Non-Cytolytic Control of Hepatitis B virus Replication and the role of Interleukin-12 (IL-12)

M.D. thesis

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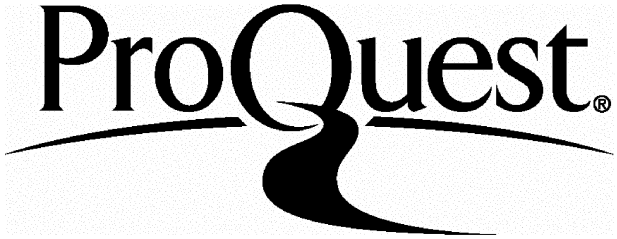

Addendum

Points arising from MD viva

Critical Summary of contribution of transgenic mice to our knowledge of HBV biology.

There are key differences between mouse and human HBV infection which should be borne in mind when considering the relevance of transgenic mouse data with reference to human HBV infection. Firstly, in the mouse model HBV is produced from a transgene that is integrated into the mouse's host genome. Although integration of HBV does occur in human hepatocytes this step is not necessary for viral replication. Secondly, the integrated transgenic source of HBV results in the absence of ccc DNA which in human HBV infection is the template for on-going viral replication. In addition the clearance of ccc DNA is likely to be important for long-term control of HBV infection. Overall these limitations mean that the mouse model does not allow investigation of the actual process of hepatocyte infection and the early part of HBV replication. Better information about these parts of the infection can be gained from other animal models such as the chimpanzee and Duck which can be truly infected. Despite these limitations, the transgenic mouse is an excellent model for studying the interaction between the host immune system and HBV and has provided useful information about the role of CD8 cells, cytokines and non-cytolytic processes in HBV infection. These studies have suggested new therapeutic approaches involving immunomodulation as being important in the control of HBV.

General Methods
Selection criteria of patients.
The patients selected for the in vitro studies (chapter 4 and 6) were consecutive patients seen in the general liver clinic at UCL hospitals. The patients for the adoptive transfer of immunity study (chapter 5) were the group of 8 patients with chronic HBV and an underlying haematological malignancy who had been treated at the Bone Marrow Transplant Unit, Queen Mary Hospital, Hong Kong, China.

Elispot Counting.
Counting elispot wells manually as described in Chapter 5.2.3 may result in observer bias, variation and inaccuracy. An alternative method of counting is with an imaging device and computer software, which takes a picture of the plate (including control wells) and enumerates the spots to provide a more accurate count.

CD4 depletion was carried out with anti-CD4 labelled magnetic beads. The final purity (as measured by FACS analysis) was over 95%.

HBcAg peptide sequences used in adoptive transfer of immunity experiments (Chiron Technologies, Clayton, Australia).

AYW (Galibert) Genotype D

1	1-20	MDIDPYKEFG	ATVELLSFLP
2	11-30	ATVELLSFLP	SDFFPSVRDL
3	21-40	SDFFPSVRDL	LDTASALYRE
4	31-50	LDTASALYRE	ALESPEHCSP
5	41-60	ALESPEHCSP	HHTALRQAIL
6	51-70	HHTALRQAIL	CWGELMTLAT
7	61-80	CWGELMTLAT	WVGVNLEDPA
8	71-90	WVGVNLEDPA	SRDLVVSYVN
9	81-100	SRDLVVSYVN	TNMGLKFRQL
10	91-110	TNMGLKFRQL	LWFHISCLTF
11	101-120	LWFHISCLTF	GRETVIEYLV
12	111-130	GRETVIEYLV	SFGVWIRTPP
13	121-140	SFGVWIRTPP	AYRPPNAPIL
14	131-150	AYRPPNAPIL	STLPETTVVR
15	141-160	STLPETTVVR	RRGRSPRRRT
16	151-170	RRGRSPRRRT	PSPRRRRSQS
17	161-180	PSPRRRRSQS	PRRRRSQSRE

Chapter 6

Expression of IL-12R on unstimulated PBMC. Preliminary experiments to measure IL-12R expression were carried out on unstimulated PBMC on healthy volunteers. PBMC were incubated 10%AB serum/RPMI for 10 days and IL-12R expression measured at days 1, 3, 5, 7, 10. At all time points the level of receptor expression measured was less than 1.5%.

The binding affinities of rIL-12 has been documented on human PHA activated lymphoblasts. These have been documented as high affinity (Kd = 5-20 pM, 100 – 1000 sites/cell), medium affinity (Kd = 50-200pM, 200-1000 sites/cell) low affinity (Kd = 2-6 nM, 1000-5000 sites/cell).

Chapter 7

Background: Carreno's study was an uncontrolled multicenter prospective randomized phase I/II study. It consisted of forty-six patients with chronic hepatitis B, who were HBV DNA positive and raised aminotransferase levels.

Methods: Recombinant human IL-12 injections were administered twice weekly (Wednesday and Sunday). Blood samples were drawn 24 hours later on Thursday and Monday. The PBMC were isolated and IL-12 Receptor expression experiments were started immediately. The patients receiving rhIL-12 were also being simultaneously treated with lamivudine, and IL-12R measurement experiments were therefore carried out on patients being treated with both.

The numbers recruited into the study were small and furthermore the division of these 15 patients into three treatment arms would mean that a clinically significant difference in the outcomes of the three treatments was unlikely to occur. In retrospect, the trial should have recruited a larger number of patients using power calculations. Furthermore the three groups of patients were not well balanced at baseline (according to ALT levels, liver histology and HBV DNA levels and prior treatment). This would have resulted in limited conclusions being drawn from the results

Results: Overall, the trial results showed small changes in elispot numbers, HBV DNA levels and T-cell proliferation counts. Although some of these may achieved statistical significance these did not translate into clinically important end-points.

Abstract

Hepatitis B is a non-cytopathic virus that causes major morbidity worldwide. Data from animal models suggest that T lymphocytes can control hepatitis B virus (HBV) replication without killing infected hepatocytes through interferon-γ (IFN-γ). Furthermore, IFN-γ production is regulated by interleukin-12 (IL-12), which not only has a direct anti-viral effect but also promotes T helper-1 type cell-mediated immune responses, which are important in the control of HBV.

The aim of this thesis was to investigate non-cytolytic control of HBV in human infection, and the role of virus-specific CD4+ T-cells in the resolution of chronic HBV infection. Furthermore, the role of IL-12 in human HBV infection and the potential of combining anti-viral and immunomodulatory drugs for the treatment of chronic HBV infection was investigated.

Activated peripheral blood mononuclear cells (PBMC) from chronically infected HBV carriers reduced cytoplasmic HBV DNA in a liver cell line by releasing IFN-γ, and without killing hepatocytes. Furthermore, recombinant IFN-γ reduced the levels of HBV DNA in naturally infected hepatocytes by between 0.3 to 3 $\log_{10}$ and the level of HBV transcription by up to 71% non-cytolytically.

Adoptive transfer of HBcAg-reactive CD4+T cells in bone marrow transplant recipients resulted in an ALT flare and the subsequent resolution of chronic HBV infection through the development of anti-HBs.

IL-12 receptor (IL-12R) expression was reduced in chronic HBV infection as measured by flow cytometry. This may be a cause of the Th2 immune responses seen in chronic HBV infection. IL-12R expression could be increased to normal levels by recombinant human IL-12 (rhIL-12) resulting in Th1 effector functions.

Combination therapy of lamivudine and IL-12 in chronically infected HBV patients has an enhanced anti-viral effect, which is associated with induction of HBcAg-specific CD4 T-cell reactivity and increased frequency of HBcAg-specific CD4+ T-cells, which produce IFN-γ.

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Chapter 1

INTRODUCTION

The hepatitis B virus (HBV) is a non-cytopathic DNA virus. It is a member of the hepadnaviridae family, which also includes similar DNA viruses that infect woodchuck (Summers et al, 1978), ground squirrel (Marion et al, 1980), duck (Mason et al, 1980) and heron (Sprengel et al, 1988). In humans HBV causes a wide spectrum of liver diseases including acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma (Tiollais et al, 1985) as well as extra-hepatic diseases including polyarteritis nodosa and membranous glomerulonephritis (Pyrsopoulos and Reddy, 2001). The presence of ongoing HBV replication lasting many years without any parenchymal liver damage suggests that the virus itself does not damage hepatocytes. Instead it is the host's immune response to the virus that is responsible for both viral clearance and disease pathogenesis (figure 1) (Chisari and Ferrari, 1995). Furthermore the failure of host T-cell immune response to HBV is a dominant cause for persistent HBV infection (Ferrari et al, 1987a; Ferrari et al, 1987b; Jung et al, 1991;Wakita et al, 1992).

Recent studies have suggested that minute amounts of HBV DNA continue to be detectable despite serological clearance. Traces of HBV are often detectable for many years in the blood of patients who have recovered from acute hepatitis, despite the presence of serum antibodies. Analysis of HBV-specific cytotoxic T-

lymphocytes (CTL) shows that they express activation markers (HLA-DR, CD69) indicating recent contact with antigen. Hence traces of virus can maintain the CTL response for decades following clinical recovery by creating a negative feedback loop that keeps the virus under control, perhaps for life (Rehermann et al, 1996). Furthermore viral genomes are detectable in hepatocytes by sensitive PCR methods in patients who are HBsAg positive but HBV DNA negative (Cacciola et al, 2000). These genomes may be a reservoir of infection that can be reactivated when the host is immunosuppressed (Ahmed and Keeffe, 1999).

In this thesis when the term 'viral clearance' is used in the context of chronic HBV replication it describes HBeAg seroconversion and undetectable HBV DNA by standard diagnostic assays. It does not equate to a sterilising immunity in which no HBV DNA can be detected. Instead it can be better considered as a resolution of viraemia as detectable by standard solution hybridisation assays but using careful PCR techniques it is still possible to detect minute amounts of HBV DNA.

1.1 HBV Structure, Genome And Antigens (Lau and Wright, 1993; Rehermann, 1997)

In 1963 Blumberg (Blumberg et al, 1966) discovered hepatitis B surface antigen (initially described as Australia antigen (Blumberg, 1977)) which forms 22nm spherical and filamentous particles and the virion envelope. HBsAg is released

during infection but does not contain HBV DNA and is not infectious. The virion (also known as Dane particle) is 42nm in size and composed of a lipoprotein envelope containing HBsAg, three envelope polypeptides, nucleocapsid (consisting of the core antigen and HBV polymerase) and the HBV genome. In blood, the titre of HBsAg outnumbers the virion by 100-1000 fold (Rehermann, 1997).

The viral genome consists of a circular, partially double-stranded DNA molecule approximately 3200 base pairs in length (figure 2, Ganem and Varmus, 1987). One strand known as 'minus' is almost a complete circle and contains 4 overlapping open reading frames (ORF) that encode viral envelope (pre-S and S), nucleocapsid (core and pre-core) polymerase and X proteins. The complementary second strand (known as 'plus') is short and variable in length.

The nucleocapsid ORF has two in-phase start codons that encode two closely related polypeptides (HBeAg and HBcAg). The first start codon defines the pre-core polypeptide (HBeAg), which is a linear, circulating peptide derived from the core gene and is then modified and exported from hepatocytes. HBeAg is not essential for viral replication and HBV mutants that are unable to produce HBeAg but are replication competent have been described (Brunetto et al, 1990). HBeAg is believed to play a role in the establishment of persistent infection, perhaps by acting as a tolerogen in utero, because of its close resemblance to HBcAg. The second start codon defines the 183 amino acids of HBcAg. In the cytoplasm, core

protein dimer pre-cursors spontaneously assemble into nucleocapsid particles, which package the viral polymerase and pre-genomic RNA.

The envelope ORF contains three in phase start codons (S, Pre-S1 and pre-S2) that code three different sizes of proteins. The S gene produces the *small* envelope protein (also known as the major envelope protein due to its abundance in the serum, HBsAg). The product of Pre-S2 and S genes is the *middle* envelope protein, and the Pre-S1 and Pre-S2 and S genes code for the *large* envelope protein. Pre-S1 and pre-S2 are highly immunogenic and also appear to be important in binding and entry to hepatocytes. The small and middle envelope proteins are present in all viral and subviral envelope particles; the larger envelope particles are preferentially present in Dane particles.

Polymerase (P) ORF encodes the longest mRNA transcript (3.5kb), which has three functional domains: reverse transcriptase, DNA-dependant DNA polymerase and RNAse H. It has a second function acting as a template for reverse transcription of the viral pregenome ('pre-genomic mRNA'). Reverse transcription is subject to mutation, as the enzymes involved do not have proof reading capacity for correction of errors in replication. This may contribute to viral persistence by generating escape mutants.

The X gene encodes a 154 amino acid protein that functions as a transcriptional transactivator of viral and cellular promoters (c-*myc*, c-*jun*) thereby inducing

several genes including HLA-DR (Hu et al, 1990), MHC-I (Zhou et al, 1990) and ICAM-1 and aiding viral replication. It is essential for in vivo but not in vitro replication and it may also play a role in the development of hepatocellular carcinoma (Su et al, 1989).

1.2 Hepatitis B virus life-cycle (Figure 3)

HBV predominantly infects hepatocytes although it has been detected in other cells including bile duct (Blum et al, 1983), pancreas, kidney (Dejean et al, 1984), brain and lymphocytes (Yoffe et al, 1990). Replication of HBV occurs within hepatocytes following attachment and uptake of the virion into to the cell. The requirements and mechanisms of this initial process have not clearly been defined. However, transferrin (Franco et al, 1992), polyalbumin (Machida et al, 1983; Machida et al, 1984) annexin V and IL-6 (Neurath et al, 1986) receptors have all been suggested as potential sites of entry into hepatocytes. Other important molecules on the hepatocyte include apolipoprotein H and endonexin II. On the viral side the pre-S1 protein has been implicated in the attachment of HBV to hepatocytes (Neurath et al, 1986).

Following entry into the hepatocyte the virion is uncoated and the nucleocapsid and HBV DNA is transported to the nucleus where the virus uses host enzymes to repair relaxed circular DNA into supercoiled covalent closed circular (ccc) DNA. ccc DNA is a template for four viral mRNA transcripts. The longest (3.5kb) is the template both for genome replication and for the expression of polymerase and

pre-core/core proteins. A 2.4 kb transcript encodes for pre-S1, pre-S2 and HBsAg proteins while a 2.1 kb transcript encodes only for pre-S2 and HBsAg proteins. The smallest transcript (0.7kb) encodes the X protein. The pre-genomic mRNA (3.5kb) is also a template for reverse transcription to form DNA. Initially viral DNA polymerase synthesises the minus HBV DNA strand whilst concomitantly degrading the pre-genomic mRNA through its RNAseH activity. The polymerase then synthesises the HBV plus strand. The nascent nucleocapsids combine DNA and HBsAg. Depending upon the concentration the virus is either packaged in the endoplasmic reticulum where they form complete virions following binding with viral envelope proteins and are exported or relocated to the nucleus where they act as a template for further replication. Hence a stable pool of ccc DNA is maintained in the nuclei by transporting some of the newly synthesised HBV DNA back to the nucleus. It has been suggested HBsAg can inhibit the formation of ccc DNA which may represent a negative feedback mechanism (Summers et al, 1990).

Although like retroviruses HBV replication relies on reverse transcription of RNA into DNA, hepadnaviruses have important differences because the HBV genome is partially double stranded, does not require integration within host DNA for replication and functional mRNAs are produced from several internal promoters on the circular DNA genome.

1.3 Natural History Of HBV Infection

The outcome of hepatitis B virus infection may be either an acute self-limited infection or chronic long-term infection. The time at which the virus is acquired has a significant impact the outcome. Adult acquired (horizontal) chronic HBV infection has different clinical presentation, outcomes and responses to treatment than childhood or vertically acquired disease. Adult acquired HBV infection is usually self-limited and associated with an acute hepatitis. The likelihood of developing chronic liver disease is less than 5% in immunocompetant adults. In 1% of cases acute infection may also lead to fulminant liver failure with a case fatality rate of 80%. In acute infection HBsAg, HBeAg and HBV DNA appear in the blood within two to eight weeks of exposure and rise to high levels. These then start to fall as the serum aminotransferase levels rise.

The terminology of chronic hepatitis B virus infection has recently been standardised by the National Institutes of Health. The new definitions are described below.

Chronic HBV infection: Chronic Hepatitis B is defined by chronic necroinflammatory disease of the liver caused by persistent infection with hepatitis B virus. The diagnosis is made by the presence of HBsAg, serum HBV DNA levels of greater than 1.10^5 copies per ml or HBcAg detected in the liver by immunostaining in a patient with persistent or intermittent elevations in aminotransferase levels for 6 months or more. In adult-acquired chronic HBV

infection most patients usually have mild or subclinical disease. The course of the infection is not static but over time evolves from a replicative to a non-replicative phase and is highly variable. Most patients have a period of chronic hepatitis with persistent elevations of aminotransaminase levels along with high HBV DNA levels, HBsAg, and HBeAg titres. The rate of development of cirrhosis ranges between 1.3-5.9% per year in patients with chronic HBV infection and is mainly in those with on-going HBV replication.

HBeAg positive chronic hepatitis B: this is characterised by persistent elevations in serum ALT, high HBV DNA levels (greater than 1.10^6 copies per ml), HBsAg and HBeAg titres. The liver disease can be highly variable but if it is severe, cirrhosis and end-stage liver disease rapidly develop. Alternatively the infection can be insidious, resulting in progressive fibrosis and cirrhosis only after years or decades of infection. Between, 20-35% of patients with chronic HBV will develop cirrhosis and end-stage liver disease. At least a quarter of patients with cirrhosis develop hepatocellular carcinoma.

An important event in the natural history of HBV is the seroconversion of HBeAg to anti-HBe. This is usually accompanied by a dramatic fall of serum HBV DNA (to below 1.10^5 copies per ml) and is associated with normalisation of aminotransferases and long-term remission of the disease in the majority of cases. However, it is increasingly recognised that in chronic HBV replication two types

of HBeAg seroconversion can occur resulting in either inactive HBsAg carrier state or HBeAg negative chronic hepatitis B.

Inactive HBsAg carrier state: In these patients seroconversion and the formation of anti-HBe occurs. These patients usually have a long-term remission in the disease with either no or only low levels of wild-type HBV DNA (less than 1.10^5 copies per ml and usually less than 1,000 copies per ml). Long-term follow-up of inactive carriers suggests that the majority do not have progressive liver disease and do not develop end stage liver disease. However these patients are at risk of hepatocellular carcinoma and episodic reactivation of disease.

HBeAg-negative chronic hepatitis B: In some patients HBeAg seroconversion is associated with the development of pre-core mutants. In these patients the pre-core mutants replicate well enough to cause continuing liver injury and moderate to high HBV DNA levels (greater than 1.10^5 copies per ml) despite the loss of HBeAg. The proportion of patients who evolve into an HBeAg negative chronic hepatitis versus the inactive HBsAg carrier state varies in different parts of the world and may in part be due to differences in immune status, gender, age of acquisition and HBV strain and genotype.

Resolved chronic hepatitis B: A small proportion of patients with chronic HBV clear HBsAg as well as HBeAg and HBV DNA. In most individuals anti-HBs can

be detected. These patients are characterised by normal ALT, inactive liver disease and small amounts of HBV DNA in the liver and occasionally serum.

In patients who develop chronic HBV infection the disease can pass through three consecutive stages (figure 4). Stage 1 and 2 are replicative phases whereas stage 3 is non-replicative.

Stage 1. *Immune tolerance:* This stage is characterised by immune tolerance (minimal peripheral blood immune response and minor histological changes), normal liver function tests and the presence of high viral load (HBV DNA). It can last decades in neonatally acquired infection whereas in adult acquired infection this may only last two to four weeks.

Stage 2. *Viral clearance:* Later in the replicative phase immune tolerance is lost the emergence of reactive T-cell clones occurs and HBV replication is reduced. The duration of the immune response phase is only 3-4 weeks in acute adult infection whereas in chronic disease this can persist for up to 10 years. The duration of this immune response phase in chronic HBV infection determines the extent of liver damage that occurs. During viral clearance the immune system is activated resulting in cytokine release, hepatocyte lysis and a fall in HBV DNA levels. An exacerbation of liver disease and deterioration of liver function accompany seroconversion to anti-HBe. Seroconversion is associated with a decrease of HBV DNA to undetectable levels by conventional assays,

normalisation of transaminases and no further liver damage. This is usually considered an end-point for successful anti-viral treatment and associated with a good prognosis. Both overall survival and survival without clinical complications is significantly longer in patients who are seronegative for HbeAg (Niederau et al, 1996). Only rarely does reactivation occur e.g. during immunosuppression. The course of adult acquired chronic HBV in the West differs from that of vertically or childhood acquired infection. The acquisition of HBV in adult life results in a shorter evolution from replicative to non-replicative phase and hence less liver damage.

Stage 3: *Inactive stage:* When the host's immune response is able to suppress HBV replication, HBV DNA levels markedly decline, HBeAg seroconversion, and normalisation of liver function tests occur. Longitudinal studies have shown that HBeAg is spontaneously lost at a rate of 7-20% per year (Lok et al, 1987). From a clinical perspective the infection has 'cleared' although many patients still have HBV DNA detectable by PCR. Naturally infected patients rarely lose HBsAg as the S gene is often integrated into the host's hepatocyte genome (Liaw, et al, 1991). Hence, although development of anti-HBe is associated with a good prognosis, the integration of HBV DNA into host genome does increase risk of hepatocellular carcinoma.

1.4 Anti-Viral Immune Response In HBV Infection

The successful elimination of free virus in the circulation and infected cells depends on the combination of the host's anti-viral immune mechanisms. Hence the combination of the innate and adaptive immune systems and also the co-ordinated activation of both the humoral and cellular limbs of the adaptive immune system is required (figure 1).

It is generally believed that HBV persistence is related to a specific failure of T-cells to respond to HBV antigens i.e. a deficiency in cell-mediated immunity rather than humoral immunity (Boyer JL, 1988). This is supported by the fact that those patients with a relative deficit of T cell function (the very young, aged and immunosuppressed) are more likely to develop chronic HBV infection. Furthermore neonates can be immunised immediately after birth successfully producing protective anti-HBs (Lee et al, 1983). In contrast adults who have a mature cell mediated immune system HBV infection usually causes an acute self-limited illness including jaundice followed by clinical recovery and viral clearance. Only 5-10% of immune-competent adult-infected patients develop chronic liver disease (Wright and Lau, 1993).

1.4.1 Innate immune response

The innate immune system is composed of granulocytes, NK cells and NKT cells which are rapidly recruited and/or activated at the site of virus infection. These cells have several direct anti-viral effector functions including killing infected

cells and the production of antiviral cytokines. They also have indirect anti-viral actions by producing chemokines that recruit inflammatory cells to infected cells and by producing immunoregulatory cytokines that enable the adaptive immune response to recognise infected cells and perform anti-viral effector functions. Granulocytes (neutrophils, eosinophils and basophils) are short lived phagocytic cells which not only have anti-bacterial effects but also have antiviral effects.

1.4.1.1 NK cells

NK cells are recruited very early in an infection by chemokines and activated resident macrophages. These macrophages produce IFN α/β that induces NK cell proliferation and NK cell mediated cytolysis, and secretion of IFN-γ (Biron, 1999; Biron, et al, 1999). Peak NK cell responses and IFN-γ production occur within hours to days of a primary infection. In contrast T and B cell responses take over a week to develop, as they are dependant upon upregulation of MHC class I. NK cells may be particularly important in the control of hepatotropic infections as hepatocytes express low levels of MHC I (Zhou et al, 1990). In the chimpanzee model of acute HBV infection it has been hypothesised that NK cells mediate the disappearance of HBV DNA (including ccc DNA) from the liver and blood. This occurs before the peak of T cell infiltration and liver disease (Guidotti et al 1999b). In addition, there is some evidence that NK cells are functionally impaired in HBV infection – the presence of HBsAg results in decreased NK cell cytotoxic activity against a liver cell line grown in vitro (de Martino et al, 1985).

Furthermore several viruses down regulate MHC I as an evasion strategy which perhaps makes the NK cell response even more important.

IL-12 is a critical cytokine in the development of NK cells' anti-viral effects. IL-12 induces NK cell secretion of IFN-γ, acts as a short term growth factor and enhances the lytic activity of these cells. Those infections eliciting NK cell IFN-γ also produce biologically active IL-12 p70 heterodimer and the NK cell response is dependent upon this endogenous IL-12 (Orange and Biron, 1996a; Orange and Biron, 1996b).

1.4.1.2 NKT cells

These are a subset of T cells that express NK cell markers. NK-T cells account for 20-30% of intrahepatic lymphocytes in the normal liver. Although they are known to be important in immunity against to intracellular bacteria and parasites their role in viral infections is less clear. However, a recent study in the transgenic mouse model has shown that NK T-cells can inhibit HBV replication via IFN-γ and IFN-α/β (Kakimi et al, 2000). NKT cells can also recruit other cells (NK cells, T cells, macrophages), which also secrete the same antiviral cytokines.

1.4.1.3 Macrophages

These are long-lived phagocytic cells that are very efficient at presenting antigen to T cells. Activated macrophages produce several factors that may have antiviral effects including TNF-α and nitric oxide. A role for macrophages in HBV

infection has been established in the transgenic mouse. Superinfection with malaria resulted in abolition of HBV gene expression and replication in hepatocytes due to activation of Kupffer cells (Pasquetto et al, 2000). Macrophages also produce IL-12, which through IFN-γ can inhibit HBV (Cavanaugh et al, 1997).

1.4.2 Adaptive immune response

Cell mediated adaptive immunity is carried out by CD4+ and CD8+ T cells (figure 5). Both CD4+ and CD8+ cells function as a result of the specific interaction of T-cell receptor with a complex consisting of processed viral protein and either HLA class II or I protein respectively. Although T lymphocytes have been described as "helper" and "cytotoxic" respectively recent evidence suggests that both types of lymphocytes are able to exert effector and immunoregulatory actions.

1.4.2 1 Dendritic Cells

Dendritic cells (DCs) are "professional" antigen presenting cells derived from monocytes DCs which stimulate B and T lymphocytes (Steinman). In vitro, monocytes treated with GM-CSF and IL-4 develop into immature myeloid DCs. DC maturation is promoted by pathogens, cytokines, and T-cells but not by IL-10 (Buelens et al, 1997). The function of DCs is dependent on their maturational state. DCs are found in most tissues where they capture and process antigens. DCs act as mobile sentinels bringing antigens to T cells and inducing immune

responses. Once DCs have captured antigen they express lymphocyte co-stimulatory molecules, migrate to lymphoid organs and secrete cytokines to initiate and modulate immune responses. DCs form clusters in lymphoid organs with antigen-specific T-cells creating a microenvironment in which immunity can develop.

During their development DCs undergo changes in cell surface phenotypic markers (figure 6). Monocytes express CD14, which is lost, as they develop into immature DCs. As the primary function of immature DCs is to capture and processing antigens to form MHC peptide complexes they express many antigen capturing Fcγ and Fcε receptors but few MHC and accessory molecules. Once an antigen is captured, DCs undergo extensive transformation – the antigen capturing devices disappear and T cell stimulatory functions increase. This is seen by the appearance of high levels of cell surface MHC II, CD54, CD58, CD80, CD86, CD40, and CD25. CD40, CD54 and CD86 are particularly important in the activation of T cells. Activated T cells then complete the immune response by interacting with other cells, such as B cells to induce antibody production, cytokine release, and lysis of target cells

DCs are particularly potent antigen presenting cells because they express very high levels of MHC and MHC-peptide complex (which is 10-100 times higher on DCs than other antigen presenting cells). Hence, one dendritic cell is potentially being able to stimulate between 100-3000 T cells (Banchereau and Steinman, 1998). Similar to the Th1/Th2 dichotomy, DCs have been divided into two

subgroups DC1 and DC2 according to the pattern of cytokine secretion they elicit from CD4+ cells (figure 7). DC1 and DC2 are derived from monocyte and CD4CD3CD11c plasmacytoid cells precursor respectively. DC1 cultured with naïve CD4+ cells secrete IFN-γ, but little in the way of Th2 cytokines (Kapsenberg and Kalinski, 1999). In contrast CD4+ cells cultured with DC2 produce IL-4, IL-5 and IL-10 rather than Th1 cytokines. However, both DC1 and DC2 induce strong proliferation of allogeneic naïve CD4+ T-cells.

DCs have a central role in regulating CD4+ T cells through the expression of many accessory molecules that interact with T cell receptors to enhance adhesion and co-stimulation e.g. LFA-3/CD58, ICAM-1/CD54, B7-2/CD86 (Caux et al, 1994; Inaba et al., 1994). Dendritic cell MHC expression, IL-12 secretion and co-stimulatory molecule expression are upregulated within a day of exposure to appropriate stimulants. Mature DC1 cells produce IL-12, which is critical in initiating the development of Th1 cells and inducing IFN-γ production. IFN-γ initiates the antimicrobial activities of macrophages and together with IL-12 promotes the differentiation of T cells into killer cells. DCs function simultaneously as antigen presenting cells and IL-12 producing cells to induce the development of Th1 cells. Engagement of CD28 by CD80 or CD86 is needed for T cell priming by DCs and the IL-12 from DCs is a potent inducer of differentiation of IFN-γ producing T cells in vivo. IL-12 from monocyte-derived DCs plays a central role in Th1 priming: Th1-prone monocytes derived DCs produce large amounts of IL-12 within 24 h of CD40L activation whereas Th2-

prone plasmacytoid – derived DCs do not. The contribution of DC in Th2 activation remains unclear- perhaps Th2 differentiation occurs as a default pathway in the absence of IL-12.

In addition to the immunoregulatory effects on CD4+ cells DCs also induce CD8+ proliferation (Bender et al, 1995; Bhardwaj, et al, 1994) and promote B cell growth and immunoglobulin secretion through CD4+ cells. IL-12 produced by DCs stimulates B cell antibody production and proliferation if stimulated by CD40L on activated T cells. This direct modulation of B cells is in addition to their indirect action on immunoglobulin production through CD4+ cells.

The communication between T cells and DCs is a two way process. T cells not only respond to DCs but also talkback to DCs thereby modulating DC function. Activated and memory T cells express CD40L and RANKL which through the ligation of the CD40 and the TRANCE/RANK receptor (Caux et al, 1994; Wong, et al, 1997) promote DC survival (Anderson et al, 1997). CD40 also upregulates CD80 and CD8+ (Caux et al, 1994), promotes the secretion of IL-12 (Koch et al, 1996)and the release of chemokines such as IL-8 and MIP-1α. DCs secrete a large variety of anti-viral and immunoregulatory cytokines which probably exert antiviral activities (Bender et al, 1998; Stockwin et al, 2000).

1.4.2.2 Class II Restricted T-cell response

CD4+ T-cells act as central regulators of the adaptive immune response to viral infections by modulating the activity of antigen-specific B cells and (to a lesser extent) CD8+ cells via cytokines. The central role of CD4+ cells has been demonstrated in CD4+ lymphocyte deficient mice which are more susceptible to chronic viral infection (Battegay et al, 1994). CD4+ cells are HLA class II restricted which means that they recognise viral antigens presented by HLA class II molecules. These are extracellular antigens that have undergone endocytosis and processing by specialised "professional" antigen presenting cells (APC) such as macrophages, dendritic cells and B-cells. APC proteolytically cleave the phagocytosed antigen into short peptide fragments, which are delivered into the lysosomal pathway and displayed at the antigen presenting cell membrane by HLA class II molecules to CD4+ T-cells.

The direction an immune response follows is influenced by the nature of the T helper (Th) response based on the pattern of cytokine secretion (Mosmann et al, 1986). Naïve CD4+ T-cells (Th0) develop into Th1 or Th2 (Sad and Mosmann, 1994) as defined by the cytokines the cells secrete. This is known as Th1/Th2 dichotomy but as CD8+ and dendritic cells produce similar patterns of cytokine secretion it can also be referred to as type 1 or 2. Th1 cells are characterised by their ability to secrete IFN-γ and IL-2 and promote cell-mediated immune responses, whereas Th2 cells are characterised by the secretion of IL-4, IL5, and IL-10 and enhance the humoral immune response (Paul and Seder, 1994).

Development of the appropriate Th subset during an immune response is important as different pathogens are most effectively controlled by either a predominantly Th1 or Th2 immune response (Sher and Coffman, 1992). A Th1 response appears to be important in HBV seroconversion (Rossol et al, 1997).

The switching of naive T-cells to either Th1 or Th2 is influenced by cytokines, antigen dose, route of antigen administration and the APC type (in the form of co-stimulatory molecules). IL-12, IFN-γ, IFN-α and IFN-β promote Th1 responses (Finkelman et al, 1991; Hsieh et al, 1993). In contrast IL-4 promotes Th2 immune responses (Seder et al, 1992). IL-12 induces differentiation of Th1 cells from uncommitted T cells and hence initiates cell mediated immunity (Hsieh et al, 1993). Theoretically therapies that enhance Th1 may favour viral clearance with as poor Th1 responses are associated with increased viral load and progressive disease.

The strength and multi-specificity of the T cell response is the most significant factor in determining whether viral clearance occurs during acute and chronic HBV infection. Patients with acute self-limited HBV infection mount vigorous, polyclonal and multi-specific CD4+ responses to epitopes within HBeAg, nucleocapsid and polymerase (see table) that is readily detectable in the peripheral blood (Chisari and Ferrari, 1995). These immune responses coincide with maximal ALT levels and precede clearance of HBeAg and HBsAg antigens and the development of neutralising antibodies (Rehermann et al, 1995). Between 4

and 8 weeks after disease onset the HBc-specific CD4+ T-cell response diminishes (Diepolder et al, 1996). During successful serological clearance there is enhanced production of Th1 cytokines (such as IFN-γ) as well as a significant T-cell proliferative response (representing clonal expansion) (Ferrari et al, 1987).

In contrast patients with chronic HBV infection only mount a weak HLA class I and class II restricted T-cell responses. Significant HBcAg-specific responses are seen only during an exacerbation of chronic hepatitis B (Tsai et al, 1992). Furthermore these increases in HBcAg correlate with ALT levels and eventual seroconversion to anti-HBe in IFN-α treated patients (Marinos et al, 1995). Hence, analogous to acute HBV infection, T-cell responses to nucleocapsid antigens are crucial for elimination of HBV in chronic HBV infection (Jung et al., 1991).

Studies in acute and chronic hepatitis B have shown that HBcAg is the major antigenic stimulus (Mondelli et al, 1982). Furthermore a study of 20 patients with acute hepatitis B showed that within the HBcAg there is an immunodominant epitope between residues 50-69, which is recognised by over 95% of patients with acute hepatitis regardless of their HLA background (Ferrari et al., 1991). This response is temporally associated with viral clearance in acutely infected patients. In contrast those who fail to clear the virus have a poor HBV-specific immune response as measured by proliferation to this peptide. Another study of acute hepatitis B infection identified a similar region that was immunodominant

between residues 60-85 which reveals immunodominant epitopes from different overlapping regions of the two peptides (Diepolder et al, 1996).

Significant changes in the CD4+ cell response to HBcAg occur during the natural history of the infection (Marinos et al, 1995). During the immunotolerant phase of chronic HBV infection HLA class II restricted PBMC response to all viral antigens is considerably reduced in comparison to patients with acute hepatitis. The nucleocapsid T-cell response is accentuated in an acute exacerbation that is often preceded by increased serum HBV DNA and HBeAg concentration. These levels then dramatically start to drop as the ALT starts to rise (Tsai et al, 1992). In contrast, during these flares serum HBsAg titres are unchanged and the proliferative response to envelop antigens is undetectable. Class II-restricted nucleocapsid-specific T cell response and perhaps the temporally associated flare of liver disease may be dependent on the expression of critical threshold levels of HBV nucleocapsid antigens (Maruyama et al, 1993; Maruyama, et al, 1993). These cells play a key immunoregulatory role while persistently elevated levels of circulating HBsAg suppress envelope-specific T-cell activity.

Significant class II restricted T cell proliferative responses to HBV nucleocapsid antigens persist for many years after successful clearance of HBsAg. HLA Class II CD4+ restricted T-cell responses were detected in 82% of patients studied for up to 13 years after clinical resolution of disease (Penna et al, 1996). Fine-epitope mapping confirmed that the peptides recognised during the acute phase and follow-up were the same. These long-lasting T-cell responses were

predominantly CD45RO+, and showed markers of recent activation. Interestingly limiting dilution studies showed that HBV specific T cell numbers were comparable to that observed in acute infection and higher than in patients with chronic HBV infection. HBV DNA was detected by a sensitive nested PCR with primers for the core gene in half of the patients, suggesting that small amounts of virus persist despite serological "clearance". These small amounts of persistent HBV DNA may be important in priming memory T-cells and thereby keeping the virus under control.

High viral load has been suggested as a cause of impaired CD4+ T cell responses in patients with chronic HBV infection. Furthermore restoration of the proliferative response to HBcAg can occur in some patients who have had a therapeutic reduction of viral load by lamivudine (Boni et al, 1998) although this is not a universal finding (Marinos et al, 1996).

Further evidence that HBV impairs the immune response has been demonstrated in *in vitro* studies. In patients with high serum HBV DNA levels (>300 pg/ml) suppressed co-stimulatory effects of Interleukin-12 on the HBV-induced immune response are seen. In theses patients HBsAg and HBcAg induced a Th2 immune response from PBMC. Culture of PBMC with IL-12 had marked Th1 effects on lymphocytes from patients with undetectable and low levels of HBVDNA (<300pg/ml) but these Th1 responses were absent in lymphocytes from patients with high levels of serum HBV DNA (Schlaak et al, 1999). The same authors also

found that HBeAg+ patients produced the lowest amounts of IFN-γ in response to envelope and nucleocapsid antigens even in the presence of IL-12. The reasons for the impaired co-stimulatory responses seen have not been identified. In other chronic intracellular infections deficiency in IL-12 receptor has been documented (Altare et al, 1998). Hence in hepatitis B virus infection it is unclear as to whether the deficiency is due to impaired CD4+ T-cell reactivity or impaired DC function.

In addition to their role as helper T-cells, CD4+ cells can exert anti-viral effector functions in three ways. B cells with immunoglobulin specific for HBcAg can process HBcAg and present it by HLA class II to HBcAg-specific CD4+ T cells. These in turn stimulate envelope B cells to secrete anti-HBs (this process is termed "intermolecular help") (Milich and McLachlan, 1986). Hence nucleocapsid specific T-cells stimulate envelope B cells to produce neutralising antibodies. Secondly, CD4+ cells induce HBV specific CD8+ cells, the kinetics of which parallels the CD4+ response (Missale et al, 1993; Penna et al, 1991). These CD8+ cells have direct cytotoxic effects as well as indirect antiviral effects by secretion of cytokines. Thirdly CD4+ cells secrete cytokines such as IFN-γ that have immunomodulatory and anti-viral effects.

CD4+ cells have been shown to have an immunopathogenic function in viral infections. CD4+ cells can function as effector cells in LCMV, influenza virus induced pneumonia as well as type 1 diabetes and experimental allergic encephalitis. In a transgenic mouse model of HBV infection transfer of envelope

specific Th1 cells resulted in recognition of viral antigen expressed by hepatic non-parenchymal cells, cytokine release and transient necro-inflammatory disease. Furthermore overexpression of HBsAg resulted in increased sensitivity to the pathogenic effects of CD4+ cells (Franco et al, 1997). This is likely to be of relevance to human HBV infection as hepatic overexpression of large envelope protein is very common in chronic HBV infection resulting in ground glass hepatocytes. Therefore CD4+ cells can contribute directly to disease pathogenesis as well as inhibiting viral replication. CD4+ cells can also be cytotoxic against CD8+ cells. Hence this may be important in inhibiting the activity of CD8+ cells and limiting the immune response thereby providing an important immunoregulatory function (Franco et al, 1992).

Interestingly the CD4+ response to envelop antigens (HBsAg) is weak even in those patients who successfully clear HBV. The reason for the poor envelope response is not known however this may be as a result of T cell exhaustion or T cell anergy following a strong response in the incubation phase (Rehermann, 1997).

1.4.2.3 Class I-Restricted T-lymphocyte response

CD8+ T cells represent the main effector limb of the immune response against intracellular pathogens. CD8+ T cells recognise endogenously synthesised peptides presented in the antigen-binding groove of HLA class I molecules on the surface of virus infected cells including hepatocytes. HLA class I-peptide

interactions are HLA allele specific in that the peptide is between 8 and 11 amino acids long and contains an HLA-specific binding motif (figure 5, Germain, 1994). This allows any viral protein regardless of whether or not it is normally expressed on the cell surface or on the viral envelope to be presented. The majority of CTL are CD8+ and recognise antigen presented on class I. Occasionally, such as in measles virus infection, CD4+ and HLA class II restricted CTL are important (Male D, 1996). Once identified these anti-viral CTL kill infected cells by apoptosis or lysis. CD8+ cells also secrete cytokines such as IFN-γ, TNF-α, and IL-2 that have anti-viral effects. Hence in addition to cytotoxic effects CD8+ T cells have immunoregulatory and anti-viral actions.

The destruction of infected cells by HLA class I restricted CD8+ cytotoxic T-lymphocytes can occur via two main pathways. Firstly, granules containing the enzymes perforin and granzyme can be released at the target cell/CTL-binding site. These enzymes punch holes in the cell membrane allowing fluid in and triggering intracellular pathways resulting in cytolysis and cell death. Secondly apoptosis can be triggered by Fas ligand expressed on CTL which can bind to the target cell TNF receptor (Male D, 1996; Squier and Cohen, 1994).

As with the CD4+ response patients with acute hepatitis B who clear the virus have a persistent CD8+ response to HBV that is vigorous, polyclonal, and multispecific (Penna et al, 1991; Rehermann et al, 1995; Rehermann et al, 1996). The response is weak or barely detectable in patients with chronic infection

(Bertoletti et al, 1991; Missale et al 1993), except during acute exacerbations of chronic disease or after spontaneous or IFN-α induced viral clearance. The HBV-specific CD8+ T-cell response coincides with peak ALT levels (Maini et al, 1999) and precedes clearance of HBsAg and HBeAg and development of neutralising antibodies (Rehermann et al, 1995). The strong association between liver disease and the CTL response during acute HBV infection suggests an important role for CTL in the pathogenesis of acute viral hepatitis. CTL cause some liver damage as seen by raised transaminases but recent data indicate that the majority of the liver damage is largely mediated by antigen non-specific cells rather than CD8+ cells (Ando et al, 1993; Chisari and Ferrari, 1995; Guidotti et al, 1999b).

In human HBV infection, studies have been hampered by the inability of HBV to infect cells in vitro and by the difficulty of studying the intrahepatic compartment. Hence knowledge of the HBV-specific CTL response has been mostly restricted to the circulating compartment relying on in vitro cell culture after peptide stimulation and confined to HLA-A2 patients (Chisari, 1997). Using synthetic peptides that mimic the processed antigen fragments, a vigorous polyclonal HLA class I restricted CTL response to multiple epitopes in HBV envelope, nucleocapsid, and polymerase proteins is seen in acute self-limited HBV infection (Penna et al, 1991). All except one CTL epitopes identified are HLA-A2 restricted. Only one HLA-A2 CTL epitope has been identified in the HBV nucleocapsid. In contrast 11 epitopes have been identified in the in the envelope

protein and 5 in the polymerase protein. These findings contrast strikingly with the dominance of class II restricted response to nucleocapsid antigens.

Following clinical recovery from acute hepatitis B infection, HBV-specific CTL continue to display recent activation markers for many years afterwards. This is associated with traces of HBV DNA that persist indefinitely (Rehermann et al, 1996). Hence transcriptionally active virions can maintain the CTL response after recovery. Clinical resolution does not necessarily correspond to eradication of HBV and long-lasting T cell memory can be maintained by serologically undetectable amounts of antigen. This suggests that infection of immunologically privileged sites may allow the virus to escape complete elimination even in the presence of an immune response. This may be a source of antigen that can periodically boost immune responses thereby maintaining a pool of anti-viral T cells. CTL control the spread of the virus since several of these subjects lacked anti-HBs antibodies. CD8+ proliferative responses are also seen showing that HLA class 1 restricted responses to viral envelop, nucleocapsid and polymerase antigens can last for decades following resolution of acute HBV infection.

A strong intrahepatic CTL response to HBV during acute viral hepatitis can suppress gene expression and perhaps even "cure" the majority of infected hepatocytes of the virus in addition to killing a small fraction of them. Conversely the data suggest that a weak immune response that occurs in chronically infected patients could contribute to viral persistence and chronic liver disease by reducing

the expression of viral antigens sufficiently for the cells to escape immune recognition but not sufficient for the virus to be eliminated. Therefore cytokines produced by CTL can inhibit HBV replication representing a survival strategy by the virus contributing to persistence or a tissue sparing anti-viral strategy by the host contributing to viral elimination. HBV infection is probably never completely cleared but kept under tight control by host immune responses as in other viral infections such as HTLV-1, HSV and EBV. A circulating reservoir of virus-specific CD8+ cells that is able to clonally expand, migrate to the liver and produce the correct antiviral cytokines will be necessary to maintain the virus under control and to respond quickly to alterations in the rate of viral replication.

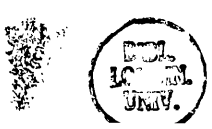
Virus specific CD8+ T-cells play an essential role in the ultimate control of HBV but the co-existence of similar numbers of virus-specific CTL with different clinical outcomes reveals that these cells can have protective or damaging effects depending on their microenvironment and functional capacity. Similarly cytokines that are able to achieve non-cytolytic clearance of infected hepatocytes can also be found in chronically infected livers where their levels correlate with liver damage (Guo et al, 2000). A threshold of IFN-γ or TNF-α production may be required for curative effects as demonstrated in the transgenic mouse model where lower numbers of CTL were unable to block viral replication (Guidotti et al, 1996). Furthermore HBV may modulate the effect of these cytokines by the production of inhibitory factors (Lalani et al, 2000).

The low level of CD8+ cells in the circulation has been reconciled with CD8+ mediated liver disease by assuming that that along with CD4+ cells they infiltrate the liver and cause damage (Ferrari et al, 1987). However a high frequency of intrahepatic CD8+ cells has been found in the presence of minimal liver damage. In contrast in patients with on-going liver disease a far greater number of non-antigen specific cellular infiltrate is present (Maini et al, 2000). Furthermore the immune response to the virus was undetectable in viraemic patients in contrast to those who had cleared the virus. Hence when CD8+ immune response is unable to control virus replication it may contribute to liver damage by recruitment of non-virus specific T-cells.

1.4.2.4 Antibody response

Antibodies are produced by B-cells following HBcAg stimulation in response to nucleocapsid and envelope proteins and their production is augmented by CD4+ cells (Milich and McLachlan, 1986). B-cell epitopes have been described in all viral proteins i.e. HBcAg, HBsAg, PreS, Polymerase and X protein. After successful clearance of acute HBV infection protective humoral anti-viral responses persist for a lifetime.

The antibody response to HBV antigens is important for several reasons. Firstly, antibodies bind to circulating free virus and may prevent attachment and uptake by susceptible cells. Secondly, B-cell immunoglobulin receptors recognise viral antigens either as soluble secreted proteins or as intact molecules expressed on the



surface of infected cells and therefore present antigen to CD4+ cells. Thirdly, different antibody patterns have been associated with different phases and outcomes of disease and therefore can be used as diagnostic tools. The presence of anti-HBs is indicative of protective immunity against HBV, as it is a neutralising antibody. It is also used to assess the efficacy of vaccination. IgG anti-HBc is a marker of past exposure to the virus. Anti-HBe is usually a marker of viral clearance except in the case of precore mutants when (usually) lower levels of viral replication continue. Antibodies may also contribute to the pathogenesis of extrahepatic syndromes associated with HBV infection such as polyarteritis nodosa, glomerulonephritis and the prodromal symptoms such as arthralgia and urticaria by immune complex deposition

1.5 Interleukin-12 (IL-12)

1.5.1 Interleukin-12 and Interleukin-12 receptor

Interleukin-12 is a heterodimeric cytokine produced by antigen presenting cells that has immunoregulatory effects on T and natural killer cells. IL-12 promotes cell-mediated immune reactions and is therefore critical for efficient immune responses to intracellular pathogens in viral, bacterial and parasitic infections by generating Th1 responses and IFN-γ production (Gately, 1997; Trinchieri, 1995).

1.5.2 Structure of IL-12

IL-12 is comprised of two sulphide-linked subunits, p35 and p40, which are coded by two unrelated genes on different chromosomes. There is no sequence homology between the two subunits. The 35-kDa subunit contains a α-helix rich structure and has homology with IL-6, G-CSF. The 40-kDa subunit belongs to the haemopoeitin receptor family and has no structural similarities to other cytokines. Neither subunit alone displays significant biological activity. Normal monocytes produce between 5-500-fold excess of p40 relative to the heterodimer. Of this, between 5-30% excess p40 exists as a homodimer ($p40_2$), which binds to the IL-12 receptor but has no biological effect and therefore may act as a physiological regulator of IL-12. However, the p40 homodimer has also been shown to stimulate CD8+ T cell differentiation and therefore facilitate some immune responses by inducing CD8+ T cells to secrete IFN-γ.

1.5.3 Actions of IL-12

IL-12 is a potent inducer of IFN-γ from activated T and NK cells (D'Andrea et al, 1992). Furthermore IFN-γ is an important stimulus for IL-12 production and this IL-12 induced production of IFN-γ provides a positive feedback loop that amplifies the response to infection. The stimulatory effect of IFN-γ on IL-12 production is due to direct induction of both IL-12 p35 gene and IL-12 p40 gene transcription (Ma et al, 1996). In contrast Th2 cytokines (IL-4, IL-10) and TGF-β down regulate IL-12 induced IFN-γ production. IL-12 also induces low levels of TNF-α, GM-CSF, IL-2, and IL-8 secretion.

IL-12 influences the cellular immune response by promoting Th1 responses. Firstly, it promotes the differentiation of naive T-cells (Th0) into Th1 cells after an initial encounter with an antigen thereby initiating cell mediated immune reactions (Hsieh et al, 1993). IL-12 mediates this differentiation through IFN-γ as depletion of IFN-γ by monoclonal antibody ablates Th1 development (Sadick et al, 1990). However IFN-γ not only differentiates Th0 to Th1 but it also enhances the expansion of Th1 cells (Manetti et al 1993). Secondly, IL-12 acts as a co-stimulus thereby maxising the secretion of IFN-γ by differentiated Th1 cells, and thirdly it stimulates the development of IFN-γ producing Th1 cells from resting memory cells interacting with an antigen to which they have already been exposed(Gately, 1997). Fourthly, IL-12 In mice enhances IgG2a, IgG2b, IgG3 antibody responses to protein antigens by 10-100 fold. These immunoglobulin isotypes are associated with Th1 responses in mice.

Other actions of IL-12 include enhancement of the lytic activity of NK (Clerici et al, 1993) and lymphokine-activated killer cells and promotion of CTL responses (Mehrotra et al, 1993). IL-12 is also a short-term growth factor to activated T and NK cells. IL-12 inhibits activation-induced apoptosis of human peripheral T cells in normal or HIV infected patients. IL-12 can upregulate IL-12R receptor expression thereby maintaining Th1 functionality in addition to initiating differentiation of Th0 to Th1.

1.5.4 Structure of IL-12 Receptor (IL-12R)

IL-12 Receptor is only expressed on T-lymphocytes, NK cells, and B cells that have been activated. Using radiolabelled rIL-12, it has been shown that there are between 100-1000 high affinity and 1000-5000 low affinity sites per cell on PHA activated lymphocytes. IL-12R is composed of two subunits IL-12Rβ1 and IL-12Rβ2 which belong to the gp130 subgroup of the cytokine receptor superfamily. Together the two subunits form a functional high affinity receptor. The main function of the β1 sub unit is to provide a high affinity binding element whereas IL-12Rβ2 functions as the signal-transducing component of the receptor.

1.5.5 Role of IL-12 in host defence

Animal models have shown that IL-12 plays a critical role for efficient immune responses the central role of IL-12 in Leishmania major, Toxoplasma gondii, Mycobacterium Avium, Mycobacterium Tuberculosis, Listeria Monocytogenes and Cryptococcus Neoformans infections. IL-12 is induced in some but not all viral infections (Orange and Biron, 1996a; Orange and Biron, 1996b). IL-12 also has a central role in immune-mediated, non-infectious conditions. In Multiple Sclerosis and Crohn's disease IL-12 is over expressed whereas in asthma IL-12 is deficient.

Impaired IL-12 secretion has been described in viral infections. In HIV positive patients decreased *Staphylococcus Aureus* Cowan (SAC) induced IL-12 production has been demonstrated (Chougnet et al, 1996). Furthermore there is

impaired upregulation of IL-12Rβ2 in PBMC from HIV infected patients in response to mitogenic stimuli. However, IL-12R expression can be restored by rIL-12. In HCV infection IL-12 has been shown to augment antigen induced proliferation and IFN-γ production (Schlaak et al, 1998), although a recent clinical trial using IL-12 as monotherapy for chronic HCV infection was disappointing (Zeuzem et al, 1999). In a mouse model of HBV infection murine IL-12 inhibits antibody production by shifting the CD4+ response from Th2 to Th1(Milich, et al 1995). In HBV infection poor Th1 immune responses are associated with increased HBV viral load and progressive disease and hence theoretically therapies that enhance Th1 may favour viral clearance.

Poorly virulent mycobacteria and salmonella infections have resulted in severe infections due to deficiencies in IL-12Rβ1 chain, IFN-γ receptor ligand-binding chain (IFN-γR1) or signaling chain (IFN-γR2) expression in otherwise healthy individuals. Despite the presence of mature granulomas, activated natural killer and T cells secreted reduced IFN-γ concentrations, which resulted in on-going infection. Thus, IL-12-dependent IFN-γ secretion in humans seems essential in the control of mycobacterial infections, despite the formation of mature granulomas due to IL-12-independent IFN-γ secretion (Altare et al, 1998). IL-12Rβ1 deficiency is homozygous and inherited in autosomal recessive manner. In these patients PHA induced and antigen induced IFN-γ production is reduced to 5% of controls however T cell activation is unimpaired compared with

controls. Hence in IL-12Rβ1 deficient patients decreased IFN-γ production correlates with impaired infection clearance.

Alternatively diminished IL-12 levels due to a genetic mutation has been documented. In a child with bacille Calmette-Guerin and Salmonella enteritidis infection, a deletion within the IL-12 p40 subunit gene precludes expression of functional IL-12 p70 cytokine by activated dendritic cells and phagocytes. This resulted in markedly impaired lymphocyte IFN-γ production suggesting that IL-12 is essential to and appears specific for protective immunity to intracellular bacteria such as mycobacteria and salmonella (Altare et al, 1998).

1.5.6 IL-12 and HBV infection

In HBV infection, *Staphylococcus Aureus* Cowan, induced lower levels of IL-12 and IL-12p40 in immunotolerant chronic HBV carriers in comparison to controls and other groups of patients with chronic HBV (Rossol et al, 1997).

Furthermore, the importance of IL-12 in HBeAg seroconversion in chronic HBV carriers undergoing IFN-α treatment has recently been documented (Rossol et al, 1997). Patients who successfully seroconverted had higher pre-treatment serum levels of IL-12 heterodimer than non-responders. There was also a 2.5 fold increase in serum IL-12 during interferon treatment or afterwards in those who seroconverted. Furthermore, the ratio between IL-12 p40 and biologically active IL-12 heterodimer was reduced in patients who seroconverted whereas this ratio

remained unchanged in those who failed to respond. In patients who developed a significant rise in serum IL-12, the peak of IL-12 occurred after the ALT flare and either preceded or coincided with the time of seroconversion suggesting a disassociation of the mechanisms responsible for hepatocyte lysis and HBV clearance. These data are consistent with the transgenic mouse model of HBV infection where IL-12 (via IFN-γ) abolishes HBV replication non-cytolytically (Cavanaugh et al, 1997).

A recently published phase I/II clinical study indicates that IL-12 has a significant antiviral effect in patients with chronic HBV infection who failed to respond to IFN-α. Recombinant human IL-12 at three doses (0.03, 0.25, 0.5 mcg/kg) given subcutaneously once a week for 12 weeks to chronic HBV carriers with ongoing viral replication resulted in a dose-dependant anti-viral effect. 25% of patients given the highest dose of IL-12 became HBV DNA negative at the end of treatment with a smaller proportion losing HBeAg (Carreno et al, 2000).

In vitro studies have confirmed that IL-12 regulates Th1 responses in HBV infection. IL-12 augments IFN-γ production and the PBMC proliferative response to nucleocapsid antigens in chronic HBV carriers. IL-12 also induces proliferation and IFN-γ production in HBsAg-stimulated cultures in both chronic hepatitis B virus patients and naturally immune subjects (Vingerhoets et al, 1998).

From the above data IL-12 and IFN-γ are important in successful HBV clearance. The reason for the low levels of IL-12 seen in patients with chronic HBV infection is unclear.

1.6 Non-cytolytic inhibition of HBV replication.

The clearance of intracellular pathogens especially non-cytopathic viruses by the immune response was widely thought to be mediated by the destruction of infected cells by HLA class I restricted CD8+ cytotoxic T-lymphocytes. However, data from animal models especially the transgenic mouse have challenged the concept of cytolysis being the predominant mechanism by which CTL control HBV. Upon antigen recognition CTL secrete potent anti-viral cytokines such as IFN-γ and TNF-α which can cure hepatocytes of their infection or destroy them via perforin-Fas ligand (Kondo et al, 1997) and TNF-α mediated pathways (figure 7).. On the other hand IFN-γ causes liver damage in the transgenic mouse model of fulminant hepatitis (Ando et al, 1993). The relative balance of these two mechanisms has yet to be determined and the characteristics and importance of non-cytolytic mechanisms along with the relevance in human HBV infection have not been defined.

In human HBV infection the assumption that viral clearance is mediated by hepatocyte lysis alone is unlikely to be correct for several reasons (Guidotti et al, 1996). Firstly CTL may not be able to reach, recognise and destroy the vast number of infected hepatocytes in the liver by direct physical contact. Secondly,

HBV infection is usually associated with clinical recovery and not patient death despite the vast majority of hepatocytes being infected. Third the number of infected hepatocytes within the liver (1.10^{11}) is similar to the total number of lymphocytes circulating in the body (1.10^{12}). Hence cytolytic clearance of HBV infected hepatocytes would require the deployment of an unrealistically high proportion of the patients' CTL. Furthermore, HBV epitope specific CTL precursor frequencies in the peripheral blood in acutely infected patients are only in the 1.10^{5} range. In addition movement of CTL within a solid viscus is constrained and some lymphocytes undergo apoptosis and die on antigen recognition. These reasons suggest that HBV clearance mediated solely by hepatocyte destruction alone is highly unlikely. Instead cytokines secreted by lymphocytes can have a powerful anti-viral effect in a paracrine fashion.

Investigation of non-cytolytic inhibition of HBV has been mainly carried out in a transgenic mouse model that produces HBV (Guidotti et al, 1995). In contrast to human HBV the transgenic mouse model lacks natural infection and ccc HBV DNA thereby limiting the investigation to only the later stages of viral replication. The transgenic mouse model has however helped to gain insight into the immunopathogenesis of HBV infection (figure 8). CD8+ lymphocytes enter liver via blood stream, attach to hepatocytes and lyse them. This direct cytopathic effect is only seen on a few cells but antigen non-specific actions result in more widespread cell damage. CTL recruit host-derived antigen non-specific cells such as macrophages, which cause cell death resulting in necro-inflammatory foci. Macrophages are recruited 4-12 hours after CTL recruitment to the liver (Ando et

al, 1993) and usually outnumber T cells by 100:1. These cells are the main source of pro-inflammatory cytokines. Further evidence that non-antigen-specific cells are responsible for the majority of liver damage is seen with HLA-peptide tetrameric complexes that directly visualise HBV-specific CD8+ cells. In immunotolerant HBV-infected patients without liver damage functionally active HBV specific CD8+ cells are seen in the liver and peripheral blood. In contrast in patients with liver inflammation fewer intrahepatic antigen-specific T-cells are seen but instead large numbers of non-antigen-specific T-cells are present (Maini et al, 2000).

1.6.1 Cytokines and the transgenic mouse

Recombinant IL-2 and type I interferons downregulate HBV gene expression in the transgenic mouse non-cytopathically (Guidotti et al, 1994). IL-2, predominantly produced by CD4+ cells, downregulates hepatocyte HBV mRNA by up to 90%. The anti-viral effect of IL-2 is mediated by TNF-α, which was confirmed by blocking experiments with antibody to TNF-α. Levels of housekeeping genes are unchanged suggesting that IL-2 has a specific anti-viral action, which is not due to global cellular transcriptional downregulation.

Treatment of the HBV transgenic mouse with murine IL-12 results in disappearance of HBV DNA replicative intermediates in the liver and kidney with minimal associated inflammation (Cavanaugh et al, 1997). IL-12 induces IFN-γ, TNF-α and IFN α/β mRNAs, however blocking antibody to IFN-γ dramatically

diminished the expression of all the above cytokine mRNAs demonstrating that IFN-γ was the prime mediator of the anti-viral effect. Immunohistochemical studies show the disappearance of cytoplasmic HBcAg suggesting that IL-12 prevents the assembly or triggers degradation of nucleocapsid particles in which HBV replication occurs. In contrast to other studies IL-12 had no effect on RNA – the 3.5 kb and 2.1 kb envelope HBV mRNA levels were unaffected. Hence although IL-12 resulted in dramatic effects on HBV replication and serum HBV DNA it had no effect on hepatic HBV gene expression in this model. The authors suggested that perhaps this was because HBV replication was exquisitely sensitive to IL-12 not allowing sufficient time for IL-12 to induce the correct quantities/ratios of other cytokines to inhibit gene expression. The authors were unable to assess the effect on ccc DNA which is the template for on-going viral replication as the mouse model does not produce it. If ccc DNA is not cleared by this process then it is likely that the visibilty of HBV to the immune system is reduced whilst the viral transcriptional template remains in place. In addition to its anti-viral effects IL-12 may also exert immunomodulatory effects by switching Th2 to Th1 phenotype. Low dose murine IL-12 given to HBeAg transgenic mice inhibited antibody production by causing a such a shift breaking HBeAg T-cell tolerance (Milich et al, 1995).

1.6.2 Adaptive immune system and the transgenic mouse

The characteristics of the adaptive immune response were investigated with adoptively transferred HBsAg positive CTL into the transgenic mouse (Guidotti et al, 1996). Following injection these CTL killed only a small fraction of HBV-positive hepatocytes resulting in mild cell-dose dependant hepatitis. Serum ALT rose within a few hours of CTL administration, reached maximum levels after 72 hours and then returned to normal within 1 week. Histological analysis showed that the majority of dead hepatocytes were found in periphery of the hepatic lobule close to the portal veins suggesting this was the site of CTL entry. In contrast the centrilobular hepatocytes that replicate high levels of HBV appeared histologically normal. Despite only mild hepatitis developing all traces of viral RNA, replicative DNA intermediates, and nucleocapsid antigen were eliminated from the liver and serum. These effects were dose dependent and occurred in a temporal sequence. HBcAg in the centrilobular hepatocytes was lost within 24 hours, and became completely undetectable by day 5 throughout the liver despite the majority of hepatocytes being morphologically normal. This effect was not due to hepatocyte turnover. HBV mRNA started to decrease by 72 hours and was completely abolished five days after CTL administration. These changes were accompanied by IFN-γ and TNF-α mRNA induction. Hence these data show that the clearance of viral nucleocapsid and replicative intermediates occurs prior to the onset of liver disease. The loss of cytoplasmic nucleocapsids and replicative DNA intermediates prior to decreases in viral mRNA suggests that two pathways are activated. One in which IFN-γ can have a direct antiviral effects on

hepatocytes to prevent the formation of nucleocapsid particles or accelerate their breakdown and promoting elimination of viral intermediates elimination. The second pathway downregulates HBV gene expression by post-transcriptional mechanisms resulting in viral RNA destabilisation and increased degradation. Overall, this non-cytolytic antiviral effect is 10-100 times more potent than the mechanism involving liver cell destruction.

In certain circumstances IFN-γ can be cytotoxic. In the HBV transgenic mouse that overproduces the large envelope polypeptide IFN-γ is cytopathic for ground glass hepatocytes suggesting that Th1-induced cytokine mediated inflammatory processes could play a role in the development of liver disease in chronic HBV infection (Franco et al, 1997). IFN-γ can recruit non-antigen specific cells and this is a characteristic feature in the transgenic mouse model of fulminant hepatitis (Ando et al, 1993). Finally, in an influenza model of infection CTL could either promote virus clearance or cause lung pathology depending on the size of viral innoculum and tissue damage could be prevented by anti-IFN-γ (Moskophidis and Kioussis, 1998).

1.6.3 Other animal models of non-cytolytic clearance

Other animal models including woodchuck, duck and chimpanzee support the existence of non-cytolytic mechanisms for dealing with chronic viral infections. rDuIFN-γ inhibits DHBV replication in primary duck hepatocytes in a dose dependant fashion non-cytolytically. Southern blot analysis showed IFN-γ

reduced cccDNA and replicative intermediates. In contrast to the transgenic mouse this naturally infected model has shown that IFN-γ has an important effect on ccc DNA and pre-genomic RNA (Schultz and Chisari, 1999).

During acute HBV infection in the chimpanzee HBV DNA disappears from the liver and the blood of acutely infected chimpanzees long before the peak of T cell infiltration and the onset of the liver disease. Noncytopathic antiviral mechanisms contribute to viral clearance during acute viral hepatitis by purging HBV replicative intermediates from the cytoplasm and covalently closed circular viral DNA from the nucleus of infected cells (Guidotti, 1999b). Hence, the intracellular inactivation of HBV can also be the predominant mode of control of viral replication in an animal model closely related to man.

Superinfection by other hepatotropic viruses such as Lymphocytic Choriomeningitic Virus (Guidotti et al, 1999a), as well as adenovirus and CMV (Cavanaugh et al, 1998) can induce IFN-γ and TNF-α resulting in clearance of HBV in transgenic mice.

The cytokine-inducible downstream events that inhibit HBV replication have not been identified. However Nitric Oxide (NO) a pleiotropic free radical with anti-viral activity may be important. In an iNOS deficient HBV replicating transgenic mouse model HBV-specific cytotoxic T-cells were unable to exert an anti-viral effect via IFN-γ. Interestingly IFN-α/β anti-viral effects were mediated in the NO

deficient mice suggesting that the anti-viral effect of type 1 interferon is NO independent (Guidotti et al, 2000). Furthermore HBV upregulates iNOS gene expression in human hepatocytes, suggesting that NO may mediate important pathogenic events in the course of chronic viral hepatitis (Majano et al, 1998).

In conclusion the above data from animal models suggest HBV replication is inhibited by non-cytolytic mechanisms through IFN-γ. Indirect evidence also exists that non-cytolytic mechanisms may be relevant to human HBV infection however no direct studies have been done in human HBV infection to directly study the relevance of such mechanisms.

1.7 Therapeutic options for chronic HBV infection

Treatment of chronic HBV infection aims to achieve sustained inhibition of HBV replication and gene expression resulting in resolution of hepatic inflammation and thereby preventing progression of liver damage and fibrosis. Seroconversion is an important event in HBeAg+ hepatitis B virus infection as it is associated with a change in the natural history of the disease and reduced complications of chronic HBV infection. Anti-HBe+ patients (without development of pre-core mutant) have undetectable HBV DNA by hybridisation or HBV DNA levels less than 10^4-10^5 copies per ml by PCR.

Current treatment of patients with chronic hepatitis B is rarely successful unless the patient exhibits immunoreactivity to the virus (as revealed by raised

transaminases and by moderate to severe inflammation on the liver biopsy). Hence, successful treatment strategies need to account for both the replication cycle of the virus and the host immune response, as the latter is critical for sustained long-term control of viral replication.

1.7.1 Current treatment options

Patients who should be considered for treatment include patients who are HBeAg positive and HBV DNA positive, with evidence of on-going liver damage and patients who are anti-HBe positive, HBV DNA positive with pre-core mutant and on-going liver damage. In patients with the pre-core mutant variant the aim is to have undetectable HBV DNA by hybridisation or 10^4-10^5 copies per ml by PCR. Patients with compensated cirrhosis can be treated with either lamivudine or IFN-α with careful monitoring. Patients with decompensated HBV infection should be considered for transplantation, although lamivudine has been used as a bridge to transplantation with considerable improvements in liver function. Those patients who are inactive HBsAg carriers do not need treatment but require monitoring for potential complications such as hepatocellular carcinoma.

There are two currently licensed treatments – IFN-α or Lamivudine. Both are effective in patients with immunoreactivity to HBV. Retrospective analyses suggest that ALT levels can be used as a basis for recommending therapy. Response rates of greater than 50% are seen with IFN-α and lamivudine if the ALT is greater than 5 times the upper limit of normal. The responses are less (20-

35%) in patients with ALT levels between 2-5 times the upper limit of normal. In these patients the liver histology is an important factor in deciding whether to initiate anti-viral therapy. In patients with normal or minimally raised ALT levels therapy is probably best deferred and the patient carefully monitored.

Lamivudine and IFN-α have different advantage/disadvantage profiles. With lamivudine the advantages include potent inhibition of viral replication, easy administration, minimal side effects. The disadvantages include resistance induced by long-term therapy and a question mark over the durability of serological responses. The advantages of IFN-α are the limited time course of treatment, absence of viral resistance, and the excellent quality and durability of responses should they occur.

1.7.1.1 IFN-α

Interferon-α has anti-viral effects on viral RNA and the production of viral proteins (Chisari and Ferrari, 1995). In addition it also has immunomodulatory activity. Current therapy with IFN-α is 10Mu thrice weekly for 16 weeks or 5Mu daily for 24 weeks. The response rate to IFN-a is 20-40% in highly selected patients (Feinman et al, 1992; Hoofnagle et al, 1998; Korenman et al, 1991). A meta-analysis of IFN-α therapy showed that IFN-α increased the likelihood of HBeAg loss from 9.1% to 45.6% (Wong et al, 1993). There is some evidence to suggest that patients who are HBeAg positive but HBV DNA negative by hybridisation assays at 16 weeks benefit by prolonging treatment to 32 weeks

(Janssen et al, 1999). IFN-α alters the natural history of the disease by accelerating HBeAg seroconversion. A meta-analysis of the efficacy of IFN-α showed that loss of HBeAg (with or without interferon treatment) was higher in patients with high aminotransferase levels and those with adult acquired acute hepatitis. Interferon-α therapy increased HBeAg disappearance rate in chronic HBV by a factor of 1.76 (Krogsgaard et al., 1994). Those patients who are most likely to respond to IFN-α are HBeAg positive, have moderate levels of HBV DNA (less than 200 pg/ml), raised transaminases (>100 IU/ml); moderate to severe inflammation seen on liver histology without evidence of cirrhosis. Patients with HIV/HDV co-infection, immunosuppression, pre-core mutant, or HBV recurrence following liver transplant respond particularly poorly to IFN-α.

1.7.1.2 Lamivudine

Lamivudine is the only nucleoside analogue currently licensed for treatment of HBV. Nucleoside analogues compete with natural nucleotides for incorporation into growing viral DNA chain during replication. The major mechanism of action is by chain termination thereby blocking the extension of new viral DNA. Nucleoside analogues have little activity against ccc DNA which may explain the rapid reappearance of serum HBV DNA on stopping nucleoside anti-viral therapy.

Lamivudine inhibits RNA-dependant DNA polymerase (reverse transcriptase) of HBV suppressing HBV replication. Treatment with 100mg of lamivudine once

daily results in undetectable HBV DNA measured by hybridisation or bDNA assays in 90% of patients by four weeks. The loss of HBV DNA is accompanied by marked decreases of serum transaminases. After 12 months therapy with lamivudine, HBeAg seroconversion occurs in 16-21% of patients (Dienstag et al, 1999; Lai et al, 1998; Schalm et al, 2000), which rises to 27% after 24 months and 40% after 36months (Leung et al, 1999; Liaw et al, 2000). However, resistance to lamivudine in the form of the appearance of mutation in the YMDD region in the HBV DNA polymerase occurs. The mutation rate increases with the duration of treatment with lamivudine - after 1 year the YMDD resistance is 14% but this increases to 53% at 3 years.

1.7.2 Other HBV antiviral compounds

Adefovir dipivoxil. (bis-POM-PMEA). This is a nucleotide analogue with broad spectrum antiviral activity against HBV, HIV, herpes and adenoviruses. It primarily acts as a DNA chain terminator but may also have some immunomodulatory activity. It has been used at doses ranging between 5mg and 120mg – the higher doses being for HIV infection. Phase 2 studies confirmed its antiviral effect in human HBV infection. High doses (60mg and 120mg a day for 12 weeks) resulted in reductions of HBV DNA by over 4 $\log_{10}$ and HBeAg seroconversion in 20% within the following 6 months (Heathcote et al, 1998). However these high does are associated with nephrotoxicity (renal tubular acidosis, phosphate wasting and reversible renal impairment). An ongoing study of low dose adefovir will assess whether 10mg has significant antiviral effect and

can be safely administered for prolonged periods without nephrotoxicity. Significantly Adefovir has antiviral activity against lamivudine resistant strains of HBV potentially making it potentially very useful either when lamivudine resistance has developed or from the outset of treatment thereby reducing the risk of mutant development and enhancing clearance rates.

Famciclovir (Penciclovir). Famciclovir is the oral form of penciclovir which has a similar structure to aciclovir. It has antiviral effects both in vitro (Shaw et al, 1994) and in vivo (Main et al, 1996). Monotherapy with famciclovir results in development of mutants after 4-6 months. Its use is therefore limited as the development of mutants occurs earlier than lamivudine.

Emtricitabine: This nucleoside analogue is structurally similar to lamivudine. It has powerful anti-HBV effects in vitro and in woodchucks but lacks activity against the YMDD mutant of lamivudine. In a pilot study of HBeAg positive patients reductions of 2-3 logs were seen after 8-24 weeks (Gish et al, 2000). Large-scale placebo controlled trials of 1 year's therapy with emtricitabine are under way at present.

Entecavir: This has been shown to have potent antiviral effects in against hepatoma cell lines and is the most potent antiviral against Woodchuck hepatitis B virus. It is currently undergoing phase I/II human clinical trials.

Clevudine: This is a pyrimadine nucleoside analogue that has both in vitro and in vivo anti-viral activities against HBV. It suppresses WHV DNA levels in blood and HBcAg levels in the liver and the anti-viral effects continue for several months after cessation of therapy. Human pilot studies are underway at present. Its efficacy against YMDD mutant HBV is unclear at present.

1.7.3 Immunomodulatory strategies:

As an adequate immune response is needed for effective control of HBV the combination of anti-viral and immunomodulatory drugs is attractive conceptually for treatment of chronic HBV. Several immunomodulatory approaches have been tried with limited clinical effectiveness.

Thymosin α-1. This is an immunomodulatory agent derived from the thymus, promotes Th1 immune responsiveness (Mutchnick et al, 1991). Thymosin α-1 administered for between 26 and 52 weeks suppresses HBV DNA by between 50-70% and is associated with biochemical improvement. HBeAg seroconversion rates in Thymosin α–1 are similar to those seen with IFN-α albeit occurring often after treatment has been completed (Andreone et al 1996; Chien et al, 1998).

Therapeutic vaccines. The correlation between strong, polyclonal T-cell responses to HBV antigens and recovery from acute HBV infection suggest that immunisation with vaccines containing potent T-cell epitopes, core peptides and DNA may be an alternative method of overcoming immune tolerance to HBV and

thereby stimulate T-cell responses. Animal studies have shown that hepatitis B surface and core epitopes (Hervas-Stubbs et al, 1997; Mancini, et al, 1993) or DNA immunisations (Mancini et al, 1996; Rollier et al, 1999)stimulate immune responses. However, in humans clinically important immune responses to vaccination have yet to be seen. Although the combination of HBsAg and anti-HBs as immune complexes has been shown to be promising (Wen et al, 1995) other preliminary studies of vaccines in humans have proved to be disappointing. A CTL epitope derived from the hepatitis B core protein amino acids 18-27 and incorporated into a vaccine also comprised of a T-helper cell epitope and 2 palmitic acid residues (CY-1899) was investigated in a pilot study. This vaccine did initiate CTL activity but the response seen was insufficient to induce viral clearance because the CTL response was lower than that seen in spontaneous HBV clearance (Heathcote et al, 1999). A further placebo controlled study investigated the effect of intramuscular injections of pre-S2/S and S antigens. Although vaccination resulted in more patients having undetectable HBV DNA than the controls there were no significant changes in HBeAg seroconversion rates (Pol et al, 2000).

Approaches to improve therapeutic HBV vaccines include the use of multiple HBV antigens, more immunogenic routes of delivery or stronger adjuvants. An adjuvant of potential therapeutic usefulness is CpG DNA, a synthetic oligonucleotide which preferentially stimulates Th1 responses. Addition of CpG to HBV vaccine (Energix B) increases anti-HBs titres. In the transgenic mouse

immunisation of HBsAg and CpG induced anti-HBs and reduced HBsAg levels. Plasmid DNA vaccines can also induce strong Th1 responses. In the transgenic mouse DNA vaccines have been shown to down-regulate HBsAg production although human studies are awaited.

The success of multi-drug regimes of highly active retroviral therapy (HAART) in HIV suggests that monotherapy as is currently used for HBV infection is unlikely to be the optimal treatment. Todate the only study to investigate the combination of nucleoside analogues was with famciclovir and lamivudine in 21 Chinese patients. The first phase decline was more rapid in the patients treated with the combination than with Lamivudine alone. However, its effect on the long-term outcome of HBV infection has not been determined (Lau et al, 2000). Combination treatment of interferon and lamivudine has also been investigated. A recent head-to-head study of combination treatment with lamivudine and IFN-α versus monotherapy with either agent was disappointing. Combination therapy resulted in a 36% response rate (by per protocol analysis) in comparison with monotherapy with either agent (19 and 18% respectively) (Schalm et al, 2000). This study did show that a sub-group of HBV patients with intermediate immune responsiveness may benefit from immune modulation with improved seroconversion rates. Studies of pegylated interferon and lamivudine are currently under investigation.

Table: HBV antigen T-cell response

HBV Antigen	T-cell response	AVH PBL	CAH PBL	CAH Liver
Envelope	CD4+	-	+/-	+
	CD8+	+++	+/-	+
Nucleocapsid	CD4+	+++	+/-	-
	CD8+	+++	+/-	+

Table showing T cell response in acute viral hepatitis (AVH) and chronic hepatitis (CAH) to nucleocapsid and envelope antigens. The T-cell responses are subdivided into CD4+ and CD8+ responses. The responses in chronic hepatitis are subdivided into peripheral blood lymphocyte (PBL) responses and liver responses.

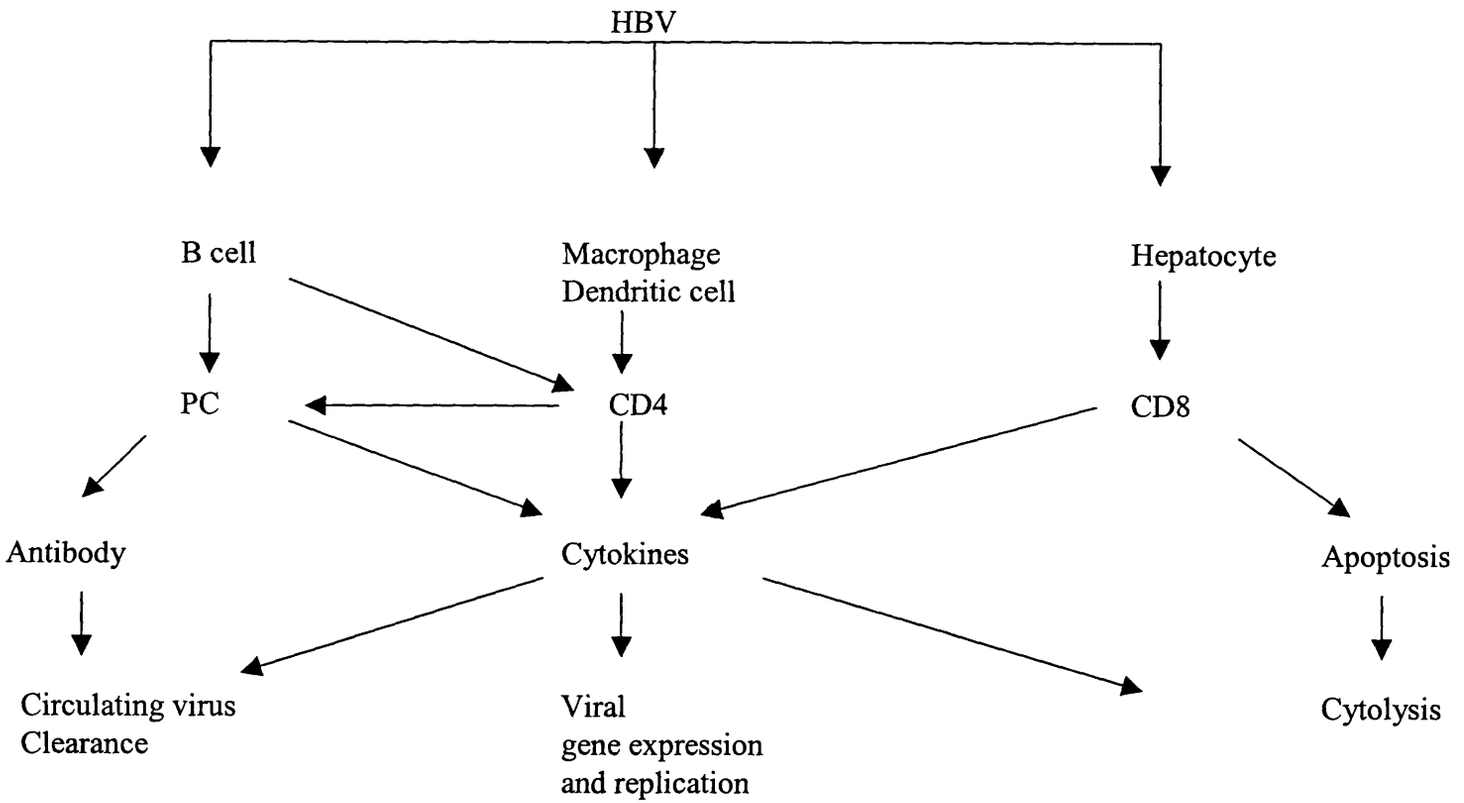


Figure 1 Hepatitis B virus immunopathogenesis. Activation of both the cellular and humoral limbs of the immune system are required for viral clearance. The cellular immune response is involved in disease pathogenesis through apoptosis and cytolysis. Non-cytolytic intracellular viral inactivation by cytokines released by virus activated lymphonuclear cells plays an important role in the clearance of the virus without killing the infected cell. The humeral immune system produces antibodies which help to clear the virus.

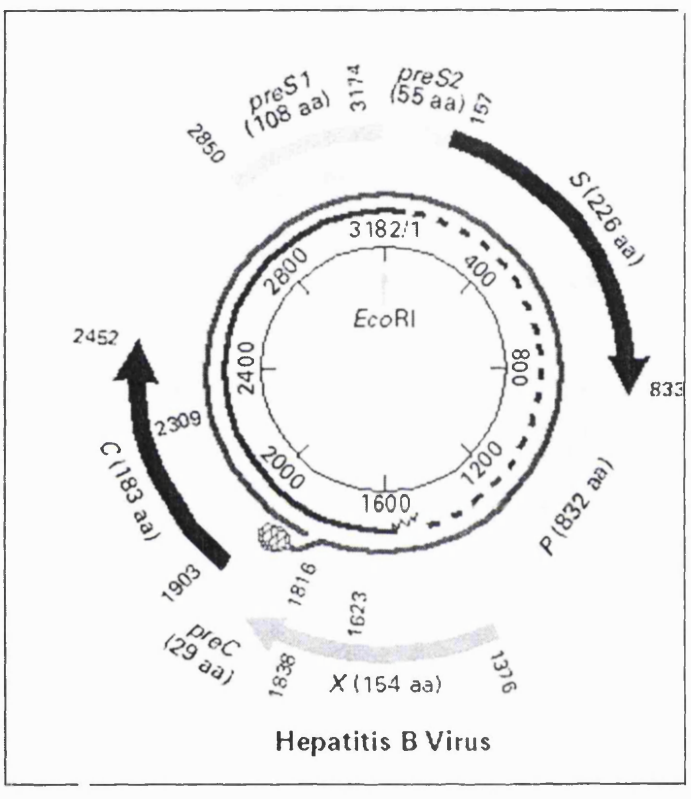


Figure 2. The DNA is partially double stranded (incomplete inner circle). The long strand of fixed length (with hatched head) encodes seven proteins from four overlapping reading frames (surface [S], Core [C], polymerase [P], and the X gene [X] shown in large arrows, and three upstream Regions (preC, preS1, and preS2). A protein is covalently linked to the 5' end of the long strand (hatched oval) and a short oligoribonucleotide at the 5' end of the short strand (zigzag). The EcoRI restriction enzyme binding site is included as a reference point. The size of each segment is shown in parentheses; aa denotes amino acids.

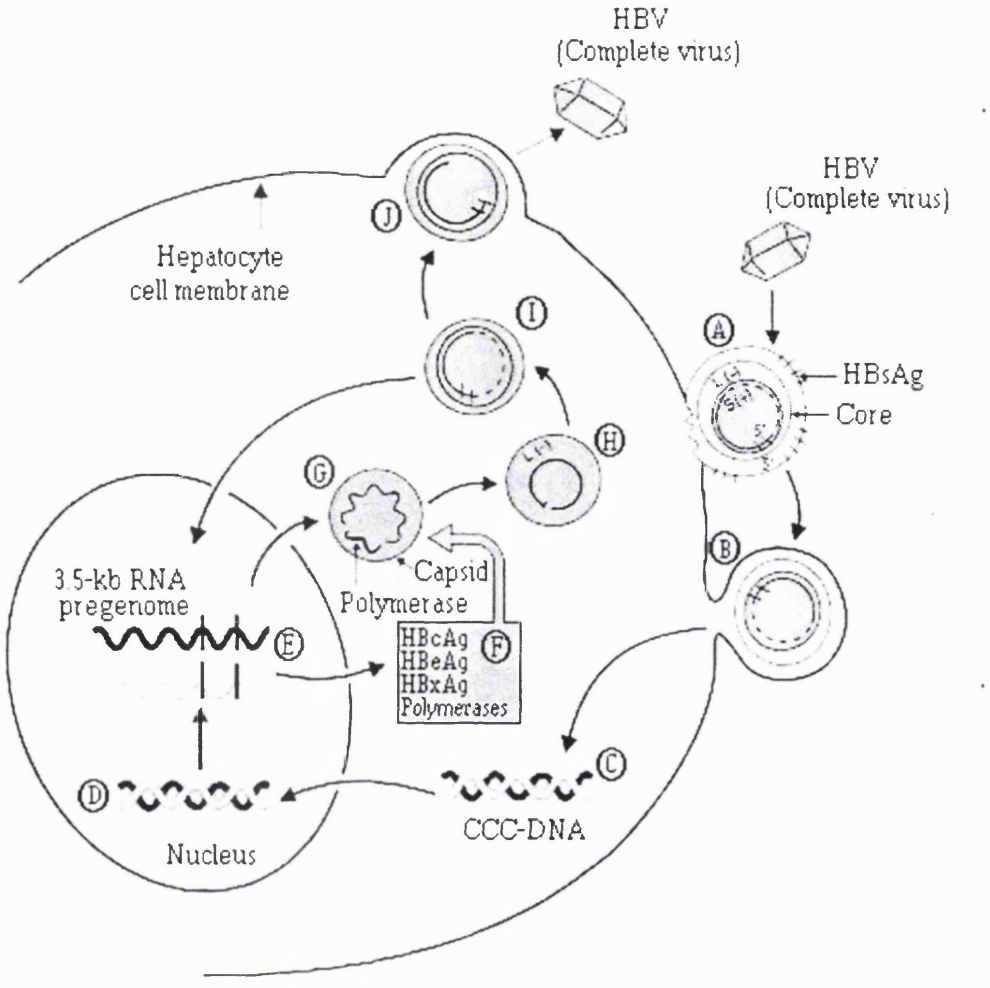


Figure 3 Hepatitis B virus replication in hepatocytes:
HBV binds to surface receptors (A). As the virus is taken up by the cell (B), the envelope is removed and enzymes extend the + strand to form a ccc-DNA (C). The HBV genome in its core migrate; to the nucleus (D), where cellular polymerases transcribe it into a long RNA. The 3.5-kb strand constitutes the pregenome containing all viral DNA sequence information. Additional viral structural protein mRNAs (E) pass into the cytoplasm and are translated (F). The pregenome and viral DNA polymerase are packaged into new capsids (G). The polymerase reverse transcribes the RNA pregenome into a new - DNA strand. The pregenome is then destroyed. The - strand (H) is then used as a template for formation of the + strand (I). Some capsids containing double-stranded DNA return to the nucleus to amplify the pool of ccc-DNA. Subsequently, the mature cores (I) are packaged into HBsAg particles, which accumulate in the endoplasmic reticulum and exit the cell (J). When the virus leaves the cell, elongation of the + strand stops immediately.

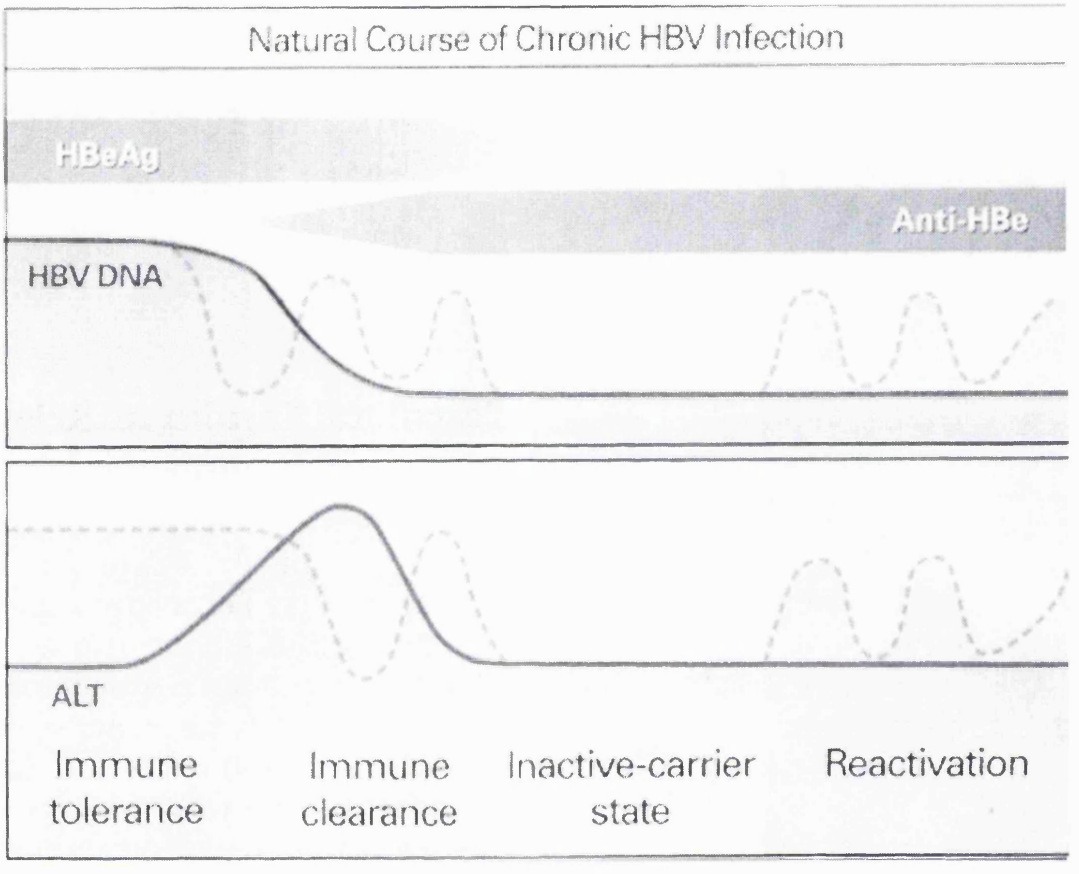


Figure 4: phases of chronic HBV infection
In patients with perinatally acquired disease the first phase is the immunotolerant phase characterised by the presence of HBeAg, and high levels of HBV DNA but normal ALT levels. These patients have very low levels of spontaneous or treatment induced clearance of HBeAg. The first phase of adult acquired and second phase of perinatally acquired HBV infection are characterised by HBeAg, high levels of HBV DNA and raised ALT (as a result of destruction of hepatocytes). HBeAg seroconversion is more common and related to flares of ALT. The development of anti-HBe is usually Accompanied by low HBV DNA levels and normal ALT levels. However some patients continue to have high levels of HBV DNA and raised ALT – HBeAg-negative chronic hepatitis (pre-core mutants). Should clearance be successful the inactive carrier develops (presence of anti-HBe, low HBV DNA and normal ALT levels). This usually persists although reactivation can occur.

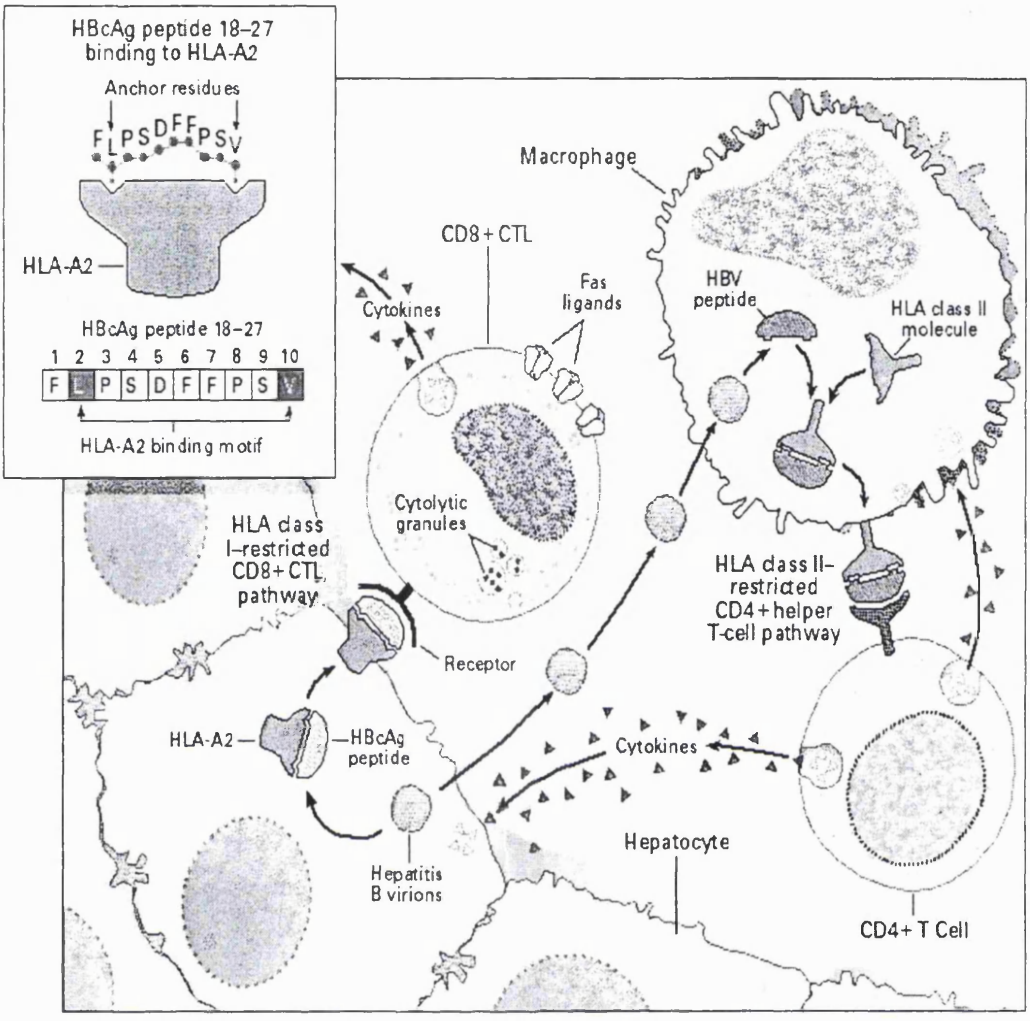


Figure 5. Pathogenesis of the immune response in HBV infection. The HLA Class 1 pathway involves the internal processing of the HBcAg peptides within hepatocytes, leading to their display on the hepatocyte surface. The fit of the most immunogenic HBV-derived peptides is determined by the host T-cell-receptor repertoire (groove configuration upper left), which in turn determines the likelihood of an effective immune response. CD8+ cell recognition of peptides displayed in the HLA binding groove initiates apoptosis mediated by Fas ligand cytokines and perforin. Externally-derived HBV antigens may also be processed by macrophages and presented to CD4 cells in a similar way. The result is increased cytokine production which augments T-cell proliferation, increases the display of HLA class 1 molecules on hepatocytes and decreased viral replication. CD4 cells can also display cytotoxic effector functions

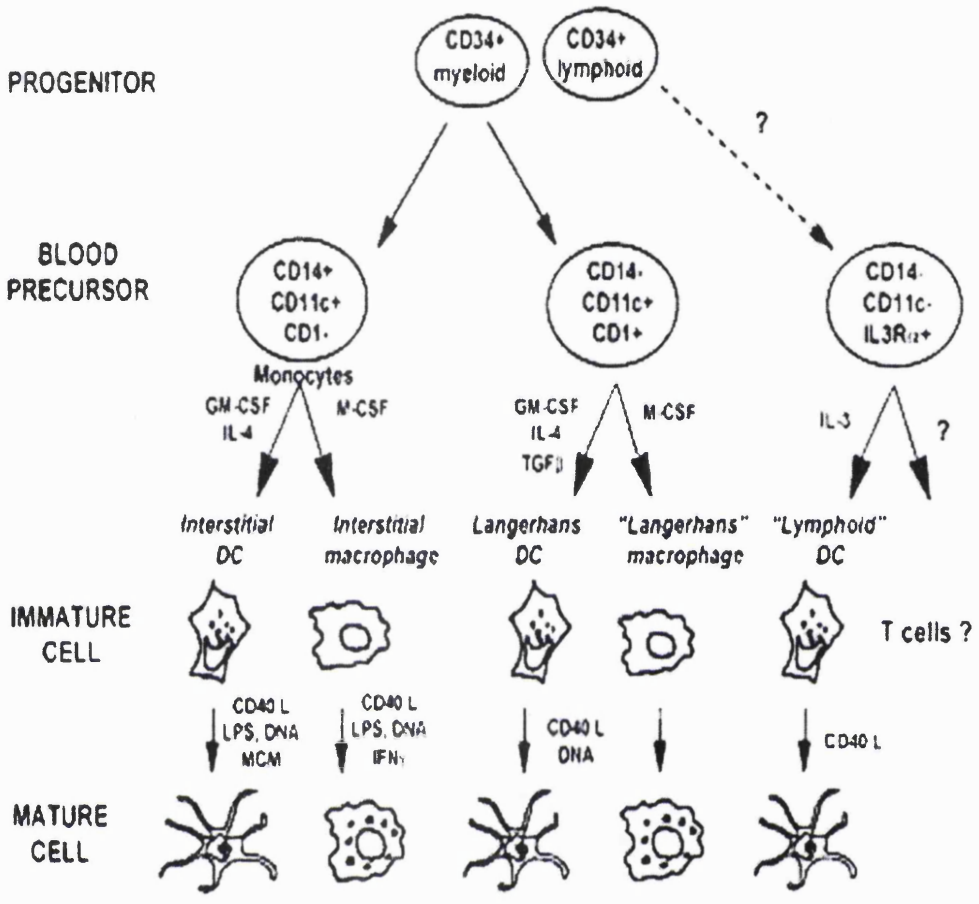


Figure 6 Several DC subsets may be produced by culture of CD34+ bone marrow or cord blood populations. The CD34+ progenitors further differentiate in to CD1a+ precursors which further differentiate into Langerhans type DCs. The CD34+ C1a- progenitors differentiate into CD14+ precursors which may differentiate either into macrophage-like cells or DC resembling interstitial type DC depending on the cytokine milieu. Uniquely, Interstitial DCs are able to stimulate B-cells to produce antibody directly. Immature cells mature in the presence of various substances as shown above.

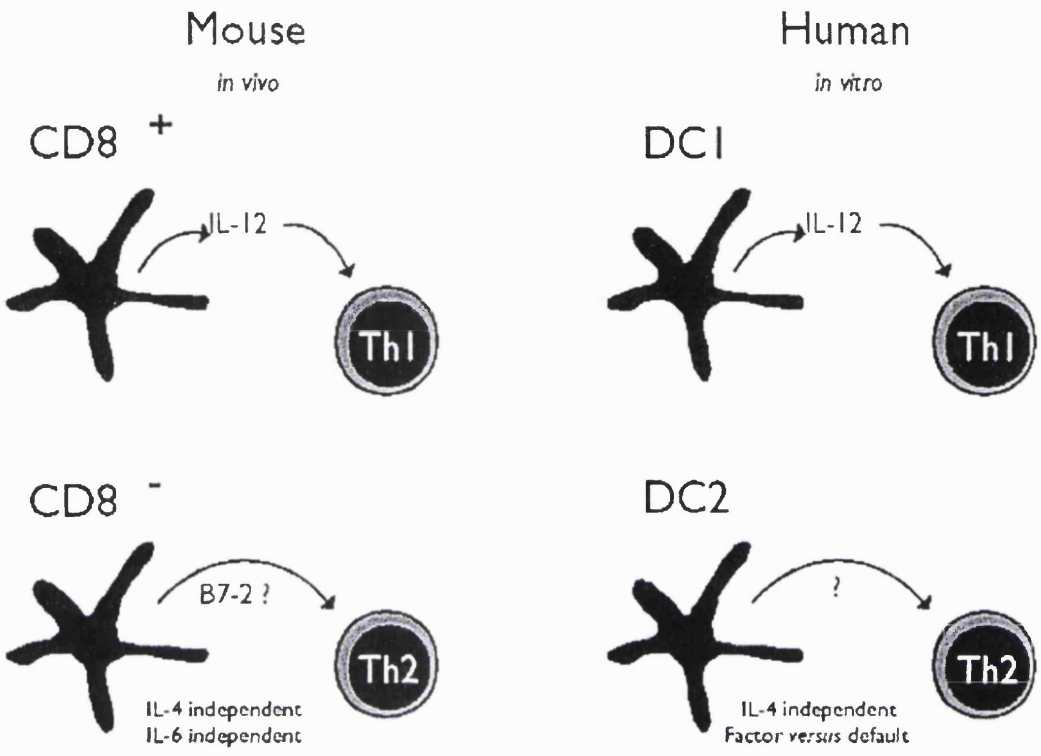


Figure 7 Dendritic cells and Th dichotomy. Mouse and human DC classes regulate the type of T-cell mediated immune response. IL-12 secreting DC subsets induce the development of Th1 cells in vitro (in humans) and in vivo (in mouse). Murine CD8α- DCs and human plasmacytoid-derived "DC2" drive Th2 development in a neutral environment. The contribution of the DC in Th2 activation remains unclear, suggesting that the Th2 pathway may occur as the "default" in the absence of IL-12.

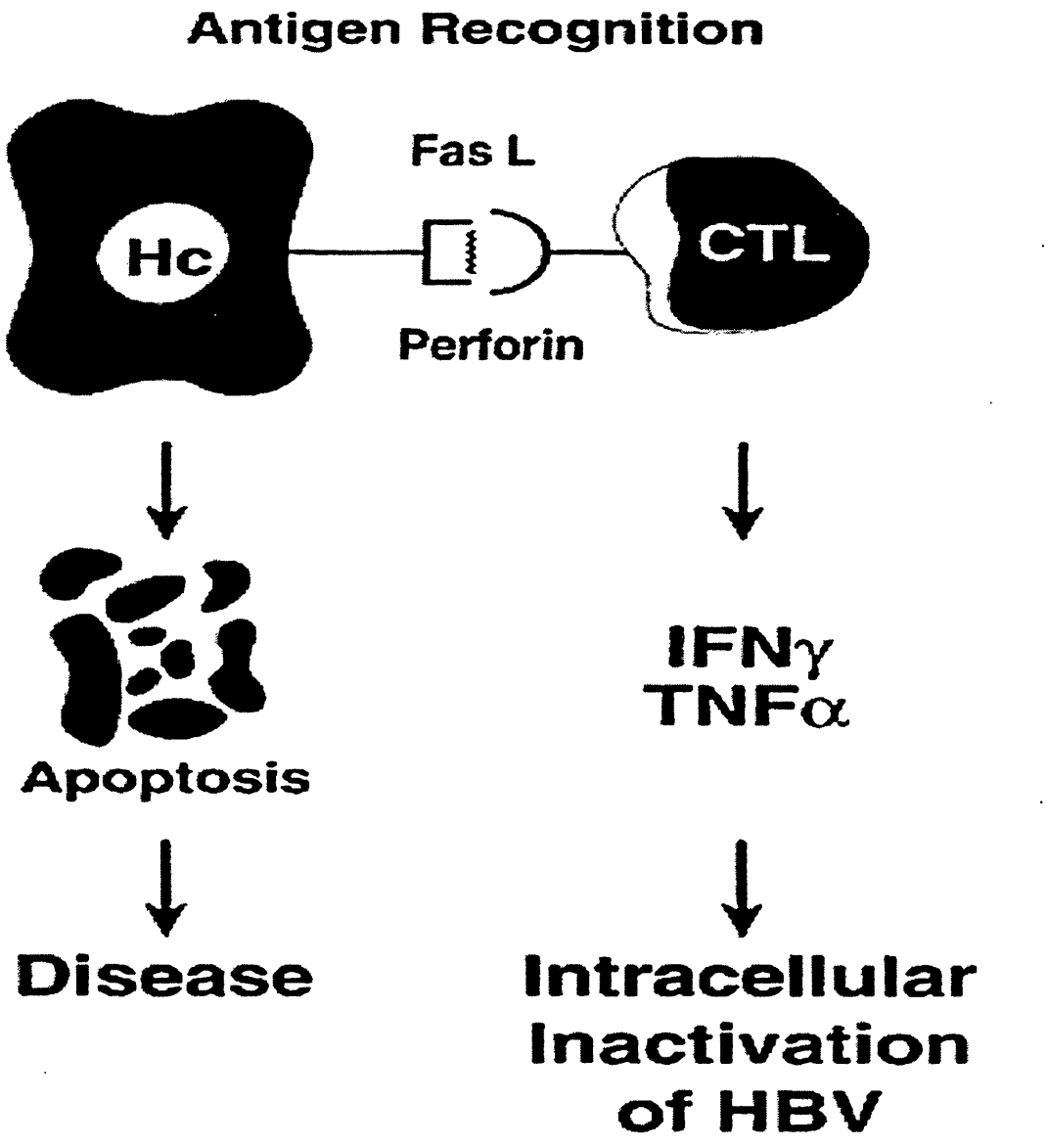


Figure 8 Non-cytolytic control of HBV infection. Upon antigen activation, CTL deliver an apoptotic signal to their target cells, killing them. They also secrete IFN-γ and TNF-α, cytokines which have been shown to abolish HBV gene expression and viral replication in vivo, curing them. The curative effect of the CTL response is several orders of magnitude more effeicient than its destructive effect. The outcome of an infection may depend on the Relative balance of these two effects with a predominantly curative response leading to viral clearance and a predominantly destructive response leading to viral persistence and chronic liver disease.

Chapter 2

Hypothesis to be tested

The hypothesis to be tested is that IFN-γ released from activated T lymphocytes represents an effector mechanism for non-cytolytic control of HBV replication in human hepatocytes. This IFN- γ production is dependent on efficient IL-12 receptor expression and adequate IL-12 production from antigen-presenting cells. Furthermore, the administration of recombinant human IL-12 would stimulate virus-specific T-cell reactivity and therefore be beneficial in the treatment of patients with chronic HBV infection.

2.1 Objectives

1. To investigate in vitro the characteristics of IFN-γ mediated non-cytolytic control of HBV infection in human hepatocytes.

2. To determine the proportion of T-cells expressing IL-12R in patients with chroi ic hepatitis B virus infection and to investigate the functionality of IL-12/IL-12R interaction.

3. To characterize the immune mechanisms involved in the resolution of chronic HBV replication HBsAg clearance following adoptive transfer of immunity to HBV in bone marrow transplant recipients.

4. To assess in vivo the anti-viral and immunostimulatory effects of IL-12 as an adjunct to lamivudine in the treatment of patients with chronic HBV infection.

Chapter 3

Materials and Methods

3.1 Patients Details

3.1.1 Human hepatocyte, co-culture, IL-12 studies and lamivudine and IL-12 trial

These patients were seen in the Liver Clinic at UCL hospital for assessment, follow-up and management of hepatitis B virus infection. No patient had received anti-viral treatment in the twelve months prior to this study. Full patient details are described in Chapters 4, 6, 7, respectively.

3.1.2 Adoptive Transfer of Immunity experiments

These patients' clinical details have been published elsewhere (Lau et al, 1998) and are described fully in Chapter 5.2.1. Briefly 8 patients with chronic HBV infection and a haematological malignancy received HLA matched allogeneic bone marrow transplant from a sibling with natural immunity to HBV. After engraftment all eight patients developed a hepatitis flare followed by clearance of HBsAg. PBMC were collected throughout the treatment course and follow-up and stored in liquid nitrogen until these experiments were carried out. In addition, 1 patient was followed prospectively.

All experiments were approved by UCL Hospitals Ethics Committee.

3.2 Tissue Culture

3.2.1 Co-culture of Peripheral Blood Mononuclear Cells and 2.2.15 cells

To investigate the anti-viral effect of PBMC from patients with HBV we developed a co-culture system of PBMC and 2.2.15 hepatoma cells that replicate HBV. As 2.2.15 cells are infectious, approval for experiments was obtained from the UCL genetic modification safety officer and all tissue culture experiments were carried out under containment level 3 conditions.

2.2.15 cells are derived from the human hepatoma cell line HepG2 and have been stably transfected with an HBV plasmid. Each cell contains four 5'-3' tandem copies of the hepatitis B virus genome such that two dimers of the genomic DNA are 3'-3' with respect to one another (Sells et al, 1987). The nuclear DNA of 2.2.15 cells includes both chromasomally integrated and full length ccc HBV DNA. Following transcription multiple HBV RNAs are secreted. The nucleic acid profile produced by these cells is similar to that of human HBV-infected hepatocytes. Like naturally infected human hepatocytes, 2.2.15 cells also secrete hepatitis B surface antigen particles, nucleocapsids, and whole virions (Sells et al, 1988). Furthermore, the 2.2.15 cell line is an accurate model of chronic HBV infection and is a predictive model of anti-viral response (Korba and Milman, 1991).

2.2.15 cells were grown in RPMI/10% foetal calf serum (Gibco, Glasgow, UK) including geneticin to select out those cells with the G418 mutation i.e. only those

cells which produce HBV DNA and other viral products. The cells were grown in $75cm^2$ tissue culture flasks and checked daily to assess growth and confluence. The cells were treated with trypsin (Gibco, Glasgow, UK) to separate the monolayer into individual cells and seeded at a concentration of 1×10^4 cells per well of a 24 well plate. The cells were grown to confluence at 37°C in 5% CO_2 which usually took approximately 14 days. The cells were then allowed to stabilise for another 2 days prior to commencement of the experiment. The presence of confluent cultures is important as they provide a cell population in a relatively homogenous state of the cell growth cycle and a consistent cell density in each separate culture and experiment. HBV replication and virus production is maximal and most stable in confluent cultures. The medium was changed for fresh sRPMI/10% foetal calf serum just prior to commencement of each experiment.

All co-culture experiments were performed in duplicate with PBMC freshly isolated from heparinised peripheral blood using standard density gradient centrifugation. A co-culture model using PBMC ($1.2.10^6$/well) and 2.2.15 cells, already grown to confluence in a 24-well plate, was set up (figure 1). The two cell types were separated by a tissue culture insert with a 0.4μm membrane, which allows the passage of soluble factors only, thereby eliminating direct cell-cell cytotoxicity. PBMC were stimulated with recombinant HBcAg (1μg/ml, American Research Products, Belmont MA, USA), recombinant human interleukin-12 (rhIL-12, 10μg/ml, Genetics Institute, Munich, Germany),

recombinant HBcAg and rhIL-12 or phytohaemagglutinin (PHA, 1μg/ml, Sigma, Poole, UK) and incubated for 6 days at 37°C in 5% CO_2. The antigen concentration and the duration of incubation was based on the experience of HBV-specific CD4+ T-cell proliferative responses and cytokine production (Penna et al, 1996; Rossol et al, 1997). Blocking experiments with anti-IFN-γ (Chemicon, Harrow, UK) were carried out to confirm that the anti-viral effect of stimulated PBMC was mediated through IFN-γ.

At the end of the experiment the supernatant was carefully aspirated to ensure no disruption to the cell monolayer and frozen at -20°C until analysis. To harvest 2.2.15 cells the monolayer was washed twice with PBS and then 100μl of Trypsin was added. The cells were incubated for 5 minutes at 37°C in 5% CO_2 and the cells then harvested by gentle aspiration. The cell suspension was centrifuged at 2000 rpm for 2 minutes to form a cell pellet. The supernatant was aspirated and discarded. The cells were immediately frozen at -20°C until further analysis.

3.2.2 Human hepatocyte isolation and culture

To investigate the effect of recombinant human IFN-γ on naturally infected human liver cells, tissue from 10 patients undergoing liver biopsy for staging of hepatitis B virus infection was used to isolate hepatocytes. Percutaneous liver biopsy was carried out using a Menghini needle. The tissue was immediately placed in RPMI/10% fetal calf serum and 0.2% EDTA to promote degradation of the connective tissue and thereby ease the isolation of the hepatocytes from

specimen. Hepatocytes were isolated in a single cell suspension by gentle mechanical disruption using two 21g needles to scrape the cells off the biopsy specimen as previously described (Lau et al, 1991). The suspension of cells was centrifuged at 1000 rpm for seven minutes to form a pellet of hepatocytes. The pellet was washed twice in 10% FCS/RPMI. The hepatocytes were resuspended in 1ml of 10%FCS/RPMI and counted in a counting chamber. The number of hepatocytes/ml was calculated by multiplying the number of cells seen by 2500.

Aliquots of 1.10^5 hepatocytes were cultured for 4 or 18 hours alone or with either 300 or 3000 pg/ml of recombinant human IFN-γ (Chemicon, Harrow, UK) at 37°C. After incubation the cells and supernatants were stored separately at -80°C.

3.3 HBV anti-viral response

3.3.1 Isolation and detection of HBV DNA in 2.2.15 cells by dot-blot hybridisation

The anti-viral effect of IFN-γ secreted by PBMC on HBV DNA was determined by measuring the cytokine's effect on cytoplasmic HBV DNA in 2.2.15 cells. DNA from 2.2.15 cells was extracted by incubating the cells with 500 µl of lysis buffer containing 80 mmol Tris/HCl pH 8.0, 150mmol NaCl, 10mmol EDTA, 1% NP40 (IGEPAL, Sigma, Poole, UK) for 10 minutes at room temperature to break down the cell membrane. The cytoplasmic fraction was separated from the nuclear fraction by centrifugation at 3,500 rpm for 5 minutes. The cytoplasmic

fraction was carefully aspirated and then incubated with 50 μg/ml Proteinase K (Sigma, Poole, UK) and 0.5% SDS for 2 hours at 56°C. DNA was extracted using Phenol/Chloroform. 300μl phenol was added to each sample to purify the DNA by absorbing proteins. The solution was centrifuged at 15,000rpm for 5 minutes to separate the aqueous phase from the phenol. This phase (containing HBV genomes) was aspirated and 300 μl chloroform/isoamyl alcohol added to remove any residual phenol. The solution was again centrifuged, the aqueous phase was aspirated and 800μl of 100% ethanol added. HBV DNA was precipitated with 40 μg tRNA and 1/10 vol 1M sodium acetate pH 5.2 and left overnight at 4°C.

The next morning the samples were centrifuged at 13,000 rpm for 30 minutes. The ethanol was aspirated and 100μl of 75% ethanol added. The water helps dissolve any salts thus improving the purity of the extracted DNA. The pellets were dried at room temperature and then dissolved in 50μl of TE buffer (pH 8.0).

DNA extracted from the cytoplasm was transferred onto a nylon membrane (Hybond, Amersham, Bucks, UK) using a manifold apparatus (Bio-Rad, Hemel Hempstead, UK) attached to a low pressure suction machine and the HBV DNA fixed to the membrane by ultra-violet light. The blot was dried at room temperature.

For hybridisation, a full-length HBV DNA probe was produced by PCR, as previously described (Gunther et al, 1995). The probe was labelled with

digoxigenin using a PCR-digoxigenin labelling kit (Roche Diagnostics, Lewes, UK). The HBV digoxigenin probe (25 ng/ml) was prepared for hybridisation by heating it to 95°C for 5 minutes. This separates probe into two strands one of which can then anneal with complementary HBV DNA fixed on the blot. To prevent annealing within the probe it was rapidly chilled to 4°C until ready for use. The blot was hybridized with the HBV-digoxygenin probe in a rotating cylinder overnight at 65°C.

For detection the blot was incubated with the alkaline phosphatase labeled anti-digoxygenin antibody solution for 60 minutes on rocking machine. The blot was washed stringently with a buffer that included 0.3% Tween 20 for fifteen minutes three times on a rocking machine. For chemoluminescence detection the blot was incubated for 5 minutes with the chemoluminescent substrate CDP-star (Roche Diagnostics, Lewes, UK) at a concentration of 1:10,000. For quantitation of HBV DNA the chemoluminescence signal from the membrane was read in a Fluor-S MultiImager (Bio-Rad, Hemel Hempstead, UK). The blot was reincubated for five minutes in a higher concentration of CDP-star (1:500) prior to exposure to X-ray film for up to 60 minutes.

3.3.2 Human hepatocytes

Because of the relatively small number of human hepatocytes that can be obtained from a liver biopsy, the effect of IFN-γ on the number of HBV DNA copies per cell was measured by quantitative PCR (Amplicor Monitor, Roche, Basle,

Switzerland). 250 µl of lysis buffer (provided in the kit by the manufacturer) was added to the hepatocyte cell pellet to break up the cell membrane. Oligo dT beads (Dynal mRNA DIRECT, Dynal, Norway) were used as per manufacturer's instructions to extract mRNA because of the high specificity of these beads for mRNA only. The remaining cell lysate was used to measure HBV DNA with the Amplicor Monitor kit.

3.3.2.1. HBV DNA extraction from human hepatocytes

The lysate was treated with 250µl of phenol/chloroform to remove excess proteins as described in 3.3.1. 75µl of 1M Na Acetate was added to precipitate HBV DNA from the lysate and the tube vortexed briefly. 750µl of 100% ethanol was added and the tubes were chilled overnight at -20°C. The following morning the tubes were centrifuged at 15000 rpm for 15 minutes. 100 µl of 70% ethanol was added to the pellet. The tubes were centrifuged again as above. The supernatant was aspirated and the tubes left to dry in air. The pellets were resuspended in 75µl of TE buffer. 50µl of TE buffer and then used for HBV DNA quantitation using the Amplicor kit in place of serum but otherwise according to manufacturer's instructions.

3.3.3.2 Isolation of HBV RNA from human hepatocytes

During the entire procedure, care was taken to ensure that there was no contamination with RNAses by using certified RNAse free tips and regular changing of gloves. mRNA was extracted from the cell lysate by adding oligo dT

magnetic dynabeads and incubating for 5 minutes. The dynabeads (with mRNA attached) was removed by applying the magnetised separator for 30 seconds. The beads were washed several times with buffers provided. The mRNA samples were treated with 1µl RNAse-free DNAse (Roche Diagnostics, Lewes, UK) for 60 minutes at 37°C prior to rt-PCR to ensure that there was no contamination with HBV DNA prior to amplification. The enzyme was inactivated by EDTA treatment and the RNA was immediately used for cDNA synthesis.

3.3.3.3 Reverse Transcription Polymerase Chain reaction (RT-PCR) for HBV mRNA

The reaction mixture for the reverse transcription (total volume 20 µl) comprised: 6 µl of mRNA, 2µl 10X PCR buffer (Gibco, Glasgow, UK), 2µl dNTP (5mmol, Gibco, Glasgow, UK), 2µl random hexamers (Promega, Southampton, UK), 1µl RNAse inhibitor (Roche Diagnostics, Lewes, UK), 6 µl RNAse free water and 1µl Omniscript RT enzyme (Qiagen, Crawley, UK). The mixture was incubated at 37°C for 60 minutes.

The cDNA was then amplified using a nested PCR with primers specific for the HBV core gene. The first round was carried out using the following primers: sense 5'-ACG ACC GAC CTT GAG GCA TAC TTC-3' (nt 1686-1709) and antisense 5'-CCC ACC TTA TGA GTC CAA GG-3' (nt 2394-2371). The reaction mix used was as follows (per sample, µl):_10X Taq buffer, 5, 25mMol

dNTP's, 0.4, 50mMol $MgCl_2$, 2, Primers 2. Taq polymerase, 0.25, water, 35.3, cDNA, 5 (Total, 50μl). The reaction mixture was heated at 94°C for 2 minutes and cycled as follows: 94°C 1 minute, 60°C 1 minute, 72°C 2 minutes for 35 cycles. The elongation step was 72°C for 10 minutes. The second round PCR was undertaken for 8 cycles with 2μl of the first round product as a template with the following primers: sense 5'-GGA GGC TGT AGG CAT AAA TTG GTC TGT TC-3' (nt 1776-1804), antisense 5'-CTG CGA GGC GAG GCA GTT CTT CTT-3' (nt 2069-2041). All PCR experiments were carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer, Warrington, UK).

The PCR products were quantitated following visualization on an ethidium bromide stained 2% agarose gel. The light emitted by the amplified cDNAs from the gel was quantitated using the BioRad imager and expressed in arbitrary units. Using serial dilutions of HBV DNA standards (from 40 to 2600 ng/ml) we confirmed that the imager is able to quantitate the amount of DNA detected in a linear range (figure 2). The signal intensity of HBV cDNA was measured and expressed as percentage change in comparison to control (untreated hepatocytes from the same patient). To determine the degree of cellular gene induction the PCR product signal strength was measured as above and this was standardised against the signal strength of β-actin run in parallel (3.5.1).

3.4 Immune response

3.4.1 T cell proliferation assay

The T-cell proliferation assay is an established method of assessing the antigen specific CD4 response (Marinos et al, 1995). When T cells recognize a ligand specific to their T cell receptor they become activated and undergo clonal expansion (proliferation). The degree of proliferation can be measured by the uptake of a radiolabelled nucleotide (^{3}H-thymidine) as this is used in DNA replication and cell division.

3.4.1.1 Reagents used for T-cell proliferation assay

RPMI 1640: This is a neutral nutrient solution used for cell culture (Gibco BRL, Life Technologies, Paisley, Scotland, UK). It was supplemented with 11.5ml of 1M HEPES (Sigma, Poole, Dorset, UK) as a buffer, 4 ml of 10M NaOH to maintain neutral pH, 0.5ml of L-glutamine to allow for any breakdown of the essential amino acid during transport and storage, and 5ml of penicillin/streptomycin (Sigma, Poole, Dorset, UK). These solutions were prepared regularly, stored at 4°C and filtered using a 0.2 micron filter (Acrodisc, Gelman Sciences, Ann Arbor, MI, USA) to ensure purity.

Lymphoprep (Nycomed, Nyegaard, Norway): Lymphoprep separates peripheral blood mononuclear cells from other blood constituents upon centrifugation according to their density.

Counting Solution: 450 ml of distilled water, 50 ml of 100% acetic acid and 5 drops of methylene blue. This solution disrupts the cell membrane, stains the cells blue allowing them to be seen and counted under a microscope.

Phytohaemagluttinin (PHA, Sigma, Poole, UK)). Used at a concentration of 1μg/ml. A positive control used to confirm cell viability and assess suitability of culture media and conditions. PHA causes non-antigen specific proliferation of all cell types in extracted PBMC.

Recombinant HBcAg (American Research Products, Belmont, USA). Used at a concentration of 1μg/ml to determine the HBV-specific CD4+ T-cell reactivity to nucleocapsid antigen.

Tetanus toxoid (Connaught Int. Laboratories, Ontario, Canada): A recall antigen used as a positive control at a concentration of 1μg/ml. Previous vaccination will generate reactive T-cell clones from memory T-cells. Used to confirm cell viability over 6 days culture.

rhIL-12 (Genetics Institute, Munich, Germany): This is a key cytokine in determining the differentiation of Th0 cells into Th1 cells. PBMC were incubated with rhIL-12 (10 ng/ml) for 6 days to determine the effect on cytokine production and proliferation.

AB serum (Gemini Bioproducts, Calabasas, California): Serum is needed to supplement the culture medium to provide essential nutrients to support cell culture and provide optimal conditions to allow cell proliferation and growth. Foetal calf serum has been used but can cause non-specific activation of T lymphocytes, which is not usually seen with human AB serum. Six different batches of human AB serum were tested with six normal volunteers' PBMC to determine which serum produced the lowest background count. The lot that produced the lowest background counts along with the highest stimulation indices in response to positive control antigens was subsequently used in all further cell culture work at a concentration of 10% - i.e. 5mls of AB serum was added to 45ml of supplemented RPMI (sRPMI).

Tritiated thymidine solution: Tritiated thymidine (^{3}H-T) stock solution (1mCi/ml) was kept in the radioactive isotope fridge and obtained from Amersham International, Little Chalfont, UK. A dose of 0.5μ Ci (micro Curie) was added to each well by adding 1 part of ^{3}H-T stock solution to 100 parts of RPMI/10% AB solution and by then adding 50μl of this solution to each well. Thymidine is taken up by any cell undergoing nuclear division and incorporated into new DNA – hence only dividing cells will incorporate ^{3}H-T. Comparison of ^{3}H-T incorporation after intervals of 8,16,24 hours was made and all these intervals resulted in similar stimulation indices being obtained.

3.4.1.2 PBMC Separation

Lymphocytes were separated from heparinised whole blood by density gradient centrifugation. 40mls of peripheral blood was taken from each patient in glass vacutainer tubes containing 100u of heparin. The blood was diluted in a 1:1 ratio with 0.9% saline. 20mls of the blood/saline mixture was carefully poured over 10mls Lymphoprep in a Sterilin plastic tube taking care to ensure a distinct interface between the two layers was maintained. The blood was spun at 1400rpm (500g) for 20 minutes at 20°C with no brake applied to ensure separation of the PBMC and the formation of a discrete layer above the lymphoprep. The top plasma layer was siphoned off and discarded. The lymphocytes were gently removed with a sterile pipette and placed in a fresh Sterilin tube.

The PBMC were washed twice with supplemented RPMI and centrifuged at 1600rpm (750g) for 10 minutes. The cell pellet was finally resuspended in 3-4mls of RPMI/10% AB serum. The cells were counted by adding 25µl cell suspension to 475µl of Counting Solution. 20µl of solution was loaded in a haemocytometer. Cells were counted in all 4 (4x4) squares. The concentration of cells ($x10^6$/ml) was calculated by dividing number of cells by 20 (dilution factor). The cell concentration was adjusted to $2x10^6$ cells/ml for the proliferation study and $3x10^6$ for the cytokine cultures by diluting with sRPMI/10%AB serum.

3.4.1.3 T-cell proliferation

For proliferation 100μl of mitogen at the appropriate concentration was added to 100μl of the cell suspension (2.10^5 cells) in a 96-well, flat bottomed microtitre-tissue culture plates (Nunclon, Life Technologies, Paisley, Scotland) at 37°C in a humidified atmosphere of 5% CO_2 in air for 6 days. Replicates of six wells were used for the background and 4 wells for each mitogen. Mean values of labeled nucleotide uptake were used to even out well-to-well variation.

In four donor/recipient pairs of patients undergoing bone marrow transplantation the HBV-specific T-cell responses to HBcAg was further defined by epitope mapping using a panel of 17 synthetic 20 amino-acid peptides (final concentration 10 μg/ml), spanning the entire HBV nucleocapsid protein. In the bone marrow transplant patients the CD4+ T-cell response to pre-S1 and pre-S2 regions of the envelope was also investigated with two 20-mer overlapping peptides covering the immunodominant area in the pre-S1 region between aa 21 to 48 (aa 21-40 PLGFFPDHQLDPAFGANSNN and aa 29-48, QLDPAFGANSNNPDWDFNPI); and two 20-mer overlapping peptides covering the immunodominant area in the pre-S2 region between aa 146 to 174 (aa 146-165, SSSGTVNPAPNIASHISSSS and aa 156-174, NIASHISSSSARTGDPVTN).

3.4.1.4. Cell harvesting

Cells were harvested onto a glass fibre filter (Wallac, Tuyku, Finland) using a matrix harvester. The 96 well microtitre plates were placed in the harvester and

aspirated by 96 nozzles using a vacuum pump. The content of each well is sucked through the filter and the glass fibre strands in the filter trap cells whilst solution passes straight through and is discarded. In this way the 96 areas on the filter correspond to each well of the 96 well plate and only ^{3}H-Thymidine that has been incorporated into cells will be retained by the filter.

3.4.1.5 Calculation of stimulation index (SI)

The filter was air-dried overnight. The following morning a layer of melted wax applied (which acts as a scintillant) and the amount of radiolabel incorporated into the DNA was measured by an automatic direct beta-counter (TriLux, Wallac, Tuyku, Finland). The β-radiation emitted by each of the 96 areas on the filter was measured and expressed as a count per minute (CPM). The CPM for the six wells cultured with medium alone was averaged to calculate the background count. Similarly the mean CPM for each antigen was calculated. The stimulation index (SI) was calculated by dividing the mean count of each antigen tested by the mean background count. The filters were kept as a hard copy of the data as the half-life of ^{3}H-Thymidine is sufficiently long to allow re-reading of the filter several months later if necessary. A proliferative response was considered significant if the stimulation index in mitogen-cultured cells was greater than 2.5 (which was higher than the mean plus two standard deviations in 20 HBV negative subjects). Positive controls in each experiment included PHA and tetanus toxoid, which act as an internal check of the viability of cultured cells.

3.4.1.6 Confirmation of HLA class II restriction of lymphoproliferation

CD4+ T-cell restriction of lymphoproliferative responses was confirmed by progressive CD4+ cell depletion using immunomagnetic beads (Dynabeads, Dynal, Wirral, UK). PBMC were incubated with on ice for 20 minutes with magnetic dynabeads coated with anti-CD4+ monoclonal antibody and then placed in magnetic separator to remove the dynabeads with CD4+ cells attached (negative selection). The remaining PBMC were decanted into a new container. The percentage of CD4+ PBMC in the decanted solution was measured by Fluorescent activated cell sorting (FACS, Cellquest, FACScan, Becton and Dickinson, Oxford, UK). CD4 depleted PBMC were then cultured to see whether lymphoproliferation occurred in response to standard antigens.

3.4.2 Antigen-specific T-cell IFN-γ production

This was assessed by in vitro cytokine production by PBMC or by the elispot assay to determine the number of HBV specific IFN-γ producing CD4+ and CD8+ T cells.

3.4.2.1 In vitro IFN-γ measurement

IFN-γ level in cell culture supernatants was measured using a commercial solid phase ELISA kit as per manufacturers instructions (Biosource Watford, UK). Cell culture supernatant and peroxidase-labelled monoclonal antibody to IFN-γ was added to each well of a 96 well flat bottomed plate coated with monoclonal antibody to IFN-γ. After incubation and washing, the enzymatic reaction was

terminated using a peroxidase specific substrate. IFN-γ concentrations were calculated by comparing the absorption characteristics measured with a multiwell ELISA reader (Dynex Technologies, Ashford, UK) with a 490 nm filter against standards of known cytokine concentrations.

3.4.2.2 Elispot assay for HBV-specific IFN- γ CD4+ and CD8+ T cells

Frozen PBMC were used for all elispot assays. PBMC were cryopreserved in a freezing mixture of complete culture medium (RPMI) with 75% fetal calf serum and 10% dimethyl sulfoxide (Sigma, Poole, UK). The cells were aliquoted in Nunc cryotube vials ($5x10^6$ per vial), placed in a purposely designed Nalgene cryocontainer (Merck/BDH, Poole, UK) with isopentane and kept at –80°C for 24 hours. The vials were subsequently transferred in liquid nitrogen for long-term storage. After thawing the cell viability was greater than 95%, as assessed by trypan blue exclusion. Cryopreserved PBMC were used to determine the number of HBV-specific, IFN-γ producing CD4 positive T cells, as described (Jung MC, 1999). Briefly, 96-well millititer plates (Milipore, Bedford, MA) were coated overnight at 4°C with a primary antibody to human IFN-γ (Mabtech, 1-DIK, Nacka, Sweden) at a concentration 10 μg/ml. In parallel, PBMC at $2x10^5$/well were cultured in triplicate in RPMI/10% human AB serum with rHBcAg (final concentration 2 μg/ml), rHBsAg (10 μg/ml) and PHA (2 μg/ml) or medium only. After 28 hours the cells were transferred to the coated plates and cultured under the same conditions for 20 hours. The plates were washed and 100 μl of biotin conjugated anti-IFN-γ antibody (Mabtech) was added to each well for 2 hours.

Next, the plates were washed and incubated for further 2 hours with 100 μl of streptavidin-alkaline phosphatase (Mabtech). The enzyme reaction was developed with freshly prepared nitro-blue tetrazoilum chloride/bromo-chloro-inolyl-phosphate toluidine salt (NBT/BCIP, Roche Diagnostics Ltd., Lewes, UK). The reaction was stopped with distilled water and the spots were counted under a dissection microscope Nikon SMZ800 (Nikon Ltd., Kingston upon Thames, UK) equipped with a graticule eyepiece. The number of specific spot-forming cells (SFC) was determined as the mean number of spots in the presence of an antigen minus the mean number of spots in the wells with medium only and expressed per $1x10^6$ PBMC.

For the CD8+ T cells, the 96-well plates (Milipore) were coated overnight at 4°C with an antibody against human IFN-γ (Mabtech) at concentration 15 μg/ml. PBMC ($2x10^5$/well) from HLA A2 positive individuals were cultured for 18 hours at 37°C with two synthetic peptides, corresponding to the known HLA A2-restricted CTL epitopes in the HBV core - aa 18-27 (FLPSDFFPSV) and the HBV surface - aa 335-343 (WLSLLVPFV) (Chisari and Ferrari, 1995) were used to determine the number of HBV-specific CD8+ T cells in the Elispot assay.- for HBV nucleocapsid aa 18-27, and for HBV surface aa 335-343(Chisari and Ferrari, 1995). The peptide concentration and that of anti-CD3 (used as a control) was 10 μg/ml. The plates were washed and the detection was the same as in the CD4 Elispot assay with biotin-conjugated anti- IFN-γ, followed by streptavidin-

alkaline phosphatase and the enzyme substrate NBT/BCIP (Roche Diagnostics Ltd., Lewes, UK).

3.4.3 Flow cytometry

Flow cytometry is a technique for making measurements on cells as they flow singly in a fluid stream through a sensor. An important feature is that measurements are made separately on each cell within the suspension and not just average values for the whole population. The laser flow cytometer measures cellular parameters on the basis of light scatter and fluorescence. Fluorescent dyes can be conjugated to monoclonal antibodies to identify cell surface markers. If more than one cell surface marker is to be measured then the fluorochromes used to label each cell surface marker must have non-overlapping emission spectra to prevent optical cross-talk from occurring.

Fluorescence occurs when a molecule excited by light of one wavelength returns to the unexcited state by emission of a light of a longer wavelength. Optical filters can separate the exciting and emitting light from one another. The technique is highly sensitive because a positive signal is detected against a negative background. The ability to detect fluorescence simultaneously in more than one compound fluorescing at different wavelengths enable several parameters to be measured. The goal of flow cytometric immunofluorescence analysis is to use light scattering and antigenic characteristics of each cell in a sequence to assign a

cell to a specific group of cells having similar properties. The detection of multiple cell surface markers can be used to precisely identify T-cell subsets.

Flow cytometry was used to investigate changes in the expression of CD4+ T cell surface markers at different time points in the patients undergoing bone marrow transplantation. We compared the expression of various markers at two time points – at the time of hepatitis flare and also at last follow-up to determine the phenotypic changes in CD4+ cell surface markers that accompany HBsAg seroconversion. We also measured the expression of these markers in the donor. To determine the effect on CD4+ activation changes in CD25 and HLA-DR expression were measured. To investigate whether the changes in CD4+ cells seen were on naïve or memory cells CD45RO (a marker for memory T cells) and CD45RA (a marker for naïve T cells) expression were measured.

Unstained cells were used to define the T cell population according to forward and side scatter characteristics. CD4 PE and CD8 FITC monostained cells were used to set the compensations. On each patient the phenotypic cell surface marker expression was analyzed by flow cytometry on cryopreserved PBMC after dual staining with phycoerythrin (PE) and fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibodies. The following combinations were measured: anti-CD4-PE/anti-CD8-FITC; anti-CD4-FITC/anti-CD25-PE; anti-CD4-FITC/anti-HLA DR-PE; anti-CD4-FITC/anti-CD45RO-PE and anti-CD4-FITC/anti-CD45RA-PE (all antibodies from Pharmingen, CA, USA).

Frozen PBMC were isolated from patients by density gradient centrifugation as described above and stored in liquid nitrogen as described in 3.4.2.1. 2.10^5 cells were suspended in 20 µl of PBS/1% foetal calf serum. They were added to alternate wells in a round-bottomed 96 well plate to minimise contamination. Fluorochrome labeled antibodies in the combination described above were added to the appropriate well at a final concentration of 1:20. The cells were incubated for 30 minutes on ice in the dark.

The cells were washed twice with 150µl PBS/1% FCS. Each wash was followed by centrifugation at 1500 rpm for 5 minutes to pellet the cells. The cells were finally resuspended in 125µl PBS/FCS for analysis.

3.4.4 Measurement of IL-12 receptor expression by FACS

All experiments were undertaken on freshly isolated PBMC. Activation of lymphocytes is required for high affinity binding of labelled IL-12, as IL-12R is not expressed on non-activated lymphocytes. We confirmed that there was no expression of IL-12R on unstimulated PBMC.

For activation PBMC at a concentration of 1 X 10^6/ml per well were incubated with 10% human AB serum/RPMI in a sterile flat-bottomed 96 well plate. Antibody to CD3 (Chemicon, Harrow, UK) at a concentration of 1µg/ml was

added and the PBMC incubated for 3 days at 37°C in a humidified atmosphere of 5% CO_2 in air.

As no fluorochrome-labelled anti-IL-12R antibody was commercially available we developed a two-layer antibody system – a mouse anti-human IL-12R antibody followed by a fluorochrome-labelled rabbit anti-mouse antibody as a second layer. 200,000 PBMC were incubated with 25μg/ml of mouse anti-human IL-12Rβ1-specific monoclonal antibody (Genetics Institute, Munich, Germany) for 40 minutes. The cells were washed twice in PBS/1% FCS and then incubated for 30 minutes with an anti-mouse FITC (Sigma, Poole, UK) antibody as a second layer. As a control normal mouse immunoglobulin (Chemicon, Harrow, UK) was used as a first layer. During all incubations the cells were kept on ice and in the dark. The cells were finally washed twice and then resuspended.

All acquisition and analysis were performed immediately by FACScan analyser (Becton Dickinson Immunocytometry system, CA, USA) and with CELLQuest software (version 1.0, Becton Dickinson Immunocytometry system, CA, USA), respectively on an Apple Macintosh computer.10, 000 gated events were counted. The number of cells binding the antibody was divided by the total number of gated cells and expressed as a percentage.

The in vivo effect of exogenous recombinant human IL-12 on IL-12R expression was evaluated in 9 HBsAg/HBVDNA positive patients. These patients were

administered rIL-12 at 200 or 500ng/kg (Genetics Institute) twice-weekly subcutaneously as a treatment for chronic HBV infection. PBMC isolated before and after 4 weeks of treatment and the expression of IL-12R on activated PBMC was analysed as described above.

3.5 Host Markers

3.5.1 Detection of mRNA expression of IFN-γ inducible genes

Interferon regulatory factor-1 (IRF-1), inducible nitric oxide synthase (iNOS), and intracellular adhesion molecule-1 (ICAM-1) are genes induced by IFN-γ. Hence the responsiveness of 2.2.15 cells and human hepatocytes to IFN-γ was monitored by the induction of these genes. The responsiveness of these genes to recombinant IFN-γ was compared with the anti-viral effect of this ytokine as measured by the effect on HBV mRNA.

In 2.2.15 cells, reverse transcription PCR was carried out with 10μg of total RNA using the RNAeasy kit (Qiagen). mRNA was extracted with oligo dT dynabeads and reverse transcription with random hexamers carried out in the same conditions as human hepatocytes. 2.5 μl of template was added to 20μl reaction mixture for each PCR (as described in 3.3.3.3).

For detection of IRF 1 the following primers were designed on the basis of human interferon regulatory factor 1 mRNA sequence (GeneBank acc. no. NM_002198): 5'-GGG GAT GGG CCT CCC TCC TTG GGT C-3' (nt 1299-1324), 5'-GCG

AGA CCC TCT CCA CCA GGC AGC TGG G-3' (nt 1495-70). The amplification reactions for IRF-1 consisted of 50 cycles of 95°C for 1 minute, 65°C for 1 minute and 72°C for 1 minute. The elongation step was 72°C for 10 minutes. For iNOS we used primers and conditions as described previously (Majano et al, 1998). ICAM-1 was detected with commercial primers in the conditions suggested (R & D, Abingdon, UK). The amplicons were 196 bp, 370 bp and 750 bp for the three PCRs respectively (IRF-1, iNOS, and ICAM-1).

PCR for β-actin. Reverse transcription PCR for β-actin mRNA was carried out in parallel to confirm that changes seen were not due to sample loss. The following primers were used; sense: 5'-GCT CTA GAG CGGG TCA GAA TTC CTA-3', antisense: 5'-GGA ATT CCG TGC CAG ATT TTC TCC AT-3'. The reaction mixture was as follows (per sample, μl): 10X Taq buffer, 5; 25mMol dNTP's, 0.4; 50mMol $MgCl_2$, 2; Primer 1, 1; Primer 2, 1; Taq polymerase, 0.25;water, 35.3; cDNA, 5; Tot 50. The samples were run using the same conditions as the HBV PCR.The GeneAmp 9600 thermal cycler (Perkin Elmer, Warrington, UK) was used for all PCR experiments.

3.5.2 Evaluation of cytotoxicity

The potential cytotoxic effect of IFN-γ on human hepatocytes and on 2.2.15 cells in the co-culture system was assessed by measuring LDH in cell culture supernatants. LDH is an intracellular enzyme that is released on cell death which catalyses the conversion of lactate to pyruvate. This process is accompanied by the conversion of NAD^+ to NADH & H^+. This LDH cytotoxicity assay (Roche Diagnostics, Lewes, UK) uses this reaction to convert a yellow dye tetrazolium to tarmazan (red). The experiment was carried out as per manufacturer's instructions. Briefly, 100µl of cell supernatant was mixed with 100µl of assay solution and incubated for 30 minutes in the dark at room temperature. The plate was then read by a multiwell ELISA reader (Dynex Technologies, Ashford, UK) with a 490 nm filter. Controls included, assay medium alone (background), untreated cells (low control) and cells treated with 1% Triton-X solution to determine maximal LDH release by the cells (high control). Cytotoxicity was calculated using the following equation.

$$\text{Cytotoxicity (\%)} = \frac{\text{experiment value - low control}}{\text{high control - low control}} \times 100$$

3.6 Standard Methods

3.6.1 Routine viral serology

All routine viral serology was carried out by the department of Virology University College London. Serum HBsAg, HBeAg and IgM anti-HBc were detected by Murex G15, (Abbott Diagnostics, Maidenhead, UK); enzyme immunoassays were used to test for antibodies to HCV and HIV 1/2 (Abbott Diagnostics) and to HDV (Sorin, Wokingham, UK). Serum HBV DNA level was quantified by a signal amplification assay (Quantiplex^{TM} bDNA assay, Chiron Corporation, Emerville, CA). Using this assay, the threshold for HBV DNA detection in serum is $0.7x10^6$ viral copies/ml (0.7Meq/ml).

3.6.2 Y-chromosome PCR in bone marrow transplant patients

In 5 of 8 patients there was a bone marrow transplant from a male donor to a female recipient. We took advantage of this sex mis-match to determine origin of the recipients' PBMC following bone marrow transplantation using a Y chromosome PCR assay. Fresh PBMC, collected at the last follow-up, from these five male patients were analyzed. CD4+ T cells were purified by immunomagnetic separation using Dynabeads (Dynal, Oslo, Norway), according to manufacturer's instructions. The purity of the CD4+ T cells was confirmed to be greater than 90% by FACS analysis using a PE-labeled anti-CD4 antibody. DNA was extracted from the purified CD4+ T cells and microsatellite PCR for detection of the Y chromosome was performed using 6 different sets of primers. The six Y-chromosome microsatellite loci - DYS19, DYS388, DYS390,

DYS391, DYS392 and DYS393 were amplified in single 10 µl multiplex PCR reactions using 1 µl of DNA template, as previously described (Thomas et al, 1999). One primer in each primer pair was labeled with one of three ABI dyes (HEX, TET and 6-FAM) and PCR products were run on an ABIÅ-310 genetic analyzer using GeneScan using POP-4 polymer and a 36 cm POP-4 capillary. The size of the PCR products ranged from 123 bp to 208 bp and individual microsatellites were identified by a combination of size range and dye label. This was kindly carried out by Dr Mark Thomas (Department of Anthropology, UCL).

3.6.3 Assessment of virological responses in lamivudine and IL-12 study (chapter 7)

Serum HBV DNA level was quantified by the signal amplification assay (Quantiplex^{TM} bDNA assay, Chiron Corporation, Emerville, CA). Using this assay, the threshold for HBV DNA detection in serum is $0.7x10^6$ viral copies/ml (Hendricks et al, 1995). Samples between weeks 4 and 16 were then re-tested for HBV DNA using a quantitative polymerase chain reaction assay (SuperQuant, NGI, Los Angeles, USA).

3.6.4 Statistics

Data was analysed using appropriate tests as described in each chapter

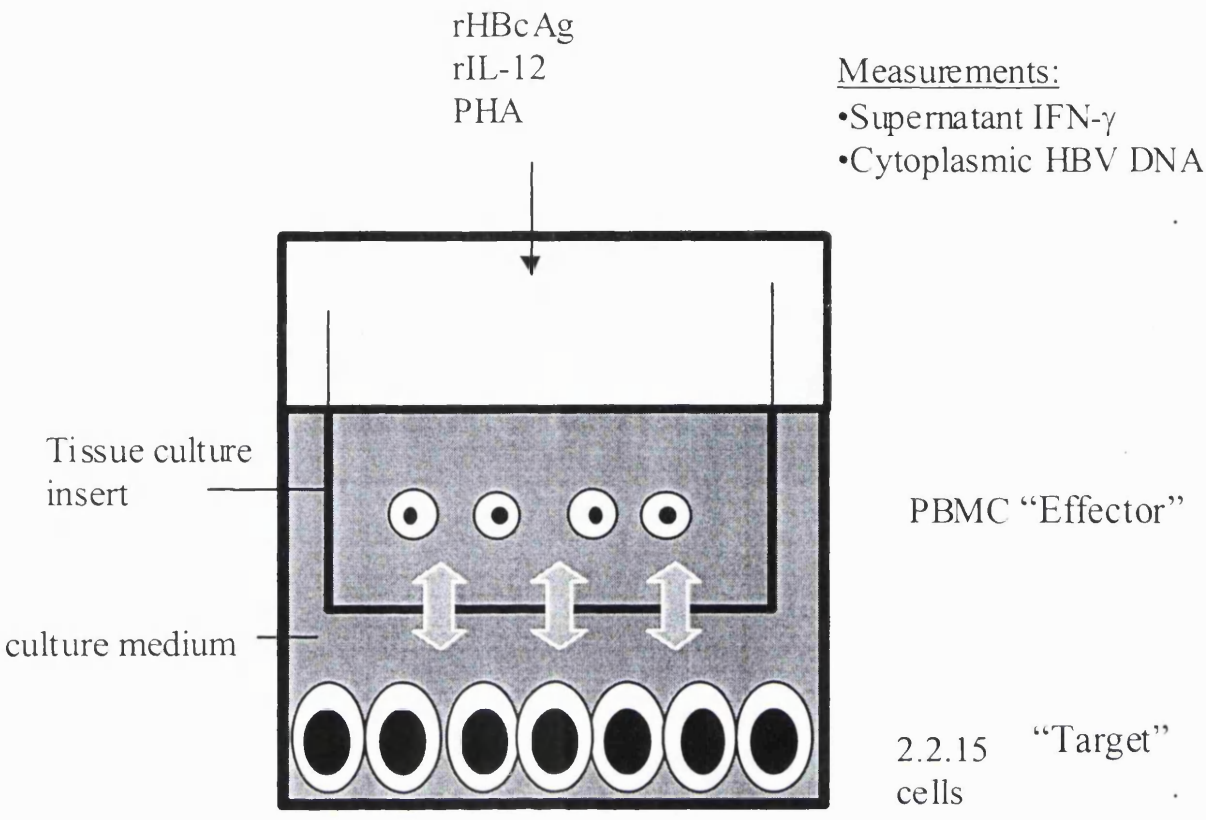


Figure 1 Co-culture model of PBMC from patients with HBV and 2.2.15 hepatoma cells. This diagram represents a single well of a 24 well plate. 2.2.15 cells grown to confluence at the base of a single well of a tissue culture plate ("target cells"). Above these cells, are PBMC from patients with hepatitis B virus infection ("effector cells"). The two cell types are separated by a tissue culture insert (0.4μm) which allows the passage of soluble factors (such as cytokines) thereby eliminating direct cell-cell cytotoxicity. PBMC are stimulated by HBcAg, and/or rhIL-12, or PHA and the cells cultured for six days.

The anti-viral effect of the PBMC was assessed by the effect on cytoplasmic HBV DNA levels in 2.2.15 cells. The levels of IFN-γ and LDH released was measured in cell culture supernatants.

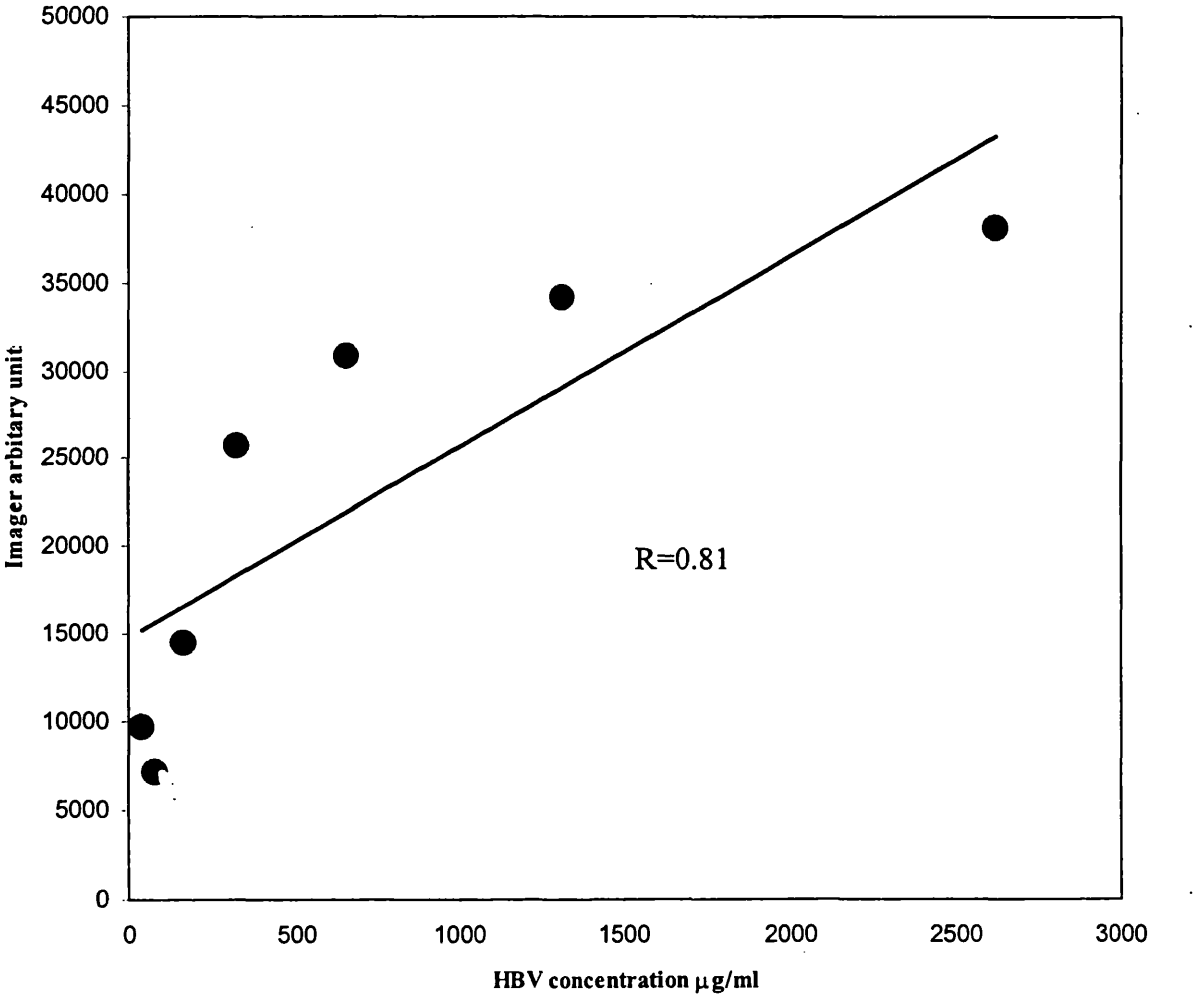


Figure 2 Bio-Rad imager arbitary units compared with serial dilutions of HBV product. This graph shows that the Bio-Rad imager is able to read diliutions of HBV product in a linear range in the concentrations produced by the co-culture experiments.

Chapter 4

Non-Cytolytic Inhibition of Hepatitis B Virus Replication In Human Hepatocytes

4.1 Background

The classical concept that hepatitis B virus (HBV) clearance is mediated by cytotoxic T-lymphocytes (CTL) which destroy the infected hepatocytes, has been challenged by recent studies which revealed that virus-specific CTL can abolish HBV gene expression and replication in the liver without killing the infected cells (Chisari, 1995; Chisari and Ferrari, 1995; Guidotti et al, 1996). This non-cytolytic antiviral effect, first demonstrated in an HBV transgenic mouse model and more recently in duck HBV infection, is primarily mediated by IFN-γ, secreted by T lymphocytes (Cavanaugh et al, 1997; Guidotti et al, 1996; Schultz and Chisari, 1999). Indirect evidence from healthy subjects with past exposure to HBV and from patients with chronic hepatitis B indicate that non-cytolytic immune control of HBV replication may also be relevant for humans. Firstly, traces of HBV DNA usually persist in healthy individuals after recovery from acute self-limited hepatitis B, but the maintenance of strong T lymphocyte responses, keep the virus under control (Penna et al, 1996; Rehermann et al, 1996). Secondly, in patients with chronic hepatitis B undergoing interferon-α treatment, it has been shown that effective control of HBV replication occurs in those who develop a substantial

rise of interleukin-12 and IFN-γ production (Rossol et al, 1997). Apart from an antiviral effect, IFN-γ is cytotoxic for various cells (Nagao et al, 1997; Toyonaga et al, 1994). In particular, hepatocytes with intracellular accumulation of HBsAg are more sensitive to IFN-γ induced cell damage (Gilles et al, 1992). The distinction between the antiviral and the cytotoxic effects of IFN-γ on HBV infected human hepatocytes is not well characterised. In this chapter the non-cytolytic inhibition of HBV replication in human liver cells was investigated using two approaches. Firstly, an *in vitro* model was developed using lymphocytes from patients with HBV and a hepatoma cell line, which produces HBV to determine whether IFN-γ secreted upon stimulation from these cells can inhibit HBV replication. Secondly, the direct antiviral effect of recombinant human IFN-γ on HBV replication was assessed in naturally infected human hepatocytes. The release of LDH was used to determine whether the changes in the HBV DNA were due to cell cytotoxicity.

4.2 Materials and Methods

4.2.1 Patient details

Fourteen patients (aged 22-45) with HBV infection were studied. One patient had acute hepatitis B (IgM anti-HBc positive) and was in the icteric phase, while the rest were chronic HBsAg carriers (serum HBsAg positive for greater than 12 months). All 13 patients with chronic hepatitis B were seropositive for HBeAg and HBV DNA. All patients were seronegative for hepatitis C virus, hepatitis Delta virus and autoantibodies.

Routine hepatitis B virus serology was measured by the Department of Virology UCL hospitals as described in chapter 3.6.1.

4.2.2 Co-culture of Peripheral Blood Mononuclear Cells and 2.2.15 cells

Full experimental details are described in Chapter 3.2.1 but briefly, fresh PBMC (13 patients with chronic HBV and 1 with acute HBV) were placed in a tissue culture insert with a 0.4μm membrane (which allows the passage of soluble factors only) above 2.2.15 cells already grown to confluence. The PBMC were stimulated with recombinant HBcAg (1μg/ml), rhIL-12 (10μg/ml), or PHA (1μg/ml) and incubated for 6 days at 37°C in 5% CO_2. All experiments were performed in duplicate. Blocking experiments with an antibody to human IFN-γ (Chemicon, Harrow, UK) were also carried out.

IFN-γ produced by PBMC in the cell culture was measured supernatants by commercial ELISA as described in Chapter 3.4.2.1.

4.2.3 Human hepatocyte isolation and culture

Full experimental details are described in 3.2.2, but briefly hepatocytes were isolated from diagnostic liver biopsies from 10 patients as previously described (Lau et al, 1991). Hepatocytes were incubated for 4 and 18 hours in RPMI/10% foetal calf serum containing two concentrations of recombinant human IFN-γ

(300 and 3000 pg/ml). After incubation the cells and supernatants were separated and stored separately at -80°C until analysis.

4.2.4 Isolation and detection of HBV DNA

4.2.4.1 Human hepatoma cells

The effect of IFN-γ produced by PBMC on HBV DNA levels was assessed by dot-blot hybridisation. Full experimental details are described in Chapter 3.3.1. Briefly, after removing the culture supernatant, 2.2.15 cells were harvested and the cell membrane lysed to release cytoplasmic DNA. The extracted cytoplasmic DNA was purified and transferred onto a nylon membrane (Hybond, Amersham, Bucks, UK) using a manifold apparatus (Bio-Rad, Hemel Hempstead, UK) and fixed by UV-light.

A full-length digoxigenin labelled HBV DNA probe was produced by PCR (Gunther et al, 1995). The blot was hybridised overnight at 65°C with 25 ng/ml of the probe and HBV DNA was detected using the DIG chemiluminescence protocol with CDP-star (Roche Diagnostics, Lewes, UK) as a substrate. The effect of IFN-γ on cytoplasmic HBV DNA was quantitated by measuring the strength of the chemoluminescence signal from the membrane in a Fluor-S MultiImager (Bio-Rad, Hemel Hempstead, UK). The membrane was then exposed to X-ray film as a hard copy.

4.2.4.2 Human hepatocytes

Due to the limited number of human hepatocytes available for each experiment, the number of copies of HBV DNA copies in each hepatocyte was measured by quantitative PCR as described in Chapter 3.3.2. The hepatocytes were lysed and mRNA was extracted using oligo dT magnetic beads (mRNA DIRECT kit, Dynal, Wirral, UK), as recommended by the manufacturers. Having removed the mRNA the lysate was used to quantitate HBV DNA with Amplicor Monitor according to manufacturer's instructions.

4.2.4.3 Isolation of mRNA and RT-PCR.

The mRNA samples were treated with RNAse-free DNAse (Roche Diagnostics, Lewes, UK) to ensure no contaminating HBV DNA was present prior reverse transcription and amplification by PCR. The enzyme was inactivated by EDTA treatment and the RNA was immediately used for cDNA synthesis.

4.2.4.4 PCR for HBV mRNA

To determine the anti-viral effect of IFN-γ on HBV replication the cDNA was amplified with primers specific for the HBV core gene using a two round nested PCR as described in 3.3.3.3. As a control reverse transcription PCR for β-actin mRNA was carried out in parallel as described in 3.5.1.

4.2.4.5 Detection of mRNA expression of IFN-γ inducible genes

Interferon regulatory factor-1 (IRF-1), inducible nitric oxide synthase (iNOS), and intracellular adhesion molecule-1 (ICAM-1) are cellular genes induced by IFN-γ and can be used to monitor the responsiveness of human hepatocytes and 2.2.15 cells to IFN-γ. For 2.2.15 cells total RNA was extracted with RNAeasy kit (Qiagen, Crawley, UK). mRNA was then extracted from the total RNA using oligo dT Dynabeads (Dynal). Reverse transcription was carried out with random hexamers as described in chapter 3.3.3.3 and 2.5 μl of template was added to 20μl reaction mixture for each PCR. IRF-1, iNOS and ICAM-1 were detected in the conditions described in 3.5.1.

4.2.5 Immunostaining of liver biopsies

To determine the proportion of hepatocytes infected with HBV, sections of diagnostic liver biopsies were stained for HBcAg, as described (Naoumov et al 1990). The primary antibody was rabbit anti-HBc (DAKO, Ely, UK) detected with DAKO EnVision+™ system HRP kit. The enzyme reaction was developed with diaminobenzidine substrate. The proportion of hepatocytes expressing HBcAg was assessed independently by two observers and was graded as a percentage i.e. <25%, 26-50%, >51%.

4.2.6 Evaluation of cytotoxicity

The potential cytotoxic effect of IFN-γ on human hepatocytes and on 2.2.15 cells in the co-culture system was assessed by measuring LDH released in cell culture supernatants using a LDH detection assay (Roche Diagnostics, Lewes, UK) as described in Chapter 3.5.2.

4.3 Results

4.3.1 Co-culture of PBMC and 2.2.15 cells

Unstimulated PBMC from patients with chronic HBV infection produced very low levels of IFN-γ between 0-4 pg/ml. Stimulation of lymphocytes from these patients induced IFN-γ (figure 1a): in response to PHA the mean IFN-γ level was 263 pg/ml (range 18-475); in response to rHBcAg the mean was 5 pg/ml (range 0-38 pg/ml); in response to rHBcAg plus rhIL-12 mean IFN-γ was 93 pg/ml, (range 39-327); in response to rhIL-12 mean IFN-γ was 10.9 pg/ml (range 0-81.6). Unstimulated PBMC from the patient with acute hepatitis B produced over 1000 pg/ml of IFN-γ.

There was no significant change in cytoplasmic HBV DNA level in 2.2.15 cells co-cultured with unstimulated PBMC from patients with chronic HBV infection (median change 5%, range 0-14%). In contrast, 2.2.15 cells co-cultured with activated lymphocytes the reduction in cytoplasmic HBV DNA seen ranged between 0 and 99 % and correlated with cell culture supernatant IFN-γ levels. IFN-γ from PBMC stimulated with HBcAg and IL-12 caused a reduction of

2.2.15 cell cytoplasmic HBV DNA ranging between 0 and 78% (R=0.91, p=0.0001, fig 1b). PHA induced IFN-γ production also resulted in 2.2.15 cell cytoplasmic HBV DNA reduction ranging between 0 and 91% with a similarly strong correlation (R=0.85). The other mitogens produced insufficient levels of IFN-γ to cause significant reductions of HBV DNA. Unstimulated PBMC from the patient with acute hepatitis B produced the highest level of IFN-γ and this was associated with the greatest antiviral effect (99 % reduction of 2.2.15 cell cytoplasmic HBV DNA).

Blocking experiments with antibody to IFN-γ considerably reduced the cell culture supernatant level of IFN-γ, which almost abrogated the antiviral effect of stimulated lymphocytes (Figure 2). Furthermore, IFN-γ induced IRF-1, iNOS and ICAM-1 expression in a concentration-dependent manner. IFN-γ at 300 pg/ml induced IRF-1, iNOS and ICAM-1 expression by 16, 25 and 3.5 fold respectively, compared to untreated cells (Figure 3). The mean cytotoxicity of 2.2.15 cells, co-cultured with PBMC stimulated with HBcAg plus IL-12 or PHA, was 5.8 and 6.4% respectively, which was similar to the levels seen in untreated cells (5.1%).

4.3.2 Effect of IFN-γ on HBV DNA in human hepatocytes

The median number of HBV DNA copies per hepatocyte calculated by Amplicor Monitor PCR assay, was 185 (range 4 to over 600). Three patients had more than 600 HBV DNA copies per hepatocyte, while the remaining 7 patients had a

median number of 33 copies per hepatocyte (range 4-583). There was some correlation between serum HBV DNA and hepatocyte HBV copy number. The three patients with low serum HBV DNA levels (<50 pg/ml) had a low number of HBV DNA copies per cell - median 12 (range 4-185). Serum HBV DNA levels in the other 7 patients were greater than 2000 pg/ml and the median number of HBV DNA copies per hepatocyte was 322 (range between 32 and over 600).

Hepatocytes isolated from 5 patients were incubated with two concentrations of IFN-γ (300 and 3000 pg/ml) for 4 and 18 hours. The number of hepatocytes obtained from the other 5 patients was sufficient only for the long incubation period, i.e. 10 cases in total for the 18 hour incubation period. Four hours exposure to both concentrations of IFN-γ reduced the HBV DNA levels in hepatocytes isolated from 1 of the 5 patients (Figure 4a). This case had the lowest viral load - serum HBV DNA was 10 pg/ml. There was no change in the HBV DNA levels in cells isolated from 3 patients and hepatocyte HBV DNA in the fifth case was reduced only in the presence of the high IFN-γ concentration (Figure 4a).

The overall reduction of HBV DNA in hepatocytes after 18 hours incubation with IFN-γ 300pg/ml, was 36 ± 41% which was statistically significant (mean ± SD, $p<0.05$, two-tailed Wilcoxon test, Figure 4b). In 7 of the 10 patients studied, the HBV DNA levels in hepatocytes incubated with IFN-γ decreased between 0.3 to 2.8 $\log_{10}$ in comparison with the HBV DNA levels in hepatocytes incubated with

medium alone. In three cases the number of HBV DNA copies per hepatocyte was not reduced with either of the experimental conditions (Numbers 5, 9 and 10, Fig. 4b).

Overall, the effect of recombinant IFN-γ on HBV DNA in human hepatocytes depended upon the duration of incubation and the viral load. The reduction of HBV DNA after 18 hours incubation was between 36 and 51%, while after four hours it was between 6 and 26% (Table). There was no difference between the antiviral effect of the two concentrations of IFN-γ used after 4 and 18 hours incubation.

The antiviral effect of IFN-γ (300pg/ml and 3000pg/ml) was considerably greater in hepatocytes obtained from the three patients with a low viral load (serum HBV DNA, <50pg/ml) - 84 ± 21% and 81 ± 17%, respectively (Table). A difference in reduction of HBV DNA was also seen when considering the copy number per hepatocyte. In hepatocyte preparations containing 4-583 viral DNA copies per cell, the reduction of HBV DNA was (mean ± sd) 76.1 ± 35.8%. In contrast the reduction in HBV DNA in hepatocytes with greater than 600 viral DNA copies per cell was 24.1 ± 49%.

Immunostaining showed that between 5 and 95% of hepatocytes expressed HBcAg (median 75%). The anti-viral effect of IFN-γ was not related to HBcAg expression. The immunostaining data show that the anti-viral effect is not due too

exquisite sensitivity of infected cells to cytolytic effects of IFN-γ. In four patients, with less than 50% of hepatocytes staining positive for HBcAg, the average reduction in HBV DNA with 3000 pg/ml of IFN-γ was 48.8%. In five patients who had more than 50% of hepatocytes positive for HBcAg the HBV DNA reduction was similar - 50.7%.

4.3.3 Effect of IFN-γ on HBV mRNA in human hepatocytes

IFN–γ markedly reduced HBV RNA levels (Figure 5). The reduction of viral mRNA ranged between 47±45% and 71±25 % (mean ± SD, Table). β-actin mRNA amplified in parallel was unchanged. The hepatocyte responsiveness to IFN-γ was confirmed by RT-PCR, which showed a concentration dependent induction IRF-1 expression (figure 5). The cytotoxicity, measured by LDH release in the supernatants, was low (5-8%) and these levels were seen in untreated hepatocytes.

4.4 Discussion

The co-culture model, employed in this study, provides the opportunity to examine the antiviral effect of cytokines produced by peripheral blood mononuclear cells from patients with HBV infection. Using this model, where the effector and target cells are separated by a membrane, we were able to determine the antiviral effect of IFN-γ produced by HBV antigen or mitogen stimulated CD4+ T-lymphocytes. Thus, the present study provides direct evidence that lymphocytes from patients with chronic HBV infection, if adequately stimulated,

are able to inhibit HBV replication in hepatocytes by releasing IFN-γ. The level of LDH release detected in the supernatant was similar to controls confirming that the reductions in HBV DNA were not due to cell lysis. In addition, the results demonstrate that IFN-γ mediated, non-cytolytic inhibition of HBV replication does occur in naturally infected human hepatocytes and results in a substantial reduction of HBV DNA and HBV mRNA in these cells.

The phenomenon of non-cytolytic inhibition of HBV gene expression and viral replication was first demonstrated and analysed in detail in HBV transgenic mice (Chisari, 1995; Guidotti et al, 1994; Guidotti 1996; McClary et al, 2000). This antiviral effect was shown to be mediated primarily by IFN-γ and TNF-α, which within 24 hours eliminate HBV nucleocapsids and their content of replicating HBV DNA from the mouse liver (Guidotti et al, 1996). The second mechanism for this antiviral effect is down-regulation of HBV RNA transcription (Guidotti et al, 1996). The induction of iNOS that we observed in 2.2.15 cells treated with IFN-γ not only shows that human hepatocytes are responding to IFN-γ, but may also represent an intracellular mechanism for the inactivation of HBV gene expression. In the transgenic mouse model of HBV infection iNOS deficient mice are insensitive to the non-cytopathic anti-viral effect of IFN-γ (Guidotti et al, 2000; Guo et al, 2000).

The present study determines for the first time the total number of HBV DNA copies per cell in naturally infected human hepatocytes - median 185 copies

(range, 4 to >600). In some cases this appear to be as high as 1000 copies per hepatocyte. These results are in line with a recent quantitation of HBV DNA in woodchuck hepatocytes (Mason et al, 1998). The analysis of liver samples from 17 woodchucks, using Southern blot assays, demonstrates that the median number of ccc DNA copies per hepatocyte is 19 (range 5 to 54 copies), while the number of total viral DNA copies per hepatocyte was calculated as median 810 (range 190 to 2,900) copies per cell. As covalently closed circular (ccc) HBV DNA was previously detected in 2.2.15 cells (Sells et al, 1988), we attempted to analyse, in the co-culture model, whether IFN-γ produced by activated lymphocytes has an effect on ccc HBV DNA. However, the number of 2.2.15 cells harvested from the two duplicate wells in the 24-well plate was not sufficient to allow the detection of ccc HBV DNA by Southern blotting. Future experiments using a specific polymerase chain reaction may provide useful information regarding this important question.

Using lymphocytes and hepatocytes from patients with HBV infection the present study demonstrates the relevance of IFN-γ induced control of HBV replication in humans. PBMC isolated from a patient with acute self-limited hepatitis B produced the highest levels of IFN-γ, even without stimulation, which practically abolished cytoplasmic HBV DNA in 2.2.15 cells in our experimental model. The efficiency of this mechanism for non-cytolytic inhibition of viral DNA in acute HBV infection was recently demonstrated in a prospective study of two chimpanzees with at least 90% of the viral DNA being eliminated from the liver

long before the development of hepatitis (Guidotti et al 1999b). Similarly, the IFN-γ levels in the liver of woodchucks with effective viral clearance were higher than in chronically infected animals (Guo et al, 2000). Interestingly, we found that lymphocytes from different patients with chronic hepatitis B markedly differ in their ability to produce IFN-γ in response to the same stimuli. This supports previous findings that individual effector functions may have different sensitivity to stimulation (Valitutti et al, 1996). In our *in vitro* model, lymphocytes which produced IFN-γ levels above 170 pg/ml were able to induce 70% or greater reduction of cytoplasmic HBV DNA within the co-cultured hepatocytes. This finding corresponds to the clinical data of IFN-γ levels measured in serial serum samples of patients in relation to the outcome of IFN-α treatment. In the majority of patients who controlled HBV replication successfully, as a result of this treatment, the peak of serum IFN-γ levels was above 150 pg/ml, while this was observed in only 3 of 23 non-responders (Rossol et al, 1997). A recent study revealed significant differences in the activation of peripheral T lymphocytes from patients with chronic HBV infection who are able to control viral replication, in contrast to HBeAg positive cases with a high viral load (Maini et al, 2000). The number of circulating HBV-specific T lymphocytes in the latter group was not only lower than in patients with effective control of HBV replication, but they also had a poor potential to expand *in vitro*.

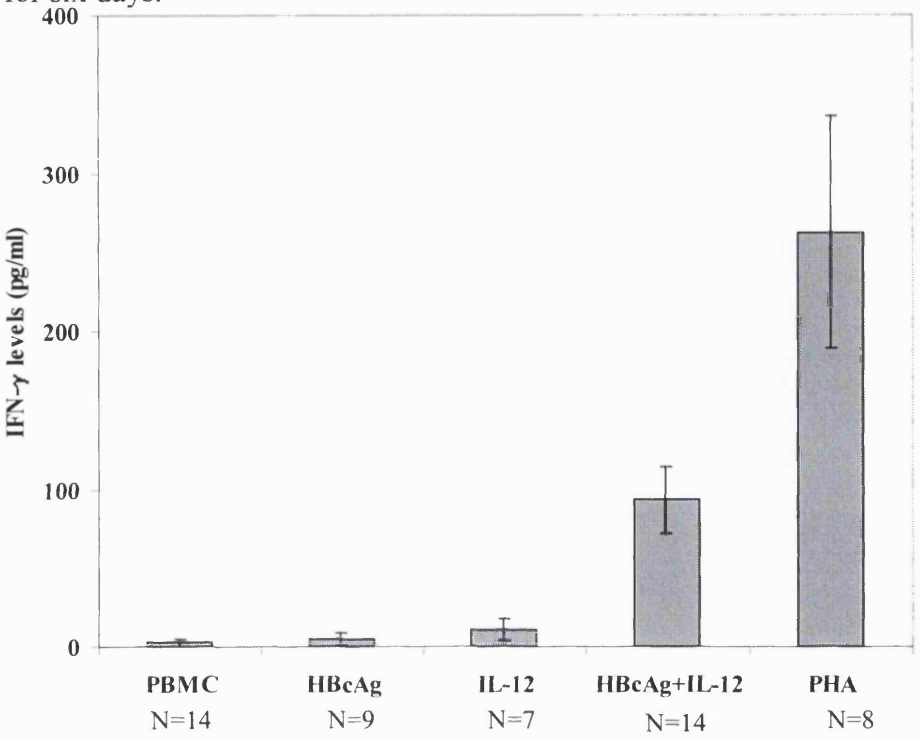
Both, antigen-specific (with rHBcAg plus rhIL-12) and mitogen-induced stimulation of PBMC from patients with chronic HBV infection produced various

levels of IFN-γ from different cases. Irrespective of the type of lymphocyte stimulation, the level of IFN-γ produced correlated with the degree of inhibition of cytoplasmic HBV DNA in hepatocytes. Similarly, LCMV infection is cleared from mouse hepatocytes when IFN-γ or TNF-α production is induced by antigen non-specific stimuli. In LCMV infection non-cytopathic clearance occurs following CTL-induced antigen-nonspecific IFN-γ production (Guidotti et al, 1999a). Furthermore anti-IFN-γ antibodies are able to inhibit immune-mediated clearance of the virus from infected tissues following adoptive transfer of immune spleen cells (Klavinskis et al, 1989).

A few clinical trials have previously examined the use of IFN-γ alone or in combination with IFN-α for treatment of patients with chronic hepatitis B (Kakumu, et al, 1991; Carreno et al, 1993). Although systemic administration of IFN-γ in these studies did cause inhibition of HBV replication, the antiviral effect was mild and the overall response to treatment was disappointing. The present study provides new insight in the understanding of the results from these clinical studies. Firstly, we found that despite the ability to induce a marked inhibition of HBV DNA and HBV mRNA in human hepatocytes, recombinant IFN-γ did not abolish HBV replication completely in any of the cases studied. Secondly, the non-cytolytic antiviral effect of IFN-γ was much more pronounced in patients with low baseline level of HBV DNA, while it had no effect in cases with a high viral load. This could be of practical importance and indicate the potential benefit

of a combination regimen involving a potent antiviral agent together with immunostimulation. Thirdly, the exposure of hepatocytes isolated from different patients to the same dose of recombinant IFN-γ showed different antiviral effect, which may indicate the presence of a genetic component in determining the efficiency of the IFN-γ dependent intracellular inactivation of HBV. Finally, systemic administration of rIFN-γ may not be the most appropriate route, as what really matters for an efficient control of HBV replication is the release of antiviral cytokines by liver-infiltrating lymphocytes, which are in close contact with HBV infected hepatocytes. Therefore, an adequate stimulation of patient's own lymphocytes or an adoptive transfer of activated lymphocytes may be a more effective therapeutic approach for chronic HBV infection.

Figure 1: Correlation between IFN-γ production and reduction of HBV DNA in 2.2.15 cells. PBMC were isolated from 13 HBsAg+ patients and co-cultured with 2.2.15 cells for six days.



1a: IFN-γ levels (mean ± SE pg/ml) produced by PBMC stimulated with HBcAg and/or IL-12 or phytohaemagglutinin in the co-culture supernatants.

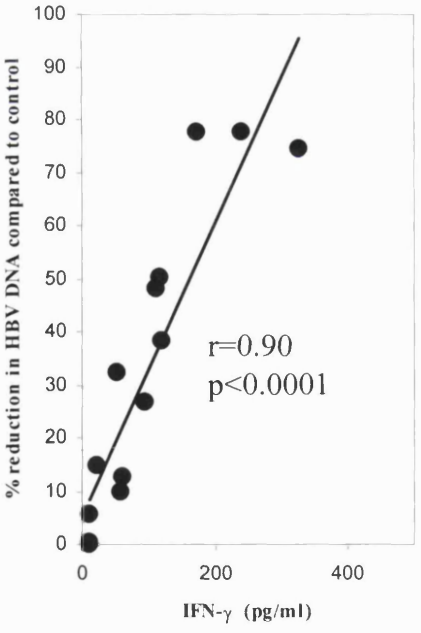


Figure 1b. Correlation between IFN-γ levels in the co-culture supernatants following stimulation with HBcAg and IL-12 and the reduction in cytoplasmic HBV DNA in 2.2.15 cells.

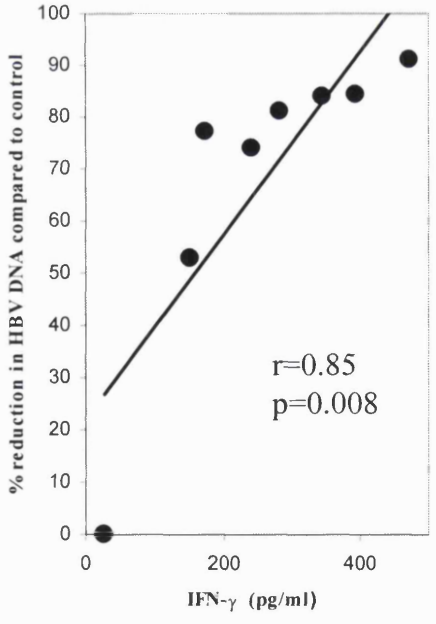


Figure 1c. Correlation between IFN-γ levels in the co-culture supernatants following stimulation with phytohaemagglutinin and the reduction in cytoplasmic HBV DNA in 2.2.15 cells

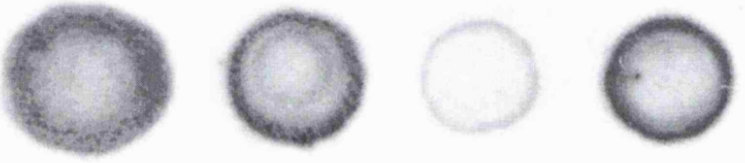
	PBMC			
	+ medium	+ rHBcAg	+ rHBcAg + rhIL12	+ rHBcAg + rhIL12 + anti-IFNγ
				
IFN-γ (pg/ml)	**0**	**0**	**172**	**13**
HBV DNA (au)	**163**	**146**	**36**	**138**
Reduction compared to medium only	**-**	**-10%**	**-80%**	**-15%**

Figure 2: Quantitation of cytoplasmic HBV DNA in 2.2.15 cells co-cultured with lymphocytes from a patient with chronic hepatitis B. Following HBV DNA hybridisation the chemiluminescence signal was quantitated by a multi-imager (Bio-Rad) and expressed in arbitrary units (au). A blocking experiment with an antibody to IFN-γ was performed to confirm that the antiviral effect is mediated by IFN-γ released from stimulated lymphocytes.

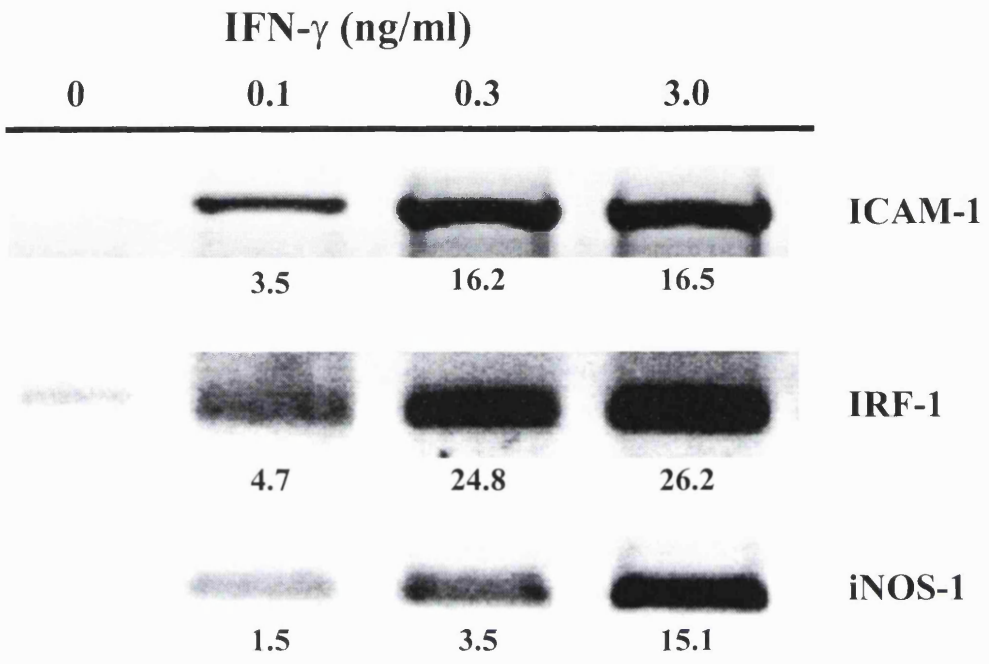
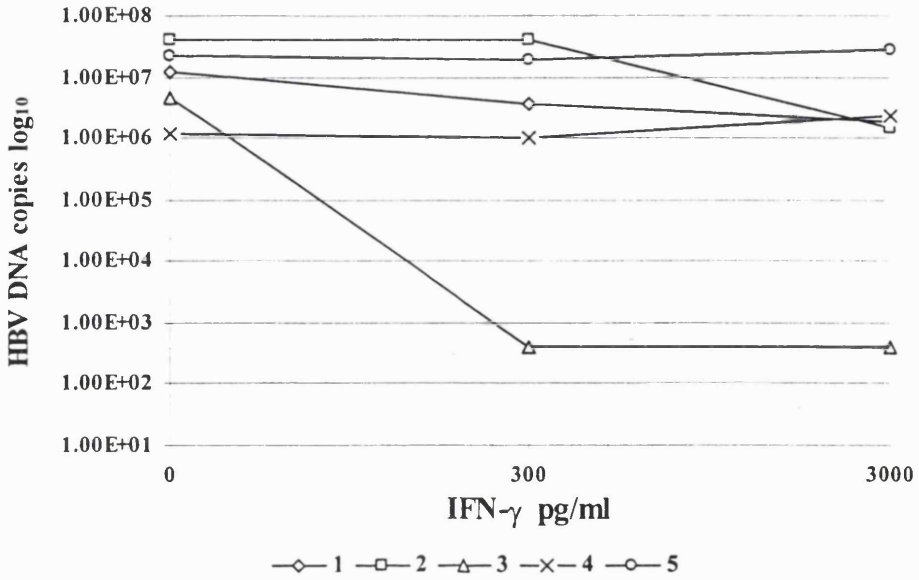


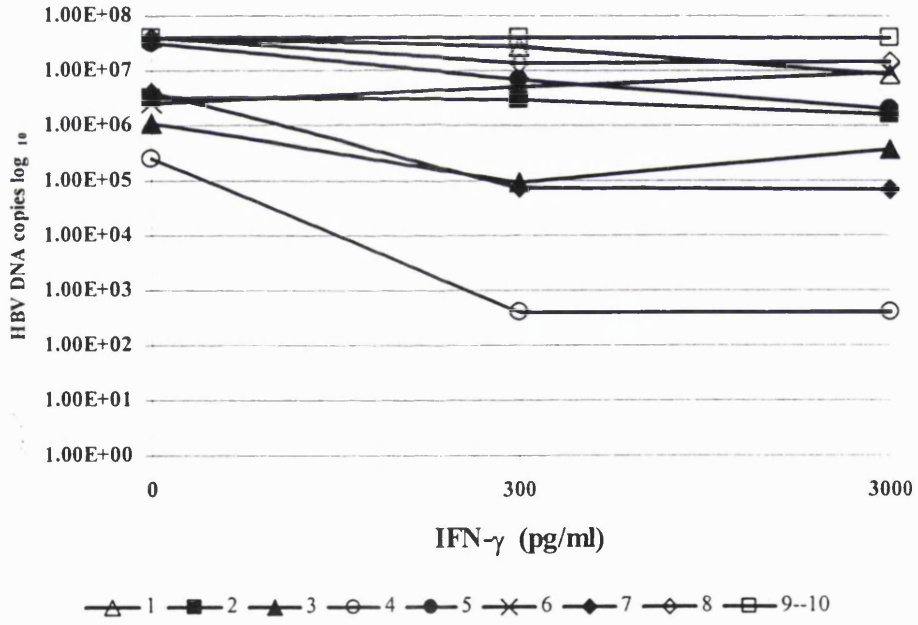
Figure 3: Effect of recombinant human IFN-γ on mRNA expression from interferon inducible genes in 2.2.15 cells. IFN-γ induced ICAM-1, IRF-1, and iNOS gene expression in a concentration dependent manner. The numbers are the ratio between the signal intensity for IRF-1, iNOS and ICAM-1respectively against the signal intensity of β-actin mRNA which was amplified in parallel.

a)



(a) Incubation of hepatocytes from 5 patients for 4 hours

b)



(b) incubation of hepatocytes from 10 patients for 18 hours.

Figure 4: Effect of recombinant human IFN-γ on HBV DNA levels in naturally infected human hepatocytes. The number of HBV DNA copies was determined by quantitative PCR using the Amplicor HBV DNA Monitor kit (Roche).

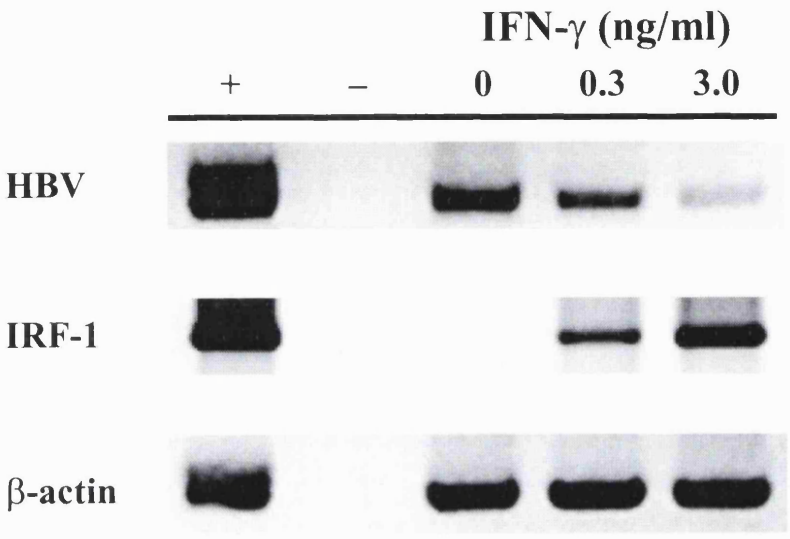


Figure 5: In vitro effect of IFN-γ on HBV RNA in naturally infected human hepatocytes. The hepatocytes (HC) were cultured with 300 and 3000 pg of recombinant human IFN-γ for 18 hours. A reduction of HBV RNA is observed with both concentrations, which is associated with a parallel increase in IRF-1. β-Actin mRNA amplified as a control shows no change.

Table. Percentage reduction (mean±SD) of HBV DNA and HBV mRNA in naturally infected human hepatocytes following incubation with recombinant IFN-γ.

	HBV DNA			HBV mRNA
IFN-γ (pg/ml)	**4 hours** (n=7)	**18 hours** (n=10)	**18 hours** (n=3)*	**18 hours** (n=8)
300	18 ± 38%	39 ± 46%	84 ± 21%	47 ± 45%
3000	26 ± 44%	57 ± 40%	81 ± 17%	71 ± 25%

Legend: The reduction of the HBV DNA and HBV mRNA levels in hepatocytes incubated with rIFN-γ is expressed as a percentage of the viral nucleic acid levels detected in hepatocytes incubated for the same period with medium only.

* This column shows the reduction of HBV DNA for 3 of the 10 cases who differed from the rest of the patients as they had a particularly low viral load (serum HBV DNA <50 pg/ml)

Chapter 5

Resolution of Chronic Hepatitis B and Anti-HBs Seroconversion in Man by Adoptive Transfer of Immunity to Hepatitis B core Antigen

5.1 Background

The development of anti-HBs is a rare event in patients with chronic HBV infection. A unique opportunity to study the underlying immune mechanisms and role of CD4+ T-cells during resolution of chronic HBV infection arose with a group of patients with chronic HBV infection who developed anti-HBs following allogenic bone marrow transplantation from siblings with natural immunity to HBV

A large body of evidence suggests that the host immune response to HBV-encoded antigens is the main determinant for the outcome of HBV infection. In patients with self-limited acute hepatitis B, both HLA class I and class II-restricted T cell responses to viral antigens are strong and multispecific, while these responses are weak or undetectable in chronically infected patients (Chisari and Ferrari, 1995). Moreover, in patients with spontaneous resolution of HBV infection, these strong antiviral T cell responses are maintained for decades following clinical recovery, which keeps the virus under control (Rehermann et al 1996; Penna et al, 1996).

In chronic HBV infection persistent viral replication is associated with ongoing necroinflammation in the liver and progressive liver damage. Spontaneous remissions, with effective control of viral replication, can occur in a small proportion of patients during the natural history of chronic HBV infection. Similar remissions are the desired outcome of antiviral treatment of chronic hepatitis B and are associated with improved patient survival (Niederau et al, 1996). However, clearance of HBsAg in patients with chronic HBV infection is unusual; 0.4% to 2% per year in Caucasian and 0.1-0.8% per year in Chinese (Liaw et al, 1991). Even in interferon treated patients with chronic hepatitis B, the rate of HBsAg clearance is increased by only 6% (Wong et al 1993).

Recently it has been shown that adoptive transfer of immunity to HBV can be achieved after transplantation of bone marrow from donors who are immune to HBV (Shouval et al, 1993; Shouval et al, 1995). Interestingly, clearance of HBsAg has been observed in individual patients with chronic hepatitis B after transplantation of bone marrow from immune donors (Ilan et al, 1993; Lau et al, 1997) however, the mechanisms of resolution of chronic HBV infection following adoptive transfer of immunity to HBV have not been studied. The aim of this investigation was to gain information for the HBV-specific T cell reactivity in patients who clear HBsAg following adoptive transfer of immunity to HBV, which would be of value in designing new approaches for therapeutic immunization in patients with chronic hepatitis B.

5.2 Materials and Methods

5.2.1 Patients

Eight patients with chronic HBV infection (serum HBsAg positive for more than 12 months) who underwent bone marrow transplantation (BMT) because of hematological malignancy and received marrow from an HLA-matched sibling with natural immunity to HBV (anti-HBs and anti-HBc positive) were studied (Table). Before bone marrow transplantation, the three patients with acute leukemia were in complete remission, induced by an appropriate chemotherapy regimen: cytarabine, daunorubicin and etoposide for acute myeloid leukaemia (Bishop et al, 1990) or vincristine, prednisolone, daunorubicin, methotrexate and cyclophosphamide for acute lymphocytic leukaemia (Hoelzer et al, 1993). The remaining 5 patients with chronic myeloid leukemia were in chronic phase and were receiving treatment with hydroxyurea. The preparative conditioning regimen for all patients involved either busulphan, cyclophosphamide and total body irradiation or cyclophosphamide and total body irradiation (Anderson et al, 1996). The clinical course in four of these patients had been described previously (Lau et al, 1997; Lau et al, 1998). All patients were treated at the Bone Marrow Transplant Unit, Queen Mary Hospital, Hong Kong SAR, China. The donors and the recipients were negative for HCV, HDV and HIV infection. Routine HBV serology - HBeAg and anti-HBe, HBsAg and anti-HBs and HBV DNA was tested 4 weeks prior to BMT and at 4 weekly intervals after transplantation. The standard immunossuppression protocol for all patients included cyclosporin A alone or in combination with prednisolone for at least 6 months after BMT when

the immunosuppression was withdrawn. Of the 8 cases described here, four were transplanted before 1996 and did not receive any antiviral treatment. Since 1996, all HBsAg positive BMT recipients were given prophylactic treatment with famciclovir or lamivudine starting 2 weeks before and continuing for six months after BMT. In this group three patients received famciclovir (500 mg three times a day) and one received lamivudine (100 mg daily) (Table).

5.2.2 T cell proliferation assay

Freshly isolated peripheral blood mononuclear cells (PBMC) were used in all experiments for testing the proliferative response to recombinant HBV proteins and synthetic peptides as described in 3.4.1. PBMC were obtained at the last follow-up visit from seven donor/recipient pairs and were tested in parallel. In one patient (Table, patient no 8), the proliferative response to viral antigens was tested prospectively before BMT, during the hepatitis flare, post-BMT and 52 weeks after transplantation; on all 3 occasions in parallel with the donor's PBMC. PBMC were cultured for 6 days at a concentration of $2x10^5$ cells/well in 96-well, flat-bottomed plates in the presence of the antigens or medium alone. The final concentration of the antigens used was 1 μg/ml for recombinant HBcAg, 2 μg/ml for rHBsAg, 1 μg/ml for tetanus toxoid and 1 μg/ml for phytohemagglutinin. In addition, the response to synthetic peptides, corresponding to immunodominant epitopes in the pre-S1 region (aa 21-48) and in the pre-S2 region (aa 146-175) was analyzed in all cases. This was done with two 20-mer overlapping peptides covering the immunodominant area in the pre-S1 region were between aa 21 to 48

and aa 29-48. The two 20-mer overlapping peptides covering the immunodominant area in the pre-S2 region were between aa 146 to 174 (aa 146-165 and aa 156-174). The proliferative response was evaluated by ^{3}H-thymidine uptake and measured by a β-counter (Wallac 1450 MicroBeta Trilux Counter, Turku, Finland) in counts per minute (cpm). A stimulation index greater than 2.5 was considered positive. In four donor/recipient pairs (patient nos. 4,6,7 and 8, Table) epitope mapping of the T cell proliferative response to rHBcAg was performed using seventeen 20-mer overlapping peptides (final concentration 10 μg/ml), spanning the entire HBV nucleocapsid protein (Chiron Technologies (Clayton, Australia).

PBMC were also obtained from eleven healthy subjects with no previous exposure to HBV (seronegative for anti-HBc), who have received HBV vaccination (Engerix-B, Smith Kline Beecham). Serum anti-HBs titer in all these individuals was greater than 100 U/L and the median interval between their last immunization and the time of mononuclear cell isolation was 15 months (range 6-60 months).

5.2.3 Elispot assays for CD4+ and CD8+ T cells

Elispot assays for CD4+ and CD8+ T cells were carried out as described in 3.4.2.1. Cryopreserved PBMC were used to determine the number of HBV-specific, IFN-γ producing CD4+ T cells (Jung et al, 1999). PBMC were cultured with rHBcAg (final concentration 2 μg/ml), rHBsAg (10 μg/ml) and PHA (2

μg/ml) or medium only. The number of specific spot-forming cells was determined as the mean number of spots in the presence of an antigen minus the mean number of spots in the wells with medium only and expressed per $1x10^6$ PBMC.

Elispot for CD8+ T-cells was carried out using similar methods for HLA A2 positive individuals. The two synthetic peptides used to determine the number of HBV-specific CD8+ T cells corresponded to the known HLA A2-restricted CTL epitopes in the HBV core - aa 18-27 (FLPSDFFPSV) and the HBV surface - aa 335-343 (WLSLLVPFV, Chiron Technologies (Clayton, Australia) (Chisari and Ferrari, 1995). The peptide concentration and that of anti-CD3 (used as a control) was 10 μg/ml.

5.2.4 Fluorescein-activated cell sorter (FACS) analysis

Phenotypic cell-surface marker expression was analyzed by flow cytometry on cryopreserved PBMC after dual-staining with phycoerythrin (PE) and fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibodies as described in chapter 3.4.3. The expression of the following combination of markers was determined: anti-CD4-PE/anti-CD8-FITC; anti-CD4-FITC/anti-CD25-PE; anti-CD4-FITC/anti-HLA DR-PE; anti-CD4-FITC/anti-CD45RO-PE and anti-CD4-FITC/anti-CD45RA-PE (all from Pharmingen, CA, USA). Briefly, $1x10^6$ PBMC were incubated with the respective combination of PE- and FITC-labeled antibodies at a concentration of 1:20 for 30 min on ice. Subsequently, the cells

were washed twice in PBS with 1% fetal calf serum. The acquisition and analysis were performed by FACScan (Becton Dickinson Immunocytometry system,CA,USA) and CELLQuest (version 1.0, Becton Dickinson Immunocytometry system, CA, USA), respectively.

5.2.5 PCR for the Y chromosome

Fresh PBMC, collected at the last follow-up, from 5 male patients who received bone marrow from a female donor were analyzed for the presence of Y chromasome (chapter 3.6.2). CD4+ T cells were purified by immunomagnetic separation using Dynabeads (Dynal, Oslo, Norway), according to manufacturer's instructions. DNA was extracted from the purified CD4+ T cells and microsatellite PCR for detection of the Y chromosome was performed using 6 different sets of primers. These experiments were kindly carried out by Dr Mark Thomas, University College London.

5.2.6 Hepatitis serology and HBV DNA quantitation

Serum HBV markers, including HBsAg, anti-HBs, HBeAg and anti-HBe, were tested by commercially available enzyme immunoassays (Abbott Laboratories, Chicago, IL, U.S.A.). The presence of anti-HBc was tested by radioimmunoassay (Corab; Abbott). Commercial enzyme immunoassays (Abbott) were used to test serum samples from all donors and recipients for antibody to hepatitis C virus, antibody to hepatitis Delta virus and antibody to human immunodeficiency virus. Serum HBV DNA level was quantified by the signal amplification assay

(Quantiplex™ bDNA assay, Chiron Corporation, Emerville, CA). Using this assay, the threshold for HBV DNA detection in serum is $0.7x10^6$ viral copies/ml (Hendricks et al, 1995).

5.2.7 Statistics

Wilcoxon rank sum test was used to analyze paired values in the same patients at different time point. The Statistical Package of the Social Sciences (SPSS; SPSS Inc, Chicago IL, USA) was used.

5.3 Results

After engraftment of the bone marrow, all eight HBsAg positive recipients developed a hepatitis flare (defined as elevation of serum alanine aminotransferase greater than 3 fold above the upper level of normal) at median 3.3 months (range 1.6 - 13.3). Between 0.5 to 6 months later all eight BMT recipients cleared serum HBsAg (Table). In six cases, the seroconversion to anti-HBs was sustained at the last follow up visit - between 1.7 and 7.9 years after bone marrow transplantation (Table). The other 2 cases lost HBsAg and seroconverted to anti-HBs for a period of 3 and 6 months respectively, but became HBsAg positive again. Despite this, in one of the latter two cases, a partial response with sustained control of HBV replication was maintained, as he cleared serum HBeAg and HBV DNA (Table).

5.3.1 Proliferative T cell response to HBV in donor and recipients

Initially, we determined the HBV-specific proliferative responses of peripheral blood mononuclear cells (PBMC), taken at the last follow up visit from 7 donor/recipient pairs (Table, cases No 1-7), using recombinant hepatitis B core antigen (rHBcAg), rHBsAg, pre-S1 and pre-S2 peptides. Significant CD4 positive T cell proliferation to HBcAg was found in 6 of 7 (86%) donors, while there was no response to HBsAg, pre-S1 or pre-S2. Proliferative response to HBV nucleocapsid protein was detected also in 4 of 7 (57%) recipients, while none showed reactivity to the envelope antigens. A significant proliferative response to rHBsAg was present in 4 of 11 healthy HBV vaccine recipients with a stimulation index varying between 3.3 and 12.2. The response to tetanus toxoid, which was used a positive control, was similar in the group of seven BMT recipients and in the group of healthy individuals - the stimulation index (mean±SD) was 28.9±23.8 and 36.4±29.7, respectively.

Next, the specificity of T cell reactivity to the nucleocapsid protein in three donor/recipient pairs (patients 4,6,7; Table) with a significant proliferative response to HBcAg was analysed. The epitope mapping was performed with a panel of 17 synthetic 20-amino acid peptides, spanning the entire nucleocapsid protein (Figure 1a). The results revealed similarity in the fine specificity of the CD4+ T cells from the donor and the recipient, as within each pair they recognized one or two identical epitopes of HBcAg within three immunodominant regions between amino acid 1-30, 61-90 and 141-170.

5.3.2 CD4 positive T cells in the recipients are of donor origin

To confirm that the CD4 positive T lymphocytes in the recipients are of donor origin, we used the sex-mismatch in 5 of these 7 pairs, where the donor was female and the recipient was male. CD4 positive T cells were isolated from the recipients' PBMC, taken at the last follow up visit, and multiplex polymerase chain reaction (PCR) was performed using six sets of Y chromosome-specific primers (Figure 1b). The CD4 positive T cells showed no positive signal for the Y chromosome with any of the primers, thus confirming that they were of donor origin.

5.3.3 Direct enumeration of HBV-specific T cells

To further characterize the T cell reactivity to HBV, we determined the frequency of IFN-γ producing CD4+ and CD8+ T cells in response to HBcAg and HBsAg using Elispot assays. PBMC obtained at the last follow up from two donor/recipient pairs with an HLA-A2 haplotype were used for this analysis. The number of IFN-γ producing CD4 positive T cells was determined in response to recombinant nucleocapsid (rHBcAg) and envelope (rHBsAg) proteins, while synthetic peptides corresponding to two HLA-A2 restricted epitopes (Core - aa 18-27; Surface - aa 335-343) were used to define the number of IFN-γ producing CD8 positive T cells (Figure 1c). Both in the donors and in the recipients, the number of CD4 positive T cells specific for HBcAg was substantially greater than those specific for HBsAg. Similarly, the frequency of CD8+ T cells, recognizing

the HBV core peptide in the two BMT recipients was 22 and 27 SFC/10^6 PBMC, which was considerably higher than the frequency of the CD8+ T cells recognizing the envelope peptide - 7 and 3 SFC/10^6 PBMC, respectively (Figure 1c).

In the group of HBV vaccine recipients (n=11), the mean frequency of IFN-γ producing CD4+ T lymphocytes in response to rHBsAg was 137.2 specific spot forming cells per 10^6 PBMC (range 35-258).

5.3.4 CD4 positive T cells are activated during hepatic flare

We next studied the changes in CD4+ T cell activation and the proportions of naive and memory CD4+ T cells in these seven recipients following the engraftment of bone marrow with immunity to HBV. For this purpose, PBMC obtained at two time points were analysed for each patient - the first sample was taken at the time when the recipients had a hepatitis flare, and the second sample was taken at the last follow up visit. Cryopreserved PBMC from these two time points were analyzed simultaneously by dual flow cytometry to determine the expression of early (CD25) and late (HLA DR) T cell activation markers, as well as markers for naive (CD45 RA) and memory (CD45 RO) phenotype on CD4+ T lymphocytes (Figure 1d). The percentage of CD25 positive/CD4 positive T cells was significantly higher at the time of hepatitis flare than at the end of follow up (mean ± s.e.m. 22.1±9.9% versus 6.6±4.5%, respectively; p=0.04, Wilcoxon test). The activation of CD4+ T cells during the hepatitis flare was also shown by the

higher expression of HLA DR at this time point, in comparison with the last follow up visit - 36.7±8.6% versus 14.4±7.8% (p=0.04). There was a significant increase in the proportion of memory CD4 positive T cells during the time of hepatitis flare as the percentage of cells expressing CD45RO was 96.1±1.6% versus 83.6±2.9% at the last follow up (p=0.04).

5.3.5 Prospective analysis of HBsAg clearance after bone marrow transplantation

We further investigated the virus-specific T cell reactivity after adoptive transfer of immunity to HBV in one prospectively studied patient who was HBeAg and HBV DNA positive (No 8, Table). Liver histology before BMT showed mild/moderate inflammatory infiltrates in the portal tracts with focal interface hepatitis. The immunostaining for HBcAg and for HBsAg was positive in 5% and 10% of hepatocytes, respectively. The patient received bone marrow from an HLA identical (HLA A2 positive) male donor and 24 weeks post BMT developed hepatitis flare - ALT peak 461 IU/L (Figure 2a). HBeAg to anti-HBe seroconversion occurred at the time of ALT peak, with loss of HBsAg four weeks later, followed by seroconversion to anti-HBs. PBMC, taken from the recipient prior to starting the pre-transplant conditioning regimen, showed no proliferative response to any HBV antigen (stimulation index, SI to rHBcAg 1.2), while PBMC from the donor showed significant proliferation to HBcAg (SI 4.6). Epitope mapping with 17 core peptides revealed consistent proliferative responses to 3 peptides, corresponding to amino acids 41-60, 51-70 and 141-160 of the HBV

nucleocapsid protein. Elispot assays with PBMC from the donor also revealed predominance of CD4+ and CD8+ T cells specific for the HBV nucleocapsid, in comparison to the envelope antigen. For IFN-γ producing CD4+ T cells: 168 spot forming cells (SFC)/10^6 PBMC with HBcAg and 25 SFC/10^6 PBMC with HBsAg; for IFN-γ producing CD8+ T cells: 13 SFC/10^6 PBMC with Core peptide (aa 18-27) and 3 SFC/10^6 PBMC with Surface peptide aa 335-343 (Figure 2b). At the time of hepatitis flare there was a significant proliferative response only to HBcAg (SI 2.6) with activation of CD4+ T cells and increased proportion of CD45RO+ T cells (Figure 2c). At 52 weeks after BMT the recipient maintained HBcAg-specific T cell proliferation (SI 2.6) and the numbers of IFN-γ producing T cells in response to HBV antigens have risen significantly. For IFN-γ producing CD4+ T cells: 245 SFC/10^6 PBMC with HBcAg and 78 SFC/10^6 PBMC with HBsAg; for IFN-γ producing CD8 positive T cells: 18 SFC/10^6 PBMC with the Core peptide and 5 SFC/10^6 PBMC with the Surface peptide (Figure 2b). Liver histology at this time point (52 weeks post-BMT) showed complete resolution of interface hepatitis and portal tract inflammation, with undetectable HBsAg and HBcAg by immunohistochemistry.

5.4 Discussion

The phenomenon of HBsAg clearance and seroconversion to anti-HBs following adoptive transfer of immunity in patients with chronic hepatitis B provides a unique model to understand virus-specific T-cell reactivity associated with resolution of chronic HBV infection. By studying the largest series of patients, who cleared HBsAg following the engraftment of an HLA-identical bone marrow from a donor with past exposure to HBV, we found that resolution of chronic HBV infection is associated with a transfer of CD4+ T lymphocyte reactivity to HBcAg, rather than to HBV envelope proteins. The present study also demonstrates that the CD4+ T cells are of donor origin and activation of the memory subset, CD45-RO+ T cells, occurs during the hepatitis flare, which precedes the seroconversion to anti-HBs. These results explain our earlier clinical observation that HBsAg clearance occurs only after adoptive transfer of naturally acquired immunity to HBV (anti-HBs and anti-HBc positive donors) and not in patients who received marrow with a vaccine-induced immunity (anti-HBs alone) (Lau et al, 1998).

The ability of HBcAg-primed T helper cells to provide functional T cell help and to elicit anti-envelope antibody production, when challenged with the hepatitis B virus, has been demonstrated in mice (Milich DR, 1987b). In the present study, HBcAg-specific CD4+ T cells were found in 7 of 8 donors and the transfer of such primed T helper cells seems to have a pivotal role for seroconversion to anti-HBs after the adoptive transfer of immunity, as they would provide intermolecular

T-cell help upon challenge with HBV in the recipients. Further examples of such functional intrastructural/intermolecular help come from experiments with chimpanzees and woodchucks immunized with the HBV nucleocapsid protein. Upon a subsequent challenge with the whole virion, the HBcAg-immunized animals reacted with a rapid production of anti-envelope antibodies and protective immunity (Murray et al, 1987; Schodel et al, 1993; Iwarson et al, 2002).

Studies of patients with acute self-limited hepatitis B also emphasize the notion that activation of HBcAg-specific CD4+ T lymphocytes is a prerequisite for resolution of HBV infection (Ferrari et al, 1990; Jung et al, 1995). The HBV nucleocapsid protein has been shown to be the strongest immunogen for HLA class II restricted T cell responses during acute infection, with the peak of T cell proliferation to HBcAg usually coinciding with the loss of serum HBeAg and HBsAg. These T cell responses to HBV nucleocapsid are long-lasting and are sustained by CD45-RO+ cells (Penna et al, 1996). Instead, the T cell proliferative response to HBV envelope antigens is very weak or undetectable also in acute HBV infection (Ferrari et al, 1990). A recent analysis of circulating HBV-specific CD8+ T cells in patients with acute hepatitis B, using HLA-A2/peptide tetramers, revealed different frequencies of core, polymerase and envelope-specific CD8+ cells (Maini et al, 1999). The frequency of core epitope 18-27-specific, CD8+ T cells was considerably higher than the proportion of cells specific for the envelope epitope 335-343, which supports the bias toward HBcAg seen in CD4+ T helper

cell reactivity during acute self-limited hepatitis B (Ferrari et al, 1990; Jung et al, 1991).

The reasons for the weak virus-specific T cell reactivity in patients with chronic HBV infection are not fully understood. Induction of HBcAg-specific T cell reactivity in patients with chronic hepatitis B has been demonstrated in both, spontaneous or interferon treatment-induced seroconversion to anti-HBe with an effective control of HBV replication (Tsai et al, 1992; Marinos et al, 1995). The present study further indicates that long-lasting reconstitution of T cell reactivity to the HBV nucleocapsid protein in such patients results in resolution of chronic hepatitis B and development of natural immunity to HBV, an immunological profile analogous to cases with previous exposure and spontaneous viral clearance. So far, the attempts to achieve this through therapeutic immunization have produced disappointing results. Immunotherapy using recombinant preS2/S protein has elicited CD4+ T cell proliferation to HBV envelope proteins in 7 of 27 immunized patients with chronic hepatitis B and some cases showed a reduction in serum HBV DNA levels (Couillin et al, 1999). However, this protocol did not increase the rate of HBeAg/anti-HBe seroconversion and serum HBsAg clearance did not occur in any of the immunized patients. These results were confirmed in a multicentre controlled trial involving 118 patients (Pol S et al, 2001). Another approach using a vaccine, which involves a CTL epitope - the core peptide aa 18-27, has induced only a low-level of CTL activity and there was no resolution of

HBV infection and chronic hepatitis B in any of the patients studied (Heathcote et al, 1999).

Recent experimental data revealed the essential role of the CD4+ T cell help for effective function of CTL and viral elimination (Zajac et al, 1998). The practical implication of the present findings of successful HBsAg clearance following adoptive transfer of immunity to HBcAg, is that therapeutic immunization of patients with chronic HBV infection should include the HBV nucleocapsid protein (or the core gene for DNA immunization) and aim to induce both HBcAg-specific CD4+ and CD8+ T cell responses. Apart from priming the T helper cells, which will then provide intermolecular help for anti-envelope antibody production, HBcAg is approximately 100-fold more efficient, than HBsAg, in its ability to activate T cells (Milich DR, 1987a). Th2 predominance and low CTL activity are characteristic for patients with chronic hepatitis B (Livingston et al, 1999) and a potent immunogen, like particulate HBcAg, may help to revert this in favor of Th1, break the tolerance at the CTL level and hence result in resolution of chronic HBV infection. The success of the adoptive transfer of immunity to HBV nucleocapsid protein in the present series of Chinese patients with chronic HBV infection, in whom the annual rate of HBsAg clearance is as low as 0.1 to 0.8% (Liaw et al, 1991), shows its potential power for resolution of chronic HBV infection.

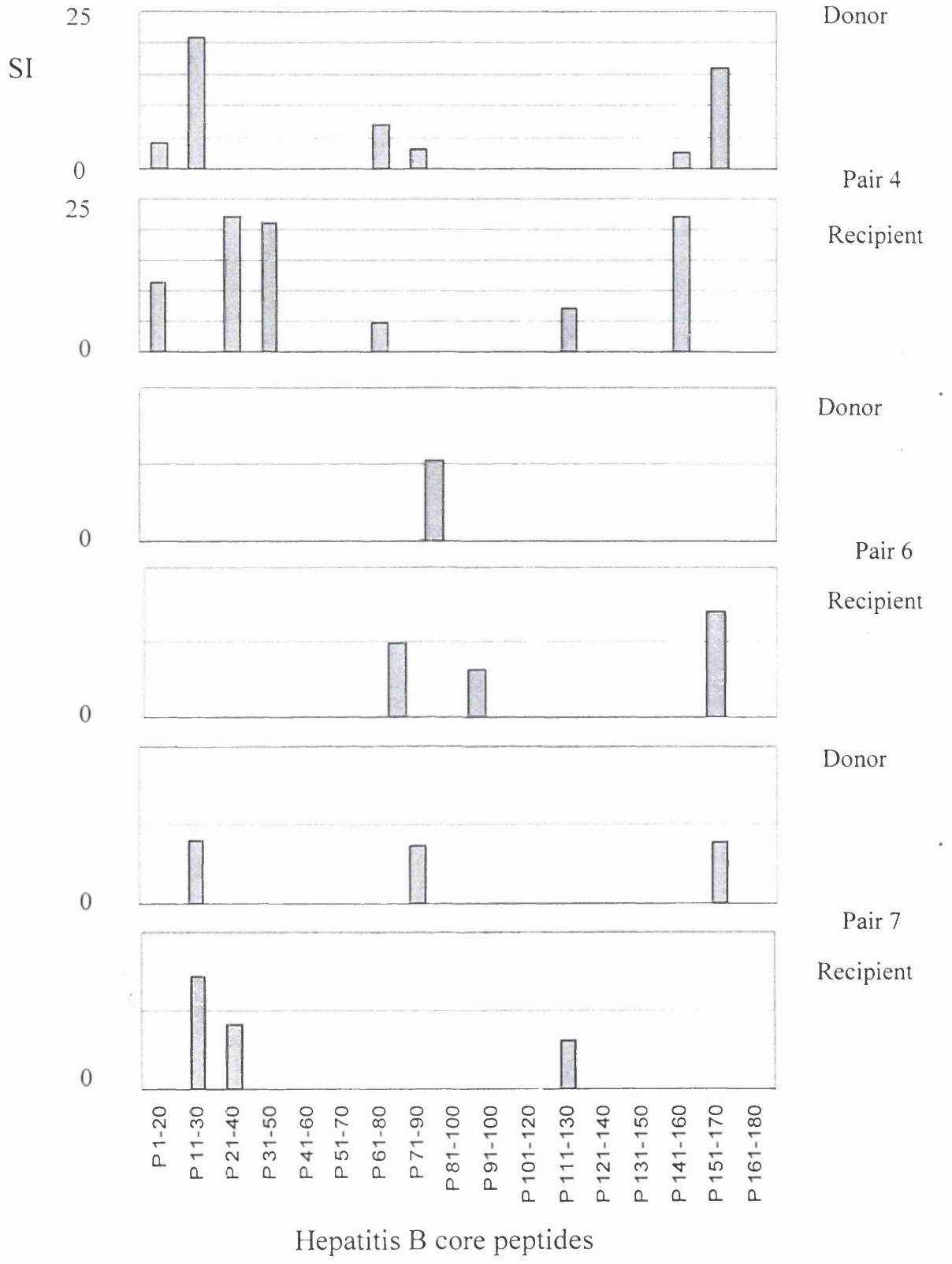


Figure 1a Characterization of T lymphocytes in bone marrow transplant recipients and their corresponding donors. Fine specificity of CD4 positive T cell reactivity in three donor/recipient pairs, who have shown a significant proliferative response (Stimulation Index, SI >2.5) to recombinant HBcAg, using a panel of 20-mer overlapping, synthetic core peptides. D, donor; R, recipient. The numbers assigned to the pairs correspond to those on the Table.

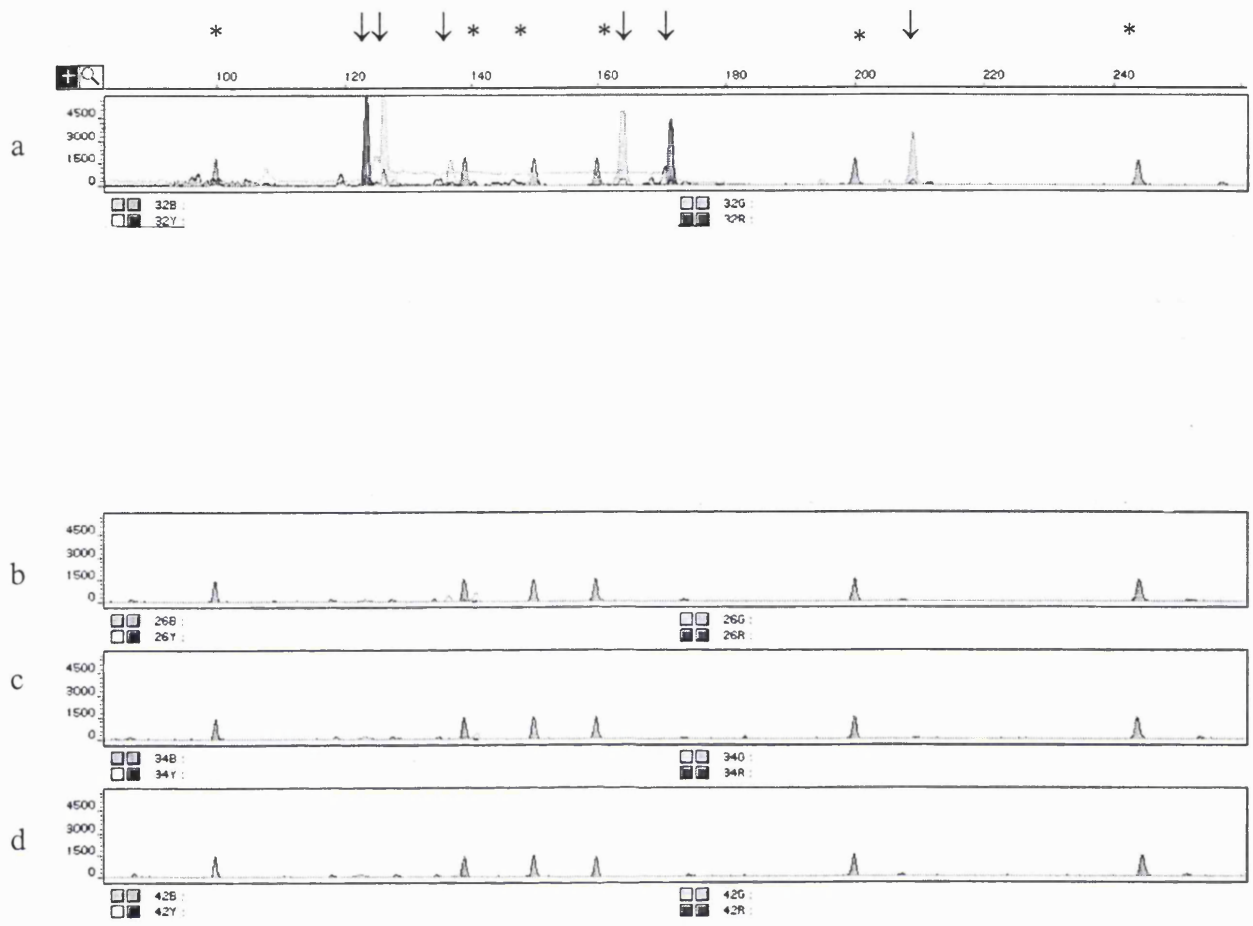
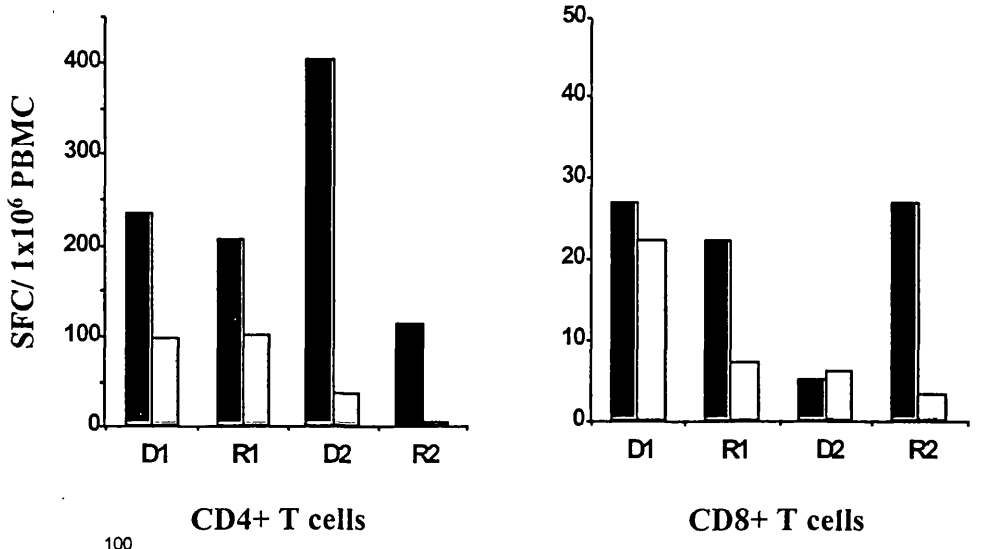


Fig. 1 Characterization of T lymphocytes in bone marrow transplant recipients and their correspondin; donors.

1b, Multiplex PCR for the detection of Y chromosome in the recipients' CD4 positive T lymphocytes. Using six sets of Y chromosome-specific primers, male CD4 positive T cells (a) show the presence of 6 positive signals (↓) between 123 and 208 base pair. The standard size markers are shown as *. Amplification of DNA from purified CD4 positive T cells of male recipients who have received bone marrow from a female donor (b, c and d) show no signal for the Y chromosome, thus confirming the donor origin of their CD4 positive T cells.

Figure 1 Characterization of T lymphocytes in bone marrow transplant recipients and their corresponding donors.

c



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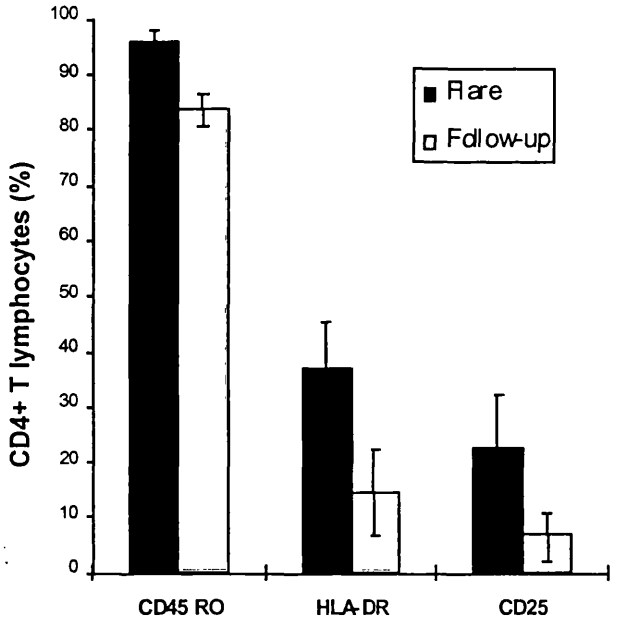
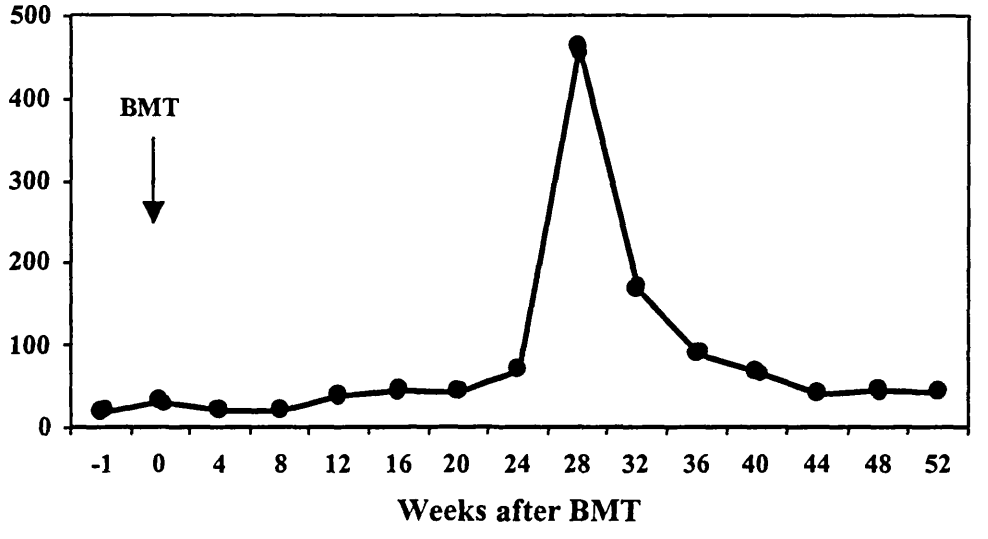


Figure 1c, Direct *ex-vivo* analysis (IFN-γ Elispot assays) of the number of HBV-specific CD4 positive and CD8 positive T lymphocytes in PBMC from two HLA A2 positive donor/recipient pairs (D1/R1 and D2/R2). D, donor; R, recipient. Recombinant HBcAg (■) and rHBsAg (□) were used as antigens to determine the frequency of HBV-specific CD4 positive T lymphocytes, while Core (aa 18-27, ■) and Surface (aa 335-343, □) peptides were used to determine the frequency of HBV-specific CD8 positive T cells. SFC, specific spot-forming cells per $1x10^6$ PBMCs.

Figure 1d, Changes in the proportion of CD4 positive T lymphocytes, which express activation markers (CD25 and HLA DR) and memory phenotype (CD45RO), at the time of hepatitis flare (■) and at the last follow-up (□) in seven bone marrow transplant recipients. The bars show the mean ± s.e.m.

Fig. 2 Prospective analysis of recipient No 8 (Table 1) after adoptive transfer of immunity

HBsAg/sAb	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	-/-	-/+	-/+	-/+	-/+	-/+
HBeAg +/eAb	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	-/+	-/-	-/+	-/+	-/+	-/+	-/+



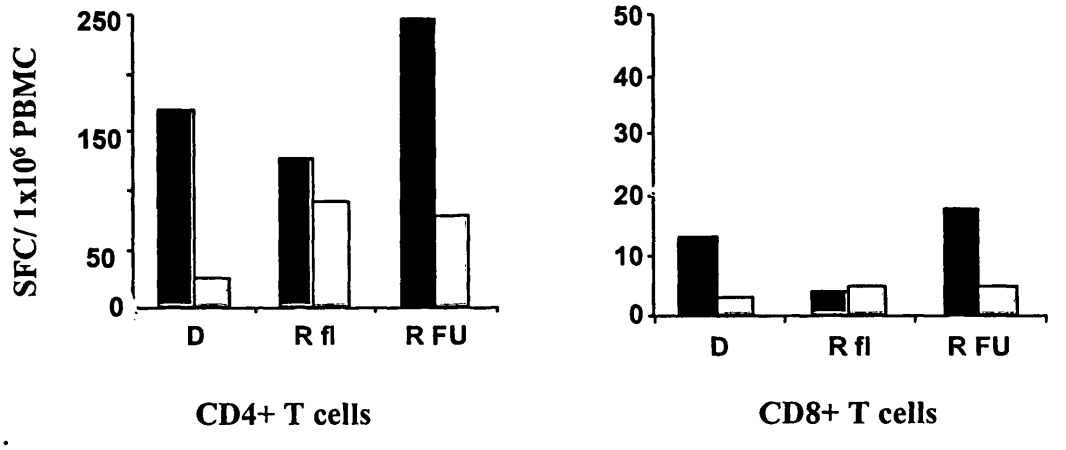


Figure 2a. Time course of serum alanine aminotransferase levels (ALT, normal level < 53 IU/L) and serum HBV markers after bone marrow transplantation (BMT). The hepatitis flare occurred 24 weeks after BMT and serum HBsAg was cleared 8 weeks later.

Figure 2b. Analysis (by IFN-γ Elispot assays) of the number of HBV-specific CD4 positive and CD8 positive T lymphocytes in the donor (D); the recipient at the time of hepatitis flare (R-fl) and the recipient at 52 weeks post-BMT (R-FU). The nucleocapsid (■) and surface (□) antigens used for CD4 positive and for CD8 positive T lymphocytes respectively, are the same, as shown in Fig. 1c.

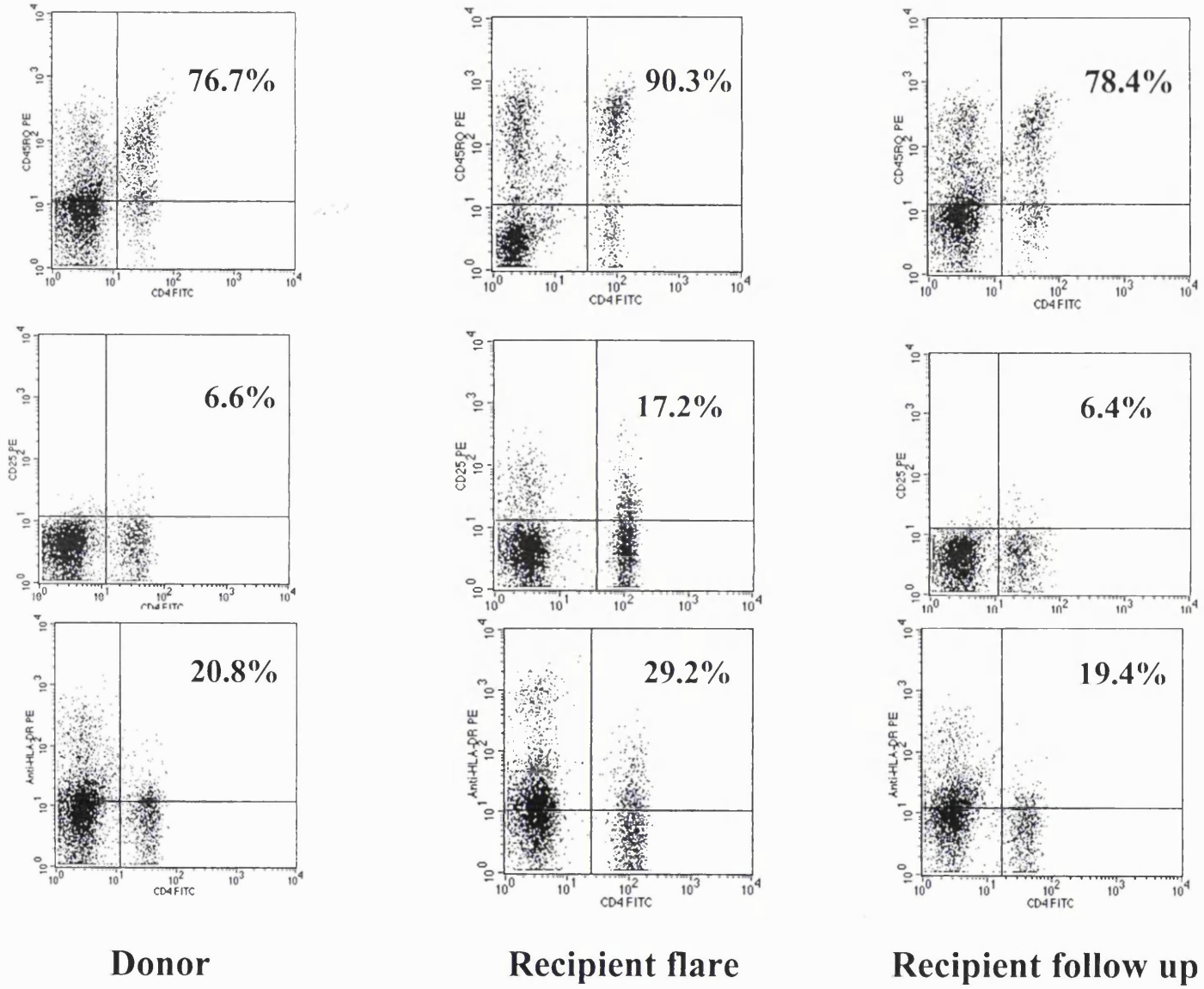


Figure 2c. Comparison of the proportions of activated CD4 positive T lymphocytes (CD25 or HLA DR positive) and the memory subset (CD45RO positive) in the donor, the recipient at the time of hepatitis flare and the recipient at 52 weeks post-BMT.

Table Clinical characteristics of BMT recipients

No	Age Sex	Hematologica diagnosis	HBV serology before BMT			Hepatitis flare and HBsAg clearance after BMT			Serum markers at the last follow-up after BMT			
			HBeAg	HBV DNA	antiHBe	Time of hepatitis flare (months)	Peak ALT (IU/l)[a]	Time of HBsAg clearance (months)	Time (months)	ALT (IU/l)	HBsAg HBs	anti HBs
1	16/M	ALL	neg	pos	neg	3.4	388	4.4	95	24	neg	pos
2	39/F	AML	neg	pos	neg	3.7	316	4.7	77	16	neg	pos
3	47/M	CML	pos	neg	pos	1.7	227	3.0	51	28	pos	neg[b]
4	26/F	CML	neg	pos	neg	1.8	145	2.5	38	18	pos	neg[b]
5[c]	29/M	CML	neg	pos	neg	3.3	197	4.0	38	21	neg	pos
6[c]	15/M	CML	neg	pos	neg	1.6	1530	2.0	34	5	neg	pos
7[c]	36/M	AML	pos	neg	pos	13.3	150	19	36	30	neg	pos
8[d]	34/M	CML	pos	neg	pos	6.0	461	7.8	20	18	neg	pos

Legend: ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; BMT, bone marrow transplantation; pos, positive; neg, negative; ALT, alanine aminotransferase (upper level of normal -53 IU/l for male and 31 IU/l for female). Serum HBV DNA was measured by Chiron bDNA assay.

[a] The peak of ALT levels after BMT occurred between 2 and 24 weeks before the loss of HBsAg;

[b] In both patients the other HBV markers are HBeAg negative, anti-HBe positive and HBV DNA undetectable;

[c] Received famciclovir 250-500 mg three times daily, starting 2 weeks before and continuing for six months after BMT;

[d] Received lamivudine 100 mg daily, starting 2 weeks before and continuing for six months after BMT.

Chapter 6

Deficient Expression Of Interleukin-12 Receptor On T Lymphocytes From Patients With Chronic HBV Infection

6.1 Background

The host immune response to HBV antigens is important in both viral clearance and disease pathogenesis (Chisari and Ferrari, 1995). Furthermore, chronic HBV is probably related to a specific failure of T-cells to recognize HBV antigens and is associated with in Th2 immune responses. In this chapter data is presented in which the possible role of IL-12 and IL-12 receptor (IL-12R) in the cause of Th2 immune responses seen in chronic HBV infection are investigated.

IL-1? is a 70 kDa heterodimeric cytokine produced by antigen presenting cells and is critical for efficient cell mediated immune responses to intracellular pathogens by inducing Th1 responses (especially IFN-γ production). IL-12 is comprised of two disulphide linked subunits of 35 and 40kD and binds to a specific β1/β2 heterodimeric IL-12R which is expressed by activated T lymphocytes and NK cells (Trinchieri, 1995). IL-12 can enhance the expression of its receptor resulting in increased IFN-γ production (Thibodeaux et al, 1999). Hence, IL-12 not only initiates differentiation of Th0 cells to Th1 phenotype also

maintains the functional responsiveness of Th1 lymphocytes. Impaired cell-mediated immunity has been seen in IL-12R deficiency resulting in Th2 immune responses causing severe disseminated infections by (usually) poorly virulent salmonella and atypical mycobacteria organisms (Altare et al, 1998; de Jong et al, 1998).

Th1 cytokines are important in the control of HBV infection. IFN-γ has potent anti-viral effects against HBV in the transgenic mouse (Guidotti et al, 1996) and in human liver cells (Romero and Lavine, 1996). IL-12 (through IFN-γ) can downregulate HBV gene expression (Cavanaugh et al, 1997) and increased levels are seen at the time of HBeAg seroconversion (Rossol et al, 1997). Hence IL-12 has direct anti-viral effects through IFN-γ in HBV infection but also has important immunomodulatory effects (Yue et al, 1999). Clinical studies have confirmed the effectiveness of recombinant IL-12 against HBV (Carreno et al, 2000).

The cause for impaired Th1 responses and diminished IFN-γ production in chronic HBV infection is unclear but the relative balance between Th1 and Th2 immune responses to HBV may be important in determining whether viral clearance occurs. IL-12R expression, which is critical for efficient cell mediated immunity in other infections and Th1 immune responses has not been investigated in HBV infection.

In this study the hypothesis that T lymphocytes from patients with chronic hepatitis B have reduced IL-12Rβ1 expression, which could result in deficient T-helper 1 responses resulting in impaired cell-mediated immunity was tested. IL-12 receptor expression on PBMC by FACS analysis on CD3 activated T lymphocytes in patients with HBV infection was determined. The functionality of PBMC from patients with chronic HBV infection was assessed by measuring the proliferative response and IFN-γ production following culture with recombinant IL-12. Finally the effect of rhIL-12 on IL-12 receptor expression in vivo was determined.

6.2 Methods

6.2.1 Patients and serological markers

We studied 3 patients with acute HBV (seropositive for HBsAg and IgM anti-HBc), 14 patients with chronic hepatitis B virus infection (seropositive for HBsAg, HBeAg and HBV DNA for greater than 6 months) and 4 patients who were HBsAg positive but anti-HBe positive and HBV DNA negative. The patients with acute hepatitis B virus infection were in the icteric phase with a bilirubin of greater than 250, and a mean ALT of 2760 iu/l when the experiments were carried out. We used 12 healthy subjects and 14 patients with chronic hepatitis C (anti-HCV and HCV RNA positive) as controls.

All patients (aged 22-48) were followed at the Institute of Hepatology, University College London between January and December 1998. The hepatitis B patients were seronegative for markers of HCV and HDV infection. No patient had

received anti-viral treatment in the past twelve months. Auto-antibodies and antibodies to HIV 1/2 (Abbott Diagnostics, Maidenhead, UK) were not detected. HBsAg, HBeAg, IgM anti-HBc was measured by qualitative enzyme immunoassays (Abbott Diagnostics). HBV DNA was measured by solution hybridisation assay (Digene 2, Abbot Diagnostics).

6.2.2 Detection of IL-12 receptor expression on T-cells

Full experimental details are described in Chapter 3.4.4. All experiments were undertaken on freshly isolated PBMC. Preliminary experiments undertaken confirmed that no measurable IL-12R is expressed on resting T-cells. We therefore used antibody to CD3 to activate T-lymphocytes to express IL-12R (Wu et al, 1996). PBMC at a concentration of 1.10^6/ml per well were incubated with 10% human AB serum/RPMI in a 96 well flat-bottomed tissue culture plate with antibody to CD3 (Chemicon, Harrow, UK) at a concentration of 1μg/ml. The plate was incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for three days as preliminary experiments showed that the maximal proliferative effect of CD3 on T-cells occurred was at this point.

IL-12R expression was measured by flow cytometry. 2.10^5 lymphocytes were incubated with 25μg/ml of mouse anti-human IL-12Rβ1-specific monoclonal antibody for 40 minutes. The cells were washed twice in PBS/1% FCS and then incubated for 30 minutes with a FITC-labelled anti-mouse IgG (Sigma, Poole, UK) as a second layer. As a control normal mouse immunoglobulin (Chemicon)

was used. During the incubation the cells were kept on ice and in the dark. The cells were finally washed twice and then fixed in 1% paraformaldehyde for analysis. The acquisition and analysis were performed by FACScan (Becton Dickinson Immunocytometry system, CA, USA) and CELLQuest (version 1.0, Becton Dickinson Immunocytometry system), respectively.

6.2.3 PBMC proliferation assay

Freshly isolated PBMC at a concentration of 2.10^5 per well were incubated with recombinant human IL-12 (10ng/ml), alone or with rHBcAg (1 μg/ml) as described in Chapter 3.4.1.3. Tetanus toxoid (1 μg/ml) and PHA (1 μg/ml) were used as positive controls. All experiments were run in four replicates.

6.2.4 HBcAg-specific in vitro IFN-γ production

For cytokine measurement, 3.10^5 PBMC were incubated with rhIL-12 (10 ng/ml) and/or HBcAg (1μg/ml) as described in 3.4.2.1. The supernatants were collected and stored at -20°C until analysis. IFN-γ levels were measured by ELISA as described in Chapter 3.4.2.1

6.2.5 In vivo effect of rhIL-12

The effect of in vivo administration of rhIL-12 on IL-12Rβ1 expression was evaluated in 9 patients with chronic HBV infection (HBsAg, HBeAg and HBV DNA positive). These patients were administered rhIL-12 at 250 or 500ng/kg (Genetics Institute, Munich, Germany) twice a week by subcutaneous injection as

treatment for chronic HBV infection. IL-12Rβ1 expression was measured on activated T-cells at before administration of rhIL-12 and four weeks after the first injection by FACS analysis as described above.

6.3 Results

6.3.1 IL-12 receptor expression

IL-12Rβ1 expression on CD3-activated T-lymphocytes was lower in patients with chronic HBV infection compared to healthy subjects or patients with chronic HCV infection (figure 1). The percentage of T-cells (mean ± SD) expressing IL-12Rβ1 in chronic HBV patients with on-going viral replication (HBeAg, and HBV DNA positive) was 6.6 ± 4.7% which was significantly less than IL-12Rβ1 expression in healthy controls which was 14.7 ± 7.2% (p=0.0002, Mann Whitney U). The level of IL-12Rβ1 expression in anti-HBe positive patients was 21.8 ± 8.8 which was significantly greater than the expression of IL-12Rβ1 expression in HBeAg+ patients (p=0.0026, Mann Whitney U). In contrast, the level of IL-12Rβ1 expression in patients with chronic hepatitis C virus infection was 23.7 ± 18.2 % which was similar to level seen in controls, (p=0.41, Mann Whitney U). IL-12Rβ1 expression in the 3 patients with acute hepatitis B infection (who all subsequently developed anti-HBe) was 30.5 ± 18.5%.

6.3.2 Effect of rhIL-12 on IL-12Rβ1 expression

Nine of the 14 chronic HBeAg, HBV DNA positive patients underwent treatment with rhIL-12 for chronic HBV infection. Four weeks after treatment with rhIL-12, IL-12Rβ1 expression increased from (mean ± SD %) 6.2 ± 5.1 % to 24.1 ± 16.4 % (p=0.0076 Wilcoxon Matched Pairs Test, figure 2).

6.3.3 rhIL-12-induced T-cell proliferation

In 14 patients with chronic hepatitis B virus infection and active HBV replication the effect of rhIL-12 on the T-cell proliferative response to HBcAg was assessed (figure 3). PBMC from these patients showed a weak proliferative response to HBcAg alone with a stimulation index (mean ± SD) 2.8 ± 1.7. IL-12 alone resulted in significantly greater proliferation with a stimulation index of 22.9 ± 19.2 (p=0.0004, Wilcoxon Matched Pairs Test). The proliferative response of PBMC to HBcAg and IL-12 was not significantly different to that of rhIL-12 alone (23.9 ± 22.1, p=0.16 Wilcoxon Matched Pairs Test).

6.3.4 rhIL-12 induced IFN-γ production

To assess whether the IL-12R on T-cells from patients with chronic HBV remains functional rhIL-12 induced IFN-γ production from PBMC in vitro was measured. IFN-γ produced by lymphocytes from patients with chronic HBV infection (HBeAg+, HBV DNA+) was similar to levels produced by normal subjects, (526.2 ± 362.2 pg/ml and 318.2 ± 257.4 pg/ml respectively, p=0.743, Mann

Whitney U). The levels of IFN-γ produced by stimulated PBMC from HBeAg+ patients was similar to 4 anti-HBe positive was 287.6 ± 224.8 pg/ml (p=0.99 2-tailed, Mann Whitney U). In anti-HCV positive patients the level of IFN-γ in response to rhIL-12 was 287.5 ± 259 which was similar to the levels of IFN-γ produced by healthy controls (Table).

Addition of recombinant HBcAg to PBMC cultured with IL-12 produced significantly higher concentrations of IFN-γ in comparison to PBMC cultured with IL-12 alone. In the 14 chronic HBV patients PBMC cultured with IL-12 alone produced (mean ± SD) 331.31 ± 308.6 pg/ml of IFN-γ, whereas PBMC from the same patients cultured with both HBcAg and IL-12 produced 838.2 ± 167.2 pg/ml (p=0.0009 Wilcoxon Matched Pairs Test, figure 3).

6.4 Discussion

The host immune response to HBV infection is critical to the resolution of HBV infection. Impaired virus-specific Th1 immune responses are associated with chronic HBV infection but the cause for these impaired immune responses is unclear. Furthermore, induction of HBcAg specific T-cell reactivity associated with Th1 immune responses has been demonstrated in both spontaneous and IFN-α induced seroconversion to anti-HBe (Tsai et al, 1992; Marinos et al, 1995). IL-12 is a key cytokine involved in the differentiation and maintenance of a Th1

immune response (Gately et al, 1998). IL-12 is important in the clearance of HBV. In addition to data from the transgenic mouse model of chronic HBV infection (Cavanaugh et al, 1997) we have previously shown that HBV clearance in chronic carriers undergoing interferon-α treatment occurs only in those who develop a substantial rise in IL-12, the peak of which coincides with the time of anti-HBe seroconversion (Rossol et al, 1997).

This study shows that T lymphocytes from patients with chronic HBV infection have reduced expression of IL-12Rβ1 as compared to control subjects and patients with HCV infection. In contrast patients who have serological resolution of viraemia (either those with acute HBV infection or patients with chronic HBV who are anti-HBe positive, HBV DNA negative) have levels of IL-12Rβ1 expression similar to healthy controls. IL-12 receptor deficiency has been documented in non-viral infections (de Jong et al, 1998; Altare et al, 1998) where it is associated with the development of serious infections to organisms which are usually poorly virulent. In these patients although IL-12 activates PBMC (as measured by antigen specific and non-specific proliferative responses), the genetic lack of IL-12Rβ1 expression results in considerably decreased IFN-γ production (antigen primed T-cells produce only 5% of IFN-γ compared to controls).

Systemic treatment with rhIL-12 restored IL-12Rβ1 expression on PBMC from chronic HBV patients to normal levels which confirms similar findings in

sarcoidosis (Rogge et al, 1999). Furthermore, we have shown that this rhIL-12 induced upregulation of IL-12Rβ1 is associated with Th1 effector functions. The upregulated PBMC produce normal levels of IFN-γ in vitro suggesting that the PBMC retain the capability to exert effective anti-viral effector functions. Our study confirms other in vitro investigations that have shown IL-12 can augment IFN-γ production in response to nucleocapsid antigen (Vingerhoets et al, 1998) and the reduced level of IL-12R expression may explain this.

Therapy with recombinant IL-12 can restore impaired host immune responses in viral infections. In HIV infection rIL-12 normalises SAC induced responses by increasing IL-12Rβ1 expression (Chehimi et al, 1994). Similarly IL-12 reverses deficient Th1 responses in HIV (Clerici et al, 1993). In chronic HCV infection impaired PBMC proliferation and IFN-γ production can also be restored by rIL-12 (Schlaak et al, 1998). However, a recent clinical study of rhIL-12 failed to show benefit as a treatment with chronic HCV infection (Zeuzem et al, 1999). Our studies showing that IL-12Rβ1 expression is normal in patients with chronic HCV infection suggest that the Th1/Th2 imbalance seen in these patients (Cramp et al, 2000) is not as a result of IL-12Rβ1 deficiency and explains the poor results seen rIL-12 as a treatment for chronic HCV infection.

In human HBV infection, lower levels of serum IL-12 are seen in immunotolerant HBV carriers compared to uninfected controls and patients with active chronic HBV infection. This may explain the differences seen in in vitro IFN-γ production

in response to nucleocapsid antigens (Marinos et al, 1995). However, HBV itself can impair the host immune responses to the virus as high levels of HBV DNA are associated with suppressed co-stimulatory and immunoregulatory effects of IL-12 as seen by lower levels of IFN-γ and TNF-α production, and T-cell proliferation (Schlaak et al, 1999). Although we did not specifically investigate the effect of HBV DNA levels on IL-12 responsiveness our findings that those patients who are HBV DNA negative have higher IL-12Rβ1 levels are consistent with this.

The restoration of Th1 effector functions of PBMC can be augmented by the presence of nucleocapsid antigen. Our data showed that in vitro stimulation of PBMC of patients with chronic HBV by HBcAg and rIL-12 results in significantly greater IFN-γ production than culture with IL-12 alone. The significantly higher levels of IFN-γ seen is in keeping with recent data showing that HBcAg is considerably more immunogenic than envelope antigens and elicits Th1 like responses (Livingston et al, 1999). In mice HBcAg-primed T-helper cells are able to provide T-cell help and elicit anti-envelope production possibly as a result of enhanced IFN-γ production. This may be sufficient to switch the Th2 phenotype to Th1 thereby encouraging appropriate immune responses to clear the virus. Gene thereapy has been used to enhance Th1 immune responses in the woodchuck model of HBV infection. Immunisation with genes for both WHBcAg and IL-12 resulted in a Th1 immune profile and control of woodchuck HBV replication (R.Garci'A-Navarro et al, 2001).

Our study provides insights into how IL-12 induced Th1 responses may be important therapeutically in the serological clearance of HBV infection and this has recently been demonstrated in human chronic HBV infection treated with rhIL-12 showed that anti-HBe seroconversion was associated with increased in vitro IFN-γ production (Barth et al, 2001). It would also be interesting to study IL-12Rβ1 expression and response to rhIL-12 in patients who fail to clear the virus – either those who have pre-core mutants or following acute infection. Furthermore, we have not investigated the cause of the low level of IL-12Rβ1 expression. As IL-12Rβ1 is under autoregulation its low expression may be as a result of low IL-12 levels. This could be genetic in origin such as a result of IL-12p40 subunit gene deletion (Altare et al, 1998). Activated dendritic cells and phagocytes are therefore unable to produce functional IL-12 p70 cytokine resulting in markedly impaired lymphocyte IFN-γ production. Although a genetic cause for this is possible the ready inducibility of IL-12Rβ1 by IL-12 is against this. The immunological defect may be at an earlier level of the pathway perhaps as a result of impaired dendritic cell function resulting in impaired antigen presentation (Wang et al, 2001). Dendritic cell function in chronic HBV carriers would be interesting to investigate further.

In conclusion, this is the first data to show that IL-12Rβ1 expression is diminished in chronic HBV infection, which may result in the Th2 immune responses seen. However, PBMC remain functional as expression can be up-

regulated by IL-12 and they can proliferate normally and secrete normal levels of IFN-γ. Recent clinical studies suggest that IL-12 may have an important anti-viral effect in patients with chronic HBV infection by promoting Th1 immune responses and IFN-γ which may be through up-regulation of IL-12Rβ1.

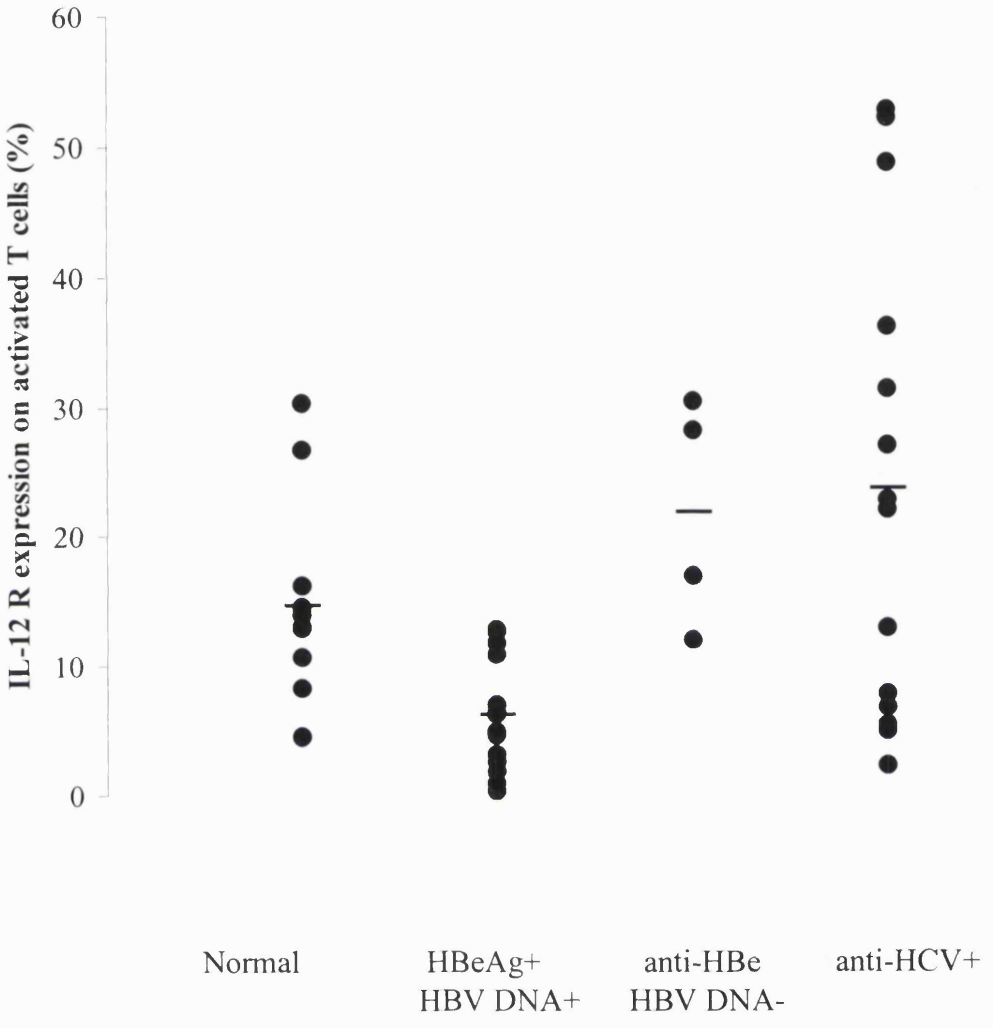


Figure 1. IL-12 receptor expression measured by FACS analysis on T-cells activated following culture with anti-CD3 for 3 days. Patients with chronic hepatitis B (HBeAg positive with on-going viral replication) are compared to those who are anti-HBe positive HBV DNA negative, and to patients with chronic hepatitis C virus infections and healthy controls.

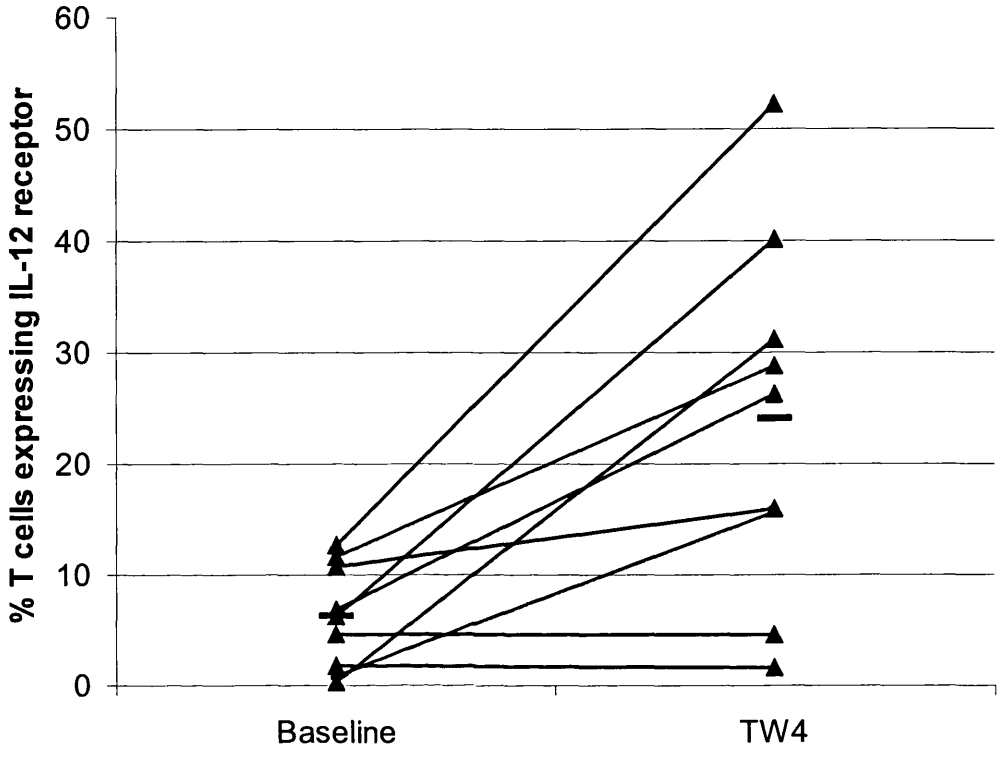


Figure 2 Effect of in vivo rhIL-12 treatment on IL-12R expression. IL-12R expression on CD3 activated T-cells was measured by FACS at baseline and after 4 weeks treatment with rhIL-12. The results are expressed as percentage of T-cells expressing IL-12R.

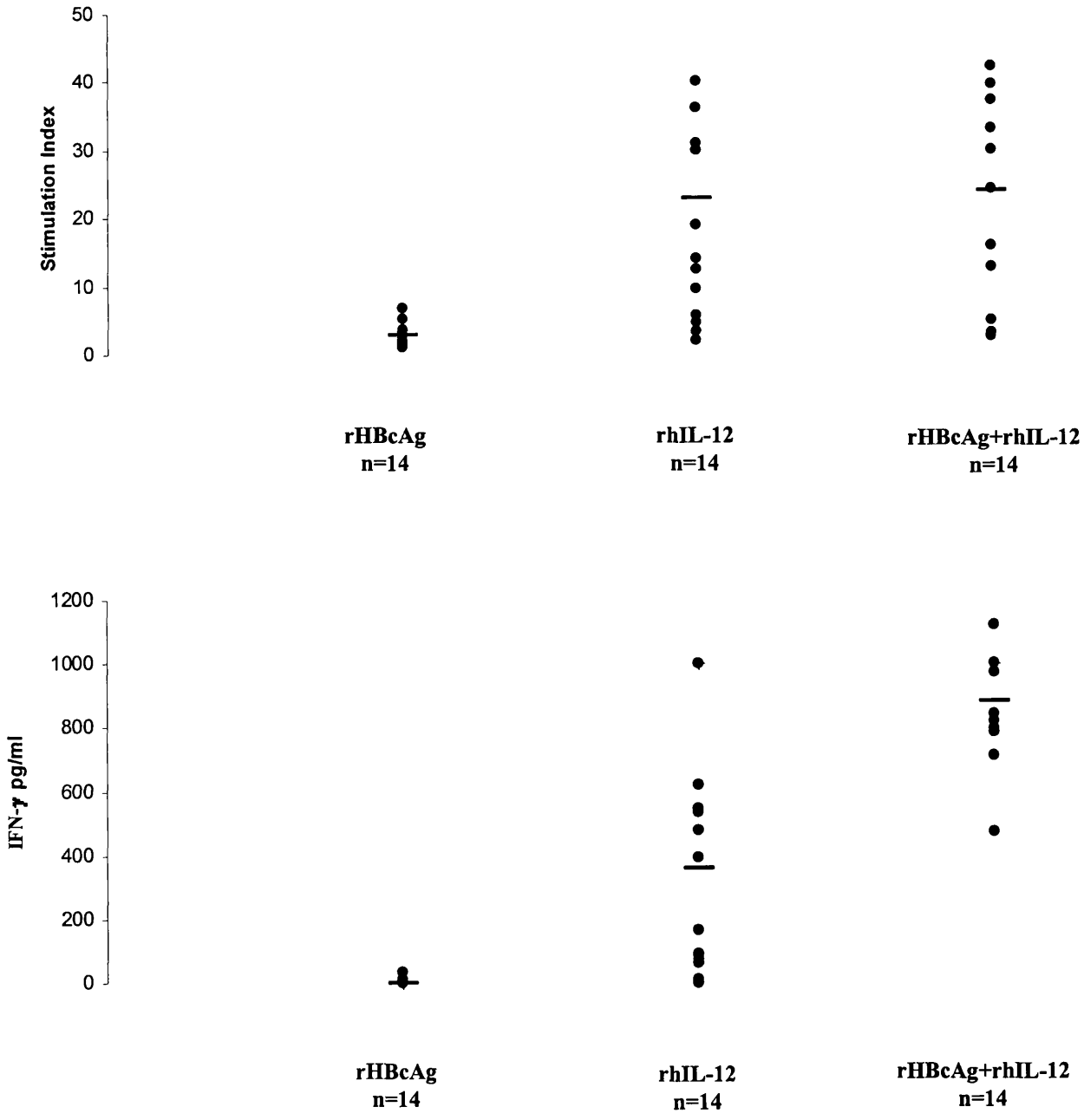


Figure 3: T-cell proliferation and in vitro IFN-γ production of PBMC from 14 chronic HBV carriers in response to HBcAg, rhIL-12 and HBcAg + rhIL-12. T-cell proliferation is expressed as a stimulation index compared with cells incubated in medium alone. IFN-γ levels (pg/ml) were measured using a commercial ELISA.

		Proliferation		IFN-γ (pg/ml)	
	IL-12Rβ1 (%) Mean ± SD	**IL-12** Mean ± SD	**HBcAg+IL-12** Mean ± SD	**IL-12** Mean ± SD	**HBcAg+IL-12** Mean ± SD
HBV (eAg+ n=14)	6.6 (4.7)	22.9 (19.1)	23.9 (22.1)	526 (362)	882 (183)
HBV (eAb+ n=4)	21.8 (8.8)	9.1 (9.4)	-	299.5 (187)	759.9 (187.1)
HCV	23.7 (18.2)	5.0 (4.9)	-	287 (259)	-
Control	14.7 (7.2)	6.2 (4.9)	-	318 (257)	-

Table Expression of IL-12 receptor measured by FACS compared to proliferation to IL-12 in the presence of HBcAg in patients with chronic HBV infection (HBeAg+, HBV DNA +) compared to patients with with HBV infection who are HBV DNA negative, chronic HCV infection and normal controls.

Chapter 7

The effect of recombinant human interleukin-12 in vivo in chronic human HBV infection

7.1 Introduction:

The data presented in the thesis so far has focussed on the non-cytolytic control of HBV and the role of IL-12. The in vitro experiments (Chapters 4 and 6) have shown that IL-12 inhibits HBV replication in human hepatocytes via induction of IFN-γ and this anti-viral effect was more pronounced in cells with a lower viral load. We therefore investigated whether addition of recombinant human interleukin-12 (rhIL-12) to standard lamivudine treatment will enhance the anti-viral effect in patients with chronic HBV infection.

IL-12 is an important cytokine involved in the clearance of HBV (Cavanaugh et al, 1997; Rossol et al, 1997). In addition case reports and pilot studies of rhIL-12 monotherapy has been shown be successful in the treatment of chronic HBV infection (Barth et al, 2001; Carreno et al, 2000)

The aim of this study was to investigate the effect of rhIL-12 in vivo in patients with chronic hepatitis B virus infection. This chapter presents data on the anti-viral and immunomodulatory effects of rhIL-12 in addition to standard

lamivudine therapy. The effect of rhIL-12 on viral load and the host immune response during the treatment was evaluated. Furthermore the effect on serum transaminase levels as well as its safety and tolerability was also investigated.

7.2 Methods

7.2.1. Patient characteristics

The study consisted of 15 patients (11 male, 4 female) aged between 21 and 44 with chronic hepatitis B virus infection followed at the Institute of Hepatology, University College London (table). All patients were HBsAg, HBeAg, and HBV DNA positive for over six months. Liver auto-antibodies, anti-HCV (third generation ELISA), anti-HIV 1/2 antibodies and anti-HDV antibodies were not detected. No patient had received anti-viral treatment for hepatitis B virus infection in the past twelve months. However, 8 of the 15 patients had failed previous attempts at treatment including interferon monotherapy at standard doses (6), famciclovir (1), adefovir (1), and interferon/lamivudine combination therapy (5). Five patients had more than one course of anti-viral treatment.

Alanine aminotransferase levels were normal in four patients, slightly raised (less than twice above the upper limit of normal) in six patients, and markedly raised in the remaining five patients (between 2 and 5 times the upper limit of normal).

A liver biopsy was performed in each patient as part of the diagnostic evaluation of his or her chronic hepatitis B virus infection with informed consent. Portal tract inflammation, interface hepatitis, and lobular inflammation were each graded on a 0 to 3 scale, and a necroinflammatory score was calculated to a maximum value of 9. Fibrosis was scored between 0 and 6 (Ishak et al, 1995).

Prior to randomisation the patients were divided into two subgroups according to their histological activity index and alanine aminotransferase level. In sub-group 1, patients had mild liver disease i.e. inflammation 0 or 1 and fibrosis 0 respectively and alanine aminotransferase levels less than twice upper limit of normal. In sub-group 2 the patients had more advanced liver disease as defined by grade of inflammation 2-5 and stage of fibrosis 1-5. The baseline alanine aminotransferase levels in these patients were greater than twice the upper limit of normal. The patients were randomised to one of three treatment regimens described below (groups 1,2,3).

7.2.2 Study design and monitoring

This was an open label, randomised study comparing lamivudine monotherapy versus a combination of lamivudine and two doses (200ng/kg or 500ng/kg) of rhIL-12 (figure 1). Patients were assigned to either lamivudine monotherapy (100mg daily, Glaxo Smith-Kline, Greenford, UK, group 1) for 24 weeks; or lamivudine (100mg daily) for 16 weeks with rhIL-12 given twice weekly at a dose of 200 ng/kg sub-cutaneously (Genetics Institute, Munich, Germany) for 20

weeks starting four weeks after initiation of lamivudine (group 2); or lamivudine for 16 weeks with rhIL-12 given twice weekly at a dose of 500 ng/kg sub-cutaneously for 20 weeks starting four weeks after initiation of lamivudine (group 3). All patients received a total of 24 weeks therapy. The patients were seen fortnightly for the first eight weeks of the study and then monthly for the following twelve weeks. The patients continued to be seen every other month for six months following cessation of treatment (figure 2). At each visit the patients were asked about tolerability of treatment and adverse events. A physical examination and routine biochemistry and haematology testing was also carried out.

The aims of the study were to compare the antiviral effect of lamivudine and rhIL-12 with lamivudine monotherapy on HBV DNA levels, to characterise the HBV-specific T-cell reactivity during treatment and finally to investigate safety of the combination treatment.

The primary end-points were loss of serum HBV DNA (by signal amplification assay) and the degree of reduction is serum HBV DNA (measured by quantitative PCR). Secondary end points assessed were: loss of HBeAg and development of anti-HBe; normalisation of ALT; induction of HBV-specific T-cell reactivity; and safety and tolerability.

The patients were enrolled in a clinical trial protocol carried out with UCL hospitals ethical committee approval.

7.2.3 Immunological assays

7.2.3.1 PBMC proliferation assay.

The response to HBcAg, tetanus toxoid and PHA were assessed at the time points shown in figure 2. All T-cell proliferation assays were carried out on freshly isolated PBMC as described in chapter 3.4.1.

7.2.3.2 Elispot assays for CD4+ and CD8+ T cells.

The ELISPOT assay was used to quantitate the number of HBV-specific, IFN-γ producing CD4+ and CD8+ T lymphocytes as described in Chapter 3.4.2.1. All experiments were carried out on cryopreserved PBMC. After thawing the cell viability was tested and was greater than 95%, as assessed by trypan blue exclusion. PBMC were tested at baseline, weeks 4, 8, 16, 24, and 48.

7.2.3.3 IL-12 Receptor Expression

In 9 patients treated with combination treatment the in vitro effects of rhIL-12 on IL-12R expression was analysed prior to commencement of rhIL-12 and 4 weeks later (full experimental details and results are described in Chapter 6).

7.2.4 Virological assays

7.2.4.1 Routine serology

HBsAg, HBeAg, IgM anti-HBc were measured by qualitative enzyme immunoassays (Abbott Diagnostics, Maidenhead, UK) by the department of Virology UCL hospital.

7.2.4.2 HBV DNA viral load

Serum HBV DNA level was measured by the Department of Virology, UCL hospitals with a signal amplification assay (Quantiplex^{TM} bDNA assay, Chiron Corporation, Emerville, CA). Using this assay, the threshold for HBV DNA detection in serum is $0.7x10^6$ viral copies/ml (0.7Meq/ml). To determine the antiviral effect further serum samples between weeks 4 and 16 were re-tested for HBV DNA using a quantitative polymerase chain reaction assay (National Genetics Institute, Los Angeles, USA) with a lower limit of detection of 400 copies/ml. Samples for this assay were taken weekly between weeks 4 and 8 and then at weeks 12 and 16.

7.2.4.3 Statistics:

Mann Whitney U and Wilcoxon Matched Pairs Test were used to calculate the significance of the non-parametric data generated using the Statistical Package of the Social Sciences (SPSS; SPSS Inc, Chicago IL, USA).

7.3 Results

7.3.1 Effect of combination treatment on HBV DNA

The mean pre-treatment HBV DNA levels measured by Quantiplex^{TM} bDNA assay were (mean ± sem) 2581 ± 1018, 3042 ± 1016, 3336 ± 881 Meq/ml in the lamivudine, lamivudine and IL-12/200 and lamivudine and IL-12/500 groups of patients respectively (figure 3). All patients received lamivudine monotherapy for the first four weeks, which substantially reduced HBV DNA to (mean ± sem) 25 ± 20.5, 65 ± 48 and 7.4 ± 3.5 Meq/ml respectively.

After 16 weeks of treatment several patients had undetectable levels of HBV DNA as measured by the Quantiplex^{TM} bDNA assay. In the lamivudine monotherapy group and the lamivudine and IL-12/200 groups two patients had undetectable HBV DNA. In contrast in the lamivudine and IL-12/500 group four patients had become HBV DNA negative.

Serum samples between weeks 4 and 16 were re-tested using the NGI quantitative PCR assay to assess whether there was difference in the anti-viral effect of the three treatments (figure 4). The reduction of HBV DNA with lamivudine monotherapy was (log_{10} mean ± sem) 0.6 ± 0.2 (p=0.19, Wilcoxon Matched Pairs Test); with lamivudine and IL-12/200 was 1.4 ± 0.3 (p=0.09, Wilcoxon Matched Pairs Test); with lamivudine and IL-12/500 2.2 ± 0.3 (p=0.03, Wilcoxon Matched Pairs Test).

7.3.2 Serological response to combination treatment

There was transient loss of HBeAg in 2 patients whilst taking the combination treatment (1 patient on lamivudine/IL-12/200 and 1 patient on lamivudine/IL-12/500). However, there was no sustained loss of HBeAg or development of anti-HBe, either during treatment or follow-up.

7.3.3 Effect on ALT levels

Lamivudine treatment reduced serum ALT levels (figure 3). In the five patients treated with lamivudine monotherapy, the mean ALT fell from 185iu/l to 42 iu/l after 16 weeks treatment ($p=0.02$, Wilcoxon Matched Pairs Test). Following treatment cessation a flare in the transaminases was seen in three of the five patients (mean ALT level 366 iu/l between weeks 32 and 40). These treatment flares were not associated with any clinical deterioration in the patients.

In the patients treated with lamivudine and IL-12/200 the ALT dropped from a mean of 106 at baseline to 63 at week 16 ($p=0.03$, Wilcoxon Matched Pairs Test) although this was still above the upper limit of normal. In the lamivudine and IL-12/500 group the mean ALT was similar at the two time points (86 iu/l and 106 iu/l, respectively $p=0.5$, Wilcoxon Matched Pairs Test). However this rise in mean ALT was largely as a result of one patient developing a flare in ALT during treatment with lamivudine and IL-12/500 at week 16 (peak ALT 273). This flare was associated with constitutional symptoms but no decompensation in liver disease. In the other four patients in this group ALT values all fell during

treatment. Following cessation of treatment ALT levels gradually rose to peak levels of 91 and 160 respectively for IL-12/200 and IL-12/500.

7.3.4 Effect on host immune response

7.3.4.1 HBcAg –specific T-cell proliferation.

The HBcAg-specific CD4+ T-cell proliferative response in all three groups increased during the course of treatment compared to baseline (figure 5). The peak T-cell proliferative response was seen at week 16. The stimulation index following lamivudine, lamivudine and IL-12/200 and lamivudine and IL-12/500 increased from (mean ± SEM) 2.8 ± 1.0, 4.0 ± 1.0 and 3.9 ± 1.8 to 4.7 ± 0.7 ($p=0.03$, Wilcoxon Matched Pairs Test), 6.0 ± 1.4 ($p=0.03$, Wilcoxon Matched Pairs Test) and 9.6 ± 4.9 ($p=0.03$, Wilcoxon Matched Pairs Test), respectively. In addition, patients on lamivudine monotherapy had an increase in the HBcAg T-cell proliferation at week 48, mean stimulation index 9.6 ± 8.7.

7.3.4.2 Quantitation of the number of HBV-specific, interferon-γ producing T lymphocytes.

CD4+ T-lymphocytes:

The number of spot forming cells (SFC) seen during treatment remained unchanged during lamivudine monotherapy. At weeks 4 and 16 the number of SFC in the lamivudine treated group was 218 and 250 respectively ($p=0.5$, Wilcoxon Matched Pairs Test, figure 6). In contrast, significant increases in SFC were seen during the combination treatments. In the lamivudine and IL-12/200

Wilcoxon Matched Pairs Test, figure 6). In contrast, significant increases in SFC were seen during the combination treatments. In the lamivudine and IL-12/200 group the mean number of SFC increased from 49 to 198 (p=0.03 Wilcoxon Matched Pairs Test) and in the lamivudine and IL-12/500 the mean number of SFC increased from 124 to 356 (p=0.03, Wilcoxon Matched Pairs Test). Furthermore the peak number of SFC was seen earlier with the lamivudine and IL-12/500 treated patients at week 8 rather than week 16.

CD8+ T-lymphocytes:

In the four patients who were HLA-A2 restricted CD8+ elispot was carried out (figure 7). Two of these patients were treated with lamivudine monotherapy, one patient was treated with lamivudine and IL-12/200, and one patient treated with lamivudine and IL-12/500. The number of SFC increased in all three groups during the treatment, but was greatest in the patients receiving rhIL-12. In the lamivudine treated patients the baseline number of SFC increased from 15 and 18 to peak at 70 and 73 SFC at week 8. In contrast the patients treated with the two concentrations of IL-12 the baseline number of SFC increased from 2 and 30 to peak at 130 and 163 at week 8 and 24 respectively for lamivudine and IL-12/200 and IL-12/500 respectively.

7.3.5 Safety/Tolerability

IL-12 was well tolerated as 9 of the 10 patients completed all doses of treatment without any dose reduction or ommisions. One patient was reduced from 500ng/kg to 200 ng/kg after three doses due to headaches, myalgia and flu-like symptoms. He completed the rest of the course without any further difficulties.

No serious adverse events were noted during the treatment. Acceptable and expected side effects after the first two injections were seen (which were transient and mainly grade 1 or 2 in severity). Very few symptoms were seen thereafter apart from tiredness. Most frequent adverse events included, mild pyrexia, headache, myalgia, arthralgia, asthenia, flu-like symptoms and insomnia. One patient on the Lamivudine and IL-12/500 experienced a prolonged productive cough commencing at week 12 and lasted 3 weeks. Investigations revealed a normal chest x-ray and sputum culture grew no pathogens. The symptoms subsided spontaneously. Overall IL-12 was felt to be easier to tolerate than IFN-α by those patients who had previously taken IFN-α.

Blood test abnormalities were rare but lymphopenia was seen in 2 patients on combination treatment, which was asymptomatic and resolved on treatment cessation. Three of the five patients on lamivudine monotherapy developed a flare in transaminases as described in 7.3.3.

7.4 Discussion

Current treatment strategies for chronic hepatitis B virus infection are unsatisfactory as only a small proportion of selected patients benefit by developing long-term control of viral replication. Therefore new treatment strategies are required for control of chronic HBV infection in the majority of patients. As the host immune response is critical to resolution of viraemia, strategies that involve augmenting it are likely to be important and therefore are conceptually attractive. Data from animal models of HBV infection and also from patients with chronic HBV have shown that IFN-γ and IL-12 are important in viral clearance (Cavanaugh et al, 1997; Chisari and Ferrari, 1995; Rossol et al, 1997). Case reports and pilot studies have shown that pharmacological doses of rhIL-12 is effective in the treatment of chronic HBV (Barth et al, 2001; Carreno et al, 2000)

In this study the anti-viral efficacy, the immune and biochemical responses, safety and tolerability of lamivudine and rhIL-12 was assessed. The quantitative PCR assay demonstrated that the addition of rhIL-12 to lamivudine resulted in greater reductions in HBV DNA levels than lamivudine monotherapy. Furthermore, a dose response was seen with IL-12/500 having a more pronounced anti-viral effect than IL-12/200 with a mean reduction of 2.2 $\log_{10}$ compared to 1.4 $\log_{10}$. The degree of reduction in HBV DNA is important as this is related to the likelihood that the host will gain long-term control and resolve HBV infection. An absolute reduction of HBV DNA levels to $<1.10^4$ genomes per ml has been

associated with HBeAg seroconversion (Gauthier et al, 1999) but no patient in this trial achieved this sustained level of HBV DNA which probably explains why no patient had sustainted HBeAg loss.

Both lamivudine and the combination treatments reduced ALT levels. After 16 weeks of lamivudine monotherapy a biochemical response was achieved with a normalisation of ALT levels. In contrast although combination treatment reduced serum ALT compared to pre-treatment levels the ALT did not completely normalise perhaps as a result of immune activation by rhIL-12. One patient treated with lamivudine and IL-12/500 developed a flare in transaminases during treatment however this was well tolerated with no clinical deterioration. HBeAg seroconversion did not occur with this transaminase flare. In contrast three of the five patients treated with lamivudine monotherapy developed flares of ALT after discontinuation of lamivudine whereas no post-treatment flares in ALT were seen in any of the patients treated with combination therapy. The post-treatment ALT flares in the lamivudine monotherapy treated patients may possibly have been as a result the rapid rise to pre-treatment levels in HBV DNA levels when lamivudine was stopped. In contrast patients treated with combination continued with rhIL-12 for 2 months after the cessation of lamivudine. Although the levels of HBV DNA started to rise after termination of lamivudine the on-going treatment with rhIL-12 continued to have some anti-viral effect.

We assessed the effect of treatment on the hosts' immune response by measuring HBcAg-specific CD4+ T-cell reactivity with the T-cell proliferation assay and the Elispot assay. We also assessed changes in HBcAg-specific CD8+ reactivity with the CD8+ Elispot assay. Lamivudine and rhIL-12/500 produced the greatest increases in T-cell proliferation from 4 at baseline to 9.6 at week 16. The Elispot assay showed a significant increase in the number of IFN-γ producing HBcAg specific CD4+ T-cells with both doses of combination treatment but not with lamivudine monotherapy. After the treatments had finished the number of IFN-γ producing spot forming cells fell back towards pre-treatment levels. These changes were seen slightly earlier than in the proliferation assay at week 8, suggesting that this may be a more sensitive assessment of HBcAg-specific CD4+ T-cell reactivity than conventional proliferation assays. In the four patients who were HLA-A2 positive IL-12/500 resulted in the greatest rise in CD8+ spot forming cells during treatment. Overall the elispot and proliferation assays showed that combination treatment resulted in enhanced HBcAg-specific CD4+ (and possibly CD8+) T-cell reactivity than lamivudine monotherapy.

Transient HBeAg loss was observed in only two patients. This is not surprising when considering the type of patients who were treated. Eight of the 15 had undergone previous attempts at treatment (including combination treatment) without success and hence could be considered as 'difficult'. In addition 4 of the 15 could be considered as immunotolerant with normal transaminase levels and these types of patients tend to show poor responses to currently licensed therapies.

Only one of the patients had an ALT greater than five times the upper limit of normal which has been shown to be an excellent predictor of HBeAg seroconversion in response to lamivudine (Dienstag et al, 1999; Lai et al, 1998).

This study demonstrated that the combination of lamivudine and rhIL-12 is safe and well tolerated. Nine of the ten patients who received either dose of interleukin-12 as part of combination treatment successfully completed the course of treatment – only one patient required a reduction from 500ng/kg to 200ng/kg after two doses and he successfully completed the rest of the course. Transient lymphopenia was seen in 2 patients but this reversed on treatment cessation. Furthermore most of the side effects seen were mild severity and most pronounced after the first two doses of rhIL-12. These symptoms resolved with simple analgesia and did not affect the long-term tolerability.

Although combination anti-viral therapy of HIV infection is well established few clinical trials have investigated its role in the treatment of chronic HBV infection. Treatment with a combination of two nucleoside analogue drugs has greater short-term anti-viral efficacy in chronic HBV infection than monotherapy (Lau et al, 2000). The largest study of combination therapy todate has been with IFN-α and lamivudine with a lamivudine monotherapy lead-in phase. Overall there was an improved HBeAg seroconversion rate with combination treatment. However, from the original 230 randomised patients there were 50 protocol violations making it difficult to gauge the clinical significance of the combination

treatment's borderline statistical significance (Schalm et al, 2000). Furthermore, subgroup analysis suggested that those patients with moderately raised transaminase levels (2-5 X upper limit of normal) were the ones to benefit the most from combination treatment rather than those with very high transaminase levels or those with near normal transaminase levels. This suggests that IFN-α can augment an existing mild immune response but is not effective if the hosts' immune response is non-existent. A study of lamivudine and IFN-α given concurrently, showed enhanced HBeAg seroconversion rates and histological improvement (Barbaro et al, 2001). Another study in previous non-responders, showed a combination of lamivudine and IFN-α resulted in high rates of HBeAg seroconversion although this study was uncontrolled (Serfaty et al, 2001). Hence, current data suggests that combination therapy of anti-viral and immunomodulatory drugs may be more effective than monotherapy.

We commenced our regime of therapy with a one month 'lead-in phase' for lamivudine to reduce HBV DNA levels prior to rhIL-12. We envisaged that lamivudine would reduce the levels of HBV DNA enhancing the host immune response, which could then be augmented by rhIL-12 in a similar way that IFN-α is more effective in patients with lower levels of HBV DNA. Furthermore, high HBV DNA levels inhibit the immunomodulatory effects of IL-12 in vitro (Vingerhoets et al, 1998) and the reduction of HBV DNA enhances the action of IL-12 in vitro (Schlaak et al, 1999). However, a theoretical drawback of pre-treatment with lamivudine reduces ALT levels and reduced ALT levels are

associated with decreased efficacy of IFN-α (and therefore potentially with rhIL-12).

The data presented in this chapter suggest that combination therapy with higher dose rhIL-12 has greater anti-viral and immunomodulatory effects than lamivudine monotherapy. Furthermore combination treatment reduced transaminase levels in 9 of 10 patients during the treatment course it would be interesting to see if longer treatments resulted in complete normalisation of LFTs, greater reductions in HBV DNA, enhanced immunoreactivity, HBeAg seroconversion and associated improvement in histological necroinflammatory score. The safety and tolerability data suggest that a longer course of rhIL-12 would be acceptable to patients, and it would be worth investigating whether a longer course of treatment translated into greater anti-viral efficacy.

It would be interesting to measure the viral kinetics during combination treatment. Kinetic studies in HIV and hepatitis C virus infection have shown which patients are likely to clear the virus and helped us to understand the lifecycle and provided predictive information on potential responders to treatments for HCV infection.

In summary, the addition of rhIL-12 to lamivudine resulted in enhanced anti-viral effects and immunomodulatory responses as compared to lamivudine monotherapy. A further study with more patients for a longer duration would be

reasonable to see if these effects are translated into histological improvements and HBeAg seroconversion.

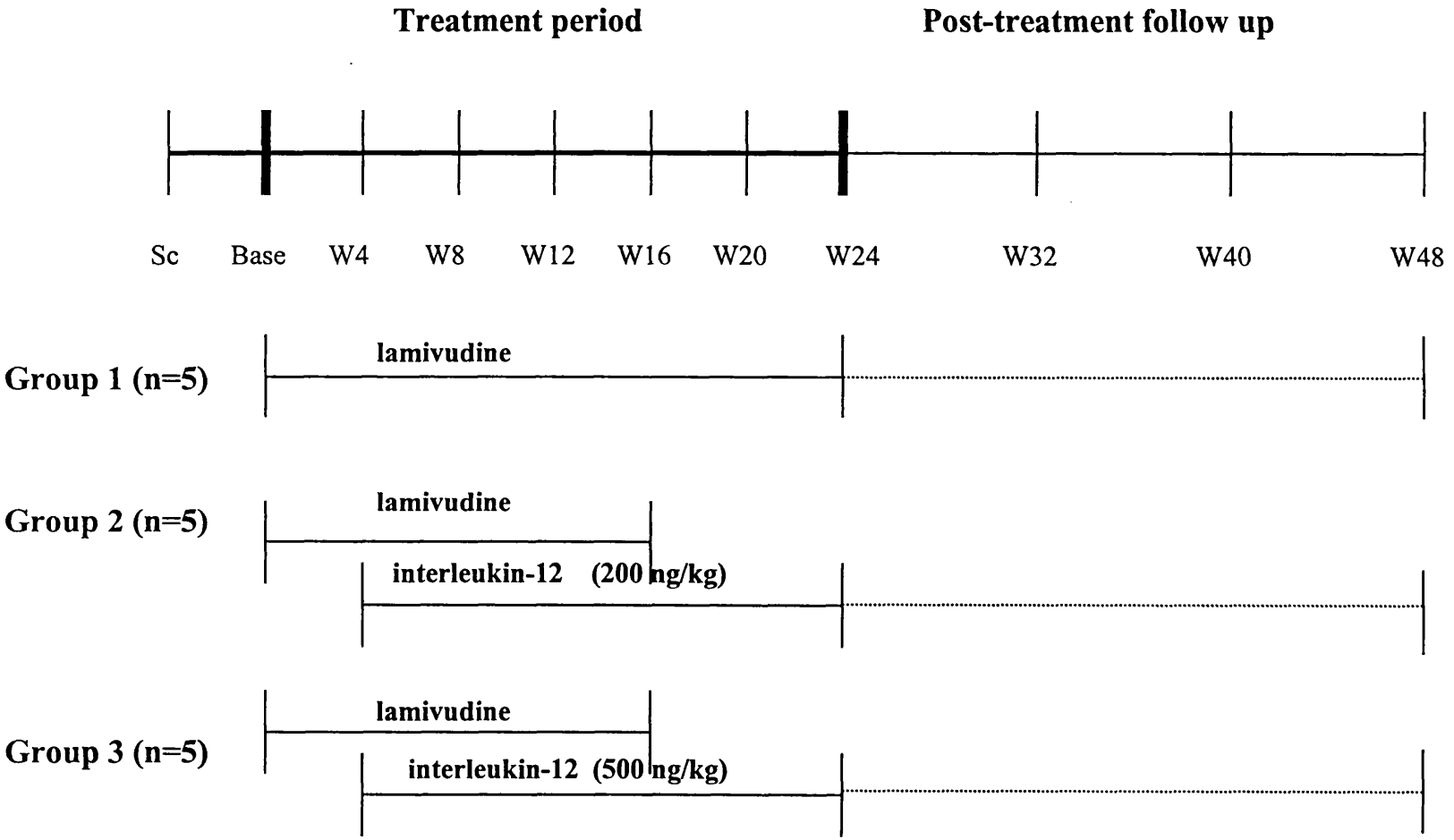


Figure 1: Schedule of treatment. 15 patients were randomised to one of three treatment groups. In group one the Patients received lamivudine monotherapy for 24 weeks. In group 2, the patients received 16 weeks of lamivudine with rhIL-12 (200 ng/kg) for 20 weeks after a four week lead-in phase. In group three the treatment was similar to group 2 Except that the dose of rhIL-12 was 500ng/kg. All patients were followed up for 24 weeks after the end of treatment.

Week	Screening	Baseline	2	4	6	8	12	16	20	24	32	40	48
Informed consent	X												
Medical history	X		X	X	X	X	X	X	X	X	X	X	X
Examination	X			X		X	X	X	X	X	X	X	X
Bloods – LFT, PT, FBC	X	X	X	X	X	X	X	X	X	X	X	X	X
HBsAg, HBeAg	X			X		X		X		X		X	
HBV DNA	X	X	X	X	X	X		X	X	X	X		X
Anti-HIV	X												
T-cell proliferation	X	X	X	X	X	X		X	X	X	X		X
CD4 depletion	X	X											
Elispot	X			X		X		X		X			X

Figure 2 Investigation schedule for patients undergoing treatment with lamivudine or lamivudine and Interleukin-12. X denotes time points at which investigations/tests/consultations were carried out.

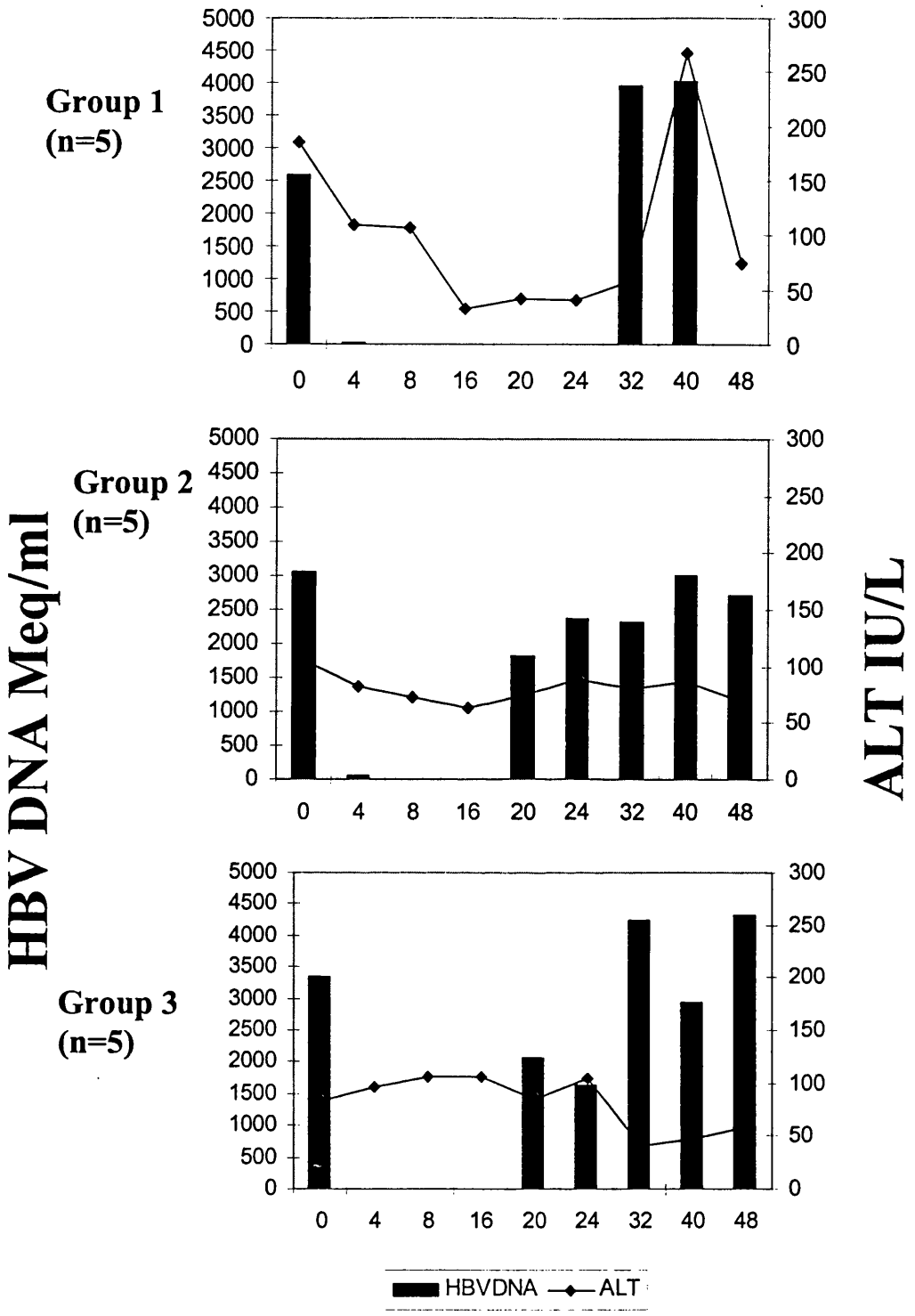


Figure 3 Mean values of serum HBV DNA and ALT during treatment and follow-up. Patients in Group 1 received lamivudine monotherapy, in group 2 received lamivudine rhIL-12 (200 ng/kg), in group 3 received lamivudine and rhIL-12 (500 ng/kg). HBV DNA levels (Meq/ml) are plotted against the left axis and shown as solid bars. The levels of ALT (IU/L) are plotted on the right hand axis as a line graph.

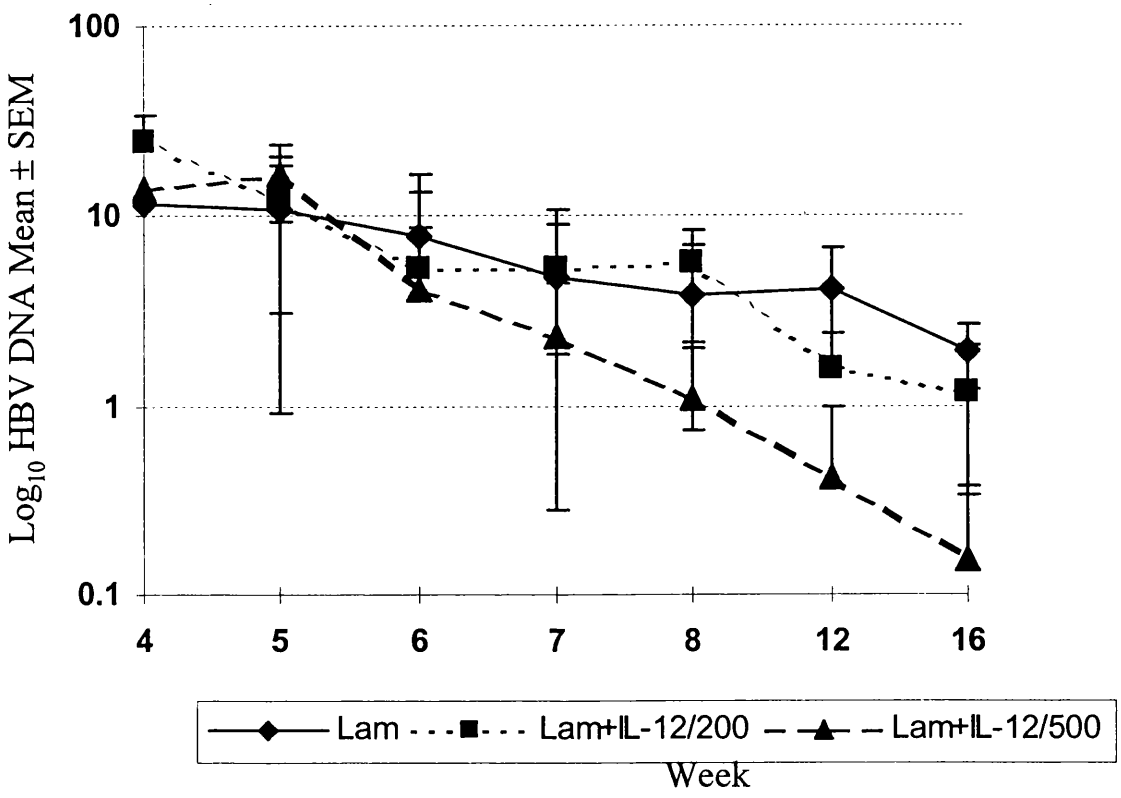


Figure 4 Changes in serum HBV DNA levels (PCR quantitation) during treatment with lamivudine alone in comparison to lamivudine plus interleukin-12 between weeks 4 and 16. The levels of HBVDNA are expressed as $\log_{10}$ (mean±sem). Patients treated with lamivudine are shown by the solid line (n=5), patients treated with lamivudine/rhIL-12 (200ng/ml) are shown by the dotted line, (n=5) and patients treated by lamivudine/rhIL-12 (500ng/ml) are shown by the dashed line, (n=5).

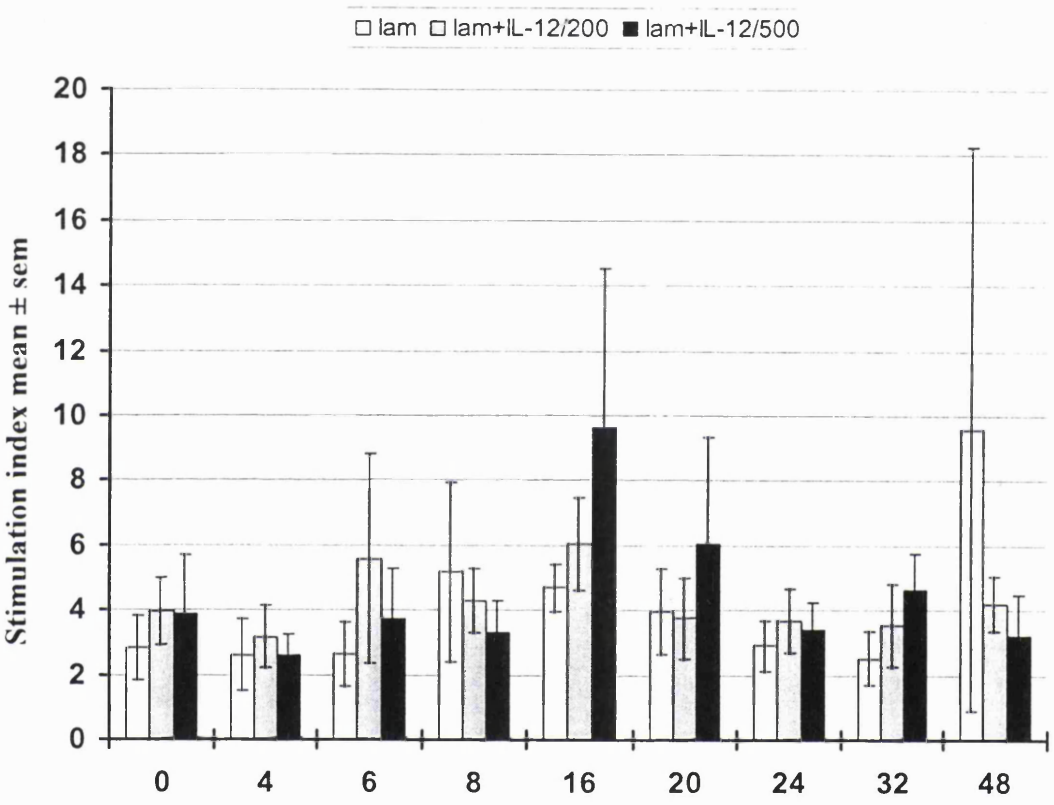


Figure 5 HBcAg specific T-cell proliferation during treatment and follow-up for patients treated with lamivudine monotherapy and lamivudine and rhIL-12. The patients treated with lamivudine monotherapy are shown as white bars, those treated with lamivudine and rhIL-12 (200ng/kg) are shown as grey bars, and those with treated lamivudine and rhIL-12 (500ng/kg) are shown as black bars. The T-cell proliferation (n=5 for all three groups treated) is expressed as a stimulation index (mean ± sem).

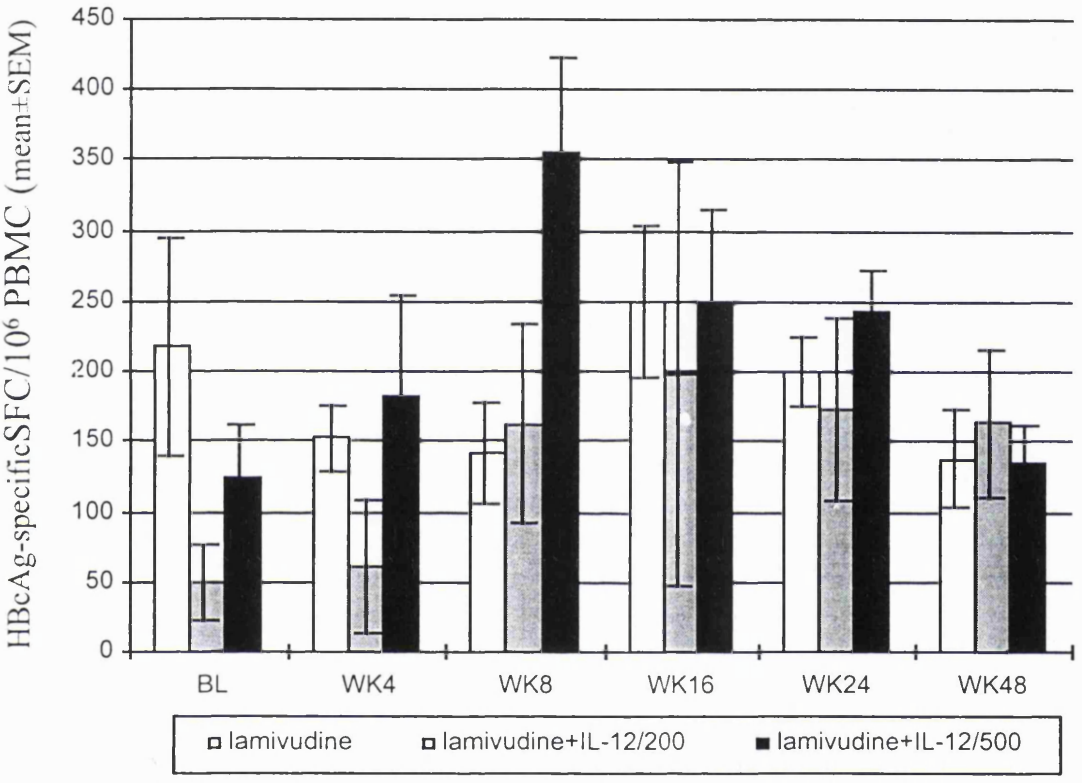


Figure 6: Changes in the number of interferon–γ producing, HBV-specific CD4+ T lymphocytes during treatment. The patients treated with lamivudine monotherapy are shown as white bars, those treated with lamivudine and rhIL-12 (200ng/kg) are shown as grey bars, and those with treated lamivudine and rhIL-12 (500ng/kg) are shown as black bars. The number of SFCs (n=5 for all three groups) is expressed as a mean ± sem.

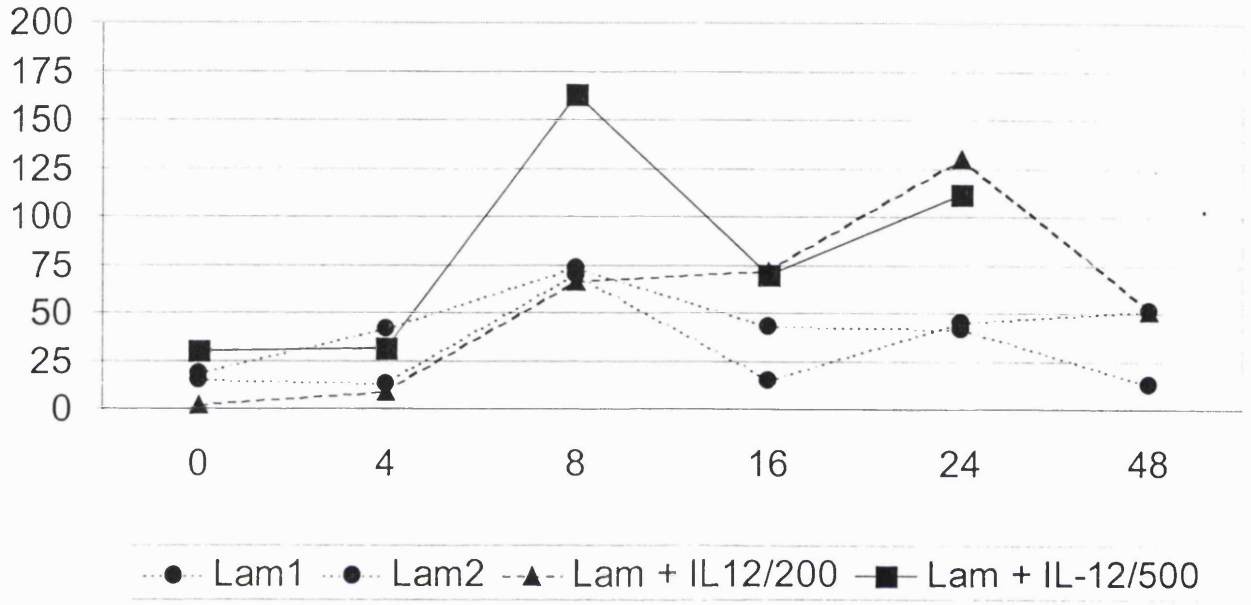


Figure 7 Changes in the number of interferon–γ producing, HBV-specific CD8+ T lymphocytes during treatment (Elispot assays). The number of HBcAg-specific CD8+ T cells (peptide 18-27) in four HLA A2 positive patients are shown During the study period. Two patients were treated with lamivudine monotherapy shown as circles with a dotted line, one patient was treated with lamivudine and IL/12 (200ng/kg) and shown as a triangle with dashed lines, and one patient was treated with lamivudine and IL-12 (500ng/kg) shown as a square with a solid line.

Table Patient Characteristics

Group Regimen	**Treatment**	**Age** (mean)	**Sex** M/F	**Baseline ALT** N/Raised	**Liver Grade** (mean)	**Histology Stage** (mean)	**Baseline HBV DNA** (mean Meq)	**Previous Treatment** number
1 (n=5)	Lamivudine100mg	28	3/2	2/3	2.2	1.2	2581.8	1
2 (n=5)	Lamivudine 100mg IL-12 (200ng/kg)	32	5/0	1/4	2.6	1.6	3041	4
3 (n=5)	Lamivudine 100mg IL-12 (500ng/kg)	38	4/1	1/4	2.8	2	3336	3

Chapter 8

Summary

Recent data from animal models has given new insights into the mechanisms involved in the control of HBV infection. In particular, IFN-γ can control HBV infection without killing infected hepatocytes. Whether such mechanisms occur in human HBV infection has not been documented.

The hypothesis that IFN-γ can non-cytolytically inhibit HBV infection in infected human hepatocytes was investigated in this thesis. It was demonstrated that peripheral blood mononuclear cells from patients with chronic HBV infection if adequately stimulated, are able to control HBV in human liver cells through release of IFN-γ. There was a striking correlation between reduction in HBV DNA levels and the supernatant levels of IFN-γ and these changes in HBV DNA occurred without significant cell death.

The CD4+ T-cell immune response is important in control of HBV and this was demonstrated during HBsAg seroconversion in patients undergoing bone marrow transplantation. The role of the Th1 immune response was further investigated by assessing the in vitro T-cell response to IL-12. This involved measuring T-cell receptor expression on PBMC and its regulation by recombinant IL-12; measuring the in vitro PBMC proliferative response and IFN-γ production in response to IL-12.

less sensitive to the anti-viral effect following IFN-γ than those with low levels of HBV DNA.

- As well as having an anti-viral effect IFN-γ also induced IFN responsive genes including iNOS in human liver cells which may represent a potential anti-viral or pathogenetic mechanism.

8.2 Adoptive transfer of HBcAg-specific CD4+ T cells is associated with HBsAg clearance in Chinese chronic HBV carriers

This chapter presents the findings from patients studied during allogeneic bone marrow transplantation. New insights into the nature of the CD4+ T-cell immune responses during seroconversion from HBsAg to anti-HBs were obtained.

- In these patients enhanced HBV-specific CD4+ T-cell immunoreactivity to nucleocapsid antigen was seen after bone marrow engraftment. Fine epitope mapping confirmed that the immune response seen in donors and recipients was against the same epitope within HBcAg. In contrast CD4+ T-cell immunoreactivity reactivity to surface antigen was absent.
- This suggests that nucleocapsid immune responses are able to stimulate anti-HBs production. This confirms the phenomenon of inter-molecular help seen in a mouse model of HBV infection.
- This CD4+ T-cell response was then further characterised with FACS analysis which showed that increased number of memory CD4+ cells

Those patients' hepatocytes with very high levels of HBV DNA, were less sensitive to the anti-viral effect following IFN-γ than those with low levels of HBV DNA.

- As well as having an anti-viral effect IFN-γ also induced IFN responsive genes including iNOS in human liver cells which may represent a potential anti-viral or pathogenetic mechanism.

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- This CD4+ T-cell response was then further characterised with FACS analysis which showed that increased number of memory CD4+ cells displaying markers of recent activation were present at the time of seroconversion.
- Finally Y-chromosome PCR demonstrated that the CD4 cells implicated in HBsAg clearance were from the donor.

8.3 IL-12 and IL-12 receptor

This chapter presents the findings of in vitro studies of IL-12 receptor expression and functionality.

- IL-12Rβ1 expression on PBMC was significantly lower in patients with chronic HBV infection compared to normal subjects and patients with chronic HCV infection.

- IL-12 induced IFN-γ production from PBMC from chronic HBV carriers was similar to that produced by normal subjects. Furthermore, IL-12 also augments antigen induced IFN-γ production. IL-12 alone resulted in marked proliferation.

- in chronic HBV infection IL-12R expression could be increased in vivo to levels similar to those found on normal controls with rhIL-12 treatment. Switching the balance to a predominant Th1 phenotype with rhIL-12 represents a possible therapeutic option for chronic HBV infection.

8.4 Therapeutic effect of rhIL-12 on chronic HBV infection.

The previous chapters demonstrated the importance of the *in vitro* anti-viral and immunomodulatory effects of IFN-γ and IL-12 and the host immune response during viral clearance. This chapter investigated the in vivo anti-viral effect of a novel combination of nucleoside analogue and immunomodulatory cytokine (lamivudine and interleukin-12). Several important findings were documented.

- This combination had increased antiviral effects compared to lamivudine monotherapy as measured by quantitative PCR.
- The combination treatment also enhanced the host immune response as measured by T-cell proliferation and Elispot assay.
- Treatment with lamivudine and IL-12 was associated with improvement in ALT levels.
- The novel combination was well tolerated.

8.5 Interpretation and importance of findings

Chapter 4 demonstrates that PBMC from chronic HBV carriers are able to control HBV infection if adequately stimulated through IFN- γ. These lymphocytes are usually unable to exert anti-viral effector functions but following in vitro manipulation they are. This is a novel finding in human HBV that may be important in the design of future therapies.

The demonstration in chapter 5 that nucleocapsid-specific CD4+ T cells provide intermolecular help to envelope B cells to produce neutralising antibodies entends

previous observations in animal studies. Furthermore intermolecular T-cell help may be important in the design of a future therapeutic vaccine for chronic hepatitis B

Chapter 6 results suggest that the predominance of a Th2 immune response in patients with chronic HBV infection may be as a result of IL-12 receptor deficiency. However this receptor is still functionally active as PBMC can still produce normal levels of IFN-γ if cultured with IL-12. Furthermore IL-12 receptor expression can be upregulated by exogenous IL-12 suggesting that this could be a therapeutic intervention by which the Th1/Th2 imbalance seen in chronic HBV infection can be corrected. Treatment with IL-12 not only upregulates the receptor but more importantly normalises Th1 immune responses as measured by T cell proliferation and IFN-γ production. Co-stimulation with both nucleocapsid antigen and rhIL-12 produces significantly greater levels of IFN-γ suggests another avenue of possible immunotherapy that could be investigated. This has important therapeutic potential as in the first chapter the central role for IFN-γ in the control of HBV was discussed.

To investigate relevance of the antiviral effect of IL-12 in human HBV infection a clinical trial of lamivudine and IL-12 was undertaken which documented important anti-viral effects and immuno-modulatory effects. Although the results on HBeAg seroconversion were disappointing the effects on viral load were

encouraging suggesting that longer courses of treatment may increase HBeAg seroconversion rates.

8.6 Further studies

These studies open up a range of possibilities for future research. As far as the non-cytolytic studies are concerned the co-culture studies were limited by PBMC numbers that could be obtained from patients. This could be overcome by using T-cell clones (thereby having a larger number of effector cells). Particularly assessment of the anti-viral effect on HBV DNA replicative intermediates in 2.2.15 cells would be possible if larger number of 2.2.15 cells (and effector cells) were used. The anti-viral effect on ccc DNA is unclear and hence further experiments using a specific polymerase chain reaction may provide useful information regarding this. In addition the intracellular mechanisms such as the role of iNOS could be studied. Similarly, further studies with naturally infected human hepatocytes should be carried out to determine whether iNOS has an important anti-viral or pathogenetic role.

The reason why patients with chronic HBV infection have a low level of IL-12 receptor is unclear. It could be as a result of a genetic defect as is seen in atypical mycobacterial and salmonella infections but the ready inducibility of receptor expression and functionality suggests that this is unlikely. As IL-12R is under autoregulation one possible cause, which should be investigated, is whether this is as a result of low endogenous IL-12 production. It is possible to measure serum

levels of IL-12 or IL-12 p40 in chronic carriers to compare to normal controls. However, more importantly it may be the production of IL-12 by antigen presenting cells such as dendritic cells. Hence further in vitro studies focussing on dendritic cell function may be useful to determine whether the defect in IL-12 receptor expression is primarily a problem at the level of the T-cell or secondary to deficient dendritic cells.

The enhanced anti-viral effect and enhanced immunoreactivty seen during combination treatment unfortunately was not translated into loss of HBeAg and long-term resolution of viraemia. This probably was because the patients treated were a difficult group (largely immunotolerant and previous treatment failure). However, the combination of lamivudine and IL-12 was very well tolerated and further studies of a longer duration would be interesting to see if the greater loss of HBV DNA with higher doses of IL-12 is translated into HBeAg seroconversion. Further studies of the effects of the combination treatment on viral kinetics would be interesting to see if these could be useful in determining likelihood of treatment response and the development of viral resistance.

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