25, 26] whereas we quantified the frequency IFNγ producing cells. One could hypothesize that after *in vitro* stimulation with HBcAg, the Th-cells of responders are more potent IFNγ-producers than those of non-responders.
At the start of treatment there was no difference in the frequencies of CD4+ CD25+ Treg between responders and non-responders. However, during PEG-IFNα therapy in non-responders there was a significant increase in the frequency of CD4+ CD25+ Treg, whereas in responders this frequency had significantly decreased both at the end of therapy and follow-up. No correlation was found between HBV DNA levels or ALT and the percentage of Treg (data not shown). Treg express the Foxp3-gene, which is important in the development and suppressive function of CD4+ CD25+ Treg [21, 22], and we and others [16] showed that they are capable of inhibiting the HBV-specific immune response. Therefore, one could hypothezise that an increment in Treg frequency during PEG-IFNα therapy may negatively influence Th-cell reactivity to the virus, and contribute to non-response to treatment. Cytotoxic T-cell responses are subjected to negative control by CD4+ CD25+ Treg [27]. Also, in mice vaccine-induced virus-specific T-cell responses were shown to be suppressed by another major regulatory T-cell population that has been implicated in the control of specific T-cell immunity, namely CD4+ IL-10+ Tregulatory-cells (Tr1-cells) [28, 29]. IL-10 could be instrumental in Tr1-mediated suppression of T-cell proliferation and cytokine production in a cell-cell contact independent manner [30]. These mouse Tr1-cells, but not CD4+ CD25+ Treg, were up-regulated under the influence of IFNαβ [28]. Also, human Tr1 cells can be generated in vitro under the influence of exogenous IFNα [31].
In our study, during the course of PEG-IFNα therapy we found that in contrast to

the responders, the non-responders showed a significant increase in frequency of IL-10 producing cells. IL-10 is important in blocking proinflammatory cytokine production, co-stimulation, MHC class II expression, and chemokine secretion [32], and increased IL-10 production has previously been described in chronic HBV-patients not responding to ribavirin - IFNα combination therapy [23]. This observation suggests a dissociation in IL-10 production in relation to response to therapy. We were unable to show a correlation between the frequencies of CD4+ CD25+ Treg and IL-10 producing cells in the individual patients. Also, despite our finding that depletion of the CD4+ CD25+ T cell population resulted in increased proliferation capacity and increased frequencies of INFγ-producing cells after stimulation with HBcAg, it did not significantly affect the frequency of IL-10 producing cells measured during the course of the treatment. Therefore, in non-responders the immunomodulatory effects of PEG-IFNα therapy may result in an increment in Treg frequency and an up-regulation of IL-10 producing cells, (potentially IFNα-induced Tr1-cells), which together may negatively influence T-cell reactivity to the virus.

Previous studies conducted in patients with chronic HBV infection treated with different treatment regimes [25] [33-35], suggest that there is an association between improved HBV-specific T-cell responsiveness and significant reductions in viremia levels. In our study, in non-responders an increment in the frequencies of Treg and IL-10 producing cells coincided with a reduction in viral load, whereas in responders both parameters decreased in parallel with decreasing viremia. Therefore, the upregulation of these immunoregulatory factors observed in non-responders is most likely not due to a reduction in the levels of HBV DNA .

In conclusion, this study indicates that there may be an important role for regulatory T-cells,in HBV-persistence during and after Peg-IFNα therapy. We found that an increasing frequency of IL-10 producing cells and CD4+ CD25+ Treg during treatment was inversely correlated to treatment response. To improve immune modulatory treatment in chronic HBV infection, future investigations should focus on strategies to manipulate the function of these regulatory T-cells, by means of their depletion or blockade of effector cytokine function.

References

1. Kane M. Global programme for control of hepatitis B infection. Vaccine 1995; 13 Suppl 1:S47-9.
2. Wright TL, Lau JY. Clinical aspects of hepatitis B virus infection. Lancet 1993; 342:1340-4.
3. Rehermann B, Fowler P, Sidney J, et al. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. J Exp Med 1995; 181:1047-58.
4. Sprengers D, Janssen HL. Immunomodulatory therapy for chronic hepatitis B virus infection. Fundam Clin Pharmacol 2005; 19:17-26.
5. van Zonneveld M, Honkoop P, Hansen BE, et al. Long-term follow-up of alpha-interferon treatment of patients with chronic hepatitis B. Hepatology 2004; 39:804-10.
6. Manesis EK, Hadziyannis SJ. Interferon alpha treatment and retreatment of hepatitis B e antigen-negative chronic hepatitis B. Gastroenterology 2001; 121:101-9.
7. Janssen HL, van Zonneveld M, Senturk H, et al. Pegylated interferon alfa-2b alone or in combination with lamivudine for HBeAg-positive chronic hepatitis B: a randomised trial. Lancet 2005; 365:123-9.
8. Paul WE, Seder RA. Lymphocyte responses and cytokines. Cell 1994; 76:241-51.
9. Guidotti LG, Ando K, Hobbs MV, et al. Cytotoxic T lymphocytes inhibit hepatitis B virus gene expression by a noncytolytic mechanism in transgenic mice. Proc Natl Acad Sci U S A 1994; 91:3764-8.
10. Rehermann B, Lau D, Hoofnagle JH, Chisari FV. Cytotoxic T lymphocyte responsiveness after resolution of chronic hepatitis B virus infection. J Clin Invest 1996; 97:1655-65.
11. Maini MK, Boni C, Lee CK, et al. The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. J Exp Med 2000; 191:1269-80.
12. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4+CD25high regulatory cells in human peripheral blood. J Immunol 2001; 167:1245-53.
13. Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. J Exp Med 2001; 193:1303-10.
14. Camara NO, Sebille F, Lechler RI. Human CD4+CD25+ regulatory cells have marked and sustained effects on CD8+ T cell activation. Eur J Immunol 2003; 33:3473-83.
15. Azuma T, Takahashi T, Kunisato A, Kitamura T, Hirai H. Human CD4+ CD25+ regulatory T cells suppress NKT cell functions. Cancer Res 2003; 63:4516-20.
16. Stoop JN, van der Molen RG, Baan CC, et al. Regulatory T cells contribute to the impaired immune response in patients with chronic hepatitis B virus infection. Hepatology 2005; 41:771-8.
17. Cabrera R, Tu Z, Xu Y, et al. An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. Hepatology 2004; 40:1062-71.
18. Lau GK, Suri D, Liang R, et al. Resolution of chronic hepatitis B and anti-HBs seroconversion in humans by adoptive transfer of immunity to hepatitis B core antigen. Gastroenterology 2002; 122:614-24.
19. Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. Immunity 2004; 21:589-601.
20. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003; 299:1057-61.
21. Walker MR, Kasprowicz DJ, Gersuk VH, et al. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. J Clin Invest 2003; 112:1437-43.
22. Wing K, Suri-Payer E, Rudin A. CD4+CD25+-regulatory T cells from mouse to man. Scand J Immunol 2005; 62:1-15.
23. Rico MA, Quiroga JA, Subira D, et al. Hepatitis B virus-specific T-cell proliferation and cytokine secretion in chronic hepatitis B e antibody-positive patients treated with ribavirin and interferon alpha. Hepatology 2001; 33:295-300.
24. Maloy KJ, Salaun L, Cahill R, Dougan G, Saunders NJ, Powrie F. CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. J Exp Med 2003; 197:111-9.
25. 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; 18:405-13.
26. Cramp ME, Rossol S, Chokshi S, Carucci P, Williams R, Naoumov NV. Hepatitis C virus-specific T-cell reactivity during interferon and ribavirin treatment in chronic hepatitis C. Gastroenterology 2000; 118:346-55.
27. Franzese O, Kennedy PT, Gehring AJ, et al. Modulation of the CD8+-T-cell response by CD4+ CD25+ regulatory T cells in patients with hepatitis B virus infection. J Virol 2005; 79:3322-8.
28. Dikopoulos N, Bertoletti A, Kroger A, Hauser H, Schirmbeck R, Reimann J. Type I IFN negatively regulates CD8+ T cell responses through IL-10-producing CD4+ T regulatory 1 cells. J Immunol 2005; 174:99-109.
29. Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. Nat Rev Immunol 2003; 3:253-7.
30. Lan RY, Ansari AA, Lian ZX, Gershwin ME. Regulatory T cells: development, function and role in autoimmunity. Autoimmun Rev 2005; 4:351-63.

31. Levings MK, Sangregorio R, Galbiati F, Squadrone S, de Waal Malefyt R, Roncarolo MG. IFN-alpha and IL-10 induce the differentiation of human type 1 T regulatory cells. J Immunol 2001; 166:5530-9.
32. O'Garra A, Vieira PL, Vieira P, Goldfeld AE. IL-10-producing and naturally occurring CD4+ Tregs: limiting collateral damage. J Clin Invest 2004; 114:1372-8.
33. 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.
34. Jung MC, Hartmann B, Gerlach JT, et al. Virus-specific lymphokine production differs quantitatively but not qualitatively in acute and chronic hepatitis B infection. Virology 1999; 261:165-72.
35. Marinos G, Naoumov NV, Williams R. Impact of complete inhibition of viral replication on the cellular immune response in chronic hepatitis B virus infection. Hepatology 1996; 24:991-5.

CHAPTER 9

Discussion: the future of immunotherapy in chronic Hepatitis B virus infection

D. Sprengers[1], H.L.A. Janssen[1]

[1] Dept. of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands

Introduction

According to the World Health Organization, an estimated 350 million people worldwide are chronically infected with hepatitis B virus (HBV). Following acute infection, approximately 10% of adults and as many as 90% of infants infected perinatally, the most common route of transmission, develop chronic infection. Although many of these individuals eventually clear the virus or achieve a state of non-replicative infection, prolonged chronic hepatitis leads to the development of cirrhosis, liver failure, or hepatocellular carcinoma in up to 40% of patients causing over 1 million deaths annually [1]. The goals of treatment in chronic hepatitis B virus (CHB) infection are sustained viral suppression, normalization of serum alanine aminotransferase (ALT), and improvement in liver histology, leading to long-term reduction of the risk of complications.
Two major classes of antiviral therapeutics have been adopted to treat the infection: drugs that directly interfere with virus replication and drugs that modulate anti-viral immune response. In most countries lamivudine, adefovir dipivoxil and recently also entecavir are the only approved inhibitors of viral replication (HBV-DNA polymerase inhibitors). Although they lead to rapid and almost absolute discontinuation of HBV replication [2], breakthrough of resistant HBV variants limits long-term use [3, 4]. Another strategy aims at the induction of immune control by a treatment course of limited duration. This approach, of which interferon alpha (IFNα) therapy is the generally used example, is to be preferred. However, in only a minority of patients conventional or pegylated IFNα (Peg-IFNα) therapy leads to sustained virological response [5, 6]. Therefore, alternative agents with enhanced potency to stimulate immune control over the virus are needed.
The study of animal models and transgenic mouse models able to express individual HBV genes or replicate the entire viral genome have clarified several aspects connected to HBV infection. Furthermore, the ability to analyse many immunological phenomena ex vivo through direct quantification of dendritic cells, antigen-specific T cells, and regulatory T cells in humans and chimpanzees has considerably increased our knowledge of HBV pathogenesis. Here, we will discuss these recent findings, reported by us and others, and their potential as a target for future immunotherapeutic strategies in CHB infection.

Targeting the immune response to Hepatitis B virus infection

Innate immunity

Innate immunity generally plays a role immediately after infection to limit the spread of the pathogen and initiate efficient development of an adaptive immune response. Innate host responses during the early phases of viral infections are mainly characterized by the production of type 1 interferon (IFN)- α/ β cytokines and the activation of natural killer (NK) cells. Production of type 1 IFNs can be triggered directly by virus replication through cellular mechanisms that detect the presence of

viral RNA or DNA [7-9], while NK cells are activated by the modulation of the quantity of major histocompatibility complex (MHC)-class I molecules on the surface of infected cells [10]. Experimental data collected, mainly in animal models but also in humans [11], show that after inoculation, HBV does not immediately start to replicate efficiently. HBV-DNA and HBV antigens are not detectable in serum or the liver until 4–7 weeks post-infection [12-14]. Following this period, HBV begins a logarithmic expansion phase and infects most hepatocytes [12, 13 15, 16]. In three experimentally infected chimpanzees, no cellular genes were activated within the liver during the lag phase of infection, and the antiviral cytokines IFNα and –β were not triggered [17]. It has been proposed that because HBV replicates within nucleocapsid particles, viral replicative intermediates of single-stranded RNA or viral DNA, generally strong activators of type I IFN genes [8, 9], are protected from cellular recognition through the Toll-like receptor system [17].
Such early events are difficult to analyse during natural infection in humans. HBV-infected patients are mainly detected after onset of clinical symptoms (nausea and icterus), which occur well after infection (10–12 weeks) [18]. Therefore, this early phase of disease is less suitable for immunotherapeutic interventions aimed at preventing persistent infection.

Initiation of the adaptive immune response

Immediately after the exponential phase of HBV expansion, chimpanzees able to control the virus show a typical acute phase of disease with a robust activation of IFN-γ, TNF-α [13] and many cellular genes linked to a T helper type 1 (Th1) type of cellular response [17]. The experimental data in animal models are consistent with the possibility that the initial burst of IFN-γ and the subsequent rapid inhibition of HBV could be mediated by NK and NK-T cells [13]. Despite these data, the only experimental evidence of NK-cell involvement in human HBV infection are represented by an analysis of NK-cell frequencies in patients studied during the incubation phase of acute hepatitis B. Here, increased numbers of circulating NK cells were concomitant with the peak of HBV replication, 2–4 weeks prior to the occurrence of HBV-specific CD8 T cells [18]. A different pattern is observed when patients or animal models develop chronicity. In humans, development of chronicity is often associated with absent or mild symptoms of acute hepatitis. In line with these clinical observations, neonatally infected woodchucks that develop chronicity lack the large cytokine production by NK-T cells observed in resolved animals [19-21] and fail to develop an efficient antiviral-specific immune response.
Drugs that are able to elicit strong cytokine responses by NK-T cells may therefore be candidates for immunomodulatory therapy in CHB infection. α-Galactosylceramide (α-GalCer), a glycolipid originally extracted from marine sponges, is a ligand of the invariant Vα14 NKT cell and is presented by CD1d molecule on antigen presenting cells (APC) [22]. Animal studies have shown that upon activation with α-GalCer, NKT cells produce a large amount of IFN-γ, which can modulate the immune responses to autoimmune diseases and infections [23, 24]. Ongoing clinical studies should determine whether α-GalCer is an effective immunotherapeutic approach, either as monotherapy

or as vaccine adjuvant, in the treatment of CHB.

The role of CD4 T cells

The adaptive immune response is comprised of a complex web of effector cell types, all of which play key roles in development of immunity to HBV. CD4 T-cells, classically referred to as helper T-cells, are robust producers of cytokines and are required for the efficient development of effector cytotoxic CD8 T-cells and B-cell antibody production. CD8 T-cells go on to clear HBV-infected hepatocytes through cytolytic and non-cytolytic mechanisms [25], reducing the levels of circulating virus, while B-cell antibody production neutralizes free viral particles and can prevent (re)infection [26]. There are clear differences in the adaptive immunity of patients with established chronic or resolved HBV infection. In chapter 5 we showed that significant CD8 T-cell responses are detectable in the blood and liver of subjects who clear acute HBV infection, even after resolution of the disease. Helper T cell and cytotoxic responses in acutely infected patients are quantitatively stronger than those found in patients with chronic infections, who are instead characterized by weaker or undetectable virus-specific T-cell responses [27, 28]. It is likely that CD4 T-cell help induces the maturation of a functionally efficient CD8 T-cell response. Interestingly, we showed in chapter 4 that the proportion of intrahepatic CD4-T cells increases in chronic HBV patients with improved immune control over the virus, emphasizing the important role for this cell type in the anti-viral immune response.

It is therefore a reasonable assumption that immunotherapy able to elicit potent CD4 T-cell responses, may be effective in chronic HBV patients. Indeed, in chronic HBV and chronic hepatitis C infection the efficacy of interferon-alpha therapy in inducing sustained virologic response has been attributed to the efficacy in induction and maintenance of significant T helper cell responses [29, 30]. However, recent attempts to improve response rates using vaccines containing HBV surface antigenic components to stimulate CD4 T-cell reactivity in patients, did not induce cytokine production by these cells or HBV-specific CD8+ T cells [31, 32]. Potentially, new vaccination strategies using the immunodominant HBV core protein as a substrate will prove more effective. We will discuss this further below.

Cytotoxic T-cell response.

Analysis of the HLA-class I-restricted CD8 T-cell response to HBV has been severely hampered by the inability of HBV to be propagated in cell culture [33]. The first definitive characterization of CD8 T cells specific for HBV derived from the understanding that the sequence of the processed viral antigens presented by HLA-class I molecules could be mimicked by synthetic peptides [34]. Thus, cytotoxic T cells specific for several viral epitopes within core [34], envelope [35], polymerase [36] and X [37] proteins of HBV were achieved using synthetic peptides, and not naturally processed epitopes, to expand memory cytotoxic T-lymphocytes (CTL) in vitro. These studies demonstrated that the magnitude of the HBV-specific CD8 response is stronger in self-limited than chronic

infection [34]. In chapter 3 we showed that flow cytometry of a fine-needle-aspiration-biopsy of the liver allows easy, atraumatic and reliable analysis of lymphocytes obtained from the intrahepatic compartment [38], and using this technique we showed in chapter 5 that in resolution of acute HBV infection the quantity of ex vivo derived intrahepatic HBV-specific CD8 T-cells correlated with HBV control and not with liver damage [39]. Others have revealed that an epitope hierarchy exists within the HBV-specific CD8 T-cell responses that can be altered by viral persistence [40]. The great majority of A2+ patients with self-limited hepatitis B recognize the HBc18–27, HBe183–91, HBe335–43 and HBp455–63 epitopes. The cause of immunodominance of these sequences is likely linked to their good binding affinity to the HLA-A2 molecule. A further possible explanation of the dominance of these HLA-A2-restricted CD8 responses is the finding that some HLA-class I epitopes are nested within CD4 T-cell epitopes. CD4-helper T-cells are necessary for the maintenance of functional CD8 T-cells and the covalent linkage between helper and cytotoxic epitopes has been shown to be important for the induction of CTL responses [41]. Amino acid mutations within the often immunodominant, HBc18–27 epitope able to inhibit activation of the core 18–27-specific CD8 T-cells have been shown to occur in patients with chronic hepatitis B [42]. Longitudinal analysis of HLA-A2-restricted HBV-specific CD8 T-cells in resolved and chronic hepatitis B patients have also revealed that the functional fate of epitope specificities differs markedly in chronic infection. Core 18–27-specific CD8 T cells cannot be detected in the circulation when HBV-DNA levels are >10^7 copies/ml. Amongst others, we showed in chapter 4 that the inability to detect core 18–27-specific CD8 T-cells within the circulatory compartment is not due to preferential intrahepatic localization [28]. This state of HBV-specific T-cell tolerance appears to be regulated mainly by the quantity of HBV replication present in chronic hepatitis B patients. The impact of viral load on antiviral T-cell responses has been characterized in animal models of viral infections (like LCMV), which show that sustained presence of viral antigens leads to a progressive functional decline of virus-specific CD8 responses and ultimately leads to virus-specific T-cell deletion [43]. Similarly, in HBV-infected patients, the frequency and function of circulating and intrahepatic HBV-specific CD8 T cells is inversely proportional to the level of HBV-DNA [28]. HBeAg, a secretory form of the nucleocapsid antigen, is produced in large excess during HBV replication. The tolerizing effect of HBeAg has been well characterized in mice [44] [45] and likely contributes to the low level of core-specific T-cell responses present in HBeAg+ chronic patients.

Since vaccination will likely be the least expensive way to treat chronic HBV infection, currently a lot of efforts are being put in studying the use of newly developed DNA vaccines to stimulate the impaired T-cell response in patients with chronic disease. Depending on the viral gene carrier system, DNA vaccines fall into two categories. Viral (or bacterial) recombinant vaccines are genetically engineered non-pathogenic viruses modified by inserting a foreign viral gene, encoding the desired vaccine protein. In contrast, the naked DNA (plasmid) vaccines are vectors encoding viral antigens, which become expressed in the immunized host [46]. The DNA vaccines are

designed to encode viral proteins that are important targets for neutralising antibodies and T-cell responses after *de novo* syntheses of antigen *in vivo* (fig 1). In rodent models for chronic HBV infection, such plasmid vaccines encoding one or several modified HBV envelope proteins have been shown to induce antibody production and CD4 and CD8 T-cell responses that were able to overcome tolerance to HBsAg, despite its relatively high presence in the serum [47-49]. However, recently after intramuscular immunization with a plasmid DNA encoding two envelope proteins, in patients with chronic HBV infection only transient HBV-specific T-cell responses were observed without a sustained virologic effect [50]. Therefore, new approaches are being investigated. Some of the more sophisticated DNA vaccines combine surface proteins with the immunodominant core protein to broaden the antigenic profile and induce potent HBV-specific CD4 and CD8 T-cell responses [51]. Also, co-delivery of cytokines with DNA vaccines may elicit the desired immune response. In animal models intradermal vaccination with a plasmid vaccine encoding a HBV surface antigen showed enhanced T-cell priming when plasmids encoding IFNγ, GM-CSF or IL-15 were co-delivered [52 53]. Others focus on reducing the viral load with conventional antiviral therapy to reduce the impact of high viral load on T-cell responses, prior to administering the vaccines [54]. Many other variables, like the mode of immunization, the schedule of immunization, and the antigenic composition of the vaccine and its adjuvants are currently being evaluated for their role in designing the optimal DNA vaccine for immunotherapy in chronic HBV infection.

Alternatively, in chapters 6 and 7 we have attempted to boost the impaired T-cell responses in patients with chronic HBV infection, using conventional anti-viral therapy [55]. Therapeutic vaccines usually restrict the antigenic profile to a few epitopes thought to be essential . Since immune responses in resolved hepatitis B infections are often directed against several but variable epitopes [36], the best immune stimulus may be the whole virus with a very broad antigenic profile. In *in vivo immunization (IVI)* of CHB patients, following rapid and profound virus suppression by interferon-lamivudine combination therapy, lamivudine was withdrawn intermittently during continued interferon therapy. The rationale being that profound virus suppression will reduce the antigenic pressure on the impaired T-cell response, after which it may effectively respond to the sudden, increased antigen expression during the viral rebound when lamivudine is withdrawn. Although initially *IVI* was able to transiently suppress viral replication in two patients with CHB (chapter 6) [55], in a subsequent pilot study the magnitude of the induced T-cell response was insufficient to cause a sustained virological effect in the majority of patients (chapter 7). However, since only moderate virus suppression was achieved, future attempts to reach immune control over the virus may be more successful when, prior to the lamivudine interruptions, the HBV DNA levels are more profoundly reduced, and when the periods of lamivudine interruption are shortened to limit the viral rebound.

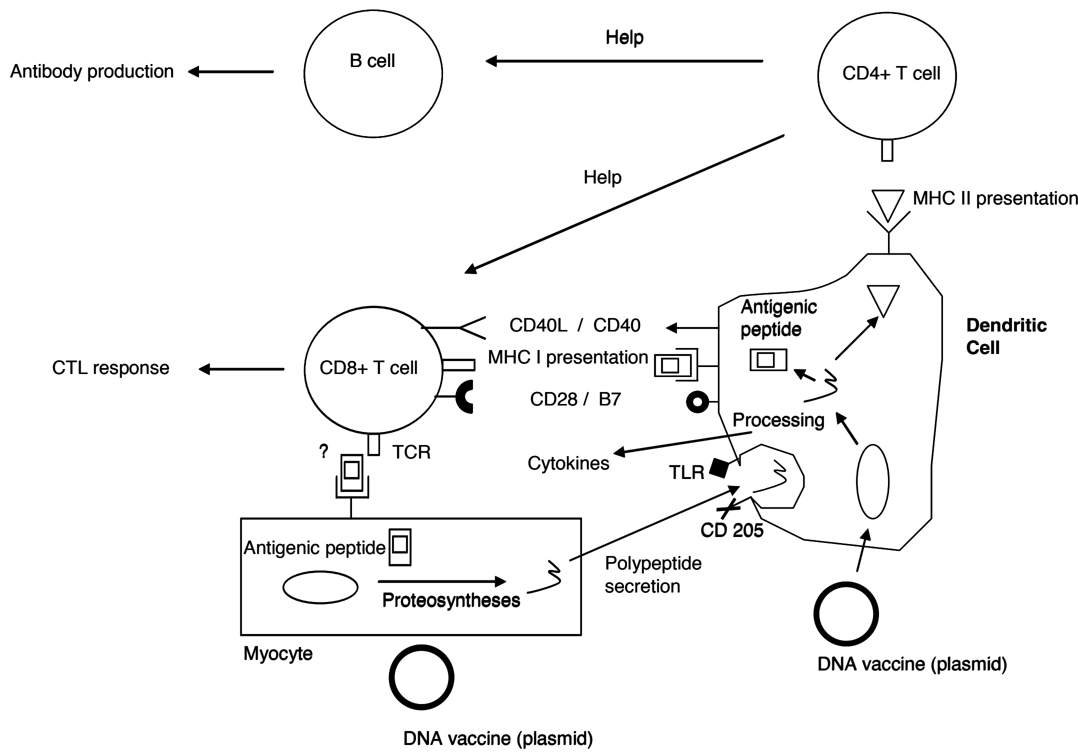


Fig 1. The possible pathways of antigen presentation following DNA-immunization (modified from Rajcani et al [46]).
The antigen synthesized in myocytes (or epidermal cells) is transferred to DC that interact with CD4 T-cells via the major histocompatibility class II (MHC II) molecule. These CD4 T-cells provide help at specific priming of CD8 T-cells and/or B-cells by means of cytokine release. Antigen is being presented by DC to CD8 T-cells via the MHC class I molecule. DC co-stimulate T-cells via the B7 and CD40 receptors that interacts with CD28 and CD40L respectively, both present on the surface of CD8 T-cells. The uptake of immunogenic peptides by DC is mediated by the CD 205 receptor. Cytokine production by DC is activated by means of toll like receptor (TLR)-signalling.

Dendritic cells

Dendritic cells (DC) represent the most potent antigen presenting cells, and thus play an important role in the induction of specific T-cell responses [56]. Functional defects in DC could therefore be an important mechanism of the virus to evade host immune responses [56] and may explain the state of T- and B-cell hypo-responsiveness present in chronic hepatitis B patients. Indeed, in chapter 2 we have shown that two major DC precursors, the myeloid (mDC) and plasmacytoid (pDC) dendritic cells, are functionally impaired in patients with chronic hepatitis B [57]. This might be an important way by which HBV evades an adequate immune response leading to viral persistence

and disease chronicity. Since productive HBV replication in DC has recently been excluded in chronic hepatitis B patients [58], further research should determine the mechanism by which HBV interferes with the DC functions.
After initial encouraging results using a mouse model for chronic HBV infection, recently a dendritic cell based therapeutic vaccine was developed for the treatment of patients with chronic disease [59, 60]. In this approach monocytes are isolated from the blood, then matured and pulsed with HBsAg *in vitro* and finally administered to the patients. This autologous DC-vaccine was able to reduce the viral load and induce HBeAg seroconversion in some patients [60]. No data on the induction of an HBV-specific T-cell response were reported. Therefore, many factors like antigenic composition and the schedule of immunization remain to be optimized, before DC vaccination can be applied as immunotherapy for CHB.

Regulatory T cells

Studies of numerous experimental models have provided evidence that a population of specialized T-cells are able to regulate the immune response. These regulatory T-cells (Treg) reside mainly within a minor population of CD4 cells that express the phenotypic marker CD25. They have been shown to suppress immunological responses against self [61] and foreign antigens [62] through suppressive cytokines or direct cell–cell contact; however, regulatory effects of CD4+ CD25+ cells have not been fully elucidated. It is possible that CD4+ CD25+ T-cells are responsible for the weak HBV-specific T-cell response in chronic hepatitis B patients and may inhibit the expansion and function of HBV-specific CD8 T-cells, precluding HBV clearance but also limiting immune mediated liver damage.
The impact of circulating CD4+ CD25+ T-cells on HBV pathogenesis has recently been analysed. Increased frequencies of circulating and intrahepatic regulatory T-cells in patients with chronic hepatitis B have been shown [63 64]. Depletion of CD4+ CD25+ cells increased the function of HBV-specific T-cells [63, 65]. Combined, these data suggest that accumulation of Treg inside the liver may contribute to inhibition of the local HBV-specific T-cell response. Furthermore, it is possible that a population of HBV-specific regulatory cells, different from the CD4+ CD25+ T-cell subset, analogous to the presence of IL-10 producing HCV-specific T-cells [66], might be induced in chronic HBV infection [67].
In chapter 8 we have shown that non-response to interferon-alpha therapy may be related to the induction of Treg during treatment. This opens up the possibility that response rates to IFNα may improve when Treg function is suppressed. In cancer, strategies that inhibit or deplete Tregs and boost anti-tumour immunity are under investigation. In mice, the removal of CD4+ CD25+ T-cells with anti-CD25 depleting antibodies, in combination with anti-CTLA-4 antibodies, led to tumour rejection [68]. However, a cautionary note comes from studies using CTLA-4 blockade along with tumour-specific peptide vaccinations in human melanoma patients, which resulted in tumour regression in some patients but also significant development of autoimmunity in six of 14 patients, thus emphasizing the delicate balance between tolerance and

immunity [69]. At present, no depleting anti-CD25 antibodies are licensed for use in humans, but eventually these findings may lead to new immunotherapeutic strategies that can also be used in the treatment of chronic HBV infection.

Concluding remarks

Even though virus-specific CD8 T-cells play a major role in HBV clearance [12], coordinated activation of the different branches of adaptive immunity seems necessary to achieve viral control. When chronicity develops, diffuse defects of helper and cytotoxic T-cell responses are apparent and are likely to be maintained by the concerted action of high levels of viral antigens, the peculiar immunological features of the liver and by the contribution of regulatory cells or dendritic cell defects. Functional restoration of the altered HBV-specific immunity during chronic infection, using newly developed immunotherapeutic strategies will therefore be a very complex process. Therapeutic DNA-vaccination may have to be combined with conventional therapy, cytokines, or the use of dendritic cells to improve the chances of success.

Up to now all preclinical and clinical studies reported here, have been invariably very safe and well tolerated. There have been no life threatening adverse events, there has been no detectable organ pathology or systemic toxicity, and there has been no autoimmunity, appearance of anti-nuclear antibodies or antibodies against double-stranded DNA [70]. Furthermore, the most important risk of DNA-vaccination, namely chromosomal integration and oncogenesis has so far only been theoretical [70]. However, it remains of great importance to carefully evaluate the long term effects of new immunotherapeutic strategies, before they are used to achieve control over chronic HBV infection.

References

1. Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. Annu Rev Immunol 1995; 13:29-60.
2. Benhamou Y, Dohin E, Lunel-Fabiani F, et al. Efficacy of lamivudine on replication of hepatitis B virus in HIV-infected patients. Lancet 1995; 345:396-7.
3. Lai CL, Chien RN, Leung NW, et al. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med 1998; 339:61-8.
4. Angus P, Vaughan R, Xiong S, et al. Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. Gastroenterology 2003; 125:292-7.
5. van Zonneveld M, Honkoop P, Hansen BE, et al. Long-term follow-up of alpha-interferon treatment of patients with chronic hepatitis B. Hepatology 2004; 39:804-10.
6. Janssen HL, van Zonneveld M, Senturk H, et al. Pegylated interferon alfa-2b alone or in combination with lamivudine for HBeAg-positive chronic hepatitis B: a randomised trial. Lancet 2005; 365:123-9.
7. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001; 413:732-8.
8. Lund J, Sato A, Akira S, Medzhitov R, Iwasaki A. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. J Exp Med 2003; 198:513-20.
9. Heil F, Hemmi H, Hochrein H, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 2004; 303:1526-9.
10. Moretta L, Bottino C, Pende D, Vitale M, Mingari MC, Moretta A. Human natural killer cells: Molecular mechanisms controlling NK cell activation and tumor cell lysis. Immunol Lett 2005; 100:7-13.
11. Fong TL, Di Bisceglie AM, Biswas R, et al. High levels of viral replication during acute hepatitis B infection predict progression to chronicity. J Med Virol 1994; 43:155-8.
12. Thimme R, Wieland S, Steiger C, et al. CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. J Virol 2003; 77:68-76.
13. 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.
14. Berquist KR, Peterson JM, Murphy BL, Ebert JW, Maynard JE, Purcell RH. Hepatitis B antigens in serum and liver of chimpanzees acutely infected with hepatitis B virus. Infect Immun 1975; 12:602-5.
15. Kajino K, Jilbert AR, Saputelli J, Aldrich CE, Cullen J, Mason WS. Woodchuck hepatitis virus infections: very rapid recovery after a prolonged viremia and infection of virtually every hepatocyte. J Virol 1994; 68: 5792-803.
16. Jilbert AR, Wu TT, England JM, et al. Rapid resolution of duck hepatitis B virus infections occurs after massive hepatocellular involvement. J Virol 1992; 66:1377-88.
17. Wieland S, Thimme R, Purcell RH, Chisari FV. Genomic analysis of the host response to hepatitis B virus infection. Proc Natl Acad Sci U S A 2004; 101:6669-74.
18. Webster GJ, Reignat S, Maini MK, et al. Incubation phase of acute hepatitis B in man: dynamic of cellular immune mechanisms. Hepatology 2000; 32:1117-24.
19. Cote PJ, Toshkov I, Bellezza C, et al. Temporal pathogenesis of experimental neonatal woodchuck hepatitis virus infection: increased initial viral load and decreased severity of acute hepatitis during the development of chronic viral infection. Hepatology 2000; 32:807-17.
20. Nakamura I, Nupp JT, Cowlen M, et al. Pathogenesis of experimental neonatal woodchuck hepatitis virus infection: chronicity as an outcome of infection is associated with a diminished acute hepatitis that is temporally deficient for the expression of interferon gamma and tumor necrosis factor-alpha messenger RNAs. Hepatology 2001; 33:439-47.
21. Menne S, Roneker CA, Roggendorf M, Gerin JL, Cote PJ, Tennant BC. Deficiencies in the acute-phase cell-mediated immune response to viral antigens are associated with development of chronic woodchuck hepatitis virus infection following neonatal inoculation. J Virol 2002; 76:1769-80.
22. Chen H, Paul WE. Cultured NK1.1+ CD4+ T cells produce large amounts of IL-4 and IFN-gamma upon activation by anti-CD3 or CD1. J Immunol 1997; 159:2240-9.
23. Gonzalez-Aseguinolaza G, Van Kaer L, Bergmann CC, et al. Natural killer T cell ligand alpha-galactosylceramide enhances protective immunity induced by malaria vaccines. J Exp Med 2002; 195: 617-24.
24. Sharif S, Arreaza GA, Zucker P, et al. Activation of natural killer T cells by alpha-galactosylceramide treatment prevents the onset and recurrence of autoimmune Type 1 diabetes. Nat Med 2001; 7:1057-62.
25. Guidotti LG, Chisari FV. To kill or to cure: options in host defense against viral infection. Curr Opin Immunol 1996; 8:478-83.
26. Grady GF, Lee VA, Prince AM, et al. Hepatitis B immune globulin for accidental exposures among medical personnel: final report of a multicenter controlled trial. J Infect Dis 1978; 138:625-38.
27. Maini MK, Boni C, Ogg GS, et al. Direct ex vivo analysis of hepatitis B virus-specific CD8(+) T cells

associated with the control of infection. Gastroenterology 1999; 117:1386-96.
28. Webster GJ, Reignat S, Brown D, et al. Longitudinal analysis of CD8+ T cells specific for structural and nonstructural hepatitis B virus proteins in patients with chronic hepatitis B: implications for immunotherapy. J Virol 2004; 78:5707-19.
29. Kamal SM, Fehr J, Roesler B, Peters T, Rasenack JW. Peginterferon alone or with ribavirin enhances HCV-specific CD4 T-helper 1 responses in patients with chronic hepatitis C. Gastroenterology 2002; 123: 1070-83.
30. 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.
31. Michel ML, Mancini-Bourgine M. Therapeutic vaccination against chronic hepatitis B virus infection. J Clin Virol 2005; 34 Suppl 1:S108-14.
32. Jung MC, Gruner N, Zachoval R, et al. Immunological monitoring during therapeutic vaccination as a prerequisite for the design of new effective therapies: induction of a vaccine-specific CD4+ T-cell proliferative response in chronic hepatitis B carriers. Vaccine 2002; 20:3598-612.
33. Chisari FV, Ferrari C. Hepatitis B virus immunopathology. Springer Semin Immunopathol 1995; 17:261-81.
34. Bertoletti A, Ferrari C, Fiaccadori F, et al. HLA class I-restricted human cytotoxic T cells recognize endogenously synthesized hepatitis B virus nucleocapsid antigen. Proc Natl Acad Sci U S A 1991; 88: 10445-9.
35. Nayersina R, Fowler P, Guilhot S, et al. HLA A2 restricted cytotoxic T lymphocyte responses to multiple hepatitis B surface antigen epitopes during hepatitis B virus infection. J Immunol 1993; 150:4659-71.
36. Rehermann B, Fowler P, Sidney J, et al. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. J Exp Med 1995; 181:1047-58.
37. Hwang YK, Kim NK, Park JM, et al. HLA-A2 1 restricted peptides from the HBx antigen induce specific CTL responses in vitro and in vivo. Vaccine 2002; 20:3770-7.
38. Sprengers D, van der Molen RG, Kusters JG, et al. Flow cytometry of fine-needle-aspiration biopsies: a new method to monitor the intrahepatic immunological environment in chronic viral hepatitis. J Viral Hepat 2005; 12:507-12.
39. Sprengers D, Molen RG, Kusters JG, et al. Analysis of intrahepatic HBV-specific cytotoxic T-cells during and after acute HBV infection in humans. J Hepatol 2006.
40. Maini MK, Boni C, Lee CK, et al. The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. J Exp Med 2000; 191:1269-80.
41. Kalams SA, Walker BD. The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. J Exp Med 1998; 188:2199-204.
42. Bertoletti A, Sette A, Chisari FV, et al. Natural variants of cytotoxic epitopes are T-cell receptor antagonists for antiviral cytotoxic T cells. Nature 1994; 369:407-10.
43. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. J Virol 2003; 77:4911-27.
44. Milich DR, Jones JE, Hughes JL, Price J, Raney AK, McLachlan A. Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance in utero? Proc Natl Acad Sci U S A 1990; 87:6599-603.
45. Chen M, Sallberg M, Hughes J, et al. Immune tolerance split between hepatitis B virus precore and core proteins. J Virol 2005; 79:3016-27.
46. Rajcani J, Mosko T, Rezuchova I. Current developments in viral DNA vaccines: shall they solve the unsolved? Rev Med Virol 2005; 15:303-25.
47. Mancini M, Hadchouel M, Davis HL, Whalen RG, Tiollais P, Michel ML. DNA-mediated immunization in a transgenic mouse model of the hepatitis B surface antigen chronic carrier state. Proc Natl Acad Sci U S A 1996; 93:12496-501.
48. Mancini M, Hadchouel M, Tiollais P, Michel ML. Regulation of hepatitis B virus mRNA expression in a hepatitis B surface antigen transgenic mouse model by IFN-gamma-secreting T cells after DNA-based immunization. J Immunol 1998; 161:5564-70.
49. Lu M, Hilken G, Kruppenbacher J, et al. Immunization of woodchucks with plasmids expressing woodchuck hepatitis virus (WHV) core antigen and surface antigen suppresses WHV infection. J Virol 1999; 73:281-9.
50. Mancini-Bourgine M, Fontaine H, Scott-Algara D, Pol S, Brechot C, Michel ML. Induction or expansion of T-cell responses by a hepatitis B DNA vaccine administered to chronic HBV carriers. Hepatology 2004; 40: 874-82.
51. Thermet A, Robaczewska M, Rollier C, et al. Identification of antigenic regions of duck hepatitis B virus

core protein with antibodies elicited by DNA immunization and chronic infection. J Virol 2004; 78:1945-53.
52. Du DW, Jia ZS, Li GY, Zhou YY. HBV DNA vaccine with adjuvant cytokines induced specific immune responses against HBV infection. World J Gastroenterol 2003; 9:108-11.
53. Kwissa M, Lindblad EB, Schirmbeck R, Reimann J. Codelivery of a DNA vaccine and a protein vaccine with aluminum phosphate stimulates a potent and multivalent immune response. J Mol Med 2003; 81: 502-10.
54. Horiike N, Fazle Akbar SM, Michitaka K, et al. In vivo immunization by vaccine therapy following virus suppression by lamivudine: a novel approach for treating patients with chronic hepatitis B. J Clin Virol 2005; 32:156-61.
55. Sprengers D, Janssen HL, Kwekkeboom J, Niesters HG, de Man RA, Schalm SW. In vivo immunization following virus suppression: a novel approach for inducing immune control in chronic hepatitis B. J Viral Hepat 2003; 10:7-9.
56. Banchereau J, Briere F, Caux C, et al. Immunobiology of dendritic cells. Annu Rev Immunol 2000; 18:767-811.
57. van der Molen RG, Sprengers D, Binda RS, et al. Functional impairment of myeloid and plasmacytoid dendritic cells of patients with chronic hepatitis B. Hepatology 2004; 40:738-46.
58. Untergasser A, Zedler U, Langenkamp A, et al. Dendritic cells take up viral antigens but do not support the early steps of hepatitis B virus infection. Hepatology 2006; 43:539-47.
59. Fazle Akbar SM, Furukawa S, Onji M, et al. Safety and efficacy of hepatitis B surface antigen-pulsed dendritic cells in human volunteers. Hepatol Res 2004; 29:136-141.
60. Chen M, Li YG, Zhang DZ, et al. Therapeutic effect of autologous dendritic cell vaccine on patients with chronic hepatitis B: a clinical study. World J Gastroenterol 2005; 11:1806-8.
61. Sakaguchi S. Regulatory T cells: key controllers of immunologic self-tolerance. Cell 2000; 101:455-8.
62. Suvas S, Kumaraguru U, Pack CD, Lee S, Rouse BT. CD4+CD25+ T cells regulate virus-specific primary and memory CD8+ T cell responses. J Exp Med 2003; 198:889-901.
63. Stoop JN, van der Molen RG, Baan CC, et al. Regulatory T cells contribute to the impaired immune response in patients with chronic hepatitis B virus infection. Hepatology 2005; 41:771-8.
64. Xu D, Fu J, Jin L, et al. Circulating and Liver Resident CD4+CD25+ Regulatory T Cells Actively Influence the Antiviral Immune Response and Disease Progression in Patients with Hepatitis B. J Immunol 2006; 177:739-47.
65. Franzese O, Kennedy PT, Gehring AJ, et al. Modulation of the CD8+-T-cell response by CD4+ CD25+ regulatory T cells in patients with hepatitis B virus infection. J Virol 2005; 79:3322-8.
66. Accapezzato D, Francavilla V, Paroli M, et al. Hepatic expansion of a virus-specific regulatory CD8(+) T cell population in chronic hepatitis C virus infection. J Clin Invest 2004; 113:963-72.
67. Hyodo N, Nakamura I, Imawari M. Hepatitis B core antigen stimulates interleukin-10 secretion by both T cells and monocytes from peripheral blood of patients with chronic hepatitis B virus infection. Clin Exp Immunol 2004; 135:462-6.
68. Sutmuller RP, van Duivenvoorde LM, van Elsas A, et al. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. J Exp Med 2001; 194:823-32.
69. Phan GQ, Yang JC, Sherry RM, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proc Natl Acad Sci U S A 2003; 100:8372-7.
70. Hanke T. On DNA vaccines and prolonged expression of immunogens. Eur J Immunol 2006; 36:806-9.

SUMMARY

SAMENVATTING

DANKWOORD

CURRICULUM VITAE

Summary

Despite the presence of an effective vaccine since 1982, chronic hepatitis B virus infection (CHB) still ranks among the highest causes of mortality from infectious diseases worldwide. The studies presented in this thesis were performed to get a better insight into the anti-viral immune response after hepatitis B virus (HBV) infection, and to identify factors in this immune response that contribute to persistent disease. These factors may function as targets for new immunomodulatory therapies that improve the low response rates to current anti-viral treatment options.
In the introduction to the thesis (chapter 1) we have described that a vigorous, polyclonal and multispecific cytotoxic (CTL) and helper T (Th) cell response to HBV is readily detectable in the peripheral blood of patients with acute self-limited hepatitis B, but is weak, antigenically restricted or undetectable in patients with chronic HBV infection. We reviewed reported data on immunotherapeutic strategies to improve this inefficient immune response, and concluded that at present the only evident effective immunomodulatory therapy is (pegylated) interferon alpha (IFN-α), which leads to sustained response in only 30-40% of treated patients.
Dendritic cells (DC) play an important role in the induction of anti-viral immune responses. In chapter 2 we compared the number, phenotype and function of two important blood precursor DC, myeloid DC (mDC) and plasmacytoid DC (pDC), of chronic hepatitis B patients with healthy volunteers. No differences in percentages of mDC and pDC in peripheral blood mononuclear cells were observed between chronic hepatitis B patients and healthy controls. However, the allostimulatory capacity of mDC, was significantly decreased in patients compared to controls. In addition, mDC of patients showed a reduced expression of co-stimulatory molecules CD80 and CD86, and reduced capacity to produce tumor necrosis factor-α. Purified pDC from patients produced less interferon-α, an important antiviral cytokine, than pDC isolated from controls. Therefore, mDC and pDC are functionally impaired in patients with chronic hepatitis B and this might be an important mechanism by which HBV evades an adequate immune response, leading to viral persistence and disease chronicity.
Information about character and grade of the intrahepatic immune response in viral hepatitis is important for evaluation of disease stage and effect of therapy. Complications like haemorrhage provide a limitation to frequently performing standard tissue needle biopsies (TB), and have made many investigators use peripheral blood as surrogate marker instead, despite the knowledge that resident intrahepatic populations of lymphocytes differ significantly from populations in the circulation. Fine-needle-aspiration-biopsy (FNAB) has proved to be an easy and atraumatic method for the diagnosis of acute rejection in kidney- and liver-transplant recipients, and patients with focal liver lesions. In chapter 3 we showed that flow cytometry of fine-needle-aspiration-biopsy of the liver allows reliable analysis of lymphocytes obtained from the intrahepatic compartment, in patients with viral hepatitis. Therefore, the FNAB is a valuable tool that enables us to study in more detail the immunological events at the site of HBV replication.

Based upon biochemical and virological parameters several potentially successive phases of chronic HBV have been well described. In the immune-tolerance phase serum hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) are detectable; serum HBV-DNA levels are high and serum aminotransferases are normal or minimally elevated. In the immune-clearance phase, serum HBV-DNA levels decrease and serum aminotransferase levels increase. A third phase, termed the inactive carrier state is characterized by low levels of HBV-DNA and normal concentrations of aminotransferases. In chapter 4 we apply the FNAB-procedure to characterize the different phases of chronic HBV infection by analysing the composition of key populations of immune effector cells, i.e. (HBV-specific) CD8+ T-cells, CD4+ T-cells and CD56+ Natural Killer (NK) cells in the liver. The findings suggest that the clinical phases of chronic HBV infection, have characteristic intrahepatic immune responses. In immune-tolerant patients NK cells are the dominant immune effector cell population in the liver, and in the immune-clearance phase the patients with low viral load had a higher proportion of intrahepatic CD4+ T-cells, than patients with high viral load. CD4+ T-cells may therefore be important in achieving immune control over the virus.
The FNAB-procedure was also used in Chapter 5. In this study we aimed to characterize the virus-specific CD8+ T-cell response in the liver of patients with acute HBV infection. During resolution of acute HBV infection in HLA-A2 positive patients tetramers were used to identify HBV-specific CD8+ T-cells in FNAB-cytology. At first presentation there was a correlation between the frequency of intrahepatic non-specific CD8+ T-cells and the degree of liver damage. During and after the disease there was sequestering of HBV-specific CD8+ T-cells in the liver, and the percentage of intrahepatic HLA-DR expressing HBV-specific CD8+ T-cells was higher than in PB. Three months after HBsAg-seroconversion the frequency of intrahepatic HBV-specific CD8+ T-cells remained high. Together with the finding in chapter 4, that low frequencies of intrahepatic HBV-specific CD8+ T-cells were also found in some immune-clearance-patients with low viral load, these data suggest that intrahepatic HBV-specific CD8+ T-cells play an important role in clearing the virus.
In chapters 6 and 7 we have attempted to boost the impaired T-cell responses in patients with chronic HBV infection, using conventional anti-viral therapy. In *in vivo immunization (IVI)* of CHB patients, following rapid virus suppression by interferon-lamivudine combination therapy, lamivudine was withdrawn intermittently during continued interferon therapy. The rationale being that profound virus suppression will reduce the antigenic pressure on the impaired T-cell response, after which it may effectively respond to the sudden, increased antigen expression during the viral rebound when lamivudine is withdrawn. Although initially *IVI* was able to transiently suppress viral replication in two patients with CHB (chapter 6), in a subsequent pilot study the magnitude of the induced T-cell response was insufficient to cause a sustained virological effect in the majority of patients (chapter 7).
Little is known about why treatment with IFNα leads to a response in only a minority of patients with chronic HBV. It was recently shown that in these patients CD4+

CD25+ regulatory T-cells (Treg) can suppress the HBV-specific immune response. In chapter 8 we aimed to investigate whether in non-responders to IFNα therapy Treg contribute to treatment failure by down-regulating the HBV-specific T-cell responses. Fourteen patients received pegylated IFNα monotherapy for 52 weeks and were followed-up for 26 weeks. Whereas there was no difference in the frequencies of CD4+ CD25+ Treg between responders and non-responders at the start of treatment, in non-responders and not in responders there was a significant increase in the frequency of these cells during treatment. In contrast to the responders, the non-responders showed a significant increase in frequency of IL-10 producing cells. Treg depletion resulted in increased proliferation capacity and increased frequencies of HBV-specific INFγ-producing cells, but did not affect the frequency of IL-10 producing cells measured during the course of the treatment. This study indicates that there may be an important role for CD4+ CD25+ Treg and IL-10 secreting regulatory cells, in HBV-persistence during and after Peg-IFNα therapy.
In chapter 9 we discuss the recent insights into the pathogenesis of chronic HBV infection, reported by us and others, and their potential as a target for future immunotherapeutic strategies.

Samenvatting

Ondanks het feit dat er sinds 1982 een effectief vaccin bestaat, blijft chronische hepatitis B virus infectie (CHB) wereldwijd een belangrijke oorzaak voor sterfte. De studies in dit proefschrift werden uitgevoerd om een beter inzicht te verkrijgen in de antivirale immuunrespons na infectie met het hepatitis B virus (HBV), en om factoren te identificeren die bijdragen aan het persisteren van de virale leverziekte. Aangezien de beschikbare antivirale medicijnen slechts in beperkte mate effectief zijn, zouden deze factoren als doel kunnen dienen voor toekomstige antivirale middelen met een beter therapeutisch effect.

In de introductie van dit proefschrift (hoofdstuk 1) wordt beschreven dat de afweerreactie in het bloed van mensen die herstellen van een acute HBV infectie wordt gekenmerkt door een hevige, policlonale en multispecifieke cytotoxische T-cel (CTL) en helper T-cel respons. In tegenstelling hiermee, wordt bij patiënten met CHB slechts een beperkte, en soms zelfs helemaal geen T-cel respons aangetoond. Tevens wordt er in dit hoofdstuk gesproken over de verschillende therapeutische strategieën waarmee in het verleden is getracht om deze zwakke immuunrespons te stimuleren. Uit de gegevens blijkt dat momenteel interferon alfa (IFN-α) de meest effectieve immuunmodulatoire therapie is, met een succesvolle klaring van het virus bij 30 a 40% van de behandelde populatie.

Dendritische cellen (DC) zijn belangrijk voor de inductie van de antivirale afweerreactie. In hoofdstuk 2 vergelijken we de aantallen, het fenotype en de functie van twee typen precursor DC, myeloide DC (mDC) en plasmacytoide DC (pDC), uit het bloed van patiënten met CHB met die van gezonde vrijwilligers. De percentages mDC en pDC waren niet verschillend, maar de allostimulatoire capaciteit van mDC was significant verlaagd bij patiënten ten opzichte van gezonde vrijwilligers. Tevens bleek dat bij patiënten de expressie van de co-stimulatoire moleculen CD80 en CD86 op mDC was afgenomen, en dat deze cellen minder goed in staat waren om tumor necrosis factor alfa te produceren. Plasmacytoide DC van patiënten produceerden minder interferon alfa, een belangrijke antivirale cytokine. Derhalve is bij patiënten met chronische HBV infectie de functie van mDC en pDC afgenomen, en dit zou kunnen bijdragen aan de beperkte afweerreactie en het persisteren van de infectie bij patiënten met chronische HBV.

Informatie over de aard van de afweerreactie in de lever is belangrijk voor een goede evaluatie van het stadium van de virale leverziekte en het effect van behandeling. Tot op heden werd frequente diagnostiek van de lever beperkt door de complicaties die gepaard kunnen gaan met het uitvoeren van een standaard leverbiopsie, waarbij vooral gedacht moet worden aan bloedingen. Veel onderzoekers maken daarom gebruik van cellen uit het bloed, ondanks de wetenschap dat de samenstelling van afweercellen in de lever enorm verschilt van die in de circulatie. De dunne-naald biopsie (DNB) is een eenvoudige en weinig invasieve procedure, die al sinds enige tijd wordt gebruikt voor het diagnosticeren van acute afstoting na een nier- en levertransplantatie, en bij patiënten met focale leverafwijkingen. In hoofdstuk 3 wordt besproken dat de DNB

eveneens geschikt is om bij patiënten met virale hepatitis afweercellen uit de lever te isoleren, en tonen we aan dat deze cellen vervolgens betrouwbaar geanalyseerd kunnen worden met behulp van flow-cytometrie. Derhalve wordt het mogelijk om via een weinig invasieve methode celmateriaal uit de lever te verkrijgen voor onderzoek naar de immuunrespons op de plaats van virale replicatie.

Op basis van biochemische en virologische parameters wordt er onderscheid gemaakt tussen 3 potentieel opeenvolgende fasen van CHB. In de immuun-tolerante fase zijn het hepatitis B surface antigeen (HBsAg) en het hepatitis B e antigeen (HBeAg) detecteerbaar in het serum, is het serum niveau van HBV-DNA hoog, en zijn de aminotransferasen in het serum van patiënten normaal of slechts minimaal verhoogd. In de immuun-klarings fase neemt het niveau van HBV-DNA af en stijgt het niveau van de aminotransferasen in het serum. De derde fase, ook wel het inactieve dragerschap genoemd, wordt gekenmerkt door een laag niveau van het HBV-DNA en normale waarden van de serum aminotransferasen. In hoofdstuk 4 wordt de dunne-naald biopsie gebruikt om bovengenoemde fasen van HBV infectie te karakteriseren met betrekking tot de samenstelling van de afweercellen die zich bevinden in de lever. Daarbij wordt met name gekeken naar de verdeling van CD4 positieve (CD4+)- en CD8 positieve (CD8+) T-cellen en CD56 positieve Natural killer (NK)-cellen, aangezien van deze celpopulaties bekend is dat ze een belangrijke rol spelen in de pathogenese van CHB infectie. De bevindingen in de studie suggereren dat de verschillende fasen van de aandoening, gepaard gaan met een karakteristieke intrahepatische immuunrespons. In de immuun-tolerante fase zijn de NK-cellen de dominante populatie afweercellen in de lever. Patiënten in de immuun-klarings fase en met lage virus titers, blijken vooral een hoge frequentie aan CD4+ T-cellen in de lever te hebben in vergelijking met patiënten uit deze fase en met een hoog HBV-DNA. De CD4+ T-cellen zijn derhalve mogelijk belangrijk voor immuun-controle over het virus. Lage frequenties HBV-specifieke CD8+ T-cellen werden aangetoond bij enkele patiënten met een laag HBV-DNA

In hoofdstuk 5 wordt de dunne-naald biopsie toegepast voor een gedetailleerde analyse van de virus-specifieke CD8+ T-celrespons in de lever van patiënten tijdens en na een acute HBV-infectie. Op het moment van eerste presentatie van de patiënt in de kliniek bleek dat er een correlatie is tussen de mate van leverschade, gemeten aan de hand van serum aminotransferasen, en de frequentie non-specifieke CD8+ T-cellen in de lever. Gedurende de infectie en erna waren er hoge frequenties HBV-specifieke CD8+ T-cellen in de lever, en een groot deel van deze cellen bracht HLA-DR tot expressie, en was dus geactiveerd. Zelfs drie maanden na HBsAg-seroconversie bleef de frequentie HBV-specifieke CD8+ T-cellen hoog. Deze bevindingen suggereren dat HBV-specifieke CD8+ T-cellen in de lever een belangrijke rol spelen bij de klaring van het virus.

In de hoofdstukken 6 en 7 hebben we getracht om de beperkte afweerreactie bij patiënten met chronische HBV infectie te stimuleren. Bij *in vivo immunization (IVI)* van patiënten met CHB wordt de virus titer verlaagd met een combinatiebehandeling van lamivudine en interferon alfa, waarna lamivudine intermitterend wordt onderbroken

tijdens continue interferon therapie. Er wordt verondersteld dat onderdrukking van virale replicatie resulteert in een afname van de hoeveelheid virale antigenen met een herstel van de geremde T-celrespons tot gevolg. Nadat aanvankelijk bij twee patiënten een tijdelijke immuuncontrole over het virus werd geobserveerd (hoofdstuk 6), bleek in een daaropvolgende grotere studie dat de geïnduceerde T-celrespons onvoldoende was om een langdurig effect te bewerkstelligen, bij de meerderheid van de onderzochte patiënten (hoofdstuk 7).

Het is onbekend waarom IFN-α therapie slechts bij een minderheid van de behandelde patiënten resulteert in genezing. Recent is aangetoond dat bij patiënten met CHB CD4+ CD25+ regulatoire T-cellen (Treg) in staat zijn om de antivirale immuunrespons te remmen. In hoofdstuk 8 van dit proefschrift wordt onderzocht of deze cellen bijdragen aan het falen van IFN-α therapie. Veertien patiënten werden gedurende 52 weken behandeld met gepegyleerde IFN-α monotherapie 52 weken, waarna ze nog eens 26 weken werden gevolgd. Voorafgaande aan de behandeling waren er geen verschillen tussen de mensen die genazen (responders) en de mensen die niet goed reageerden op behandeling (non-responders) met betrekking tot de frequenties CD4+ CD25+ Treg. Echter, gedurende de therapie bleek de frequentie Treg in het bloed van non-responders toe te nemen, terwijl deze sterk afnam bij de responders. Bovendien bleek dat er bij de non-responders een toename was van het aantal IL-10 producerende CD4+ T-cellen. Wanneer de Treg werden weggenomen bleek dat er een toename was van HBV-specifieke proliferatie en interferon-γ productie door T-cellen, terwijl het aantal IL-10 producerende CD4+ T-cellen onveranderd bleef. Deze bevindingen indiceren dat CD4+ CD25+ T-cellen en IL-10 producerende T-cellen mogelijk een belangrijke rol spelen bij het persisteren van chronische HBV infectie tijdens behandeling met IFN-α.

In hoofdstuk 9 bediscussiëren we de bevindingen van bovengenoemde studies in de context van gepubliceerde data van andere onderzoeksgroepen, en we bespreken de potentiële aangrijpingspunten voor toekomstige, nieuwe antivirale behandelingen voor chronische HBV infectie.

Dankwoord

Dit proefschrift is tot stand gekomen met de steun van velen, die ik op deze plaats wil bedanken voor hun inspanningen.

Vooral wil ik Harry Janssen bedanken voor zijn voortdurende gedrevenheid en zijn vermogen om mijn, bij tijd en wijlen, onstuimige energie in goede banen te leiden en te houden. Harry, je gaf me de ruimte om mijn ideeen te verwoorden en, indien niet te exotisch, uit te werken, zonder daarbij het einddoel uit het oog te verliezen. Dat gaf mij het vertrouwen en het enthousiasme om in de wijde wereld van het onderzoek mijn persoonlijke grenzen te verleggen, en van die ervaring maak ik ongetwijfeld ook na mijn promotie nog dankbaar gebruik.
Meerdere mensen hebben mij gedurende de promotie in meer of mindere mate begeleid. Natuurlijk Renate van der Molen…denk ik aan mijn tijd op het lab, dan denk ik aan jou. Hartstikke bedankt voor je gezelligheid, binnen en buiten de werkomgeving, je daadkracht en je gepassioneerde manier van managen. Hans Kusters wil ik bedanken voor zijn vertrouwen en de ruimte die ik ervaarde om te "experimenteren", maar met name ook voor de vlijm scherpe (en soms zelfs pijnlijke) kritieken. Jaap Kwekkeboom en Luc van der Laan wil ik graag bedanken voor de leerzame discussies en de zeer bruikbare adviezen. Professor Schalm was immer inspirerend en zal dat vermoedelijk ook altijd blijven, zoals de rust en de weloverwogen raad van Rob de Man altijd verhelderend zal zijn. Heren bedankt.
Een aanzienlijk deel van de in het proefschrift beschreven werk is uitgevoerd met de hulp van enkele mensen die ik daarvoor speciaal wil bedanken, te weten Rekha Binda. Patrick Boor, Paula Biesta, Jeroen Stoop, Shanta Mancham en last but not least Alice Kok. Dames en heren het was een groot plezier om met jullie te werken.
De grote hoeveelheid patiëntenmateriaal werd grotendeels verzameld door de dames van "het research assistenten team". Ik wil daarom Cockie, Heleen en Anneke graag bedanken voor de gezellige en vruchtbare samenwerking. De onregelmatige en soms verrassende polibezoeken van de patiënten zouden niet in goede orde zijn afgerond zonder de prettige en flexibele medewerking van de mensen op de poli MDL. Daarvoor ben ik Esther, Esther, Ronald, Minou, Ellen, Laksmie, Wilma en met name Nermin dan ook zeer dankbaar.
Wanneer een of ander administratief probleem mij weer eens tot wanhoop had gedreven, dan waren Marion en Margriet van het secretariaat Hepatologie altijd beschikbaar voor advies en hulp. Daarom mijn dank voor jullie begrip, en speciaal ook Marion bedankt voor je assistentie bij het organiseren van dit proefschrift.
De klinische studies werden ondermeer begeleid door de mensen van het Clinical Trial Bureau. Met name Elke wil ik daarom bedanken voor de prettige samenwerking.

Terwijl hier in Boston het regelmatige gekletter van de regen en het galmen van de sirenes van de onophoudelijk uitrukkende brandweerwagens en ambulances mij doet wegdromen, denk ik aan nog zo vele anderen die mijn promotietijd tot een

onvergetelijke hebben gemaakt …zoals Leon, met wie ik gelukkig nog steeds een nimmer eindigend gesprek voer over onze werkzaamheden en de rest van het leven, Thjon, Rudi en Dorine… die onze kamer op het lab tot een waar “thuis” maakten, Arnoud…die op geheel eigen wijze kritiek afwisselde met gezelligheid, Linda met wie je gezellig kon babbelen…en natuurlijk Pauline, op de werkvloer ontmoet, en sindsdien mijn allerliefste experiment.

Kortom, het was een erg prettige werksfeer waarvoor ik nog vele andere mensen ook erg dankbaar ben, en die er mede toe heeft geleid dat ik mijn “promotieproces” voorspoedig heb kunnen afronden.

Dave Sprengers.

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 1 november 1974 te Goirle. Hij groeide op in Tilburg alwaar hij aan het Theresia Lyceum het VWO voltooide. Vervolgens vertrok hij naar België om aan het Rijksuniversitair Centrum Antwerpen geneeskunde te studeren. Na het eerste kandidatuur verhuisde hij in 1994 naar Nijmegen voor het vervolg van deze studie aan de Katholieke Universiteit Nijmegen. Het doctoraal examen werd behaald in juli 1998 en het arts examen in april 2001 (cum laude). Gedurende de studie volgde hij een facultatieve klinische stage aan de afdeling Gastrointestinale Chirurgie van het Prince of Wales Hospital in Hong Kong en een klinische stage aan de afdeling Gastroenterologie van het Groote Schuur Hospital te Kaapstad, Zuid Afrika. Van augustus 1999 tot april 2000 werd in de Verenigde Staten een onderzoeksstage gedaan aan de afdeling Gastroenterologie van de University of North Carolina te Chapel Hill, onder supervisie van Prof. R.B. Sartor. Het onderwerp van dit project was: "de rol van de gastrointestinale microflora bij het ontstaan van colitis in HLA-B27 transgene ratten". Vanaf april 2001 werkte hij als assistent geneeskundige in opleiding tot klinisch onderzoeker (AGIKO) onder supervisie van Prof. dr. H.L.A. Janssen aan het onderzoek beschreven in dit proefschrift. In januari 2005 werd begonnen met de opleiding tot Maag-Darm-Leverarts in het Erasmus MC te Rotterdam (opleider Prof. dr. E.J. Kuipers).