THE ROLE OF VIRUS-SPECIFIC T CELL IMMUNITY IN THE PATHOGENESIS OF ACUTE AND CHRONIC HEPATITIS B VIRUS INFECTION.

A thesis submitted to the

University of London,

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Doctorate in Medicine

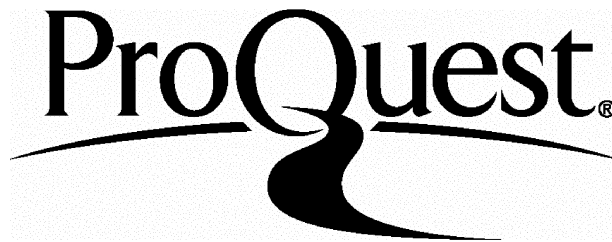by

Dr George Webster BSc MRCP

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ABSTRACT

Hepatitis B virus infection is a major cause of morbidity and mortality worldwide, but is of low endemicity in the UK, and outbreaks are rare. I report here a large outbreak of HBV infection, in which 57 of 356 patients and staff of an alternative medical clinic had markers of exposure to the virus. Using molecular sequencing and phylogenetic analysis 30 of 33 individuals with active infection were shown to have been infected from a common source. Infection was linked to a form of acupuncture, and the risk of infection was correlated with the number and timing of treatments.

The identification of patients prior to the onset of clinical disease provided a unique opportunity to study immune responses during the early phase of hepatitis B. HBV is non-cytopathic, and early host-virus interactions have been thought to be fundamental in determining disease outcome. Using new techniques to directly quantify specific cell populations, it was found that components of the innate and adaptive immune response, including HBV-specific CD8+ and CD4+ cells, were present in the circulation weeks before clinical disease, and that maximal reductions in circulating virus also occurred before the onset of jaundice.

These findings were extended to a longitudinal comparison of CD8 responses in patients with resolved or chronic HBV infection, characterised according to the degree of viral control and liver injury. Virus-specific CD8+ cells were shown to persist in the circulation, years after resolution of acute infection. In chronic infection the HBV-specific CD8+ cell responses was found not simply to be a weak version of that seen in acute disease. Instead, a completely different epitope hierarchy was found, with dominance of epitopes which were usually sub-dominant in acute disease. The findings might be of relevance to the development of therapeutic vaccines for chronic hepatitis B.

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DECLARATION

The work for this thesis was performed within the Royal Free and University College Medical School, at the Centre for Hepatology, Royal Free Campus, and the Institute of Hepatology, UCL. The work was performed by myself, unless otherwise stated in the text. It has not been submitted previously for a higher degree.

PUBLICATIONS

Full publications arising from this work..

Peer reviewed papers.

1. Escaping high viral load exhaustion: CD8 cells with altered tetramer binding in chronic hepatitis B virus infection. Reignat S, Webster GJM, Brown D, Ogg GS, King A, Seneviratne SL, Dusheiko GM, Williams R, Maini MK, Bertoletti A *J Exp Med* 2002 May 6;195(9): 1089-101

2. Incubation phase of acute hepatitis B in man: dynamic of cellular immune mechanisms. Webster GJM, Reignat S, Maini MK, Whalley SA, Ogg GS, King A, Brown D, Amlot PL, Williams R, Vergani D, Dusheiko GM, Bertoletti A. *Hepatology* 2000 Nov;32(5):1117-24

3. Kinetics of acute hepatitis B virus infection in humans. Whalley SA, Murray JM, Brown D, Webster GJM, Emery VC, Dusheiko GM, Perelson AS. *J Exp Med* 2001 Apr 2;193(7):847-54

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5. T cell receptor usage of virus-specific CD8 cells and recognition of viral mutations during acute and persistent hepatitis B virus infection.Maini MK, Reignat S, Boni C, Ogg GS, King AS, Malacarne F, Webster GJM, Bertoletti A. *Eur J Immunol* 2000 Nov;30(11):3067-78.

6. Direct ex-vivo analysis of hepatitis B virus-specific CD8+ T cells in patients who control infection. Maini MK, Boni C, Ogg GS, King AS, Reignat S, Lee CK, Larrubia, Williams R, Webster GJM, McMichael AJ, Vergani D, Ferrari C, Bertoletti A. *Gastroenterology* 1999;117:1386-96

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1. Webster GJM, Bertoletti A. Control or persistence of hepatitis B virus: the critical role of initial host-virus interactions. *Immunology and Cell Biology* 2002;80:101-5

2. Webster GJM, Bertoletti A. Quantity and quality of virus-specific CD8 cell response: relevance to the design of a therapeutic vaccine for chronic HBV infection. *Mol Immunol* 2001;38(6):467-473

3. Webster GJM, Bertoletti A. Immunopathogenesis and treatment of hepatitis B virus infection. In: Eds ME Gershwin, JM Vierling, MP Manns. *Immunology and the Liver* 2002;15:203-216

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CHAPTER 1

INTRODUCTION

1.1. Epidemiology and transmission of hepatitis B virus.

It has been estimated by the World Health Organisation that more than 2 billion people, a third of the World population, have been exposed to the hepatitis B virus (HBV) (Kane, 1995; Zuckerman, 1999). Estimates suggest that more than 350 million people worldwide are chronically infected (Lee, 1997). HBV carriage has been found in all populations studied, but there are wide geographical variations: in areas of high endemicity, such as China and sub-Saharan Africa, more than 8% of people are hepatitis B surface antigen (HBsAg) positive, but in others with low endemicity, such as the UK, carriage is estimated to be less than 1%.

HBV is transmitted by the parenteral route. In regions of high endemicity such as the Far East, where a high proportion of HBsAg mothers are also hepatitis B e antigen (HBeAg) positive, indicating a high circulating viral load, mother to neonate (vertical) transmission is an important means of spread. In Africa, non-sexual intra-familial (horizontal) infection during the first few years of life appears to be an important method of transmission (Tabor et al., 1985). HBV is readily transmitted sexually, and appears to be much more infectious by this route than either hepatitis C virus (HCV), or human immunodeficiency virus (HIV) (Thomas et al., 1994). In the UK, where prenatal blood testing and screening of blood products has significantly reduced vertical and transfusion-related infection, sexual transmission appears to have been the route of infection in 24% of the 5000 reported cases of acute hepatitis B from 1985-96, in which a source of infection could be defined (Balogun et al., 1999). In the USA, recent sexual contact with multiple partners, or a partner with hepatitis, has been reported to account for more than 40% of cases of acute hepatitis B. In

developed countries injected drug use is also an important means of infection (Balogun et al., 1999; Lee, 1997). Less frequent causes of infection include household contacts, occupational exposure, and nosocomial infection from surgeons (The Incident Investigation Teams, 1997) or haemodialysis equipment (Huang, 1997). No obvious risk factors are reported in up to 30% of patients.

1.2. Natural history of HBV infection.

HBV is reported to be the most common cause of acute and chronic liver disease worldwide. Infection may result in a range of clinical outcomes, with markedly differing implications for the long-term health of the infected individual. The natural history of HBV infection is profoundly influenced by the age at acquisition, host immunity, and environmental cofactors.

1.2.1. Acute hepatitis B.

Approximately 6 to 24 weeks after infection with HBV more than 50% of adults develop an acute clinical hepatitis, characterised by general malaise, fever, right upper quadrant pain, and jaundice. Although fulminant hepatitis, which may be fatal, occurred in nearly 10% of cases of acute hepatitis B in one large outbreak (Lettau et al., 1987), the usual frequency appears to be less than 2%, and the great majority of infected adults with acute icteric hepatitis clear HBsAg from the serum and make a full clinical recovery, with no long-term sequelae. Other patients may clear HBsAg after an anicteric, asymptomatic illness. In a study involving more than 50,000 servicemen who developed icteric hepatitis following the use of an HBV-contaminated Yellow Fever vaccine, less than 0.3% were still HBsAg positive 40 years later (Seeff et al., 1987). Overall, more than 90% of immunocompetent adults, and 70% of children infected from 6 months to 6 years of age, will clear HBsAg after acute infection (Ganem, 1982; Lee, 1997). In marked contrast, infection in neonatal

life rarely leads to icteric hepatitis, and persistence of HBsAg occurs in more than 90% of cases (Beasley et al., 1981a).

1.2.2. Chronic hepatitis B.

Chronic infection, defined serologically by the persistence of HBsAg in the serum for more than six months, is a major cause of morbidity and mortality worldwide. The overall mortality due to HBV results from chronic infection, not acute disease (Francis et al., 1981). One million deaths occur annually due to the complications of HBV-related cirrhosis and hepatocellular carcinoma (Lee, 1997), and 30-50% of chronically infected individuals can expect to die early, as a direct result of infection (Beasley et al., 1981b). In carriers of HBsAg, the relative risk of death due to cirrhosis is up to 79, and of hepatocellular carcinoma (HCC) is up to 100, compared with normal subjects (McMahon et al., 1990).

Several patterns of chronic HBV infection are recognised, defined clinically by serological markers, serum levels of HBV DNA, and markers of liver injury (including serum aminotransferase levels and liver histology). These different patterns are likely to reflect a range of interrelated factors including age at infection, duration of disease, and many aspects of the host immune response. Following neonatal infection, childhood HBV infection is characterised by high levels of HBV DNA, HBeAg positivity, near normal serum alanine transaminase (ALT), and minimal histological injury within the liver. This period has been termed the 'immunotolerant' phase, and is not associated with rapid progression to cirrhosis (Lok and Lai, 1988). After a variable length of time, often at about 30 years in neonatally infected individuals, this replicative phase of disease is characterised by increased and often fluctuating levels of ALT. Whilst these flares may result in loss of HBeAg from the serum, they are also associated with progressive liver injury, with up to 50% of a subgroup of patients with a long duration of infection progressing to cirrhosis in 5 years (Liaw et al., 1988). Following the development of cirrhosis, prospective follow-up has shown that 5 year survival in HBeAg-positive patients falls to 72%, but is well

maintained at 97% in those with HBeAg-negative disease. HBeAg seroconversion during follow-up is associated with a two fold reduction in risk of death (de Jongh et al., 1992). Once an episode of decompensation has occurred, the 5 year survival due to HBV-related cirrhosis falls to 35% (Fattovich et al., 1995). The period of chronic inflammation and repeated episodes of hepatic regeneration is also strongly linked to the development of HCC. In patients with compensated cirrhosis due to HBV, there is a 6% risk of developing HCC within 5 years of diagnosis (Fattovich et al., 1995). In epidemiological studies in Alaska, it has been shown that HBeAg loss occurs at a rate of less than 10% per year (Alward et al., 1985), and if this occurs the risk of progressive liver disease falls significantly (Fattovich et al., 1986). Whilst HBeAg loss and the development of anti-HBe usually reflects a significant reduction in serum HBV DNA, impaired production of HBeAg, due to mutations in the pre-core region of the HBV genome, may lead to anti-HBe-positive infection associated with high levels of HBV DNA and significant progression of liver injury (Carman et al., 1989). Many individuals, however, are identified in the low replicative phase of disease (anti-HBe-positive, low levels of serum HBV DNA, minimal liver injury), with no prior clinical evidence of replicating disease.

In chronic hepatitis B loss of HBsAg from the serum is rare, but may occur, in approximately 1% of adult patients each year (Alward et al., 1985; Liaw et al., 1991). This may be due to the slow turnover of hepatocytes with integrated forms of HBV, although evidence from a range of sources provides convincing evidence that even in individuals who have cleared HBsAg from the serum, small quantities of the virus continue to be present (Gandhi et al., 2000; Cacciola et al., 1999). Although over time the usual pattern of change is from a state of high HBV replication (HBeAg-positive)/minimal liver injury, to low HBV replication (anti-HBe-positive) disease, via a period of increased damage associated with flares of ALT and HBeAg seroconversion, it is well recognised that a cyclical pattern may occur, and reactivation may occur, either spontaneously, or in response to immunosuppression (Markovic et al., 1999).

1.3. Hepatitis B Virus

1.3.1. Viral Structure and antigens

The HBV genome is an enveloped, circular, partially double stranded DNA virus of approximately 3200 base pairs, belonging to the hepadnavirus family. It encodes four overlapping open reading frames (ORF): S, for the surface, or envelope gene; C, for the nucleocapsid or core gene; X, for the X gene, and P, for the polymerase gene. Each virion consists of a double-shelled structure.

Figure 1.1.   Diagrammatic representation of the four overlapping reading frames (ORF) of hepatitis B virus
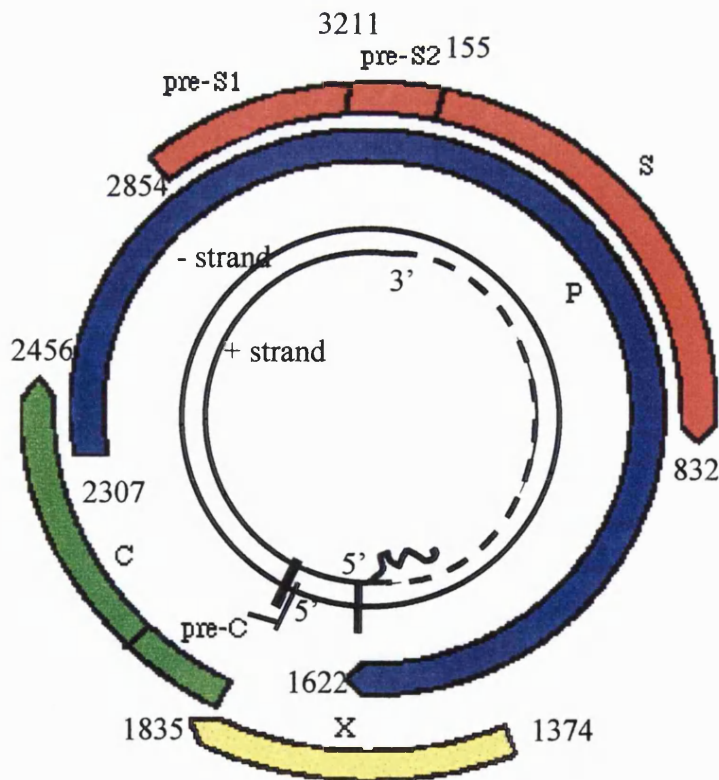
Based on figure in {Ganem & Varmus 1987}

The outer viral envelope encoded by the S gene is composed of hepatitis B surface antigen (HBsAg), which may be configured into 3 distinct forms, termed the large, middle, and small proteins, due to beginning transcription with, respectively, the preS1, preS2, or the S gene alone. The preS1 and preS2 portions of HBsAg may contain important immunogenic epitopes.

The nucleocapsid ORF encodes for two overlapping polypeptides. Hepatitis B core antigen (HBcAg) self-assembles in the hepatocytes cytoplasm to form nucleocapsid particles, that package pregenomic RNA and viral polymerase. As discussed below, they are the source of viral genomes for the nucleus, and also form the inner shell of complete virions for secretion from the cell. HBcAg expression on the hepatocyte surface is thought to be crucial step in initiating cellular immune responses, as discussed later. Non-particulate HBeAg is a secretory form of the core protein, encoded within the pre-C genome sequence. Soluble HBeAg may circulate at high levels in the blood during HBV infection, and indicates high levels of viral replication. Stop codons within this pre-C region are common, and give rise to a variant of chronic HBV infection characterised by an inability to express HBeAg (Carman et al., 1989). HBeAg functions as a signal peptide. However, the role of HBeAg remains unclear, as it is not a structural component of the virus, and is not an absolute requisite for infectivity or replication (Chang et al., 1987; Chen et al., 1992). It may be important in ensuring viral persistence, and a function in promoting neonatal immunotolerance in HBeAg-positive mothers has been suggested (Milich et al., 1990). In adults, secreted HBeAg may modify host immunity by preferentially eliciting a Th2 profile of response (Milich et al., 1997). As discussed below, immune responses to nucleocapsid antigens appear an important determinant of viral control or persistence following acute infection (Chisari and Ferrari, 1995a).

The X gene encodes for a protein, HBx, whose role is still to be fully elucidated. Although absent in some hepadnaviruses, in HBV it appears necessary for viral replication, mainly through the activation of Scr kinase. Recent work suggests that this activation may occur through the regulation, by HBx, of mitochondrial calcium channels (Bouchard et al., 2001).

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HBx may also be an activator of a range of promoters involved in hepatocarcinogenesis, and have an interaction with the p53 tumour suppressor gene (Lee et al., 1995). The P gene encodes the DNA polymerase, which also serves a reverse-transcriptase function, since replication requires RNA intermediates.

1.3.2. Life cycle

The replication cycle of HBV begins with attachment of the virion to the hepatocyte membrane. The mechanism of this is unclear, but may occur through pre-S1 (anti-pre-S1 antibodies have been shown to block infection in vitro (Neurath et al., 1989)), or through pre-S2, via receptors on the hepatocyte membrane (Pontisso et al., 1989). Virus penetration probably then occurs through endocytosis. Cores are released, which bind to the nuclear pore. The genome then enters the nucleus. After entry into the cell nucleus, incomplete virion HBV DNA is repaired and converted into covalently closed circular DNA (cccDNA). The HBV genome replicates by reverse transcription via an RNA intermediate. The episomal DNA is transcribed by host RNA polymerase II within the nucleus to produce an RNA template (the pregenome), and this is the stage at which multiplication of the viral genome occurs. The pregenome serves both as a template for reverse transcription, and as a messenger RNA for the production of nucleocapsid and polymerase proteins. This dual role appears to be performed through the production of two classes of viral RNA: genomic RNAs, which act as replication templates, and subgenomic RNAs, which act as messenger RNA, particularly for the expression of pre-S1 protein (Ganem and Varmus, 1987; Seeger and Mason, 2000). The pregenomic RNA, and nucleocapsid and polymerase proteins are encapsulated within the virus core particle, and within which reverse transcription takes place. Initially reverse transcription results in the synthesis of small amounts of a first (minus) strand of DNA, by copying pregenomic RNA, using a protein primer. Elongation then occurs, followed by the synthesis of a second (plus) strand of DNA by copying the minus strand, with an oligomer of viral RNA, probably a fragment of pregenomic RNA, as a primer. After genome maturation, nucleocapsids, with the

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partially double-stranded HBV DNA, are prepared for secretion through envelopment by a mixed aggregate of HBs antigen. These enveloped virions, and a large excess of HBs particles, are moved from the endoplasmic reticulum to the cell surface, via the Golgi apparatus. Modification of envelope proteins occurs during this migration, and enveloped virions (also known as Dane particles – 42nm in diameter) and HBs particles (22nm subviral filaments and spheres) are subsequently released from secretory vesicles. As well as being secreted from the cell in conjunction with the nucleocapsid, HBV DNA may also reenter the infected cell nucleus. This is necessary in order to maintain infection, as HBV cccDNA, which represents the pool of intranuclear HBV genomes, has a putative half life of only 2-3 days, in ducks (Civitico and Locarnini, 1994).

1.4. Overview of mechanisms of anti-viral immunity

In order to efficiently eliminate virus from both the extracellular space and from within infected cells, the coordinated action of different components of the host immune system, both innate and adaptive, humoral and cellular, appear to be important. The mechanism of these responses will be briefly discussed here, but the focus of this work is a specific analysis of adaptive immune responses in HBV infection.

1.4.1. Innate immunity

The initial host immune response to a virus may lead to immediate anti-viral defence mechanisms, and mediate immunoregulatory functions which promote the development of downstream adaptive immune mechanisms (Biron, 1999). Physical components of the innate immune response include the skin and mucous membranes. Cellular components include dendritic cells (professional antigen presenting cells (APCs)) and natural killer (NK) cells. These cells differ from those of the adaptive response, in that they cannot mature over time, and have polyspecific receptors. The mechanisms that lead to the

induction of the innate immune response to viral infection are being elucidated. The type 1 interferons, IFNα/β, can be induced by virtually any cell in response to viral infection, often leading to high and sustained serum levels (Welsh, Jr., 1978). This induction occurs within hours of viral infection, and is widely accepted to be the most immediate antiviral host response to a wide range of viral infections (Guidotti and Chisari, 2001). As well as having direct antiviral effects, IFNα/β may also trigger downstream activation of adaptive cytotoxic T cell responses and IFNγ production, upregulation of major histocompatibility complex (MHC) class 1 and II molecule expression on infected cells, and the promotion of NK cell activation and proliferation (Biron et al., 1999a).

NK cells are populations of lymphocytes which contribute to protective innate immunity to a wide range of infections. Although they display some functions overlapping with those of T cells, classical NK cells do not express T cell receptors (TCR) or the CD3 complex. NK cells can be rapidly recruited into infected organs by chemoattractant factors produced by infected cells, which are a major source of IFNα/β, and which lead to NK cell activation and proliferation (Biron et al., 1999b). NK cells recognize virally infected cells before MHC class I expression is upregulated, reinforcing their importance in the early response to infection. Although NK cells may control viral replication by direct cytolysis, it is increasingly recognised that a non-cytolytic action, probably through the production of IFNγ and TNFα, is important for viral control (Guidotti and Chisari, 2001). The development of life-threatening viral infections in a patient with NK cell deficiency supports the evidence for the role of these cells in the immune response to viruses (Biron et al., 1989). NK T cells are a subset of T cells that are neither CD4+ or CD8+ cells, but which share with classical NK cells an important role in innate immunity, express NK cell markers, and have a limited TCR repertoire (Vicari and Zlotnik, 1996). The role of NK T cells has not been well defined in viral infections, but it is of interest that they are found at high frequency in normal livers, accounting for 20-30% of intrahepatic lymphocytes (MacDonald, 1995). Activation of NK T cells leads to the rapid production of cytokines, including interleukin-4 (IL-4) and IFNγ, which have been implicated in the non-cytolytic control of HBV.

1.4.2. Adaptive immunity

Cells of the adaptive immune response differ from those of innate immunity in exhibiting antigen specificity, and by having the ability to respond with enhanced efficiency to a secondary encounter with antigen, constituting immunological memory. Lymphocytes are divided into B and T cells. Following antigenic stimulation B cells develop into plasma cells and produce antibodies to exogenous antigens, as part of the humoral response. Antibodies can block viral reinfection by inhibiting binding of virions to target cells, or viral penetration of the cell. They may also promote complement-mediated cell lysis and phagocytosis. T cells, including CD4+ and CD8+ cells, play a crucial role in controlling intracellular viral infection, and are the main focus of this work.

CD4+ T-helper cells play a key role in anti-viral immunity, both directly (through the production of antiviral cytokines), and indirectly (by providing help for B cells and CD8+ cytotoxic T cells). CD4+ cells recognise viral antigens presented on the surface of antigen-presenting cells (APC), which include dendritic cells, macrophages, and B cells. Extracellular antigens are taken up by APCs, digested into 10-20 amino acid peptide fragments, and presented on the cell surface in complexes with MHC class-II molecules, where they may interact with the antigen-specific T-cell receptor (TCR) of MHC class-II restricted CD4 T cells (Figure 1.2). These cells may be classified on the basis of their cytokine production, and thence their effector function. Th1 cells secrete IFN-γ and IL-2, which may enhance CD8+ cytotoxic T lymphocyte (CTL) activation and proliferation (Doherty et al., 1992). Th2 cells produce IL-4, IL-5, and IL-10, and support antibody-producing B cells (Vitetta et al., 1989), and may inhibit CTL.

Antigen-specific CD8+ cells are believed to play a major role in the eradication of a wide range of viral infections (Zinkernagel and Hengartner, 1997). For example, in HIV disease the early control of viraemia after acute viral infection is associated with the appearance of virus-specific CD8+ cells in the circulation (Borrow et al., 1994), and in chronic infection a negative relationship has been shown between CTL numbers and viral load (Ogg et al.,

1998). The action of CD8+ cells is based on (Lechner et al., 2000a; Goulder et al., 2001)their capacity to identify viral peptides on the surface of infected cells (Yap et al., 1978) (Rickinson and Moss, 1997), presented as a complex with MHC class 1 molecules (Yewdell and Bennink, 1992). These 9-10 amino-acid antigenic peptides are generated in the cytosol of the infected cell, and are transported into the endoplasmic reticulum (ER) via the MHC-encoded transporter with antigen processing (TAP) complex (Lorenzo et al., 2001). Peptides are loaded on to MHC class I molecules, and the resulting complex is transported to the cell surface. CD8+ cells bearing a T cell receptor (TCR) corresponding to this peptide then bind the peptide-MHC class 1 molecule complex. Considerable advances have been made in defining the epitopes which are important in eliciting a CTL response to a wide range of infections, including hepatitis B. The response is MHC class I restricted, and much work has been focussed on the study of patients with the HLA-A2 haplotype, which is present in approximately 30-50% of individuals in most populations that have been studied worldwide (Sidney et al., 1996; Fernandez-Vina et al., 1992).

1.5. Immunopathogenesis of HBV infection.

The observations that acute infection may be characterised by weeks of viraemia prior to the development of hepatitis, and that a high level of HBV replication may be tolerated for years without significant liver damage (Chu et al., 1985), suggest that the virus is not directly cytopathic tor the infected hepatocyte (Chisari et al., 1989). The pathogenetic basis of accelerated liver damage in fibrotic cholestatic hepatitis, which may occur in profoundly immunosuppressed patients with hepatitis B (Davies et al., 1991), has led to the suggestion that it might be cytopathic in this situation, but the pathogenetic mechanism of this clinical pattern is poorly understood. It is believed, therefore, that the host immune response is responsible for both controlling viral replication, and mediating liver injury. The relative contribution of the different components of the immune response to viral control and liver injury, and the interdependence of these two processes in viral infection, have been the subject of much study over the last twenty years.

1.5.1. Innate response to HBV

The role of innate immunity in the early response to HBV infection has been difficult to study, due both to the absence of a suitable *in-vitro* culture system able to sustain infection and replication of HBV, and the rarity of identifying patients at such an early stage of disease. It has not been possible to determine, for example, whether HBV is a strong or weak inducer of type 1 interferons. Nevertheless, IFNα/β inhibits viral replication in duck hepatitis B virus (DHBV) infection (Schultz et al., 1999), and has been demonstrated using a transgenic mouse model of HBV infection to control HBV non-cytopathically (Shimizu et al., 1998). Although patients with acute HBV infection have been shown to contain high levels of IFNα-producing cells within their livers (Nouri-Aria et al., 1991), these patients (as is usually the case with acute hepatitis B) were studied after the development of significant hepatic inflammation. It is therefore difficult to interpret from this data the true role of IFNα during the early phase of HBV infection. In chronic hepatitis, intrahepatic levels of IFNα and IFNβ are no higher than in normal livers (Castelruiz et al., 1999), and there is weak hepatic activation of IFNα and IFNβ-regulated genes in chronic hepatitis B (Ikeda et al., 1986).

In cytomegalovirus (CMV) infection of the liver in mice, NK cells have been shown to cause liver injury and viral control independent of cytotoxic T cell activation, but associated with IFNγ production (Orange et al., 1995) , and in hepatitis C virus in humans impaired NK cell cytotoxicity has been associated with viral chronicity (Corado et al., 1997). Increased NK cell functional activity has been demonstrated in the blood of patients with acute hepatitis B (Chemello et al., 1986). It is of interest that Guidotti et al have shown that significant reductions in HBV DNA in the livers of acutely infected chimpanzees occur concomitant with the appearance of IFNγ, but prior to the influx of CD3+ T cells, suggesting a possible role for NK and/or NK T cells in this early non-cytolytic response (Guidotti et al., 1999a). In HBV transgenic mice, the injection of α-galactosyl ceramide, a ligand for NK T cells, has been shown to abolish viral replication, through the activation of

NK T cells, and secondary activation of NK cells and cytokine production (Kakimi et al., 2000).

1.5.2. Humoral response to HBV.

Antibody responses are raised against both HBV envelope (HBsAg) and nucleocapsid (HBeAg and HBcAg) antigens. Anti-envelope antibodies are directed against envelope antigens encoded by the S, pre-S, and pre-S1 genes of HBV. Because their appearance is associated with recovery from acute HBV infection, and they are usually undetectable in patients with chronic infection, they are thought to play a role in viral clearance. This may occur through complexing with free viral particles and removing them from the circulation, or by preventing their attachment and uptake by hepatocytes (for which the pre-S1 region may be important (Neurath et al., 1986). If anti-envelope antibodies are involved in viral clearance it would be expected that impaired production would be associated with viral persistence. Although the immune response is clearly complex and multifactorial, the fact that HBV infection in patients with agammaglobulinaemia frequently leads to chronicity may support this hypothesis (Chadwick et al., 1978).

In contrast to anti-envelope antibodies, humoral responses against nucleocapsid antigens do not appear to be neutralizing, as high titres of anti-HBc may be found during acute and chronic hepatitis B, and in association with a high viral load. Despite this, passively administered anti-HBe antibodies have been shown to protect chimpanzees against HBV infection, although the mechanism for this is unclear (Stephan et al., 1984). The fact that strong anti-HBc antibody responses may be found in both acute icteric hepatitis B (when T cell responses against nucleocapsid antigens are vigorous), and in chronic infection (when T cell responses are weak), may be due to the fact that HBc may function as both a T cell dependant and independent antigen (Milich and McLachlan, 1986). In acute infection, appearance of anti-HBe antibodies is associated with control of viral replication and is a strong predictor of clearance of HBsAg from the serum and resolution of disease. In chronic infection, development of anti-HBe is usually associated with long-term reduction

in viral replication, and with this, a reduced risk of progressive liver disease and hepatocellular carcinoma (de Jongh et al., 1992).

1.5.3. CD4+ T helper cell responses to HBV

In the peripheral blood of patients with acute self-limiting hepatitis B, strong CD4 responses against multiple epitopes in the HBV nucleocapsid (HBcAg, HBeAg) have been demonstrated (Ferrari et al., 1993), but HLA class-II responses against envelope epitopes (HBsAg) have been reported as being barely detectable (Jung et al., 1991). Nevertheless, anti-envelope CD4+ cell responses have been shown in the incubation phase of acute hepatitis B (Vento et al., 1987), and are detectable in recipients of HBsAg vaccine (Celis et al., 1988). These observations have led to the suggestion that loss of this HBsAg response in highly replicating hepatitis B may be due to exhaustion, or inactivation, of HBV-specific CD4+ cells by high levels of envelope antigens (Chisari and Ferrari, 1995b).

The mechanism of action of HBV-specific CD4+ cells in acute and chronic hepatitis B has not been fully elucidated. Although CD4+ cells can be demonstrated to have cytolytic activity *in vitro*, it is unclear whether cell destruction due to these cells contributes to viral control *in-vivo* (Hahn and Erb, 1999). In the transgenic mouse model of HBV infection, passive transfer of HBV-specific Th1 cells has been shown to lead to suppression of viral replication through a direct cytokine-dependent non-cytolytic mechanism (Franco et al., 1997). Nevertheless, the main action of CD4+ cells in viral infection is likely to be through their influence on CD8+ cell responses. In acute HBV infection, high levels of the Th1 cytokine IFN-γ (Guidotti et al., 1999a), and HBV-specific CD4+ cell responses, are seen in association with vigorous virus-specific CD8+ cell responses (Chisari and Ferrari, 1995b). The maintenance of CD8+ cell responses in chronic viral infection have been shown to be dependent on cytokine-mediated CD4+ cell responses (Matloubian et al., 1994), and in chronic HBV infection, where CD8+ cell responses are often weak, HBV-specific CD4+ responses to all viral antigens are also very hard to find (Ferrari et al., 1990). It is of

interest, however, that during acute flares of chronic hepatitis B, which may be associated with HBeAg seroconversion and control of infection, CD4 responses to nucleocapsid epitopes appear to be accentuated (Tsai et al., 1992). In a recently reported study, the adoptive transfer of HBV-core-specific CD4+ cells from HBV-immune donors was felt to be the central event in precipitating resolution of chronic HBV infection following bone marrow transplantation (Lau et al., 2002).

1.5.4. CD8+ cytotoxic T cell responses to HBV

Studies to define the specific viral epitopes involved in the CD8+ response to HBV have been based on the combined use of 9-10 amino acid synthetic peptides, mimicking the processed antigen fragments, and eukaryotic expression vectors which enable these peptides to be processed and presented in the context of MHC class 1 molecules (Penna et al., 1991; Bertoletti et al., 1991). Epitope specificity has been defined using cytotoxicity assays based on the release of ^{51}chromium from target cells incubated with synthetic HBV peptide (Guilhot et al., 1992; Rehermann et al., 1996a; Bertoletti et al., 1991). These strategies have been applied to the study of peripheral blood cells in patients with self-limiting acute hepatitis, and chronic infection. It has been determined that patients with acute hepatitis develop a vigorous, polyclonal, CTL response against multiple epitope specificities within the HBV envelope, nucleocapsid, and polymerase proteins (Rehermann et al., 1995a). A hierarchy of epitope-specific responses is suggested by the fact that several HLA A-2 restricted CTL epitopes, in particular HBV core 18-27, envelope 250-258, envelope 335-43, and polymerase 455-63, are often seen (Bertoletti et al., 1993; Nayersina et al., 1993), but other specificities, such as envelope 260-69 and polymerase 61-69 (Rehermann et al., 1995b; Bertoni et al., 1997; Nayersina et al., 1993), are rarely found. These studies, and more recent analysis of the direct *ex-vivo* frequency of HBV-specific CD8 cells suggest that HBV core 18-27 epitope may elicit the numerically immunodominant CTL response during the acute recovery phase of HBV infection (Maini et al., 1999) . An overview of cellular adaptive responses in HBV infection are shown in figure 1.2.

Until recently, both control of HBV replication, and the associated liver injury, have been thought to be mediated by the action of these MHC class 1-restricted CTL (Chisari and Ferrari, 1995a; Koziel, 1997). Liver injury implies hepatocyte cell death, and it has been clearly demonstrated following HBV infection that the action of virus-specific CTL may result in viral control through direct killing of infected cells (Kondo et al., 1997). In transgenic mice, the adoptive transfer of HBsAg-specific CTL leads to the development of liver disease resembling acute viral hepatitis (Moriyama et al., 1990), and in patients with acute hepatitis B, studied at various time-points after the development of jaundice, peak circulating frequencies of HBV-specific CD8+ cells have recently been shown at around the time of maximal liver injury (Maini et al., 1999).

Non-cytolytic viral control by HBV-specific CD8+ cells.

The presence of an inextricable link between the control of viral replication and liver injury has more recently been questioned, and a number of observations suggest that hepatocyte lysis by HBV-specific CTL is unlikely, alone, to explain the usual outcome of clinical recovery and clearance of circulating HBsAg following acute HBV infection. Based on findings in woodchuck hepatitis virus infection, it appears that nearly 100% of hepatocytes are infected following acute infection (Kajino et al., 1994), yet acute hepatitis is clinically mild in many patients (suggesting the destruction of no more than 5% of hepatocytes (Chisari and Ferrari, 1995b)), and fulminant hepatic failure, implying the destruction of a large proportion of hepatocytes, is a rare clinical outcome of infection. Studying HBV transgenic mice, Guidotti and colleagues have shown that HBV replication within hepatocytes may be abolished without the necessity for cell death (Guidotti and Chisari, 1996). The demonstration in chimpanzees (Guidotti et al., 1999a) and woodchucks (Guo et al., 2000), following inoculation with HBV or WHV, that virus is controlled prior to significant liver injury, also supports the evidence for intracellular inactivation of HBV replication. Non-cytolytic control of infection is certainly not peculiar to HBV, and has been shown for other viruses that infect the liver, including murine cytomegalovirus (CMV) (Tay and Welsh, 1997), lymphochoriomeningitis virus (LCMV) (Guidotti et al., 1999b), and *Listeria monocytogenes* (Kagi et al., 1994).

Figure 1.2. Schematic representation of HLA restricted T cell responses in HBV infection.
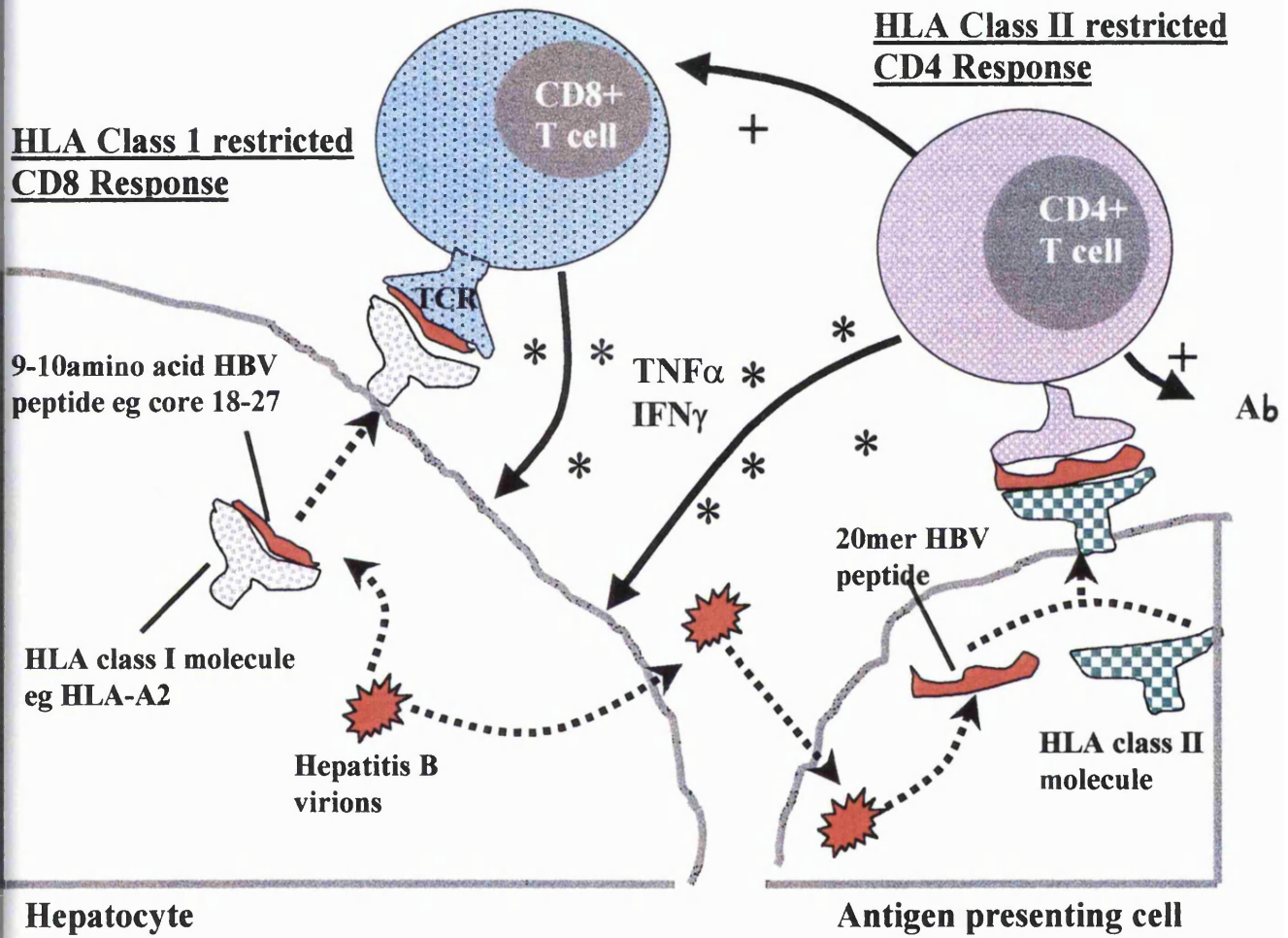
1.5.5. Role of cytokines in viral control and liver injury.

As has been eluded to, cytokines (including IL-12, IFNα/β, IFNγ, TNFα) may have an important role not only in promoting recruitment and activation of cellular components of the adaptive immune response to HBV, but also in directly controlling intracellular viral replication. In the transgenic mouse model of HBV infection IFNα/β, IFNγ and TNFα have been shown to downregulate HBV replication (Guidotti and Chisari, 1996),(Guidotti and Chisari, 1999), and treatment of these mice with an inducer of type 1 IFN leads to clearance of HBV replicative intermediates from hepatocytes (McClary et al., 2000). Furthermore, infection of transgenic mice with unrelated hepatotropic viruses leads to inhibition of HBV through IFNα/β pathways (Cavanaugh et al., 1998; Guidotti et al., 1996a). The inhibition of HBV replication following the injection of HBV-specific CTL has also been shown to be mediated by the secretion of IFNγ and TNFα (Guidotti et al., 1996b). The finding of increased levels of IFNγ and TNFα in the livers of chimpanzees at the time of maximal reduction in HBV DNA, but before significant T cell influx or liver injury, also lends support to the probable role of these, and perhaps other, cytokines in non-cytopathic control of HBV (Guidotti et al., 1999a), as discussed above.

Differential induction of cytokines may be found in different patterns of HBV infection, and may be associated with viral control or persistence. A key determinant of successful control of HBV appears to be the expansion of IFNγ -producing CD4 (Th1) (Lohr et al., 1995; Ferrari et al., 1990) and CD8 (Tc1) responses (Maini and Bertoletti, 2000). The majority of HBV-specific T cell lines from patients with acute resolved infection express a Th1 phenotype (Penna et al., 1997), and Tc1 responses have been shown in these patients (Maini et al., 1999; Jung et al., 1999), and in others with persistent infection but lower levels of viral replication (Maini et al., 2000). This response appears to be promoted by the action of IL-12 and IFNα/β. IL-12-dependent induction of a Th1 response has been observed during HBV infection, and increased serum levels have been seen in association with HBeAg seroconversion in chronic hepatitis B (Rossol et al., 1997). However, in

chronic HBV infection the majority of intrahepatic T cells do not have a Th1 phenotype, but produce Th-2 type cytokines (eg IL-4 and IL-5) (Bertoletti et al., 1997a).

1.5.6. Mechanisms of liver injury in HBV infection.

Although an inextricable link between viral control and liver injury, due to the action of HBV-specific CD8+ cells, has recently been challenged, an association between liver damage and a reduction in HBV replication undoubtedly exists. It is well recognised that symptomatic, icteric, acute HBV infection is almost always associated with clearance of HBsAg from the serum, and that this may occur even more rapidly in those with fulminant disease (Gimson et al., 1983). In chronic infection, clinical flares of liver injury (indicated by a significant rise in serum ALT, with or without jaundice), may be related to HBeAg seroconversion and a reduction in HBV replication. Apoptosis and regeneration of hepatocytes during virus control in woodchuck hepatitis virus infection suggests that lysis occurs (Guo et al., 2000), and the fact that this may occur through perforin-mediated mechanisms demonstrates the likely role of CTL (Kondo et al., 1997). In the transgenic model of hepatitis B it has been well shown that adoptive transfer of HBV-specific CTL leads to liver damage (Moriyama et al., 1990). Using various techniques a clearly detectable HBV-specific CTL response can be demonstrated in patients with acute hepatitis (associated with significant liver injury) (Rehermann et al., 1995a; Maini et al., 1999), but this response has been difficult to detect in those patients with chronic infection (Bertoletti et al., 1994a).

The degree of liver injury does not appear to correlate well with the number of virus-specific CTL within the liver. Using methods to directly quantify, ex-vivo, virus-specific cells in patients with chronic hepatitis B, it has been shown that a similar number of HBV core 18-27-specific CD8+ cells are present in the livers of those with low viral replication and minimal liver injury, compared to those with high levels of viral replication and significant liver damage (Maini et al., 2000). In chimpanzees with resolved HCV infection and no liver injury intrahepatic HCV-specific CD8+ cells display marked cytolytic activity,

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whereas in animals with chronic HCV infection and significant liver damage, very little CTL activity can be demonstrated (Cooper et al., 1999). These studies suggest that the function, as much as the number, of virus-specific CTL within the liver, may be a crucial determinant of the pattern of viral replication and liver injury.

Whilst present techniques may fail to define all the important populations of epitope-specific CD8+ cells (because the response is HLA class I restricted), it nevertheless appears that the main cause of liver injury in hepatitis B is due to the effects of non-antigen-specific CD8+ cells. The importance of these cells in the pathogenesis of liver damage has been shown in a transgenic mouse model of fulminant hepatitis (Ando et al., 1993) and in the concanavalin A-induced model of hepatitis (Tiegs et al., 1992; Kusters et al., 1996). In the HBsAg-transgenic mouse model, fulminant hepatitis is initiated by the injection of a large quantity of CTL clones specific for HBsAg. Initially these clones trigger apoptosis of HBsAg positive hepatocytes, but the destructive phenomenon that causes liver failure involves the recruitment of large numbers of other activated cells, including T cells and macrophages. Cytokines seem to play an important role in this recruitment, as this process is blocked by the injection of antibodies against IFN-γ (Ando et al., 1993). In patients with chronic hepatitis B and significant hepatic inflammation, the great majority of intrahepatic CD8+ cells have been shown to be non-antigen-specific (Maini et al., 2000).

A number of factors are likely to determine viral control, recruitment of non-antigen-specific cells, and liver injury, including a range of cytokines, but the balance between quantity of virus and the quantity and/or efficiency of virus-specific CD8 cells is likely to be important. It is possible that when virus-specific CTL are faced with a quantity of virus that exceeds a certain threshold, a cascade of events are triggered that lead to liver damage, mainly through the action of non-antigen specific cells. This scenario has been demonstrated in a mouse model of influenza virus infection, where the balance between the infectious dose of virus and the quantity of adoptively transferred virus-specific CTL was tightly controlled. The same quantity of virus-specific CTL could either promote virus clearance without any pathology, or cause fatal lung injury (Moskophidis and Kioussis, 1998). The crucial variable was the infectious dose of virus, because when the virus

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exceeded the clearance capacity of the virus-specific CTL, lung pathology resulted. Interestingly, treatment with anti-IFN-γ antibody prevented lung pathology by blocking the recruitment of large numbers of activated non-antigen specific cells.

Considering all this data together, it appears that whilst HBV-specific CD8+ cells may be the trigger for liver injury, recruitment of non-antigen-specific CD8+ cells, through the action of cytokines such as IFNγ, is the main cause of liver injury in hepatitis B. Although high levels of viral antigen may influence this recruitment, it is also increasingly recognised that different outcomes following infection, in terms of liver injury and viral control, are crucially influenced by the function of virus-specific CD8+ cells, and not simply their number. Therefore, whilst recognising that a successful immune response to HBV infection is dependent on the dynamic integration of innate, acquired, humoral and cellular immunity, the main focus of investigation in this report relates to study of the HBV-specific CD8+ cell response.

1.5.7. Mechanisms of viral persistence:

In view of the significant differences in morbidity between those individuals who control HBV after infection, and those who are chronically infected, the processes that predict persistence are of great importance. Although an extensive discussion of these is beyond the scope of this thesis, the virus must develop strategies to evade the host immune response (Ploegh, 1998; Zinkernagel and Hengartner, 1997). Possible strategies used by viruses may include downregulation of viral gene products, infection of sanctuary sites, or expression of gene products which directly alter the immune response (eg interference with MHC class I and II expression and function) (Lorenzo et al., 2001). The induction of tolerance by the adaptive immune response to HBeAg in the neonatal period has been implicated as a cause of the high rate of chronic infection following vertical transmission (Thomas et al., 1982) (Milich et al., 1990). The excess production and secretion of large amounts of envelope protein (HBsAg) may also act as a means of 'flooding' host humoral

responses, reducing the chance of neutralisation of whole viruses. Host genetic factors also appear to play a role. A range of polymorphisms within MHC class II, such as allele DRB1*1302 in subjects in the Gambia (Thursz et al., 1995), have been shown to predict susceptibility to HBV persistence following infection (Almarri and Batchelor, 1994).

Whilst many processes are likely to contribute to viral persistence, it may be that the strength of the antiviral CTL response is the most critical factor (Chisari and Ferrari, 1995b). As discussed earlier, the polyclonal, multispecific HBV-specific CTL response that characterises acute infection and viral control is frequently absent in chronic disease. Depending on their site within the CTL epitope, mutations within viral epitopes may lead to a number of downstream effects, including the loss of binding to MHC class I, binding to MHC but absent binding to TCR, or binding to MHC and TCR, but the initiation of altered subsequent CTL function (Rosenberg, 1999). Viral mutations within the immunogenic HBc 18-27 epitope of chronically infected HLA-A2-positive patients have been shown (Bertoletti et al., 1994a), but these have been found in only the minority of chronically infected patients, suggesting that viral escape does not play a major part in the chronicity of HBV. However, these natural variants have been shown to elicit a diminished CTL response following MHC class I activation, and act as T cell receptor antagonists (Bertoletti et al., 1994b). MHC class I downregulation is adopted by a range of viruses to evade CTL recognition, and may occur through disruption at a range of points along the path to cell surface expression (Lorenzo et al., 2001), (Ploegh, 1998). Downregulation of class I has not been definitively shown in hepatitis B, although reduced class I expression in patients with chronic disease (Brook et al., 1989), and the inhibition of immunoregulatory molecules by HBV (Foster et al., 1991) suggests that it may occur. Nevertheless, complete downregulation of MHC class I on hepatocytes seems very unlikely, as the absence of MHC class I is an important stimulant to NK cell-mediated cytolysis (Biron et al., 1999a).

Just as cytokine production is an important component of both the innate and adaptive immune response, a number of viruses (eg human papilloma virus, EBV) have been shown to abrogate their action, through a range of processes, including the production of cytokine

analogues which block the activity of IFN, the synthesis of soluble IFN receptors, and the encoding of genes which specifically inhibit IFN-inducible genes (Lorenzo et al., 2001). Whereas this process has been less well defined for hepatitis B, HBcAg has been shown to inhibit IFN-β transcription (Twu and Schloemer, 1989), and the *in vitro* transfection of HBV in culture renders them specifically unresponsive to IFN (Onji et al., 1989).

It has been argued that the critical period for determining viral control or persistence occurs soon after infection (Goulder et al., 2001), and that HBV persistence may result from the exhaustion of virus-specific CTL by overwhelming levels of virus during this early period of infection (Chisari and Ferrari, 1995b). This process has been shown in lymphocytic choriomeningitis infection (LCMV) in mice (Moskophidis et al., 1993). Recently, however, data from patients studied from the incubation phase of hepatitis C virus infection has suggested that failure to induce a CTL response, rather than exhaustion, may be important, and that low levels of replication (as seen in HCV infection) may fail to trigger an adequate immune response, so predisposing to chronicity (Thimme et al., 2001). The dynamic relationship between CTL and virus seems likely to be an important factor in the development of viral control or persistence.

1.6. Difficulties in studying cellular immunity of hepatitis B:

Advances in our understanding of the role of CTL in the pathogenesis of HBV infection have largely come from the study of 1: Patients with acute icteric (Maini et al., 1999), chronic, or resolved infection (Penna et al., 1996; Rehermann et al., 1996a), in whom cross-sectional analysis of CTL has been correlated with markers of viral control and disease activity. 2: Animals with HBV or related infections. 3: A transgenic mouse model of HBV infection. In the latter model, the development of embryo microinjection technology has allowed the introduction of partial or complete copies of the HBV genome into transgenic mice (Chisari et al., 1985; Babinet et al., 1985). Although these methods have led to

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significant advances in our understanding of the role of host immune responses, and CTL mechanisms in particular, in hepatitis B, a number of complicating factors exist:

1. HBV infects and replicates only in man, chimpanzees, and possibly gorillas, therefore limiting the availability of experimental models of infection. Although duck hepatitis B virus, and woodchuck hepatitis virus are hepadnaviral homologues of HBV, the immune systems within these hosts are less well defined, and extrapolation to human infection is difficult (Chisari and Ferrari, 1995a).

2. It has proved very difficult to grow the virus in culture.

3. Study of the immune response at the major site of viral replication (ie the liver), particularly sequentially over time, may pose technical and ethical difficulties in man. Even when tissue from transcutaneous liver biopsies is available, the yield of CD8+ cells, and, of course, a subset of virus-specific CD8+ cells, has made the study of HBV-specific immune mechanisms difficult.

4. Patients with acute infection are usually identified only after the onset of jaundice, therefore precluding studies of immune and viral dynamics during the early period of infection. This may be important, as it is recognised for a range of viral infections, including HIV and HCV infections, that an understanding of the early immune responses that are associated with viral control may provide insights into the processes that are deficient in chronic disease (Klenerman and Zinkernagel, 1997; Lechner et al., 2000a; Goulder et al., 2001). Some insight into the early events of acute hepatitis C have recently come from the study of a patient identified during the incubation phase of infection (Thimme et al., 2001). Study of immune responses during the incubation phase of acute hepatitis B in man are also, not surprisingly, very limited (Vento et al., 1987), because of the requirement to identify recently infected, but asymptomatic patients. Nevertheless, studies in chimpanzees (Guidotti et al., 1999a) and woodchucks (Guo et al., 2000) suggest that crucial immune events involved in the control of viral replication may occur during

the pre-clinical phase of infection. Despite the lack of detailed data on early immune mechanisms in man, the clinical observation that HBsAg may be negative at the time of presentation with acute hepatitis B, and that this is most likely to occur in those with fulminant liver disease (Gimson et al., 1983; Woolf et al., 1976), further suggests that immune events prior to the development of significant liver injury are of importance.

5. Previous methods to define the HBV-specific CD8+ cell response, using conventional chromium release assays, have depended upon single (Penna et al., 1991) or multiple (Rehermann et al., 1996b) rounds of *in vitro* peptide stimulation (Ferrari et al., 1993). Whilst this technique has been essential in order to define cells at very low precursor frequency, it precludes analysis of the true frequency of virus-specific cells within a cell population. As this approach depends on the ability of CD8+ cells to expand *in vitro*, it may underestimate the real number of antigen-specific CD8+ cells (Alexander-Miller et al., 1996), and may bias the identification of cells with particular functional capacity. For example, CD8+ cells with a memory phenotype expand more readily on re-exposure to antigen, than is the case for activated, naïve cells (Flynn et al., 1998a; Maini et al., 1999).

6. The development of a transgenic mouse model of HBV infection (Moriyama et al., 1990; Chisari, 1995; Chisari, 1995) which expresses HBV gene products, and replicates the viral genome within the hepatocytes, has undoubtedly led to significant advances in understanding of the sequential recruitment of components of the cellular immune response, and the importance of cytolytic, and non-cytolytic mechanisms. Nevertheless, a limitation of this model is that the mice are not infected by the virus, precluding the study of the dynamics of viral replication and propagation.

1.6.1. New techniques for studying virus-specific CD8+ cell responses.

The limitation of requiring rounds of peptide stimulation to identify virus-specific CD8+ cells *in vitro*, as discussed above, can now be avoided, following the important development of tetrameric HLA-peptide complexes (Altman et al., 1996). These tetrameric complexes allow the direct *ex-vivo* quantification of virus-specific CD8+ cells within the blood and tissue lymphocyte populations, and have been applied to the study of a wide range of infections (Callan et al., 1998; Gallimore et al., 1998a; Ogg and McMichael, 1999; Ogg and McMichael, 1998; Gallimore et al., 1998a; Ogg et al., 1999), including hepatitis B (Maini et al., 1999; Maini et al., 2000). The complexes consist of a structural analogue of the HLA class 1 molecule, incorporating a specific viral antigen, to which virus-specific CD8+ cells bind on the surface of infected cells, leading to the initiation of the CTL response, as discussed earlier. Each tetramer consists of four biotinylated HLA class 1 molecules, each folded with a specific peptide, and multimerized by the addition of fluorochrome-labelled streptavidin (Figure 1.3). Tetramers have been made to incorporate 9-10 amino acid peptides corresponding with previously defined HLA-A2 associated HBV CTL epitopes (including core 18-27, polymerase 335-343, envelope 575-583). The multimeric HLA class 1-peptide complex has a high avidity for CD8+ cells displaying the T cell receptor (TCR) corresponding to the specific epitope used. Fluoresence labelling of the tetramer allows these specific cells to be identified using flow cytometry. A prerequisite to using tetramers to study immune responses in patients is a knowledge of the HLA haplotype profile (most class I tetramers have been HLA-A2, but others are also now being used), and the specific epitope sequences presented by these molecules.

Figure 1.3. **Schematic representation of HLA class I tetrameric complex**

9-10 amino acid HBV epitope

eg: core 18-27 (FLPSDFFPSV)
    polymerase 575-83 (FLLSLGIHL)
    envelope 335-343 (WLSLLVPFV)
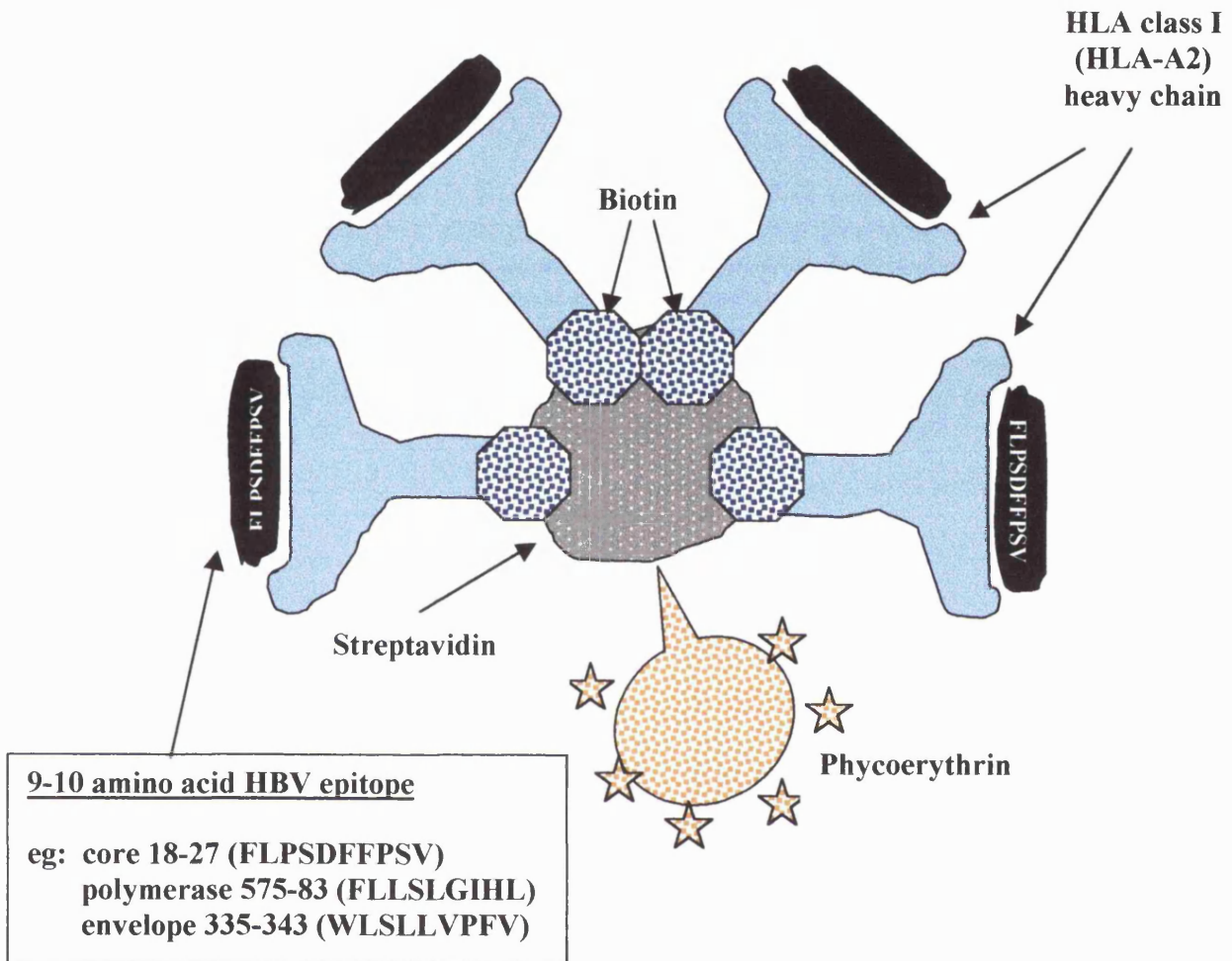
NB. Components not to scale

The great advantage of tetrameric complexes is that they allow direct quantification of antigen-specific CD8+ cells, largely independent of their functional properties. Using this method it has been observed that the frequency of circulating virus-specific CD8+ cells in acute infection with HBV (Maini et al., 1999) or Epstein Barr virus (EBV) (Callan et al., 1998), may be up to 40 fold higher than has been demonstrated by limiting dilution techniques. Remarkably more than 40% of circulating CD8+ cells during acute EBV infection have been shown, using this technique, to be specific for a single epitope (Callan et al., 1998).

The importance of these antigen-specific CD8+ cells *in-vivo* lies in their ability to lyse infected cells and produce pro-inflammatory cytokines, such as IFNγ. It has also been demonstrated *in-vitro* that the functional capacity of tetramer-specific CD8+ cells may show wide variation, with some antigen-specific cells, for example, showing minimal capacity to lyse or produce cytokines (Lechner et al., 2000b). The capacity of these cells to produce IFNγ, after exposure to the MHC/peptide complex to which their TCR specifically binds, has led to the development of ELIspot and intracellular cytokine staining assays, thus enabling the frequency of IFNγ-producing (and thus antigen-specific, *in-vitro* functionally active) CD8+ cells to be defined after peptide stimulation, both directly and following expansion (Schauer et al., 1996). With these considerations in mind, both tetrameric complexes and intracellular cytokine staining assays have been employed in the immunological studies described subsequently in this report, providing complementary information on the frequency and function of HBV-specific CD8+ cells.

CHAPTER 2

EPIDEMIOLOGY OF A LARGE OUTBREAK OF
HEPATITIS B IN NORTH LONDON

2.1. Introduction.

HBV is transmitted parenterally, and the important routes of infection within particular populations have been defined. The incidence of acute hepatitis B is low in the UK (Balogun et al., 1999) and USA (Centres for Disease Control and Prevention, 1996), and most sporadic cases follow high risk behaviour, including unprotected sexual intercourse and injecting drug use. A clear source of exposure is absent in approximately 30% of cases. As with other infectious diseases, cases of HBV infection may be linked by having arisen from a common source (ie. as part of an outbreak), but in view of the enormous number of people worldwide who have been exposed to HBV, it is surprising that more clinical outbreaks of HBV infection have not been reported. This may be due to a range of factors, including an absence of clinical suspicion of infection in patients with anicteric acute hepatitis, and the high frequency of asymptomatic viral carriage in patients with chronic hepatitis B (in contrast to other infections, such as pulmonary tuberculosis, or cholera). Social stigma surrounding HBV infection may well contribute to under-reporting of acute disease, specific risk behaviour, and contact details, all of which would hamper efforts to define the source and extent of an outbreak of infection. Importantly, characterising an outbreak of HBV infection necessitates the coordinated efforts of a range of personnel, including primary care and public health physicians, epidemiologists, and laboratory-based staff. This infrastructure is rarely available in parts of the world where spread of HBV infection within a community is most likely to occur.

Despite these limitations, outbreaks of hepatitis B have been reported from all geographical regions, arising from a range of sources. Skin piercing procedures have been identified as an important source of outbreaks, both in the community and in clinical care settings. The widespread screening of blood products, immunisation of health care workers, and the implementation of infection control regulations (UK Health Departments, 1993) has led to a significant reduction in hospital outbreaks in the developed world, but they continue to be described (The Incident Investigation Teams, 1997; Harpaz et al., 1996). Community acquired outbreaks due to a range of skin piercing procedures, including tattooing (Limentani et al., 1979) and acupuncture (Boxall, 1978; Stryker et al., 1986), were reported in the 1970s and 80s, and in the UK led to the introduction of licensing and hygiene regulations (Local Government Act, 1982).

I report here a large outbreak of acute hepatitis B linked to autohaemotherapy performed at an alternative medicine clinic. Epidemiological data and viral genome analysis allowed the relatedness of infections and the probable mechanism of transmission to be defined.

2.2. Methods.

2.2.1. Background and epidemiological investigation

In February 1998 a 43 year old woman living in Derby, in the Midlands, developed acute icteric hepatitis B. She had attended a clinic in London in October and November 1997, where she had received a treatment known as autohaemotherapy. This technique involved the drawing of approximately 1-2 ml of the patient's blood with a needle and syringe, and mixing this with an equal volume of saline. The mixture of autologous blood and saline was then injected back into the patient via the buttocks or various 'acupuncture points', such as in the web of the fingers.

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The Consultant in Communicable Disease Control (CCDC) with responsibility for Barnet Health Authority, in which the clinic was based, advised the clinic staff to cease autohaemotherapy. Directors of public health laboratories and CsCDC nationwide were sent an electronic message asking them to report any cases of hepatitis B possibly linked to this clinic. Through these case finding procedures seven further HBsAg-positive cases were identified over the next 4 weeks. Separate to this investigation, a patient was referred to the Royal Free Hospital by the North London Blood Transfusion Service in March 1998 with evidence of hepatitis C virus (HCV) seroconversion. She had received autohaemotherapy at the clinic in March 1997. As a result of this the outbreak investigation was extended to include HCV.

Patients who attended the clinic between January 1997 and February 1998 were identified, either through their clinic records, or following local media coverage (which included a number of local radio and television reports, and the setting up of a dedicated telephone 'helpline' at Barnet Health Authority). A lookback investigation was carried out between February and September 1998 by the local health authority and the PHLS Communicable Disease Surveillance Centre (CDSC). An outbreak monitoring team was convened, consisting of representatives of Barnet Health Authority, the CDSC, and the Department of Medicine at the Royal Free Hospital, under the direction of Professor Dusheiko. This outbreak team met on a monthly basis during 1998, in order to coordinate the identification of cases, discuss and define the source and mechanism of infection, provide clinical care for infected individuals, and prevent cases of secondary infection. Each patient was asked to give a serum sample for serological testing, and to complete a questionnaire detailing the dates on which they received autohaemotherapy. Clinic staff were also tested for markers of HBV and HCV infection.

2.2.2. Hepatitis B investigation

Serum samples were tested for HBsAg by an enzyme-linked immunosorbent assay (EIA) (Hepanostika Uni-Form II, Organon Teknika, Boxtel, Netherlands), and total antibody to hepatitis B core antigen (anti-HBc) using an in-house competitive radioimmunoassay.

Samples with positive or equivocal results for HBsAg were tested for hepatitis B e antigen (HBeAg), anti-HBe antibody (Hepanostika HBe, Organon Teknika), IgM antibody to HBc (anti-HBc IgM) (Murex Biotech Ltd, Dartford, England), and antibody to HBsAg (anti-HBs) (IMX Abbott GmbH Diagnostika, Wiesbaden-Delkenheim, Germany). If negative for HBsAg, samples were tested for total anti-HBc, anti-HBc IgM and anti-HBs. Antibody to hepatitis D (ETI-AB-DELTAK-2, Diasorin S.r.I., Saluggia, Italy) was tested for in all patients who were HBsAg-positive. This was done at the PHLS, Colindale, London.

The case definitions applied were: 1) 'Active HBV infection': HBsAg-positive. Those who were anti-HBc IgM-positive were defined as having acute infection. Those who were anti-HBc IgM-negative, or who remained HBsAg-positive for more than six months, were defined as having chronic hepatitis. 2) 'Resolved HBV infection': HBsAg-negative and total anti-HBc-positive and anti-HBs-positive; or anti-HBc-positive on sequential sampling, if anti-HBs-negative. In this category, those patients who were anti-HBc IgM-positive were defined as having recently resolved infection. 3) 'No serological markers of HBV infection': HBsAg-negative, anti-HBc-negative and anti-HBs-negative; or HBsAg and anti-HBs-negative, but anti-HBc-positive in a single specimen.

Phylogenetic analysis was performed by Rachel Hallett, at the PHLS Hepatitis and Retrovirus Laboratory, London. All samples were tested for HBV DNA following extraction of DNA from sera by the guanidium thiocyanate-silica method (Boom et al., 1990). Fragments from the hepatitis B core and surface genes were amplified by nested PCR (Kaneko et al., 1989); the sequence of the primers are shown in Table 2.1. The PCR products from all HBV DNA-positive samples were sequenced using both sets of inner primers. Sequencing reactions were performed using the ABI Prism DNA Sequencing kit and reactions were electrophoresed on an ABI 373 automated sequencer (PE Applied Biosystems, Warrington, UK). The resulting sequences (197 nucleotides from the core gene and 404 nucleotides from the surface gene) were aligned using the CLUSTAL V algorithm in the Lasergene Program (DNASTAR Inc, USA) (Higgins and Sharp, 1989). HBV DNA from patients with acute hepatitis B, whose serum had been referred to the Hepatitis and Retrovirus Laboratory during 1997, was also sequenced, to act as controls. The genetic

relatedness of aligned sequences was compared by phylogenetic analysis (Saitou and Nei, 1987). Serum HBV DNA was quantified using the AMPLICOR HBV MONITOR assay (Roche Diagnostic Systems, NJ, USA).

Table 2.1. Oligonucleotide primers used for the amplification of hepatitis B virus DNA.

Region	Position	Name	Primer Sequence
Surface	Outer-sense	S1-109	5'ATACCACAGAGTCTAGACTC
	Outer-antisense	S2-609	5'AACTGAAAGCCAAACAGTGG
	Inner-sense	109*	5'ATACCACAGAGTCTAGACTCGTG GTGGACT
	Inner-antisense	585R*	5'AAGCCCTACGAACCACTGAACAA TGGCAC
Core	Outer-sense	1763*	5'GCTTTGGGGCATGGACATTGACC CGTATAA
	Outer-antisense	2032R*	5'CTGACTACTAATTCCCAGGATGCT GGGTCT
	Inner-sense	1778-E*	5'GACGAATTCCATTGACCCGTATA AAGAATT
	Inner-antisense	2017R-B*	5'ATGGGATCCCTGGATGCTGGGTC TTCCAAA

* taken from (Saitou and Nei, 1987)

2.2.3. Hepatitis C investigation

Samples were analysed for antibody to HCV using one of two enzymatic immunoassays (EIAs) (MONOLISA anti-HCV PLUS, Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France; and Ortho HCV 3.0, Ortho-Clinical Diagnostics, Neckargemund, Germany). A supplemental recombinant immunoblot assay (RIBA) (Chiron RIBA HCV 3.0 SIA, Chiron Corporation, Emeryville, Ca.,USA) and repeat EIA were performed where equivocal or positive results were found. Patients were defined as serologically positive for HCV if they were EIA positive and RIBA positive. HCV RNA detection was carried out by reverse transcription-PCR. RNA was extracted from serum, using the Roche AMPLICOR Extraction Kit. Amplification of the 5' non-coding region and genotyping was performed using a restriction fragment length polymorphism assay (Harris et al., 1999).

2.2.4. Statistical analysis

Where the number of treatment visits was known, the proportion of patients with serological markers of viral hepatitis was related to the number of visits, using a Chi-squared test for trend. Trends over time in the probability of HBV or HCV infection per patient visit were investigated using data on patients with known visit dates. This analysis was performed by Conor Farrington, senior statistician at the PHLS Communicable Disease Surveillance Centre, and was complicated by the fact that many patients visited on several occasions over the 12 month period of interest, as became clear during initial patient contact and the receipt of completed questionnaires. This made it difficult to precisely define the date of infection for each individual, and required the number of visits to be taken into account. The analysis was therefore performed using a likelihood method which allowed for multiple visits. In each week (w) of the study period it was assumed that all patients undergoing treatment in that week had the same probability (P_w) of acquiring infection. All patients were considered to be equally susceptible to infection at the outset, and the outcome for different patients was

assumed to be independent. Given a patient's sequence of visits (for example, weeks $w_1,...., w_k$), the patient's probability of escaping infection is $\Pi(1-Pw_i)$. The overall likelihood is obtained by multiplying the outcome probabilities for all the patients. The weekly infection probabilities Pw were estimated by maximising the likelihood. The hypothesis of a constant infection probability $Pw=P$ was tested by the likelihood ratio test. If information on the date of onset of clinical hepatitis was available, this was incorporated by excluding visits in weeks in which infection could not have occurred (ie. more than six months prior to onset of clinical disease).

2.2.5. Clinical assessment

All patients with serological markers of active HBV or HCV infection were referred for clinical assessment. A history of acute hepatitis, and enquiry into other risk factors for exposure to viral hepatitis, were sought. Household and sexual contacts of these patients were identified and counselled, and, where appropriate, offered serological testing and HBV vaccination. Patients with active acute or chronic HBV or HCV infection were assessed on a regular basis at the Royal Free Hospital, in order to monitor clinical evidence of hepatic decompensation and the possible need for admission to hospital. Blood tests were taken at each visit to monitor biochemical and serological resolution of infection. Where this did not occur, patients were counselled as to the need for further investigation and treatment. Consideration was given on an individual basis to the merits of treatment with antiviral therapy, both in those with acute, and those with chronic infection. For those patients unable to attend the Royal Free Hospital for further assessment, advice was given to their general practitioner or hospital consultant, either directly or via the PHLS, concerning the interpretation of their clinical and serological data, and need for further assessment.

2.3. Results

2.3.1. HBV and HCV infection in clinic staff

The clinic was staffed by four members of the same family (father, mother, son and daughter). The father was a registered physician. He and his wife developed acute hepatitis B in March 1998, at which time his wife was also anti-HCV positive, but HCV RNA negative. Their daughter had resolved hepatitis B, was anti-HCV positive, but HCV RNA negative. The fourth staff member, their son, had no markers of HBV or HCV infection. The two staff members with acute hepatitis B cleared HBsAg after an acute hepatitic illness. On further questioning of the clinic staff, it is of note that autohaemotherapy had been performed by father, mother and daughter on each other on several occasions over the previous year, for the treatment of a range of conditions, including upper respiratory tract infections and influenza-type symptoms.

2.3.2. HBV infection in clinic patients

Three hundred and ninety-nine patients who attended the clinic between January 1997 and February 1998 were identified. Serum samples were received from 352 (216 female, 136 male, mean age 42 years, range 1-86 years). Including the original cases, 54 patients (15%) had evidence of HBV infection (Figure 2.1). Twenty-three patients had markers of resolved infection, and six of these had recently resolved infection. Thirty-one patients had evidence of active hepatitis B at the time of initial blood testing. Anti-hepatitis D antibodies were negative in all HBsAg-positive patients.

Figure 2.1. HBV serological status at the time of initial blood testing in clinic staff and patients who received autohaemotherapy
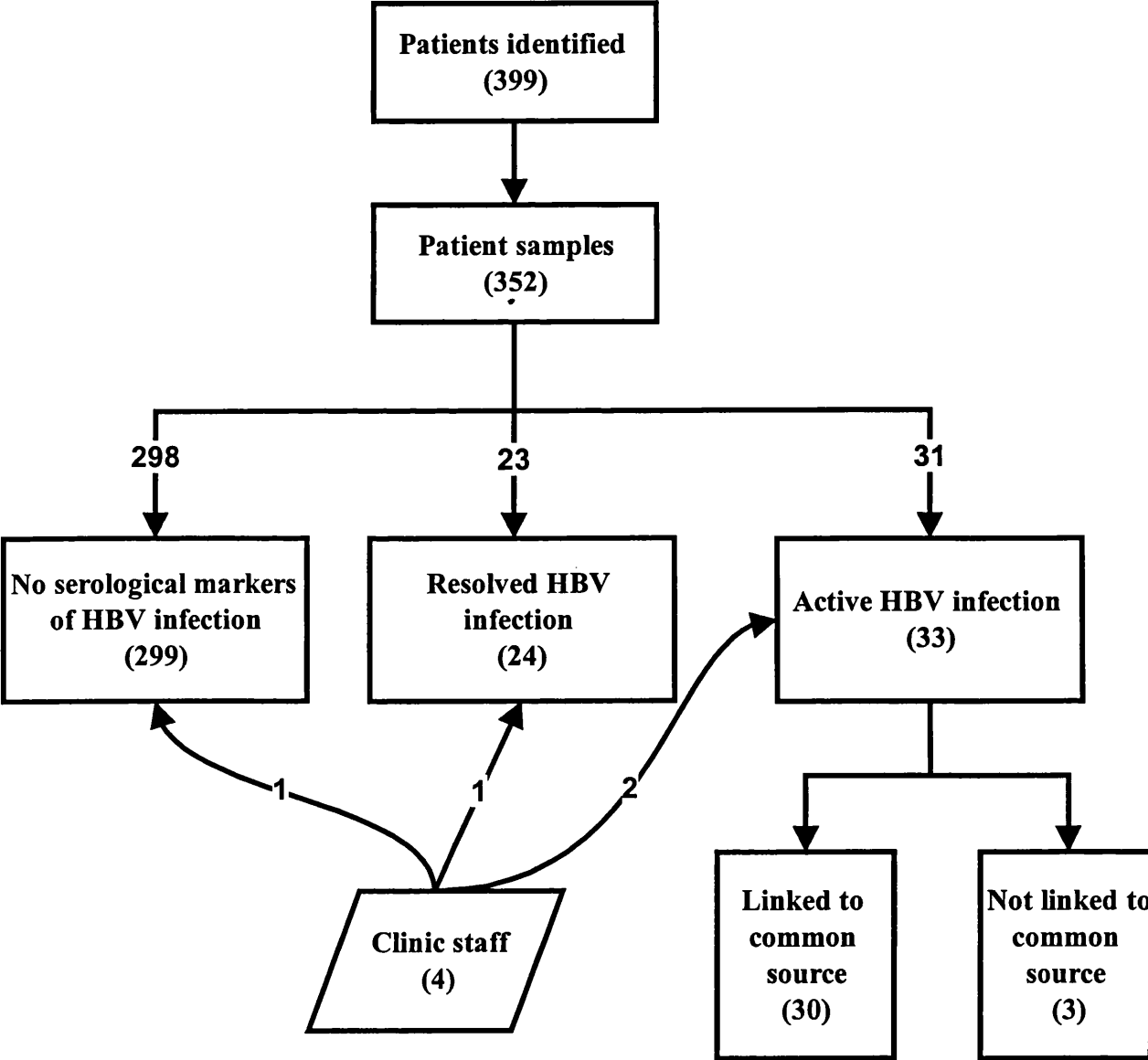
2.3.3. HBV DNA sequence analysis

HBV DNA could be amplified and sequenced from 32 of the 33 HBsAg-positive patients, including both actively infected staff members. There was complete nucleotide identity in both the surface and core genes in 30 cases (91% of HBsAg-positive cases). The sequences possessed nucleotide motifs characteristic of variants belonging to genotype D. The variant found in these 30 linked cases was different to control samples from acutely infected patients, as demonstrated by phylogenetic analysis (Figure 2.2).

Figure 2.2.

Genetic relatedness of the HBV identified in the outbreak, by phylogenetic analysis of sequences from surface gene fragment.

Analysis of 66 nucleotide sequences from a 404 base pair fragment of the HBV surface gene. Numerals designate cases identified in the investigation of this outbreak. 1 and 2 are clinic workers; 3-32 are patients at the clinic. Letters designate patients with acute hepatitis B identified in transmission incidents that occurred in the UK in 1997, but were not associated with the outbreak. Sequences M, N, P and R are derived from an outbreak among injecting drug users. Sequences F, O, X, Y and Z are generated from another outbreak principally involving tattoo clients, prison inmates and injecting drug users. The other sequences are from single transmission incidents not known to be part of any outbreak. PP designates the sequence of the plasmid control used in PCR studies. HBV genotype is displayed on the right axis.

% divergence
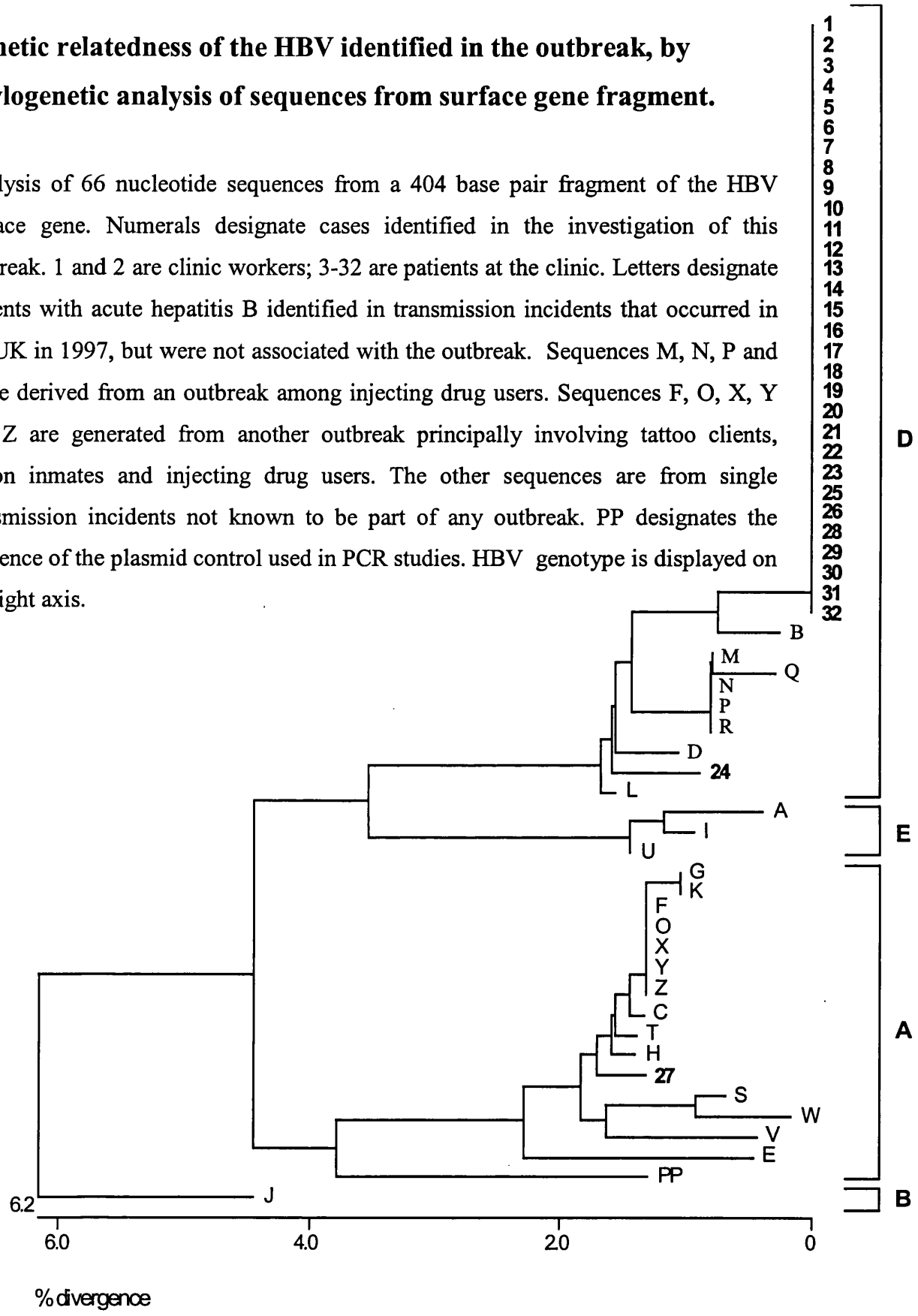
2.3.4 Clinical outcome of HBV infection

Twenty-five of the 30 cases linked by HBV DNA sequence analysis cleared circulating HBsAg over the first six months of assessment. Clinical hepatitis was documented in 23, and jaundice occurred in 16. Five of the patients with linked infection did not clear HBsAg over a six month period, and so were classified as having chronic hepatitis B. None had a history of acute clinical hepatitis, but three were anti-HBc IgM-positive at presentation. The remaining two patients were anti-HBc IgM-negative, anti-HBc IgG-positive, when first tested. One was a 79-year old man (patient 16), born in India, who was HBsAg-positive, HBeAg-positive, with HBV DNA of 1.2×10^{10} copies/ml. The other was a 79-year old woman (patient 18) with diabetes mellitus, also born in India, who remained HBsAg-positive, HBeAg-positive. Of the three patients with acute infection who became chronically infected, two were taking oral steroid therapy. One was a 72 year old woman (patient 6), who was taking prednisolone for cryptogenic organising pneumonitis, and remained HBeAg-positive, with very high HBV DNA concentrations (10^8-10^{10} copies/ml) and minimally elevated serum transaminases. The other was a 19-year old woman (patient 11) who was being treated with prednisolone and azathioprine for a relapse of ulcerative colitis. Patient 22 was an 18 year old woman who had no other significant medical problems, and took no regular medication, but failed to clear HBsAg.

Two of the HBsAg-positive patients in whom HBV-DNA was amplified (patients 24 and 27) were not linked by sequence analysis (Figure 2.2). They had serological evidence of chronic hepatitis B (anti-HBc IgM-negative, anti-HBc IgG-positive), with no history of clinical acute hepatitis, and both were born in countries with increased endemic rates of HBV carriage. Consequently, there was no evidence to definitively link their infection to autohaemotherapy.

2.3.5. Relationship between autohaemotherapy and HBV infection

The dates of 1,546 clinic visits in the period January 1997 to February 1998 were available for 315 patients (53 with markers of hepatitis B and 262 who were negative). The proportion of patients with markers of HBV infection increased with the number of treatments received (χ^2 =18, p=0.00002) (Table 2.2). The percentage of patients with markers of HBV was higher amongst those treated in each month after June 1997 than amongst those treated before this date (Figure 2.3). Analysis by period of visit, taking account of multiple visits, demonstrated a significant variation in the estimated probability of infection per visit by week of visit (p=0.011), which increased over the study period.

Table 2.2. **Frequency of serological markers of HBV infection in patients with a known number of autohaemotherapy treatments.**

Treatments received	Number of patients	Active infection	Recently resolved infection	Past resolved infection	Serological markers of HBV infection
1-4*	194	6	3	12	21 (11%)
5-9	84	12	1	4	17 (20%)
10-14*	27	9	1	1	11 (41%)
15-29	10	3	1	0	4 (40%)
Total	315	30	6	17	53

$\chi^2 = 18$, p=0.00002

Numbers in parenthesis represents percentage of patients with serological markers of HBV infection, according to number of treatments received.

* refers to the inclusion of a single patient in each group with unlinked active infection.

Figure 2.3.

The proportion of patients attending during each calendar month who were
subsequently found to have serological markers of HBV infection.
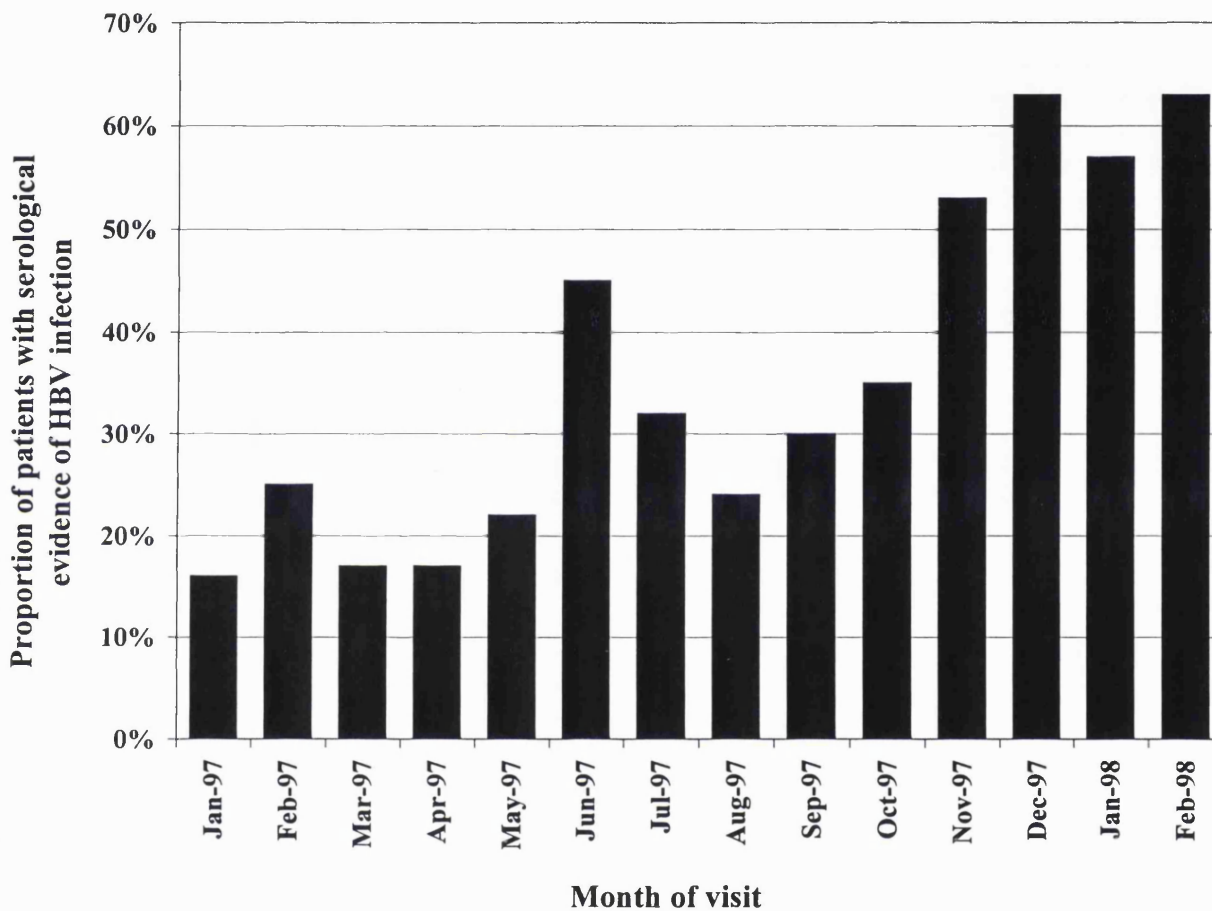
NB. Patients may have attended in more than one month.

2.3.6. Source and mechanism of HBV transmission

Except for the 23 patients with acute clinical hepatitis in whom the onset of clinical disease was known (Figure 2.4), no other cases of acute clinical hepatitis B were reported in clinic attendees over the investigation period. It was considered likely that one of the patients with chronic hepatitis B and phylogenetically linked infection was the original source. A number of factors implicated HBV transmission from patient 16, the 79 year old man referred to earlier, who only attended the clinic in June 1997, as the source of the outbreak: 1) No patient completing treatment in the five months before he attended in June 1997 was shown to be infected with the common source virus, or to have had a recent history of clinical hepatitis. 2) All patients with linked active infection, or recently resolved infection, received autohaemotherapy at least once after he had attended. 3) Assuming a maximum incubation period for hepatitis B of six months, none of the patients with known onset dates for clinical hepatitis had a predicted earliest date of infection before June 1997. 4) The hepatitis B virus characterised in this outbreak belonged to genotype D, which circulates widely in various geographical regions (Lindh et al., 1997), including India, from where he originated. 5) The patient had chronic hepatitis B, with a high level of circulating HBV DNA. Although transmission from this patient cannot be proven, we believe that transmission from the other patients with chronic hepatitis B was less likely. Patient 18 had received treatment in January 1997 and again in January 1998 (figure 2.4). As no cases of clinical hepatitis occurred for more than 12 months after this first date of treatment, it was thought more likely that she became infected in January 1998. She was not tested until June 1998, when she was anti-HBc IgM-negative. The three remaining patients with chronic hepatitis B (patients 6,11, and 22) were anti-HBc IgM-positive at initial testing in March 1998, suggesting recent infection. They all began treatment after patient 16 completed attending, and so were not considered the likely source of the outbreak.

Review of the technique used by the clinic staff, and detailed reporting from patients, revealed that new needles and syringes were used for each patient. Although the needle used for venepuncture was usually changed for a new one prior to putting it into a multi-use

bottle, the saline was always drawn directly into the syringe containing the patient's blood. This

500ml bottle was refilled from a central source of normal saline. Although the bottle was seen by inspectors from Barnet Health Authority on their initial visit to the clinic, it was not available for analysis when they returned.

Figure 2.4: Month of onset of acute clinical hepatitis B, and earliest predicted month of infection in 23 clinic patients and staff with linked infection.
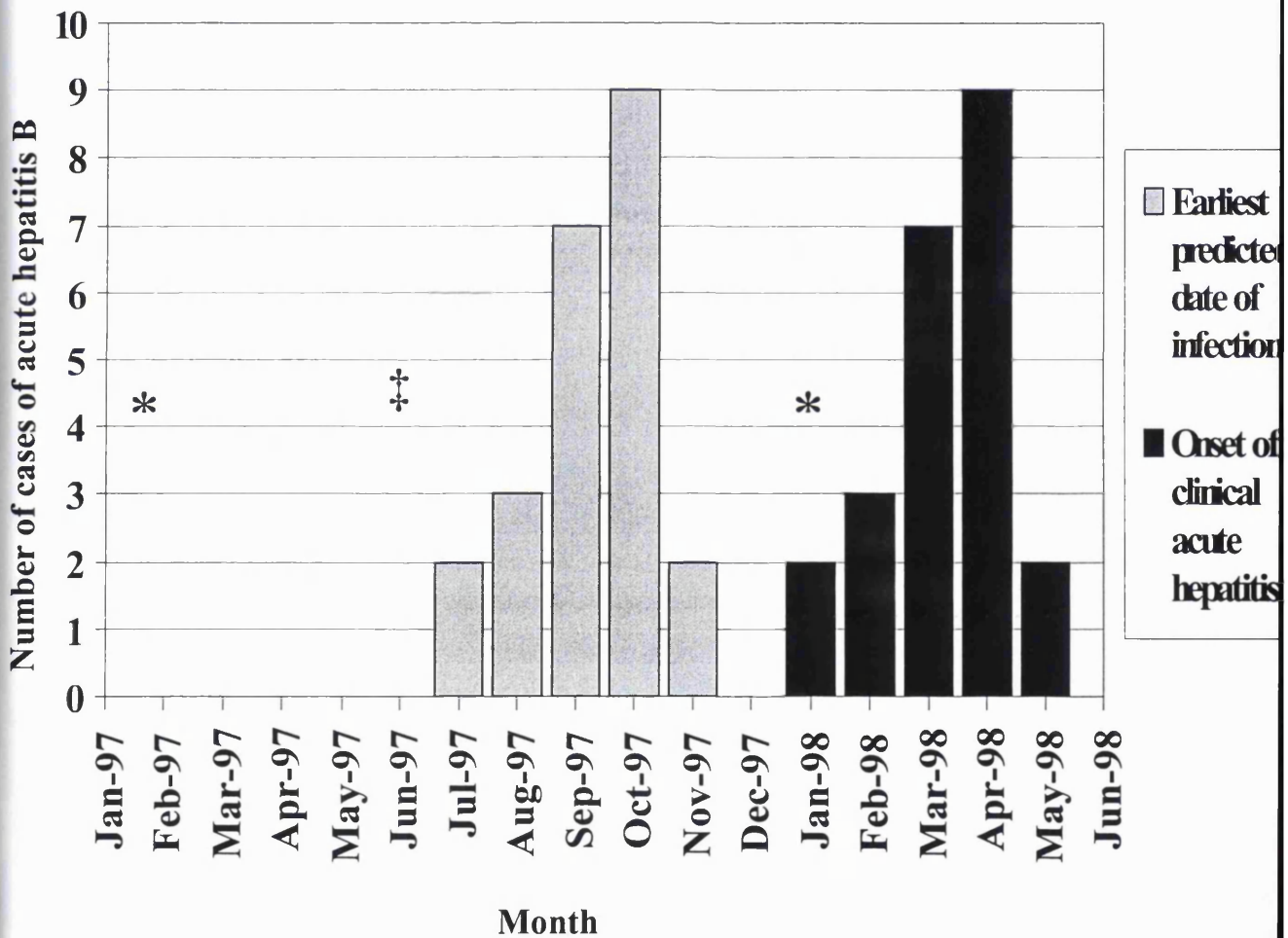
Earliest possible month of infection is shown, assuming maximum six month duration between

hepatitis B infection and acute clinical disease. Dates of autohaemotherapy of patients 16 (‡) and

8 (∗), two possible sources of HBV transmission, are indicated.

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2.3.7. HCV infection in patients

Five of the 352 patients were anti-HCV antibody positive. The index case was a 54-year old female who was found during blood donation in October 1997 to be anti-HCV antibody positive and HCV RNA positive. She was infected with HCV genotype 2b. She most recently donated blood in January 1997, when she was anti-HCV-negative and HCV RNA negative. Her only risk factor for HCV infection was having received autohaemotherapy in March 1997. Four other patients were known to have chronic hepatitis C before they attended the clinic. Further testing revealed that all four were infected with a different HCV genotype. In view of the low frequency of infection, disparate HCV genotypes, and lack of acute hepatitis C in these patients (apart from the index case), an analysis of trends over time in the probability of HCV infection was not performed.

2.3.8. Secondary transmission of HBV

Fifty household or sexual contacts of patients exposed to HBV were identified and tested. Twenty-four contacts of patients with active hepatitis B, who had no markers of previous HBV infection, were vaccinated. The sexual contact of one patient, who had been lost to follow up, developed acute icteric hepatitis B. HBV genetic analysis linked the infection to her partner.

2.4. Discussion

In this report I have described, to the best of our knowledge, the largest community-based outbreak of hepatitis B involving cases definitively linked to a single HBV variant. The findings strongly implicate autohaemotherapy as having facilitated viral transmission.

For many years skin piercing procedures have been recognised as being important causes of parenteral hepatitis virus transmission. Although a clear mechanism of transmission may often be difficult to establish, infection through this route may broadly be attributed to either the injection of products previously contaminated with virus in the donor (eg blood, immunoglobulin preparations), or due to the use inadequately sterilised, repeatedly reused, materials (eg needles). In a seminal epidemiological study of an outbreak of icteric hepatitis amongst American servicemen, Seeff et al attributed the infection of up to 330,000 servicemen in 1942 to the use of batches of yellow fever vaccine contaminated with hepatitis B (Seeff et al., 1987). The infection of more than 100 Irish women with HCV during pregnancy in 1977 arose from the use of aliquots of anti-D immunoglobulin derived from an individual with previous non-A-non-B acute icteric hepatitis (Power et al., 1995). The transfusion of blood products derived from donors infected with hepatitis viruses is, of course, the explanation for the extremely high rate of HCV infection in persons with haemophilia, who received pooled factor VIII concentrates prior to 1985 (Lee, 1995). Since the availability and introduction of procedures to screen blood-product donors, in 1970 for HBV, and 1990-1 for HCV, outbreaks of hepatitis B and C due to the use of products inadvertently derived from infected individuals have become less frequent in the developed world, although they continue to be reported (Healey et al., 1996). The transmission of viral hepatitis through the contamination of inadequately sterilised, repeatedly reused, equipment, is likely to have been an important mechanism of iatrogenic infection in the past. It has been suggested that the 15% prevalence of chronic HCV infection in Egypt may be significantly due to the mass treatment of children with parenteral anti-schistosomiasis therapy (PAT), using repeatedly reused needles for injections (Frank et al., 2000).

The introduction of infection control measures, and the use of disposable equipment, including needles, has led to a reduction in the number of reported outbreaks attributed to skin piercing procedures within 'conventional' medical settings. Nevertheless, nosocomial transmission of HBV continues to be reported, including infection following leakage of infected material into a cryopreservation tank (Tedder et al., 1995), contamination of reusable fingerstick blood sampling devices (Centers for Disease Control and Prevention, 1997); (Douvin et al., 1990) and haemodialysis equipment (Roll et al., 1995), and an outbreak linked to the use of intravenous cannulae in healthy volunteers taking part in a drug trial (Vickers et al., 1994). These examples, and the extraordinary case of a Spanish anaesthetist who infected more than 200 patients with HCV through giving himself part of the patient's post-operative opiate analgesia prior to administering the rest to the patient through the same syringe (Bosch, 2000), emphasises the potential risks wherever skin piercing procedures are performed. Inadequate awareness of risks, and low availability of disposable equipment, continues to result in outbreaks of hepatitis B occurring following medical treatment (Singh et al., 1998). An epidemiological study of 54 cases of acute hepatitis B (with an associated 33% mortality) in a community in rural India strongly linked the use of 'unnecessary therapeutic injections' to infection (Singh et al., 2000). Overall it is estimated that in less developed countries 50% of injections are unsafe (Simonsen et al., 1999), and that this may be responsible for more than 8 million new cases of hepatitis B per year, worldwide (Kane et al., 1999).

Over the last twenty years there has been a huge increase in the number of people in the USA and Europe who consult alternative therapists in addition to, or instead of, seeking conventional medical help (Eisenberg et al., 1993; Thomas et al., 1991). There has been debate recently concerning the validity of some alternative therapies, and the need for greater critical analysis of their efficacy and safety (Angell and Kassirer, 1998). Some of these procedures involve skin piercing, and so carry a risk of parenteral virus transmission. Outbreaks of hepatitis B due to acupuncture have been widely reported from all parts of the World (Stryker et al., 1986; Boxall, 1978; Slater et al., 1988). In an elegant report of an outbreak linked to an acupuncture clinic, Kent et al demonstrated a clear relationship

between the number of treatments received, number of needles used (>450 needles carrying 33% risk of infection), date of attendance, and even the time of day of treatment (Kent et al., 1988). Poor safety procedures and repeatedly reusing needles was thought to have been a major factor in the outbreak. Although vertical transmission perinatally is undoubtedly the major cause of the high prevalence of HBV infection in China, it has been argued that acupuncture may make a significant contribution (Conn, 1988).

Autohaemotherapy, which is also known as ozone therapy and ozonotherapy, is available in clinics world-wide, and seems to have originated from Central Europe in the 1950s (Bocci, 1994). The reinjection of the patient's own blood, often following oxygenation or ultraviolet irradiation, is thought by its proponents to exert immunomodulatory effects (Klemparskaya et al., 1986). It is offered for the treatment of a wide range of ailments, including allergies, malignancy, viral hepatitis, and herpes zoster (Bocci, 1998a; Olwin et al., 1997), and is claimed to be free of side-effects (Bocci, 1998b).

The point source of infection in this outbreak could not be unequivocally identified. The observation that all the patients with linked acute hepatitis B had received treatment during the latter months of 1997 may be biased because blood sampling was performed in early 1998, when recently infected patients would be more likely to be HBsAg-positive than those infected earlier in the year. However, the finding that all cases of symptomatic acute hepatitis B in this outbreak developed clinical disease after December 1997, and that the proportion of patients with markers of HBV infection increased in the last six months of that year, strongly suggests that this outbreak began during the middle of 1997. As patient 16, who had chronic hepatitis B, only attended in June 1997, the epidemiological data suggests that virus transmission occurred from that time. The possibility that he received infection at this time from another patient with asymptomatic acute or chronic hepatitis B, who was not identified in the outbreak investigation, cannot be excluded.

The source of infection in previous health care-related outbreaks has often been attributed to practitioners infected with HBV (The Incident Investigation Teams, 1997). However, although the staff in the present outbreak were infected, they presented with clinical acute

hepatitis several weeks after the first case of acute hepatitis was identified, and had themselves also received autohaemotherapy. It is of interest that infection of the practitioner during an outbreak of hepatitis B associated with poor needle hygiene has previously been reported. In that case the acupuncturist was observed to have wiped the needles between his fingers, and to have checked their sharpness by sticking the points into his finger pulp (Kent et al., 1988).

This is not the first report to highlight the risks of blood-borne infection from autohaemotherapy, as cases of HCV and HIV infection have been reported from Germany, and were attributed to viral contamination of inadequately sterilised and repeatedly reused equipment (Daschner, 1997; Gabriel et al., 1996). The infection control procedures that were carried out in the clinic at the centre of this outbreak appeared sub-optimal. However, even if new syringes and needles were used for each patient, with correct safety procedures followed from this point, the fact that viral contamination appeared to have occurred into the repeatedly reused bottle of saline meant that viral transmission was likely. Transmission of HBV through the use of multi-dose containers has been previously described, in association with a haemodialysis unit (Alter et al., 1983) and due to jet injections in a weight reduction clinic (Canter et al., 1990). Multi-use hypodermic equipment has been implicated as a possibly important cause of HIV transmission in Russia (Seale and Medvedev, 1987), and concerns about parenteral virus transmission have led to calls for the removal of multi-use vials altogether (Mortimer, 1999). Since patients infected at this clinic had received treatment up to 16 weeks apart, it is difficult to determine if transmission occurred from viable virus that persisted in the bottle from initial inoculation, or whether repeated contamination of the container occurred from the pool of reattending infected patients. Although DNA viruses, such as HBV, are believed to be more robust *ex-vivo* than RNA viruses, we do not know whether HBV would remain viable in a saline solution for this length of time.

In previous outbreaks of hepatitis related to skin piercing procedures, cases have been linked on the basis of strong epidemiological data, a history of clinical hepatitis, and serological markers of recent infection (Boxall, 1978; Stryker et al., 1986). In the study by

Kent, HBsAg subtyping by radioimmunoassay was used to provide further evidence for the relatedness of infections (Kent et al., 1988), but this technique is not sufficiently sensitive to provide definitive evidence of a common source. However, HBV sequence amplification followed by phylogenetic analysis, as used in this outbreak, is an extremely powerful tool to definitively link infections to a common source (Yusof et al., 1994; Tedder et al., 1995; Hutin et al., 1999).

The documentation of HCV seroconversion, in a patient with no other apparent risk factors, suggests that HCV was transmitted through a similar mechanism as HBV. The finding of many fewer cases of hepatitis C than hepatitis B is of interest, but remains unexplained. HCV is less easily spread during parenteral health care procedures than HBV (Petrosillo et al., 1995), and the low rate of hepatitis C in this outbreak may be a reflection of the relatively small size of inoculum, or of the poor *ex vivo* viability of HCV. It is also of note that the patient who was thought to be the source of HBV (patient 16) was not infected with HCV. Nevertheless, HCV transmission through autohaemotherapy has been described in the past (Gabriel et al., 1996).

The diligence of the public health physician involved in identifying the index cases in this outbreak probably led to many fewer individuals becoming infected than might have been the case. Particular attention was paid to the development of acute infection in a white middle aged housewife, who did not 'fit the picture' of those at apparent risk of HBV infection. Hepatitis B vaccination has been introduced into the immunization programmes of more than 85 countries worldwide, and is a component of universal infant immunization in many countries, including the USA and Italy (Zuckerman and Zuckerman, 1996). In regions with a high prevalence of hepatitis B, such as Alaska, introduction of a universal vaccination policy over the last 15 years has led to significant reductions in the incidence of new infections (Harpaz et al., 2000; Wainwright et al., 1997). In the UK, which has a low prevalence of infection, hepatitis B vaccination is not universally recommended, but is targeted towards those at perceived risk, including health care workers, intravenous drug users, individuals with multiple sexual partners, men who have sex with men, contacts of patients with known HBV infection, and infants born to mothers carrying the infection.

This report emphasises that HBV infection poses a continuing risk in non-endemic countries where universal vaccination has not been instituted. The findings are a reminder to patients and health practitioners to be aware of the hazards of blood borne virus transmission, and the need for aseptic practice during all skin-piercing procedures. In the absence of the strict regulations that are applied to conventional medicine, further outbreaks of this nature may continue to be associated with this type of alternative therapy.

CHAPTER 3

INCUBATION PHASE OF ACUTE HEPATITIS B IN MAN: DYNAMICS OF CELLULAR IMMUNE MECHANISMS.

3.1. Introduction.

There has been increasing focus over the last few years on comparing host immune responses during acute viral infection, which are often associated with effective control of replication, with those found in persistent infection. Studies in the lymphocytic choriomeningitis virus (LCMV) mouse model (Klenerman and Zinkernagel, 1997), and in patients with recent symptomatic acute infection with HCV (Lechner et al., 2000a), HIV (Goulder et al., 2001) and HBV (Maini et al., 1999) have shown that early antiviral cellular events strongly influence the subsequent course and outcome of infection. From a clinical perspective it appears likely that the early immune responses to HBV are crucial, in that icteric hepatitis virtually guarantees clearance of HBsAg from the serum (Seeff et al., 1987), but asymptomatic HBsAg carriage six months after infection is associated with a high likelihood of long-term infection.

Most studies of acute viral infection in humans have been performed in subjects after the onset of clinically apparent disease, which may develop a variable period of time after initial infection. This incubation period is generally long following infection with non-cytopathic viruses such as HBV (Chisari and Ferrari, 1995a) (hence its initial moniker of 'long-incubation hepatitis' (Sherlock, 1972)), in contrast to other viral diseases (Koup et al., 1994; Yasutomi et al., 1993; Musey et al., 1997). The development of symptoms, which may occur up to 6 months after HBV infection (Zuckerman and Zuckerman, 1996), has been assumed to mark the onset of viral control by the immune system (Chisari and Ferrari, 1995a). Recent studies using animal models of HBV infection have demonstrated that the incubation period may not simply represent a period of unchecked viral replication, but

may be associated with important immune mechanisms. In the livers of chimpanzees acutely infected with HBV (Guidotti et al., 1999a), and the serum during woodchuck hepatitis virus (WHV) infection (Guo et al., 2000), marked reductions in DNA concentrations have been shown to occur prior to the onset of liver injury or significant T cell influx into the liver (Guidotti et al., 1999a). The observation in the transgenic mouse model of HBV infection that viral clearance by HBV-specific CD8 cells may occur in the absence of cytolysis or significant liver injury (Guidotti et al., 1996b), as discussed in the introductory chapter, lends further support to the argument that immunologic control of HBV might occur prior to symptomatic disease in HBV infection. This has been very difficult to test in humans, as patients usually present only after the development of jaundice. Thus, a detailed analysis of the relationship between viral dynamics and liver injury, and whether the incubation phase of HBV infection in humans represents a period of ongoing viral replication in the presence of a deficient immune response, has not been performed. Nevertheless, in one of the very few studies of patients identified in the pre-clinical phase of HBV infection, cellular responses to pre-S antigens and HBcAg have been demonstrated up to two months prior to the onset of liver damage (Vento et al., 1987).

In the outbreak of hepatitis B described above (Webster et al., 2000a), a number of patients were identified prior to the onset of clinical hepatitis. In a prospective study of these subjects, and using recently developed techniques to directly quantify virus-specific lymphocytes, including tetrameric complexes (Ogg and McMichael, 1998; Schauer et al., 1996), there was a unique opportunity to study the interaction between HBV, clinical disease, and host immune responses from the incubation phase of acute infection.

3.2. Methods.

3.2.1 Patients

Seven patients (all female, mean age 54 years, range 37-71) were identified during the incubation phase of acute hepatitis B, and were longitudinally followed through the course of the disease. All had been contacted as part of the outbreak look back investigation, had received autohaemotherapy within the preceding 12 months, and found to be HBsAg on testing by PHLS. After receiving notification that they were infected with hepatitis B, all were seen within 3 days at the Royal Free Hospital. Infection within the incubation phase was confirmed by the findings of positive HBsAg, HBV DNA by PCR, normal or minimally raised serum alanine transaminase (ALT), a marker of hepatocyte damage (Feutren et al., 1984), and no symptoms of clinical acute hepatitis, in particular jaundice. At around the time of referral each patient was confirmed by Rachel Hallett at PHLS to be infected with the same variant of HBV as others in the outbreak, as assessed by viral genome sequencing and phylogenetic analysis (Webster et al., 2000a), and all were anti-hepatitis C and D antibody negative. Dates of receipt of autohaemotherapy were known from clinic records and a patient questionnaire, allowing the minimum duration of infection to be determined. Baseline characteristics of these patients are shown in Table 3.1.

Table 3.1. **Baseline characteristics of patients identified in pre-clinical phase of acute hepatitis B virus infection and studied longitudinally.**

Pt ID	Age	Date	Wks after last Tx.	HB sAg	HB eAg	Anti-HBc IgM	Anti-HBe	HBV DNA pg/ml	Bilirub. umol /l	ALT IU/L	Subsequent jaundice
1	45	3.3.98	10	+	-	-	-	2577	4	80	Yes
2	37	3.4.98	8	+	+	+	-	3927	14	499	Yes
3	63	22.3.98	7	+	+	-	-	499	6	17	Yes
4	52	23.3.98	7	+	-	-	-	26	8	18	Yes
5	71	27.3.98	15	+	+	-	-	7140	4	59	No
6	38	8.4.98	12	+	+	+	-	832	23	2022	Yes
7	53	16.3.98	8	+	-	-	-	174	12	16	No

Four of the patients (patients 1-4) were recruited 4-7 weeks before the onset of classical features of acute hepatitis B, which included jaundice, positive IgM anti-hepatitis B core (IgM anti-HBc) antibody, and serum ALT concentrations elevated more than 10 times above the upper limit of normal (Figure 3.1). Patient 6 was included in the incubation phase analysis because she was asymptomatic at presentation, although her serum ALT was already significantly raised (2022 U/L) at that time, and she developed marked clinical jaundice within 2 weeks of this assessment. In these cases clinical features of acute infection started 7-10 weeks after the most recent possible time-point of infection. In each, the characteristic serological pattern of acute hepatitis B, culminating in clearance of

circulating HBsAg, and the development of anti-hepatitis B surface (anti-HBs) antibodies, was seen. It should be noted that IgM anti-HBc, the usual marker of acute hepatitis B, was initially negative in the 3 patients who were identified prior to the peak of HBV DNA (patients 1,3,4). Serum ALT levels were virtually normal in 3 patients (patient 1,3,4) and elevated in patient 2 and 6, at the time of initial sampling, and reached their peak value after 3-5 weeks of observation, at the same time as (patient 4) or 1-2 weeks before the onset of jaundice (patients 1,2,3). Patient 7 was also included, as she was identified in the incubation phase, and cleared HBsAg over the subsequent 3 months. However, despite a rise in ALT, she did not develop symptomatic hepatitis or jaundice.

One 71 year old patient (patient 5), who was infected with the identical HBV variant as the others, and on the initial sample was HBsAg and IgM anti-HBc-positive, with normal ALT, at least 15 weeks after infection, did not subsequently develop an increase in ALT, or clear HBsAg. HBV DNA levels persisted at high level (around 7000 pg/ml). She was taking systemic corticosteroid therapy for a chronic pulmonary disease prior to, and during, HBV infection.

During the incubation and clinical acute phases of infection, clinical assessment and blood sampling was performed every 1-4 weeks, following informed patient consent. After resolution of acute disease monthly assessments were carried out. At each time-point 50ml of blood was drawn from an antecubital fossa vein for a biochemical screen (including serum ALT), HBV serology and HBV DNA analysis, and isolation of peripheral blood mononuclear cells (PBMCs), as described below.

3.2.2. Virological assessment

HBsAg, anti-HBc IgM, HBeAg, and anti-HBs were determined by commercially available enzyme immunoassay kits (Hepanostika Uni-Form II, Organon Teknika, Boxtel, Netherlands; Murex Biotech Ltd, Dartford, England; IMX Abbott GmbH Diagnostika, Wiesbaden-Delkenheim, Germany). The presence of HBV DNA in the serum was

confirmed by in-house PCR, and quantified using the Roche AMPLICOR Monitor assay (Roche Diagnostics, NJ), with a DNA detection threshold of 400 copies/ml (0.0014 pg/ml).

3.2.3. Isolation and storage of PBMCs.

Approximately 30ml of whole blood was drawn, mixed with 2000 units of unfractionated heparin, and carefully layered over Ficoll-hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden), at a volume ratio of 2:1, in a 50ml centrifuge tube (Nunc Brand Products, Roskilde, Denmark). Gradient centrifugation was performed by spinning at 2000rpm for 20 minutes, without a brake, using a Heraeus Megafuge 2.0 R centrifuge (Kendro Laboratory Products, Hanau, Germany). The cellular suspension was removed using a sterile 1ml transfer pipette, transferred to a 15 ml centrifuge tube (Nunc Brand Products), and washed twice in 10ml of RPMI 1640 (Autogen-bioclear, Calne, UK) + 10% foetal calf serum (FCS) (Life Technologies, Grand Island, USA). Cells were then counted using a $0.0025mm^2$ haemocytometer (Weber Scientific International Ltd, Middlesex, UK), and either studied immediately in the relevant medium, as discussed below, or frozen. For freezing, 10×10^6 cells were suspended in 1 ml vials of a freezing solution made up of 50% RPMI 1640, 40% FCS, and 10% Dimethyl Sulfoxide (DMSO) (Sigma Chemical Co., St Louis, MO). The cells were then immediately placed in a -80^0C freezer for 48 hours, in a NALGENE™ freezing container, aimed at achieving a -1^0C/min rate of cooling. After 48 hours the vials were transferred into a liquid N_2 container, until required for further analysis.

3.2.4. Phenotypic analysis of peripheral blood mononuclear cells

CD8+ cells were identified by incubating 0.3×10^6 PBMC at 4^0C for 30 minutes with saturating concentrations of directly conjugated anti-CD8/cychrome monoclonal (mAb) (Sigma Chemical Co.), and their activation status was measured by subsequent staining with one of a number of FITC conjugated antibodies, including anti-HLA-DR, anti-CD28, and anti-CD38 (Pharmingen, San Diego, CA). Cells were washed twice in PBS (Sigma

Chemical Co.) + 1% FCS, and then analysed immediately on a FACScan® using CellQuest® software (Becton Dickinson, San Jose, CA). NK cells were stained using anti-CD7 and anti-CD16 mAb (NK+ cells = CD7+/CD16+), and identified using the same technique.

3.2.5. Screening for the HLA-A2 haplotype

As the antigen-specific CTL response is HLA class I restricted, quantification of HBV-specific CD8+ cells using tetramers could only be performed in HLA-A2+ subjects, in whom relevant CTL epitopes have been previously defined. 100ul of blood, freshly drawn into a tube containing EDTA, was added to 100ul PBS + 1% FCS in a 5ml FACS tube. 1ul of mouse anti-human HLA-A2 antibody (Incstar, Stillwater, MI.) at a final concentration of 1/200, was added, and incubated at 4^0C for 25 mins without light exposure. After washing (involving the addition of 2ml PBS, centrifugation at 1500 rpm for 5 mins, and removal of supernatant), 1ul of fluorescein isothiocynate (FITC)-conjugated goat anti-mouse IgG (Sigma Chemical Co.), at a final concentration of 1/256, was added to the 200ul cellular suspension. Cells were then incubated at 4^0C for 25 mins without light exposure. 2ml of FACS™ lysing solution (Becton Dickinson) (diluted 1/10) was added to the cellular suspension, and incubated at room temperature in the dark, for 15 mins to lyse the erythrocytes. The cells were then washed 3 times, resuspended in 100ul of cold PBS + 1% FCS, and analysed for HLA-A2 positivity on FACScan®. The 3 HLA-A2+ subjects in this study (patients 1, 2, 5) were subsequently confirmed to have the HLA-A2.01 allele by PCR DNA typing (performed in the Antony Nolan Centre, Royal Free Hospital).

3.2.6. Synthetic peptides and HBV antigens

Peptides corresponding to the sequence of the core 18-27 (FLPSDFFPSV), envelope 335-343 (WLSLLVPFV), and polymerase 575-583 (FLLSLGIHL) region of HBV genotype D were purchased from Chiron Mimotopes (Clayton, Victoria, Australia). Of note, infection in this linked outbreak was due to genotype D infection, and the patients undergoing detailed CTL analysis (patients 1, 2, 5), were all shown by Rachel Hallett at PHLS to have dominant wild type sequences in core 18-27, envelope 335-343 and polymerase 575-583. Recombinant hepatitis B core antigen (HBcAg; Sorin Biomedica, Saluggia, Italy) was obtained from bacterial extracts of *Escherichia coli* K12 strain HB 101, harbouring the recombinant plasmid carrying the HBcAg coding gene, as described previously (Pasek et al., 1979). Purity was 90%, as previously reported (Ferrari et al., 1991). Recombinant yeast derived preparation of HBsAg (Amgen, Thousand Oaks, CA), containing the S/p25 protein was used. Purity was higher than 90%.

3.2.7. HBV-specific CD8+ cell enumeration.

Quantification of HBV-specific CD8+ cells was performed, in HLA-A2-positive patients, using HLA-A2/HBV peptide tetrameric complexes, as previously described (Ogg and McMichael, 1998). The tetramers were synthesised by Graeme Ogg at the Institute of Molecular Medicine at Oxford, where the technology has been developed (Ogg and McMichael, 1998; Altman et al., 1996; Callan et al., 1998). In brief, recombinant HLA-A2 heavy chains and β2-microglobulin were produced by E coli cells transformed with the relevant expression vectors. Only the extracellular domain of the heavy chain was expressed. Complexes were folded in vitro using 30mg of HLA-A2 heavy-chain protein, 25mg of β2-microglobulin, and 10mg of synthetic peptide. Wild-type sequences of HBV (genotype D) peptides core 18-27, polymerase 575-583, and envelope 335-343 were used. The HLA-A2/peptide complexes were biotinylated, and tetramers were generated by mixing the biotinylated protein complex with streptavidin-phycoerythrin at a molar ratio of 4:1 (See also Figure 1.3). PBMCs were incubated for 30 minutes at 37°C with 0.5µg of

phycoerythrin (PE)-labelled tetrameric complexes in RPMI 1640 and 10% FCS in 5ml FACS tubes. Cells were washed in PBS, and then incubated with directly conjugated anti-CD8/cychrome mAb (Sigma Chemical Co). Cells were washed twice, and analysed immediately on a FACScan®, using Cell Quest software. Due to variation in the number of cells available for each experiment, the quantity of CD8+ cells that were analysed at each time-point was not constant. However, more than 2×10^4 CD8+ cells were analysed after gating on live lymphocytes. The quantity of HBV-specific CD8+ cells/ml of blood was calculated by multiplying the frequency of tetramer-positive cells by the total CD8+ cell count/ml, determined from the percentage of CD8+ cells within the total lymphocyte count (Figure 3.1). Positive and negative controls for the tetramer staining for each epitope have been previously defined within the study laboratory, using HLA-A2 restricted clones specific for these epitopes, and staining PBMC from HBV-uninfected HLA-A2+ subjects, and acutely HBV infected HLA-A2- individuals. These experiments established the specificity of the tetramers, and a background frequency of non-specific tetramer staining of less than 0.1% of CD8+ cells (Maini et al., 1999).

Figure 3.1. Method applied to the analysis and quantification HBV-specific CD8+ cells using tetramer staining and flow cytometry.

Gated on live lymphocytes (R1)

Autofluorescent 'dead cells'

Gated on live, non-autofluorescent lymphocytes (R1 and R2)

Quad	Events	% Gated	% Total
UL	7	0.02	0.01
UR	378	1.30	0.61
LL	20397	70.38	33.00
LR	8200	28.29	13.27
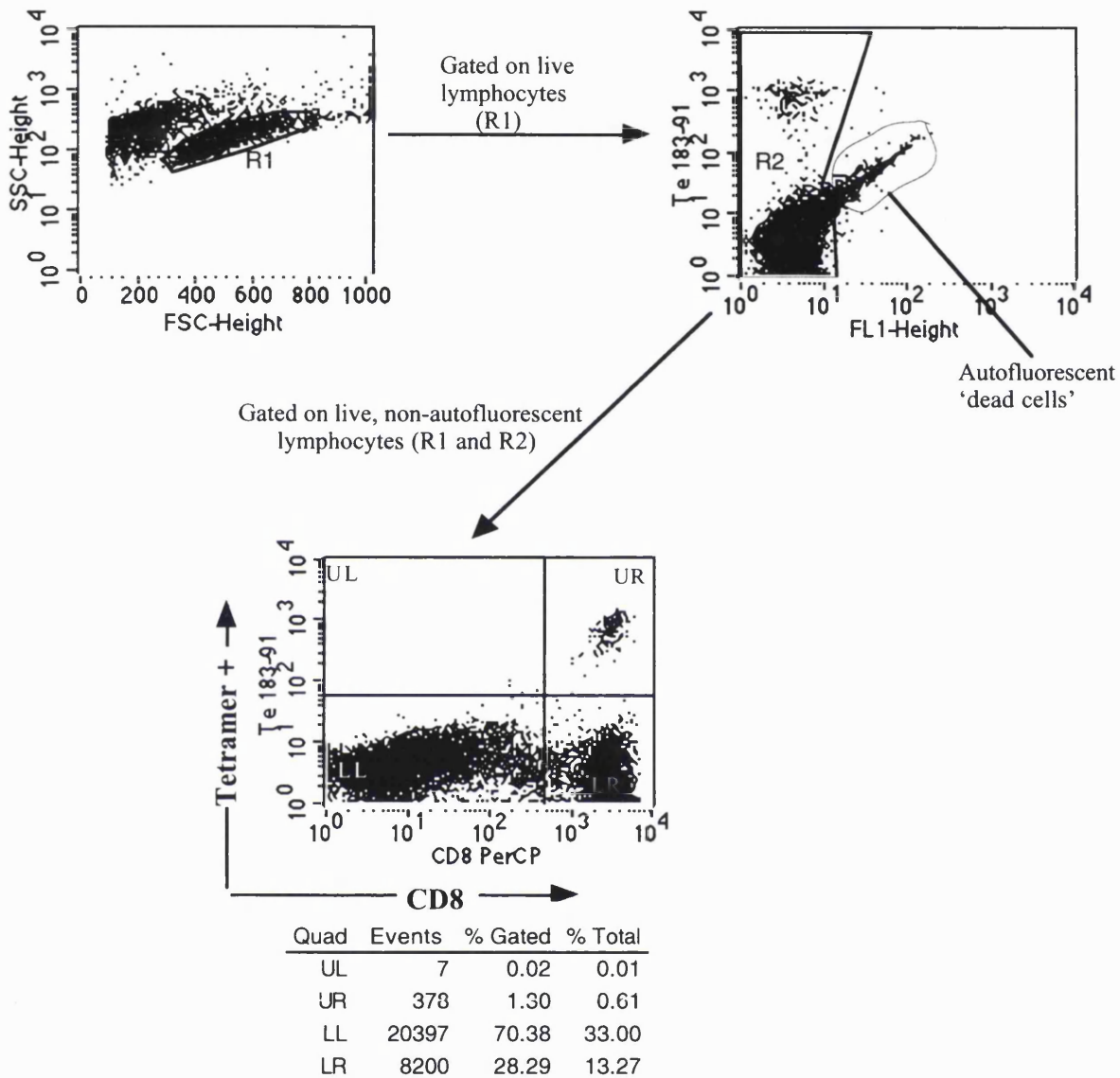
Frequency of HBV-specific
CD8 cells from FACS:

$$\frac{378 \text{ Tet+ CD8 cells}}{378 + 8200 \text{ total CD8 cells}} \times 100 = 4.4\% \text{ Tetramer+ CD8 cells}$$

Concentration of peripheral HBV-specific
CD8 cells in direct ex-vivo experiments:

→ Peripheral lymphocyte count = 1.6×10^6 cells/ml

→ % CD8 cells of total lymphocytes from FACS = $\frac{8200 + 378}{8200 + 378 + 20397 + 7} \times 100 = 29\%$

→ Peripheral CD8 cells = $0.29 \times 1.6 = 0.46 \times 10^6$ cells/ml

→ Peripheral frequency of HBV-specific CD8 cells = $0.46 \times 0.044 = 0.02 \times 10^6$ cells/ml
= 20,000 cells/ml

3.2.8. HBV-specific CD4+ cell enumeration.

PBMCs (0.5×10^6 cells/ml) were suspended in 100μL of RPMI 1640 and 10% human serum in a 96 well plate, and incubated for 15 hours at 37°C with 2μl of a 100μM concentration of HBV core or envelope antigen (Ferrari et al., 1990). 1μg/ml Brefeldin A (Sigma Chemical Co) was added after the first 2 hours. Incubation without antigen, and with 1μg/ml mouse anti-human CD3 mAb (Serotec, Oxford, UK), and 1μg/ml mouse anti-human CD28 Ab (Pharmingen, San Diego, Ca), acted as negative and positive experimental controls, respectively. Cells were then spun at 1500rpm for 5 minutes to wash, resuspended with 1ul anti-CD4 mAb (Pharmingen) in 100ul PBS + 1% FCS, and incubated at 4°C for 20mins. After a further wash, 100ul of Permeafix 1x (Ortho Diagnostic Systems) was added for 20 minutes at 4^0C, to permeabilize and fix cells, followed by 3ul FITC-conjugated anti-IFNγ Ab and its isotype matched control (Pharmingen), in 100ul PBS + 0.05% Saponin (Komanduri, V et al., 1998). After incubating for 30 minutes at 4^0C, cells were washed three times, resuspended in 50ul of PBS, and analysed by flow cytometry. The total number of HBV-specific CD4+ cells/ml was calculated as described for HBV-specific CD8+ cells/ml, using the percentage of CD4+ cells in place of CD8+ cells.

3.3. Results.

3.3.1. Dynamics of virus replication and liver injury

Figure 3.2A shows the dynamics of HBV replication, as measured by HBV DNA concentrations in the serum, and ALT concentrations, for the four patients (patients 1-4) who were clearly identified during the incubation phase of acute HBV infection, had normal or minimally elevated ALT at baseline, and who cleared HBsAg after symptomatic icteric hepatitis. After a peak in viral concentration approximately 8-10 weeks into the incubation period, HBV DNA concentrations fell sharply thereafter. Similar data have been obtained in animal models (Guidotti et al., 1999a; Guo et al., 2000; Kajino et al., 1994). In two of the patients high levels of HBV were seen in association with completely normal ALT levels (see patient 3 week 7 (ALT 17 U/L, HBV DNA 499 pg/ml) and patient 4 week 9 (ALT 23 U/L, HBV DNA 1486 pg/ml), in support of the argument that HBV is not directly cytopathic. Rapid and massive falls in HBV DNA occurred prior to the onset of jaundice, (mean reduction 99.2% (range 98.9-99.9%) from peak level), with most of the reduction occurring prior to peak ALT. High levels of ALT persisted for several weeks after control of HBV, and clearance of HBsAg from the circulation. A different profile of HBV-replication and liver damage was observed in patient 5 (Figure 3.2B), who was immunosuppressed. She had been infected at least 15 weeks previously, and high levels of HBV DNA in the serum persisted for a further 4 weeks, until lamivudine therapy was initiated. ALT concentrations never exceeded 130 U/L, and the patient did not develop symptoms of acute hepatitis or jaundice. Comparison between this immunosuppressed patient and those patients with symptomatic acute hepatitis supports the argument that immune-related events are important in the control of viral replication and seems to rule out the possibility, hypothesised in acute HIV infection (Phillips, 1996), that massive reduction in viral load could result from the saturation or spontaneous death of infected cells.

Figure 3.2. Relationship between viral load and liver injury during the incubation, clinical acute, and recovery phases of HBV infection.

A

HBV-DNA	2577	21955	564	0.83	0.24	0.0			
HBsAg	+	+	+	+	+	+	+	-	-
HBeAg	-	+	+	+	+	+	-	-	-
IgM anti-HBc	-	+	+	+	+	+	+	+	+

	3927	258	96	22	0.37	0.07	0.0	0.0	
	+	+	+	+	+	+	+	-	-
	+	+	+	+	+	-	-	-	-
	+	+	+	+	+	+	+	+	+

	499	7568	119	48	16	0.0	0.0		
	+	+	+	+	+	+	+	-	-
	+	+	+	+	+	-	-	-	-
	-	+	+	+	+	+	+	+	+

	26	2434	114	0.28	0.0	0.0			
	+	+	+	+	+	+	+	+	+	-
	-	+	+	+	+	-	-	-	-
	-	-	+	+	+	+	+	+	+	+

Onset of symptoms

Before After

B   Lamivudine

Before During

HBV-DNA	7140	4998	4890	428	30	32
HBsAg	+	+	+	+	+	+
HBeAg	+	+	+	+	+	+
IgM anti-HBc	+	+	+	+	+	+

- ○ - ALT

▨ HBV-DNA
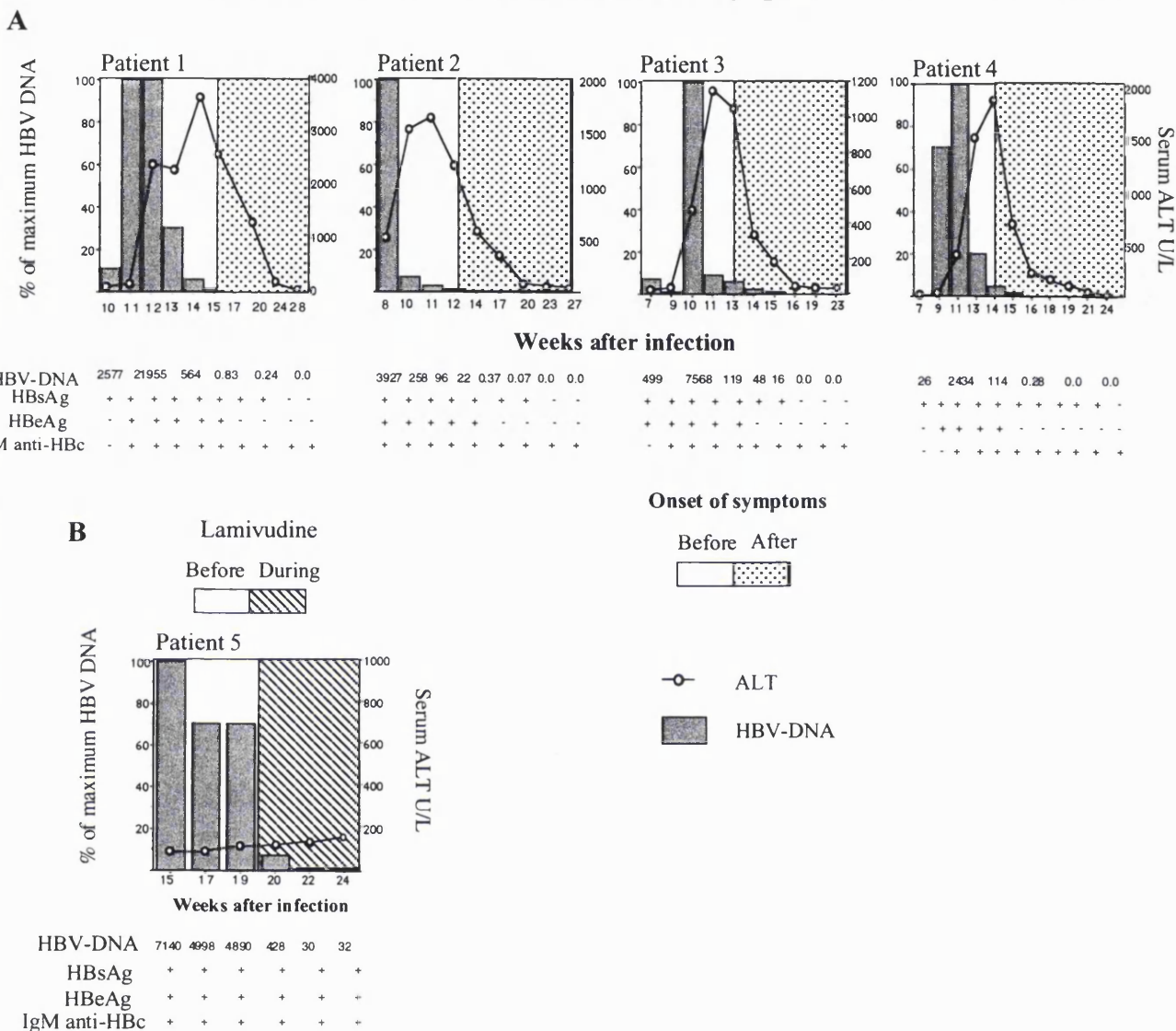
Figure 3.2A displays the results for the 4 patients who were identified with near normal ALT, who subsequently developed acute icteric hepatitis, and controlled infection. The period after the onset of symptoms is shown as the shaded area. Figure 3.2B shows the results for a patient who did not develop acute hepatitis, did not spontaneously control infection, and was commenced, and maintained on lamivudine. HBV DNA is expressed graphically as a percentage of the maximal recorded HBV DNA concentration for each patient. Absolute concentrations of HBV DNA (in pg/ml) and serological markers are displayed below each graph. As the last possible date of infection was known for each patient, the minimum number of weeks since infection is expressed on the horizontal axis.

Figure 3.3 shows the relationship between HBV DNA and maximal liver injury in all the patients who cleared HBsAg, including patient 6, who had a very significantly raised ALT at presentation, and became icteric soon afterwards, and patient 7, who was asymptomatic throughout, and did not develop jaundice (maximal serum bilirubin 23umol/l).

Figure 3.3. Correlation between HBV DNA and peak ALT in the 6 patients identified in the incubation phase who cleared HBsAg acutely.
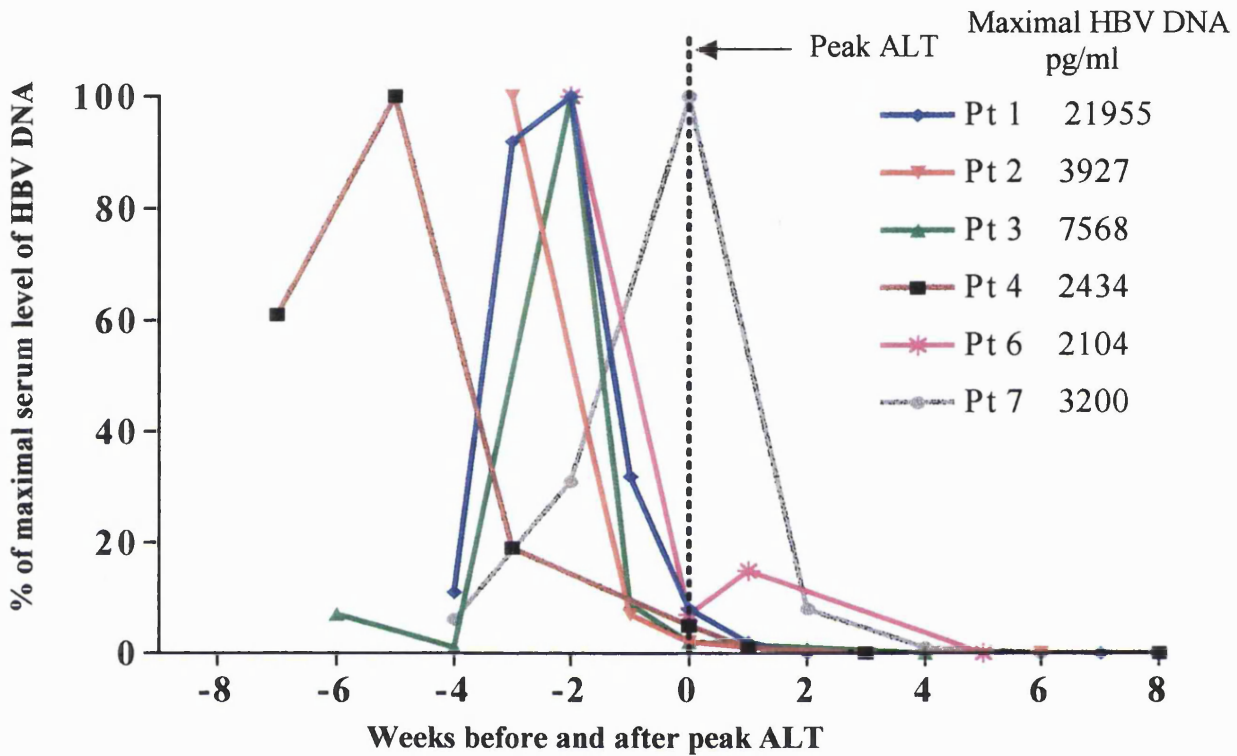
Figure 3.3 displays the dynamic change in serum levels of HBV DNA in the 6 patients who cleared HBV after acute infection, and were identified during the incubation period. Change in serum HBV DNA over time is related to the point of peak ALT for each individual. The graph includes data on patient 6, who had a significantly elevated ALT at initial testing (2022 U/L), but who was asymptomatic at this time, and patient 7, who cleared HBsAg without developing jaundice (maximum bilirubin 23 umol/l). Patients 1-4 were all identified in incubation phase, with near normal ALT, and cleared HBsAg after symptomatic, icteric hepatitis. Jaundice usually began 2 weeks after peak ALT. In view of the considerable range in HBV DNA levels between patients (see right panel), HBV DNA is expressed as a percentage of maximal levels at each time point.

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3.3.2. Dynamics of cellular immune response

Figure 3.4 shows the longitudinal change in circulating NK cells. Consistent with evidence for their role in the early phase of viral infection (Biron et al., 1999c; Welsh et al., 1991; Giavedoni et al., 2000), the highest frequency of circulating NK cells was found at the earliest point in the incubation period in the patients who developed acute hepatitis, and their number decreased in association with a fall in the level of HBV DNA. A similar pattern was seen in the immunosuppressed patient, such that, although she was persistently lymphopaenic, NK cells accounted for more than 30% of circulating mononuclear cells at the earliest time-point.

Figure 3.4. Longitudinal analysis of natural killer (NK) cells following HBV infection, related to viral load and liver injury.
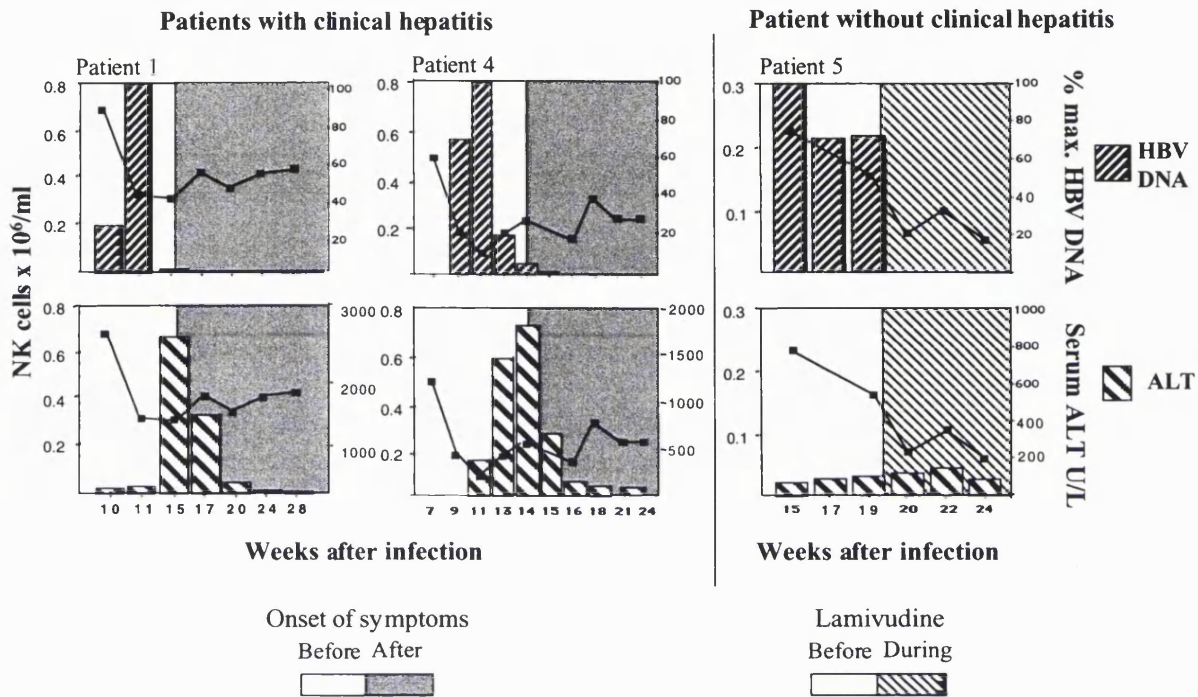
Circulating NK cell frequency (cells x 10^6/ml) over time, related to HBV-DNA level (expressed as a percentage of maximal HBV DNA concentration for each patient) (upper panels), or serum ALT concentrations (lower panels). Results shown for 2 patients who developed acute hepatitis, and in one who did not develop acute hepatitis.

Figure 3.5. Specificity of HLA-A2-restricted HBV peptide tetrameric complexes.

CTL lines specific for:
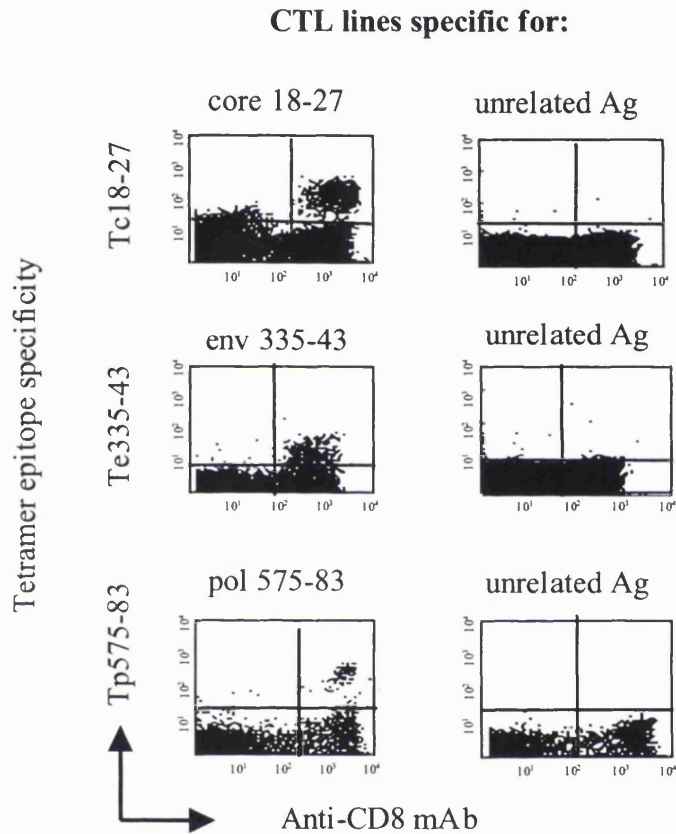
CTL lines with specificity as indicated were stained with HLA-A2 peptide tetramers folded with the core 18-27 (Tc18-27), envelope 335-43 (Te335-43) and polymerase 575-83 (Tp575-83) peptides and anti-CD8 mAb, and analysed by flow cytometry. The specificity of the lines was tested in standard chromium release assays using HLA-A2 matched target cells pulsed or unpulsed with the relevant peptide. Specific lysis of pulsed versus unpulsed targets was 35% for core 18-27 CTL lines, 12% for envelope 335-43 CTL lines, 15% for polymerase 575-83 CTL lines. No specific lysis of HBV peptides was found in lines indicated as unrelated antigen specificity.

Quantification of HBV-specific CD8+ cells.

Circulating HBV-specific CD8+ cells were analysed using HLA-A2 tetramers able to bind to CD8+ cells specific for core 18-27 (Tc 18-27), envelope 335-43 (Te 335-43), and polymerase 575-83 (Tp 575-83) HBV epitopes. The specificity of these tetramers is shown in Figure 3.5 (and previously reported in (Maini et al., 1999)). Although this method was only applicable to HLA-A2 positive patients (patient 1, 2, and 5), it allowed a direct quantification of HBV-specific CD8+ cells. HBV-specific cells were demonstrated in patients 1 and 2 before the onset of symptoms (Figure 3.6). In patient 1, a Tp 575-83-specific CD8+ cell response was the first specificity to be detectable. Since the frequency of Tc 18-27 and Tp 575-83-specific CD8+ cells at this early time-point was barely above the background level present in HLA-A2 negative patients (0.02% of CD8+ cells) (Maini et al., 1999), the specificity of these cells was confirmed by the expansion of Tc 18-27 and Tp 575-83-specific CD8+ cells after peptide-specific stimulation (Figure 3.6C). In patient 2, Tc 18-27-specific CD8+ cells were the only detectable population of HBV-specific CD8+ cells in the pre-clinical phase of disease. Therefore, these data show that at least 4 weeks before the onset of clinically acute hepatitis, and prior to the peak of ALT, HBV-specific CD8+ cells were present in the circulation of patients who subsequently developed acute hepatitis. Tetramer-positive cells continued to increase in the circulation in patients 1 and 2 after marked reduction in serum HBV DNA concentrations had occurred, and reached their highest number at around the time of maximal ALT level and the onset of jaundice in patient 1 (2400 Tc 18-27 CD8+ cells/ml, 1.1 % of CD8+ cells), and after maximal ALT level and the onset of symptoms in patient 2 (2100 Tc 18-27 specific CD8+ cells/ml, 0.6% of CD8+ cells). Circulating HBV-specific CD8+ cells decreased during the recovery phase, in parallel with settling of the biochemical hepatitis, but persisted at a lower frequency (~500-600 Tc 18-27-specific CD8+ cells/ml; 0.2% of CD8+ cells) after complete normalisation of serum ALT and clearance of HBsAg. Except at the first pre-clinical time point in patient 1, Tc 18-27 specific CD8+ cells were the numerically dominant CD8 population, in accordance with recent data in a larger population of acute hepatitis B patients (Maini et al., 1999), whereas in no patient were Te 335-43-specific CD8+ cells clearly demonstrated. HBV-specific CD8+ cells were not demonstrated in the immunosuppressed patient (patient 5), who did not develop acute hepatitis.

Figure 3.6. **Longitudinal analysis of circulating HBV-specific CD8+ cells in relation to HBV-DNA load and serum ALT concentrations.**

A

Weeks	10	11	15	17	20	24
ALT U/L	80	114	2557	1280	171	33
HBV DNA pg/ml	2577	21955	0.83	0.16	0	0
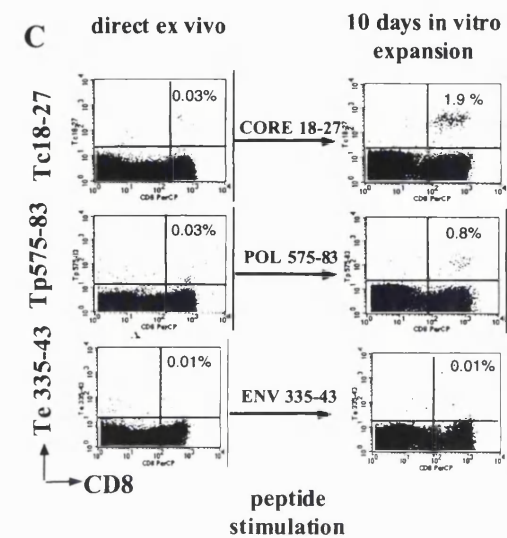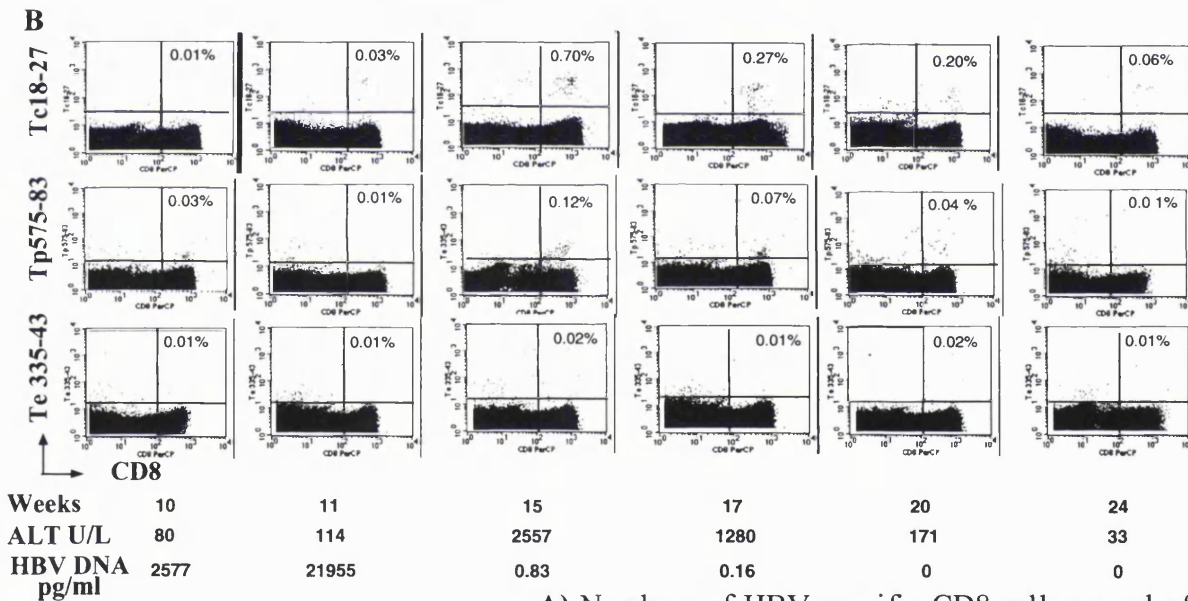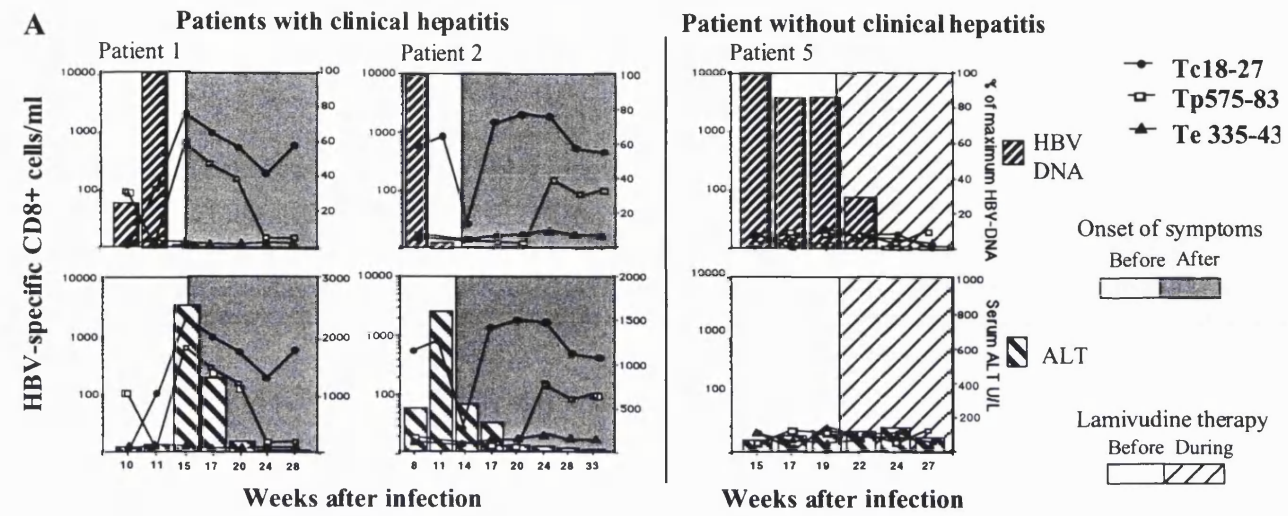
A) Numbers of HBV-specific CD8 cells per ml of blood, related to time after infection, HBV DNA, and ALT.

B) Dot plots of PBMCs of patient 1 stained with HBV tetramers and anti-CD8 Ab. The percentage of tetramer-positive cells is indicated in the upper right quadrant.

C) Expansion of HBV-specific CD8+ cells after peptide specific stimulation in patient 1. PBMCs and T cell lines were stained with tetramers and anti-CD8 Ab directly or after stimulation with the indicated HBV peptides. Experiments shown relate to PBMC from week 11 for core 18-27 and week 10 for pol 575-83 and env 335-43 peptides.

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In all patients identified in the incubation phase, irrespective of their HLA-A2 status, staining of CD8+ cells with a range of antibodies specific for T cell activation markers (including HLA DR and CD38) provided an indication of the dynamic change in the activation of CD8+ cells over the time course of infection (Figure 3.7). This technique, however, could not allow the epitope-specificity of these cells to be assessed. Nevertheless, in a pattern similar to that seen for tetramer staining, CD8+ cell activation was seen to occur during the incubation phase of disease, and to peak at the time of maximal ALT. It is of note that the 2 patients who showed no peak in CD8+ cell activation (patients 5 and 7) were also the only patients not to develop jaundice, although patient 7 did develop a raised ALT and cleared HBsAg.

Figure 3.7. Chronological change in CD8+ cell activation markers in patients identified during incubation phase of acute HBV infection.
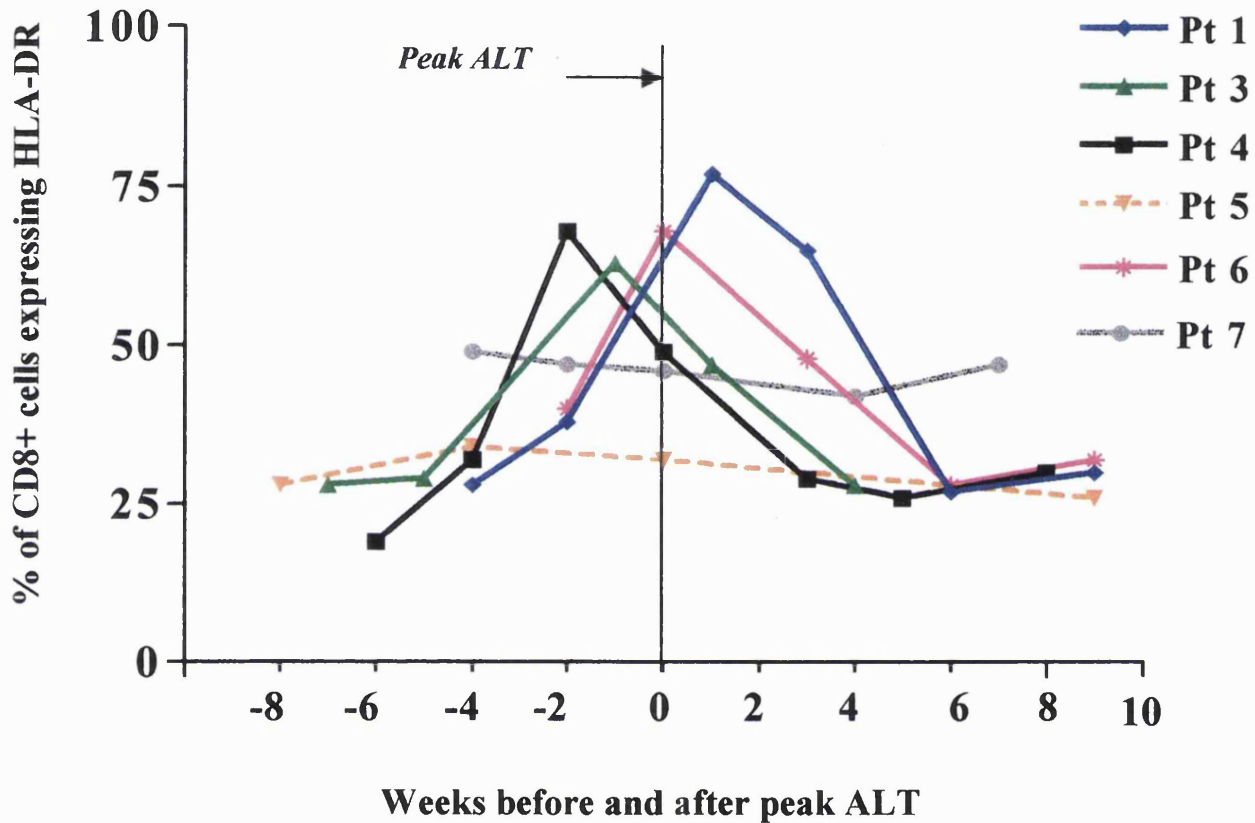
In Figure 3.7, the change over time in the activation status of CD8+ cells is displayed for each individual patient relative to the point of peak ALT. Data on patient 5 relates to the time course of study, not peak ALT, which was not observed, as she developed no acute hepatitis, and failed to clear HBsAg. Patient 7 developed a rise in ALT (peak 1628 U/L), and cleared HBsAg, but did not develop jaundice, unlike patients 1,3,4,6. Insufficient PBMC prevented assessment of activation markers in patient 2.

Quantification of HBV-specific CD4+ cells.

Experiments to directly quantify the number of CD4+ cells specific for nucleocapsid and envelope HBV proteins were performed in patients 1-4, using intracellular cytokine staining. No increase of IFNγ-positive CD4+ cells was detectable in patients 2, 3 and 4 after antigen specific stimulation at any time points, but core-specific CD4+ cells were clearly quantifiable in patient 1 (Figure 3.8). The collated data on the kinetics of the circulating HBV-specific CD4+, CD8+ and NK cells in relation to HBV-DNA level and liver damage in this patient are shown in Figure 3.9. One month before the onset of acute hepatitis, raised levels of NK cells (0.65 x 10^6 cells/ml) and the presence of core-specific CD4+ cells (900 core-specific CD4+ cells /ml) and Tp 575-83-specific CD8+ cells (80 cells/ml) were demonstrated. Serum ALT was minimally elevated (80 U/L), and HBV-DNA level was still increasing at this point. One week later the number of NK cells and core-specific CD4+ cells began decreasing in the circulation, in association with a peaking of HBV-DNA replication. After a further 4 weeks (approximately 15 weeks after infection), at the time of maximal of liver damage (ALT 2557 U/L), core-specific CD4+ cells were still present, but at much lower frequency than was seen earlier in the incubation phase. In contrast, HBV-specific CD8+ cells reached their maximal level at this point, in association with a peak in the total number of activated CD8+ cells in the circulation (figure 4, week 16). The number of core-specific CD4+ and CD8+ cells then fell, reaching a frequency of 50-100 core-specific CD4+ cells/ml and 180-400 Tc 18-27-specific CD8+ cells/ml after clinical resolution of infection.

Figure 3.8. Quantification of HBV-specific CD4+ cells during acute hepatitis B by intracellular cytokine staining and flow cytometric analysis.
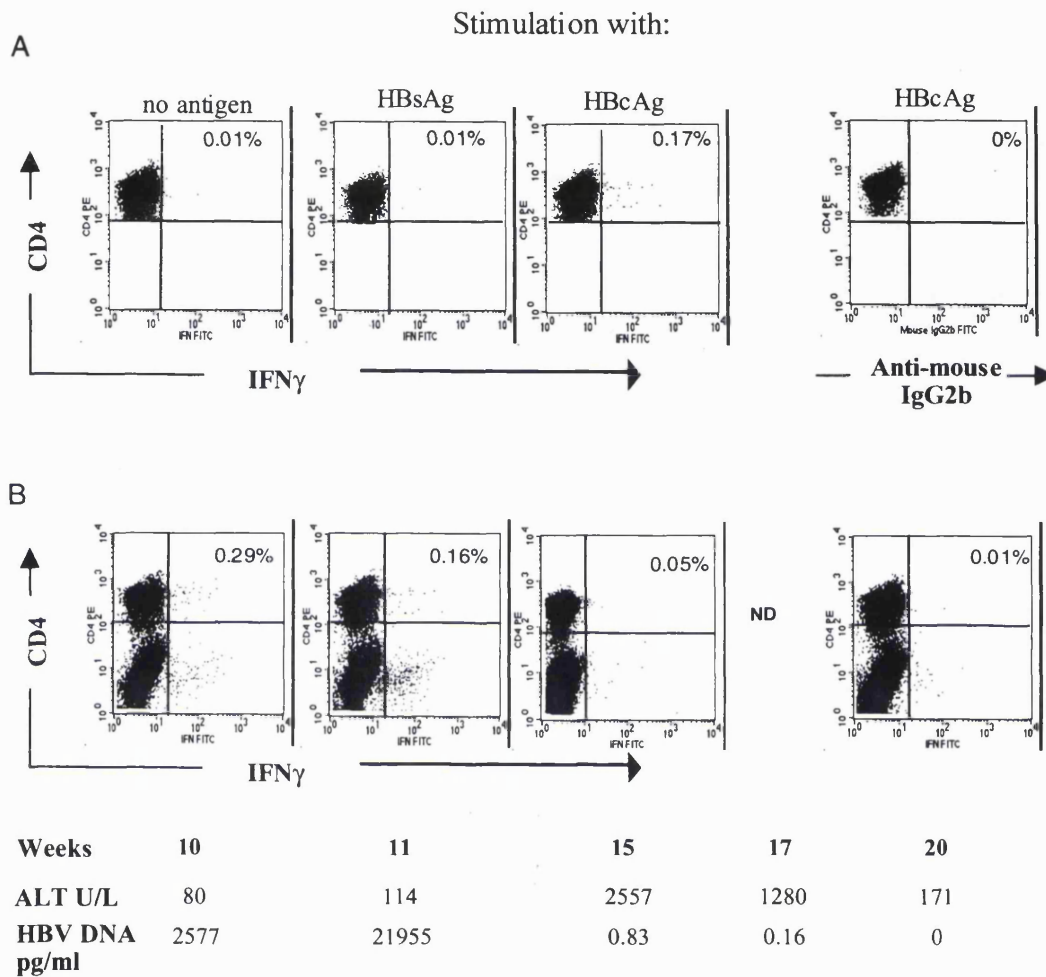
A) Dot plots represent CD4 +cells (gated on live and anti-CD4+ PE cells) from patient 1 (week 11) after stimulation of PBMC with the indicated antigens. Cells are stained with anti-CD4 PE, anti-IFNγ FITC antibodies (horizontal axis) and isotype matched FITC antibody (horizontal axis, last panel). At least 3×10^4 CD4+ cells were analysed in each sample. The percentage of IFNγ producing CD4+ cells out of total CD4+ cells is indicated in the upper right quadrant.

B) Dot plots represent IFN-γ producing PBMC (cells in the live gate are shown) of patient 1 after stimulation with core antigen during the course of acute HBV infection. The number of weeks after infection is indicated at the bottom, with HBV-DNA level (pg/ml) and ALT (U/L). The percentage of IFNγ producing CD4+ cells out of total CD4+ cells is indicated in the upper right quadrant.

Figure 3.9. Collated data on the dynamics of cellular immune responses during acute hepatitis B, in patient 1.
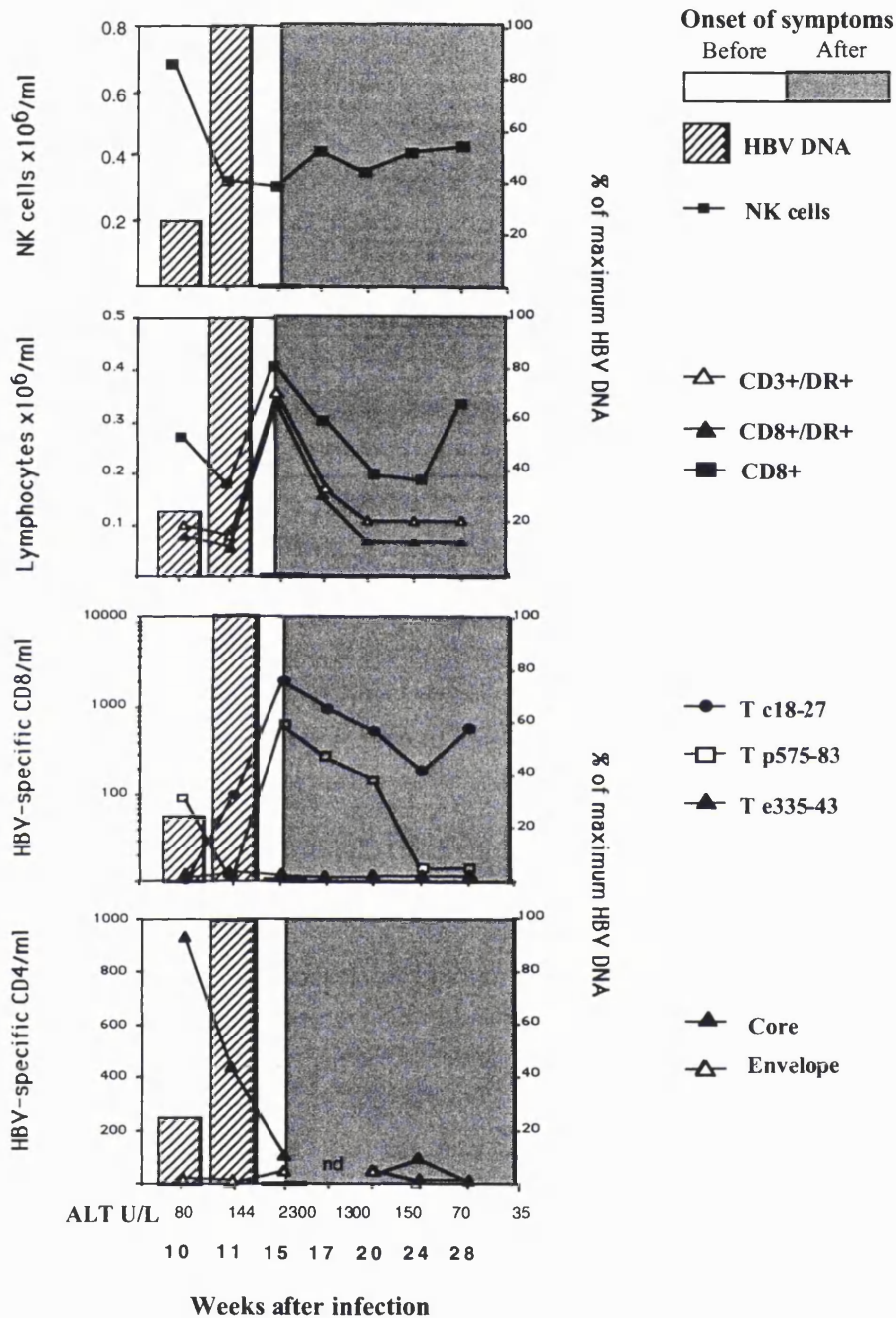
Graph shows the collated data of the changes in the numbers of circulating NK cells; total and activated (DR+) CD8+ cells; HBV-specific CD8+ cells; and HBV-specific CD4+ cells over the time course of acute HBV infection. The period before and after the onset of symptoms is shown. Viral load is expressed as the percentage of maximal HBV DNA concentration, and serum ALT is displayed numerically below the graph.

3.4. Discussion.

This is the first study in man in which the relationship between HBV replication, liver injury and the cellular immune responses, during the incubation phase of infection, has been studied in detail. Firstly, it was found that maximal reduction in circulating HBV DNA occurs before the onset of clinical disease. This observation of control of HBV replication prior to liver injury has recently also been demonstrated in chimpanzees (Guidotti et al., 1999a) and woodchucks (Guo et al., 2000). In chimpanzees, serum levels of HBV DNA and ALT have also been shown to closely parallel intra-hepatic virus levels and histological evidence of inflammation. The performance of liver biopsies was not clinically justified in our patients, but this observation in chimpanzees suggests that the measurements made from our patients' circulating compartment may be well correlated with the pattern of intra-hepatic viral control and liver injury. Only a careful longitudinal analysis of intrahepatic events in patients with acute hepatitis B would allow this assumption to be confirmed. Whether the observed fluctuations in the circulating frequency of NK cells, and of antigen-specific and total CD4+ and CD8+ cells, reflect changes in total cell number or are a consequence of redistribution between the hepatic and circulatory compartments is also uncertain, in the absence of sequential liver biopsy samples. However, it is likely that many HBV-specific CD8+ cells are preferentially sequestered in the liver, where the intrahepatic frequency of these cells can be 100 times higher than is seen in the circulation (Maini et al., 2000). The finding of HBV-specific CD8+ cells in the periphery during the incubation period in patients 1 and 2 may therefore be significant, suggesting that liver infiltration by these cells occurs at an early stage of infection, several weeks prior to maximal liver damage. CD4+ and CD8+ cell infiltration of the liver has recently been shown in woodchucks to occur within 3 weeks of infection, and prior to liver injury (Guo et al., 2000). Furthermore, virus-specific CD8+ cells are present in the livers of patients with chronic HBV, even in the absence of significant liver injury (Maini et al., 2000). It is possible, therefore, that during the incubation phase of acute hepatitis B, a selected population of HBV-specific CD8+ and CD4+ cells infiltrate the liver, where they can contribute to virus suppression, and subsequently trigger a cascade of events leading to significant liver damage. The huge increase in the circulation of activated CD8+ cells

(Figures 3.7 and 3.9), not accounted for by tetramer-staining CD8+ cells, at the time of clinical hepatitis, supports the evidence from a range of viral infections that the bulk of CD8+ cells are activated in a 'bystander' fashion involving cytokines (Tough and Sprent, 1996; Abrignani, 1997). The importance of 'non-antigen specific' cell recruitment in liver damage has been observed in the transgenic mouse model of fulminant hepatitis (Ando et al., 1993), and has been implicated as playing an important role in the development of liver injury in chronic HBV (Maini et al., 2000; Bertoletti and Maini, 2000) and HCV infections (He et al., 1999) .

The dynamic relationship between virus, liver injury and cellular immune response seen in patients who developed acute hepatitis (patients 1 and 2) is in marked contrast to that seen in the immunosuppressed subject (patient 5), who failed to control infection. The case has previously been reported of a patient unwittingly given oral steroid therapy for arthralgia associated with the prodrome of acute hepatitis B. The incubation period was prolonged, with acute clinical hepatitis and clearance of HBsAg only occurring after withdrawal of steroids (Johnson and Reed, 1983), again implicating the effects of steroids on the early immune response to HBV. The curtailment of steroids in our patient was not, however, a clinical option. Immunosuppressive therapies which suppress T cell function have been shown to predispose to chronicity following transient WHV infection (Cote et al., 1991), reinforcing the importance of T cells in the recovery from acute infection. It seems likely that an inability to mount an appropriate HBV-specific CD8+ and CD4+ cell response following infection contributed to viral chronicity in this patient. It is of interest that in a recent report of patients identified during the incubation phase of acute HCV infection, viral control was associated with a vigorous virus-specific CD8+ cell response, but the development of chronicity was associated with a failure to mount an effective CD8+ response following infection (Thimme et al., 2001).

Taken together, the results confirm in humans that the incubation phase of HBV infection is not only the period when the virus establishes a reservoir of infected cells. On the contrary, maximal reduction in HBV DNA concentration occurs before the development of symptoms. The presence of HBV-specific CD8+ and CD4+ cells during this phase suggests

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that these cells play an important early role in the control of infection and in the initiation of events that lead to liver damage. Nevertheless, our data do not allow us to evaluate whether these cells are controlling virus predominantly through cytolytic or non-cytolytic mechanisms. A rise in ALT was seen in association with the initial fall in HBV DNA in all patients with clinical hepatitis, suggesting that direct lysis of infected hepatocytes by NK and/or HBV-specific CD8+ cells during this phase cannot be discounted. However, maximal liver damage occurs after HBV DNA has been virtually cleared, and there is a lack of proportion between serum HBV clearance and the rise in ALT. This pattern is similar to that recently shown in chimpanzees acutely infected with HBV, in which viral reduction during the incubation phase was attributed to non-cytolytic mechanisms (Guidotti et al., 1999a).

From the clinical perspective, the finding of massive reductions in HBV DNA during the incubation phase casts doubt on the logic of considering antiviral therapy, as a means of reducing liver injury, in immunocompetent patients who present in the clinical phase of acute or fulminant hepatitis B (Santantonio et al., 1999). The unusual pattern of sub-acute hepatic necrosis, in which progressive liver damage and high levels of HBV occur for months after acute infection, may benefit from lamivudine (Reshef et al., 2000), but the pathogenesis of this condition is not at all well defined. These results suggest that the incubation phase of HBV infection should be considered as the critical period for the interaction between host and pathogen. This is likely to be the time when viral control or persistence is determined.

CHAPTER 4

LONGITUDINAL ANALYSIS OF HBV-SPECIFIC CD8+ CELL RESPONSES IN DIFFERENT PATTERNS OF HBV INFECTION.

4.1. Introduction

Resolved and persistent HBV infection have, in the past, been viewed as dichotomous states, in terms of viral replication and host immunity (Chisari and Ferrari, 1995b). The vigorous, multispecific CD8+ cell response seen in acute HBV infection has been believed to result in the complete eradication of HBV from the liver and circulation, contributing to lifelong immunity. In contrast, the HBV-specific CD8+ cell response in chronic disease has been reported as being weak and mono/oligospecific, insufficient to control viral replication. Recent clinical and laboratory-based studies have suggested that these divisions may not be absolute, but that a continuum may exist between virus and immunoresponsiveness. HBV has been shown to persist in the livers and circulation of humans and related animal models of HBV infection long after serological resolution (Michalak et al., 1999; Rehermann et al., 1995a; Cacciola et al., 1999), and the demonstration of a vigorous multispecific CD8+ cell response in patients many years after recovery from acute infection suggests that a lifelong interaction occurs between virus and CD8+ cell immunity (Penna et al., 1996). In support of this, it has been widely reported that patients with resolved acute infection (HBsAg-, anti-HBc+, anti-HBs+) may reactivate in the setting of profound immunosuppression, with often serious consequences (Marusawa et al., 2001; Yeo et al., 2001).

Over the last few years, circulating antigen-specific CD8+ cells have been quantified directly in a number of infections, including HIV (Ogg et al., 1998), influenza (Flynn et al., 1998b), and EBV (Callan et al., 1998). Longitudinal analysis of these cells during the acute phase of disease has also been performed for a range of infections, and has shown a

correlation between CTL frequency and viral control (Koup et al., 1994; Lechner et al., 2000a; Thimme et al., 2001; Maini et al., 1999; Webster et al., 2000b). The relationship between CTL and viral control during chronic disease appears more complex. Although longitudinal analysis of patients with chronic HIV has suggested that reduction in viral replication with anti-viral therapy leads to an increase in virus-specific CD8+ cell responses in the circulation (Goulder et al., 2001), data on the pattern of change during the evolution of natural infection remains conflicting (Ogg et al., 1998). Changes in the function and specificity of the CTL response during the natural evolution of infection have been shown for a number of infections, including HIV (Goulder et al., 2001).

In chronic HBV infection, the correlation between clinical parameters and serological markers of disease over the course of natural infection have been well described (Alward et al., 1985; Maruyama et al., 1993), but the temporal pattern of cellular immune responses have been much less widely reported (Lohr et al., 1995; Tsai et al., 1992), in particular with respect to HBV-specific CD8+ cells (Sing et al., 2001). Whilst the use of tetramer technology in the host laboratory enabled comparisons to be made of circulating and intrahepatic HBV-specific CD8+ cell frequency in patients with different patterns of chronic hepatitis B (Maini et al., 2000), this study, like most in this area of work, was cross-sectional in design, and so allowed only a 'snap-shot' of the interaction between virus, liver injury, and host immunity at a single time-point in the course of the disease. Yet it is widely recognised that the clinical 'phenotype' of chronic infection may change significantly over time. On the one hand, reactivation of clinically 'quiescent' chronic disease may occur, and be difficult to distinguish from acute infection (Maruyama et al., 1994). On the other hand, patients with long-standing chronic infection may lose HBsAg from the serum, either spontaneously or in response to treatment (Bonino et al., 1986; Lohiya et al., 1986). In these cases, HBV-specific CD8+ cell responses have been shown to be of comparable magnitude to those seen in patients who have cleared HBsAg after acute infection (Rehermann et al., 1996b). From our understanding of the noncytopathic nature of HBV, and the importance of cellular immunity in viral control and liver injury, it seems likely that the dynamic nature of chronic HBV infection is mirrored by similar dynamism in immune responses. Yet it is not clearly known how the magnitude and specificity of HBV-

specific CD8+ cell responses change during chronic disease, and whether clinical and virological events which appear similar to those in acute disease (eg hepatitis flare and HBeAg seroconversion) are associated with the same pattern of CTL response. The aim of the present work was therefore to perform a longitudinal analysis in patients with different clinical patterns of HBV infection, to determine how peripheral HBV-specific CD8+ cell responses change over time, and how they correlate with intrahepatic HBV-specific CD8+ cells, changes in disease activity, and the degree of viral control.

4.2 Materials and Methods

4.2.1 Patients.

Study participants were selected from the attendees at the viral hepatitis clinic at the Royal Free Hospital. Studies to analyse HBV-specific CD8+ cell responses longitudinally in the periphery, and cross-sectional analysis of responses within the liver, were approved by the ethics committee of The Royal Free Hampstead NHS Trust (Nos. 188-99 and 261-99). Patients aged 18-70 years were eligible for study. All had clinical, biochemical and virological evidence of chronic HBV infection, or resolved acute hepatitis B following a clear history of symptomatic disease. Potential participants were initially screened for their HLA haplotype, using the technique described below, as only those who were HLA-A2 positive were invited for further analysis (in view of the HLA class 1 restriction of CD8+ cell responses, as discussed previously). Patients were recruited into one of 5 study groups, according to their virological status and degree of liver injury (Table 4.1).

Table 4.1.   Study groups according to baseline characteristics

Group	HBsAg	HBeAg	Anti-HBe	Anti-HBs	HBV DNA copies/ml	Serum ALT U/L	Previous icteric hepatitis
A	-	-	+/-	+	Neg	<40	✓
B	+	-	+	-	$<10^5$	<40	✗
C	+	-	+	-	10^5-10^7	>40	✗
D	+	+	-	-	$>10^7$	»80	✗
E	+	+	-	-	$>10^7$	<80	✗

4.2.2 HLA-A2 typing.

2ml of whole blood were drawn into an EDTA (citrated) blood tube. The same technique as described previously (see 3.2.5) was applied. Briefly, mouse anti-human HLA-A2 antibody was added to a 200ul mixture of citrated blood and PBS, and incubated at 4^0C for 25 mins. After washing, a second layer of FITC-conjugated anti-mouse IgG (Sigma Chemical Co.) was added, prior to further incubation at 4^0C for 25 mins. After lysing the red cells using FACS™ lysing solution, the cell suspension was washed and analysed on a FACScan®. Only individuals who were anti-human HLA-A2 antibody were studied further.

4.2.3 HBV serology and biochemical analysis.

HBsAg, HBeAg, and anti-HBs were determined by commercially available enzyme immunoassay kits (Hepanostika Uni-Form II, Organon Teknika, Boxtel, Netherlands; Murex Biotech Ltd, Dartford, England; IMX Abbott GmbH Diagnostika, Wiesbaden-Delkenheim, Germany). HBV DNA in the serum was quantified using the Roche AMPLICOR Monitor assay (Roche Diagnostics Ltd, NJ), with a DNA detection threshold of 400 copies/ml. Serum ALT was measured on an automated biochemical analyser system.

4.2.4 Isolation and storage of PBMC.

Approximately 40ml of blood were drawn at each time point, and PBMC were isolated by centrifugation over a Ficoll gradient, using the technique described previously (see 3.2.3.). Cells were counted (approximately 30-100 x 10^6 cells per time-point) and either studied immediately in the relevant medium, as discussed below, or submitted to graded freezing to -80^{0}C, followed by transfer into liquid N_2, until used for further analysis. Subjects undergoing longitudinal analysis of circulating HBV-specific CD8+ cell responses had blood drawn on a two to six monthly basis, dependent upon their availability and disease

activity. All patients undergoing liver biopsy as part of the study had blood drawn for analysis within 4 weeks prior to the procedure.

4.2.5 Isolation of intrahepatic lymphocytes.

Liver biopsies were only performed where clinically indicated. Approval from the local ethics committee was obtained (No. 188-99) to allow a second pass at liver biopsy to be performed, in order to obtain liver tissue for this study. All biopsies were performed by the author. Using a Trucut biopsy needle, one 1-2 cm core of liver tissue was taken for research. The tissue was put in 20ml of RPMI, and processed immediately. A similar method for isolating intrahepatic lymphocytes to that previously reported was used (Bertoletti et al., 1997a). In brief, the biopsy was extensively washed in RPMI (Autogen-bioclear) to remove contaminating blood, and digested with collagenase type 1 (1mg/ml; Sigma Chemical Co.) and deoxyribonuclease 1 (25ug/ml; Sigma Chemical Co.) at 37°C for 60 minutes, on a shaking device. The specimen was then repeatedly and vigorously pipetted to disrupt liver architecture, allowing the release of intrahepatic lymphocytes. The cell suspension was washed twice in RPMI, and lymphocytes were recovered by gradient centrifugation over Ficoll-Hypaque. After washing twice in RPMI, cells were counted. For direct *ex-vivo* quantification of HBV-specific CD8+ cells, 0.3 x 10^6 cells per well were suspended in 100ul of PBS + 1% heat-activated FCS, in a 96 well plate. Quantification of virus-specific CD8+ cells was performed by tetramer staining, using the technique described below for PBMC.

4.2.6 Direct quantification of HBV-specific CD8+ cells using tetramers.

Quantification of peripheral and intrahepatic HBV-specific CD8 cells was performed, in HLA-A2-positive patients, using HLA-A2/HBV peptide tetrameric complexes, following a technique similar to that described earlier (3.2.7, and Figure 1.3). The tetramers were synthesised by Dr Graeme Ogg at the Institute of Molecular Medicine at Oxford, where the

technology has been developed (Ogg and McMichael, 1998; Altman et al., 1996; Callan et al., 1998). Wild-type sequences of HBV (genotype D) peptides core 18-27, polymerase 816-824, envelope 183-191, and envelope 348-357 were incorporated into the tetramers.

In brief, approximately 0.3 x 10^6 PBMCs were incubated for 30 minutes at 37°C with 0.5µg of phycoerythrin (PE)-labelled tetrameric complexes in 100ul of PBS + 1% FCS. Cells were washed, and incubated with 1ul of directly conjugated anti-CD8/cychrome mAb (Sigma Chemical Co) in 100ul of PBS + 1% FCS, at 4°C for 25 minutes. Cells were washed twice, resuspended in 50ul of PBS + 1% FCS, and analysed immediately on a FACScan®, using Cell Quest software. The quantity of HBV-specific CD8+ cells/ml of blood was calculated by multiplying the frequency of tetramer-positive cells by the total CD8+ cell count/ml, determined from the percentage of CD8+ cells within the total lymphocyte count (see Figure 3.1). Positive and negative controls for the tetramer staining for each epitope have been previously defined within the study laboratory, using HLA-A2 restricted clones specific for these epitopes, and staining PBMC from HBV-uninfected HLA-A2+ subjects, and acutely HBV infected HLA-A2- individuals. These experiments established the specificity of the tetramers, and a background frequency of non-specific tetramer staining of less than 0.02% of CD8+ cells (Maini et al., 1999).

Tetramer staining was used to directly quantify the *ex-vivo* frequency of peripheral and intrahepatic HBV-specific CD8 cells, and the expansion capacity of peripheral CD8+ cells after *in-vitro* stimulation.

4.2.7. HBV-specific CD8+ cell quantification by intracellular cytokine staining.

In a 96 well plate, $3x10^5$ PBMCs/well were added to 200ul of T cell medium (containing RPMI 1640, 10% heat-activated FCS, 25mmol/l HEPES, 2mmol/l L-glutamine, and 50ug/ml gentamycin), and stimulated with a 1uM concentration of one of 11 9-10 amino-

acid HBV peptides (see Table 5.2). These peptides have been previously reported to elicit HLA-A2 restricted CTL responses in HBV infection in man and animal models, and to express significant binding affinity (expressed as the dose of test competitor peptide yielding 50% inhibition of reference labelled peptide binding; ie. IC 50%) (Bertoni et al., 1997; Nayersina et al., 1993; Sette et al., 1994; Vitiello et al., 1997). Cells were cultured at 37^0C. On day 4-5 the medium was changed, and 100ul of 20U/ml human recombinant IL-2 (final concentration 10U/ml) (Roche Diagnostics, Mannheim, Germany) was added. On day 10-12 100ul of supernatant was removed from each of a pair of wells and the cells added into one well. Each well was stimulated again with 2ul of HBV peptide 100uM, and control wells were not restimulated. To each well was added 2ul of Brefeldin A (Sigma Chemical Co.) (1mg/ml), to a final concentration of 10ug/ml. This agent blocks the transport to the cell surface of proteins for secretion (eg IFNγ). After incubating at 37^0C for 5 hours the wells were washed by spinning at 1500rpm for 5 mins. After discarding the supernatant, 1ul of Cychrome-labelled anti-CD8 mAb (Pharmingen), and 100ul PBS + 1% FCS, were added to each well, and incubated at 4^0C for 20mins. After adding 100ul PBS + 1% FCS and spinning at 1500rpm for 5 mins the supernatant was removed. The cells were then incubated in 100ul of Cytofix/Cytoperm™ (Pharmingen) at 4^0C for 20mins, to permeabilise the cells, allowing subsequent intracellular access of anti-IFNγ Ab. Cells were washed in 100ul PBS + 0.1% Saponin (Sigma Chemical Co.), and the supernatant removed. 3ul of FITC-conjugated anti-IFNγ mAb (R+D Systems, Minneapolis, MN.) in 100ul of PBS + 0.05% Saponin, and cells incubated for 30 mins at 4^0C. After washing twice in 200ul of PBS +1% FCS, cells were resuspended in 50ul of PBS. The frequency of IFNγ + CD8+ cells in HBV peptide-stimulated and control wells was analysed on the FACScan, using CellQuest software, and the proportion of antigen-specific cells was calculated using a similar technique to that described earlier (Figure 3.1). Direct intracellular cytokine staining was also performed on fresh cells, with peptide stimulation for only 5 hours, otherwise using the technique described above.

Table 4.2   HBV CTL epitopes used for intracellular cytokine staining and tetramer staining in longitudinal hepatitis B study

Residues	Sequence	HLA-A2.01 binding affinity IC 50%nM	Corresponding tetramer available for analysis
Core 18-27	FLPSDFFPSV	3.3	✓
Core 107-115	CLTFGRETV	nd	✗
Polymerase 455-63	GLSRYVARL	71	✗
Polymerase 502-10	KLHLYSHPI	17	✗
Polymerase 575-83	FLLSLGIHL	10	✗
Polymerase 655-63	ALMPLYACI	10	✗
Polymerase 816-24	SLTADSPSV	14	✓
Envelope 183-91	FLLTRILTI	7	✓
Envelope 335-43	WLSLLVPFV	7	✗
Envelope 338-47	LLVPFVQWFV	3.2	✗
Envelope 348-57	GLSPTVWLSV	19	✓

4.2.8 DNA Sequencing of the Hepatitis B virus Core, Surface and Polymerase regions.

Analysis of week 0 samples of patients with detectable HBV DNA (ie groups B-E) was performed. This sequencing was performed by David Brown in the Centre for Hepatology. DNA was isolated from serum samples using the QIAamp Blood DNA mini kit (Qiagen, Crawley, UK). This was then subjected to nested or semi-nested PCR in the regions nt1869-2333 and nt375-861 for sequencing of core and surface/polymerase gene segments respectively. Primers used are shown in Table 4.3. PCR products were electrophoresed on 1% agarose gels and the bands excised. DNA was extracted using the QIAquick gel extraction kit (Qiagen, Crawley, UK). This was used as a template for direct sequencing using an ABI Prism model 373 automated sequencer and carried out at Babraham Bioscience Technologies, Cambridge, UK.

Table 4.3 Primers used for HBV DNA sequencing

Primer	Sense	Position	Sequence
HBV C1	+	1744-1761	5'GGGAGGAGATTAGGTTAA
HBV C2	-	2508-2489	5'GTACAGTAGAAGAATAAAGC
HBV C3	+	1869-1886	5'CCTCCAAGCTGTGCCTTG
HBV C4	-	2333-2314	5'CCGGAAGTGTTGATAAGATA
HBV S1	+	61-81	5'TGGTGGCTCCAGTTCAGGAAC
HBV S2	-	861-838	5'ACCCCATCTTTTTGTTTTGTTAGG
HBV S3	+	375-393	5'GGATGTGTCTGCGGCGTTY

4.3 Results

4.3.1 Patient groups.

122 patients with chronic or resolved hepatitis B were screened for HLA-A2 status. 22 patients were HLA-A2 positive and were available for further study (Table 4.4).

Table 4.4.   **Baseline clinical and virological characteristics**

Pt No.	ID	Age	HBs Ag	HBe Ag	Anti-HBe	Anti-HBs	HBV DNA copies/ml	ALT U/L	Liver Biop.	Longit. analysis
Group A										
1	SH	42	-	-	-	+	Neg	24	✗	✓
2	ES	52	-	-	-	+	Neg	18	✗	✓
3	AS	51	-	-	-	+	Neg	32	✗	✓
4	CB	39	-	-	-	+	Neg	32	✗	✓
Group B										
5	VM	63	+	-	+	-	2.4×10^4	26	✗	✓
6	M1	43	+	-	+	-	1.2×10^4	27	✗	✗
7	M3	38	+	-	+	-	1.0×10^4	33	✗	✓
8	MK	39	+	-	+	-	1.0×10^4	31	✗	✗
9	BG	37	+	-	+	-	1.3×10^4	31	✗	✓
Group C										
10	TC	56	+	-	+	-	2.7×10^6	74	✓	✓
11	HO	61	+	-	+	-	1.5×10^6	61	✓	✓
12	DM	44	+	-	+	-	1.0×10^6	57	✓	✓
Group D										
13	IC	43	+	+	-	-	1.0×10^8	227	✓	✓
14	SK	36	+	+	-	-	4.0×10^8	2635	✓	✓
15	RF	25	+	+	-	-	5.3×10^8	291	✓	✓
Group E										
16	SA	32	+	+	-	-	6.8×10^8	60	✓	✓
17	DC	25	+	+	-	-	5.8×10^8	66	✓	✓
18	SK	49	+	+	-	-	5.9×10^8	20	✓	✓
19	KN	24	+	+	-	-	0.5×10^8	41	✗	✗
20	DM	31	+	+	-	-	15×10^8	79	✓	✓
21	AM	50	+	+	-	-	15×10^8	33	✓	✓
22	JT	24	+	+	-	-	13×10^8	48	✓	✗

4.3.2 Direct *ex-vivo* quantification of circulating HBV-specific CD8+ cells.

In the analysis of patients during the acute phase of HBV infection, HBV-specific CD8+ cells were directly quantified from the circulation, using tetramer staining (Webster et al., 2000b; Maini et al., 1999). Similar techniques were applied to this study of 18 HLA-A2+ patients with chronic hepatitis B, and 4 with resolved acute icteric HBV infection.

In patients with resolved hepatitis B we were able to detect virus-specific CD8+ cells in the circulation on direct *ex-vivo* analysis, up to 10 years after resolution of acute hepatitis and clearance of HBsAg from the serum (Figure 4.1). As previously shown during the icteric phase of acute infection (Webster et al., 2000b), there was a hierarchy of response, with CD8+ cells specific for core 18-27 more frequent than those for polymerase or envelope epitopes (Figure 4.2). In contrast, the frequency of HBV-specific CD8+ cells in patients with chronic HBV infection was barely above background frequency for any of the epitopes (less than 0.02% of CD8 cells in HLA-A2 positive, HBV non-exposed subjects, and HLA-A2 negative, HBV infected control subjects (Maini et al., 1999). Nevertheless core 18-27-specific CD8+ cells appeared to be at slightly higher frequency in patients with low levels of HBV replication (group B), than in those with very high levels of virus (group E). These findings of very low circulating levels of virus-specific CD8+ cells in chronic HBV infection are similar to those previously reported from the laboratory using HLA class I tetramers (Maini et al., 2000), although this previous report focussed only on patients equivalent to those in groups B (low HBV DNA and low ALT) and D (high HBV DNA and high ALT).

Figure 4.1 **Frequency of circulating HBV-core 18-27-specific CD8+ cells in different patterns of HBV infection, analysed directly *ex-vivo* by tetramer staining and flow cytometry.**
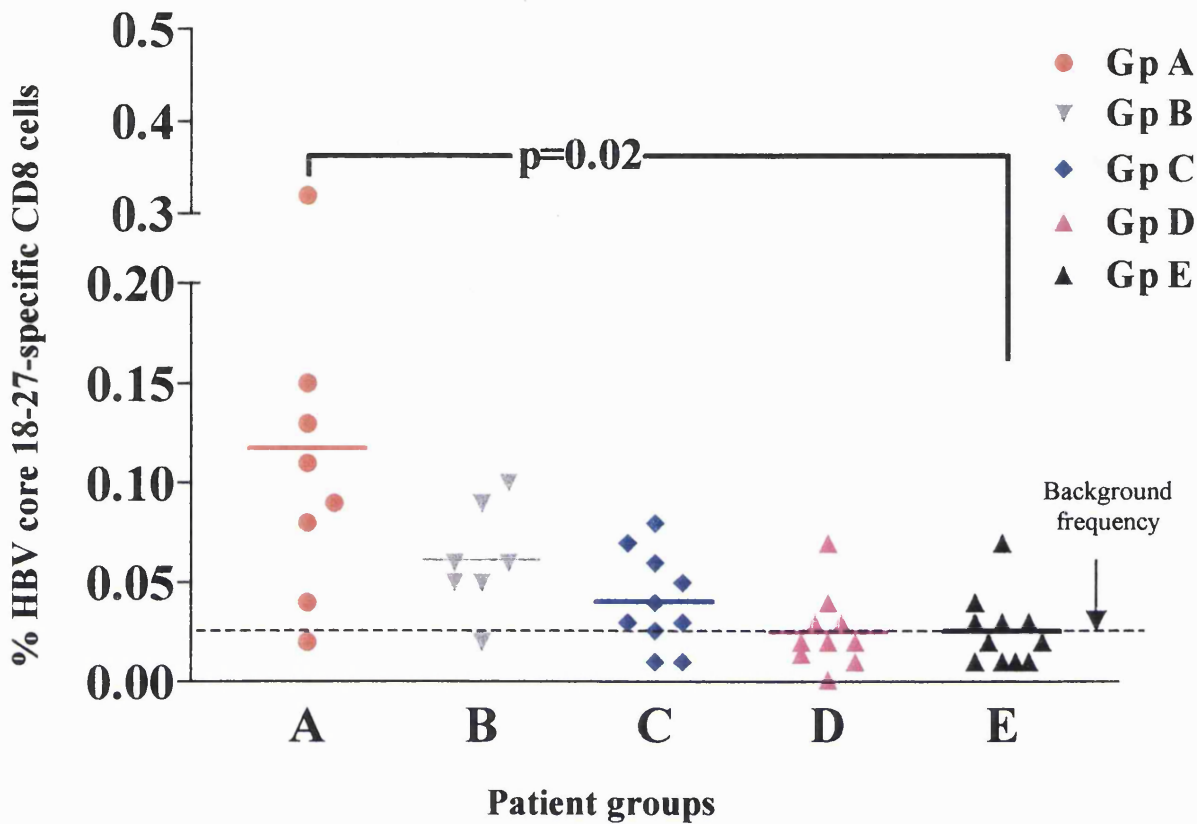
The direct *ex-vivo* frequency of circulating HBV-specific CD8+ cells was analysed using HLA-class I tetramers. Graph shows data only for core 18-27-specific cells. Polymerase 816-24, envelope 183-91 and envelope 348-57-specific cells were demonstrated above baseline levels on a number of occasions in those in group A, with resolved infection, but not in patients with chronic hepatitis B (groups B-E – see Table 4.1 for key to groups). The background frequency of tetramer staining was determined by direct *ex-vivo* staining of PBMC from HBV-infected non-HLA-A2 subjects, and HLA-A2 positive individuals who had no serological markers of exposure to HBV.

Figure 4.2.   **Direct *ex-vivo* frequency of epitope-specific CD8+ cells in resolved acute hepatitis B (Group A).**
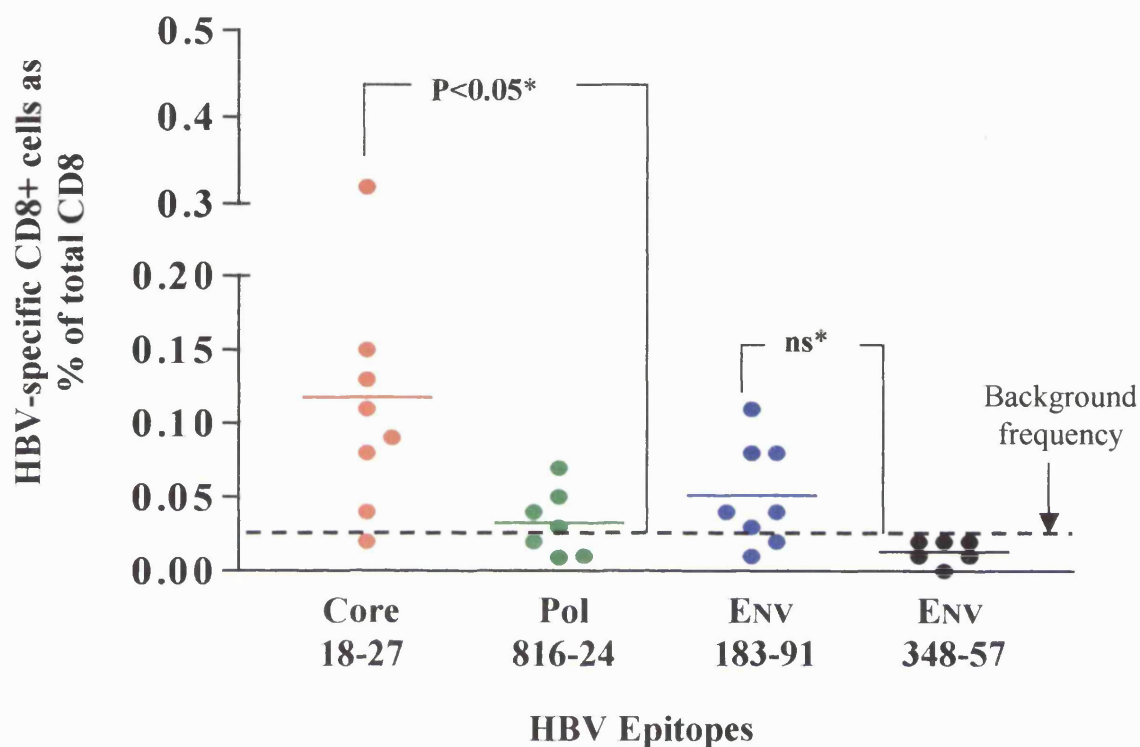
HLA class I tetramers displaying specific HBV epitopes were used to directly quantify the frequency of circulating HBV-specific CD8+ cells. Although core 18-27+ CD8+ cells were seen in resolved and chronic infection (Figure 4.1), cells specific for other epitopes were detectable only in those with resolved infection, as shown here. Each point represents the data for a specific patient at a particular timepoint. The bars indicate the mean frequency for each epitope, and the background frequency of tetramer staining was determined as described above. Although individual patients had clearly detectable CD8+ cells specific for envelope 183-91 and polymerase 816-24, only core 18-27 cells were found at statistically significantly higher frequency than controls.

4.3.3 Analysis of HBV-specific CD8+ cells after *in-vitro* expansion

In patients with chronic hepatitis B, circulating levels of HBV-specific CD8+ cells were barely above baseline frequencies on direct analysis. In order to determine whether these cells were absent from the circulation, or present at a precursor frequency too low to distinguish from the backgound, attempts were made to expand an antigen-specific CD8+ cell population by HBV peptide-specific stimulation. After ten days of *in-vitro* stimulation analysis of HBV-specific CD8+ cells was performed using intracellular cytokine staining. This technique has an advantage over tetramer staining in allowing the analysis of responses to a broader repertoire of HBV epitopes (11 epitopes, see Table 4.2) than is presently possible with HBV tetramers (maximum 4 epitopes).

In order to assess the comparability of tetramer and intracellular cytokine staining as a means to measure the frequency of HBV-specific CD8+ cells, we first compared the direct frequency of cells in the circulation using HBV peptide-specific tetramers and intracellular cytokine staining, following brief stimulation with the corresponding peptide (Figure 4.3). Patient 1 was studied, who had resolved hepatitis B, and who had been shown to have detectable HBV-specific cells directly *ex-vivo* (core 18-27 CD8+ cells 0.32% of total CD8+ cells), using tetramer staining (Figure 4.1). Using both techniques, it was shown that the frequency of HBV-specific cells for the different epitopes was similar. Just as core 18-27 CD8+ cells were clearly detected directly *ex-vivo* using tetramers, they could be similarly identified using intracellular cytokine staining. Importantly, the hierarchy of epitope-specific responses, with core 18-27-specific cells being numerically dominant over other epitopes in resolved hepatitis B, was maintained using both methods. The assessment of HBV-specific CD8+ cell responses was therefore performed using peptide-induced *in-vitro* expansion and intracellular cytokine staining.

Figure 4.3   **Direct quantification of circulating HBV-specific CD8+ cells using intracellular cytokine or tetramer staining**
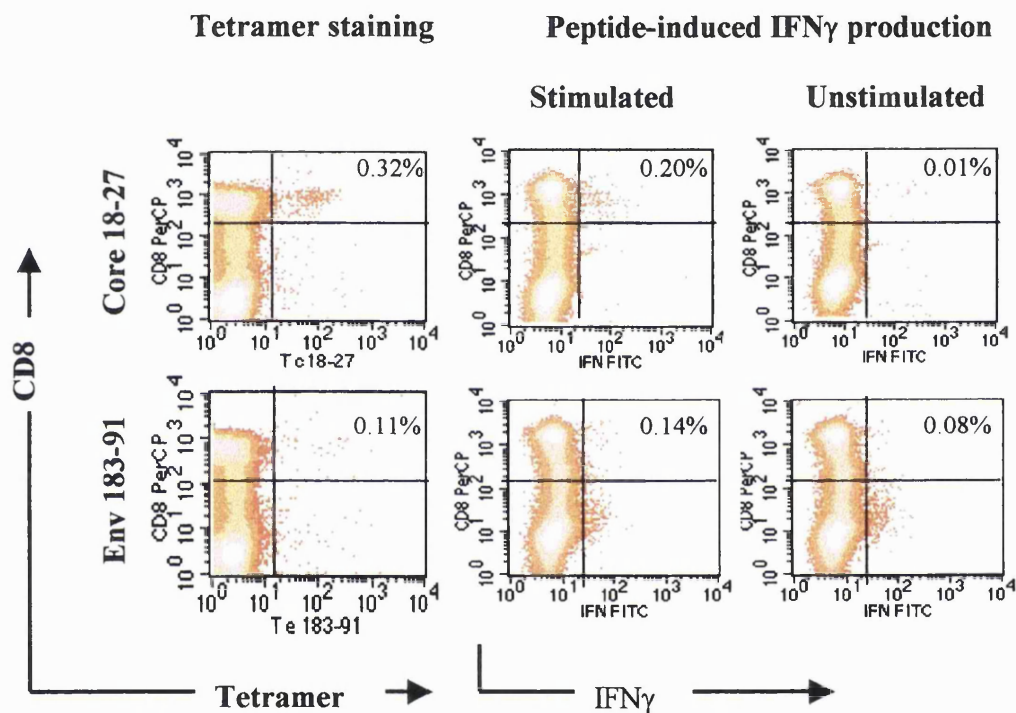
PBMC from patient 1 were stained both directly *ex-vivo* with HBV peptide-class I tetramers (left panels), and using intracellular cytokine staining following 5 hours stimulation with the corresponding HBV peptide (middle panels). Intracellular cytokine production by unstimulated PBMC are shown as controls, on the right. HBV-specific CD8+ cells, using tetramer and intracellular cytokine staining, are shown in the right upper quadrant of each dot plot, and their percentage of total CD8 is indicated.

In figure 4.4 the hierarchy of epitope-specific CD8+ cell responses, as assessed by peptide-specific stimulation and *in-vitro* expansion, is shown for the different patterns of hepatitis B. It can be seen that the hierarchy differed between patients with resolved and chronic HBV infection. In those with resolved infection (group A), significant expansion of core 18-27-specific CD8+ cells was always found, and these cells were numerically dominant over cells specific for polymerase or envelope epitopes. This pattern mirrors that found on direct *ex-vivo* analysis in this study (see Figures 4.1 and 4.2), and in previous studies during the acute clinical phase of HBV infection (Maini et al., 1999; Webster et al., 2000b), suggesting that the hierarchy of HBV-specific cells after *in-vitro* expansion may be a true reflection of the relative frequency of these precursor cells *in-vivo*. Polymerase 455-63-specific CD8+ cells were consistently found in patients in group A after expansion, reinforcing the merit of studying the broader repertoire of responses allowed using intracellular cytokine staining, as tetramers to this specificity were not available. Responses to a range of other envelope and polymerase epitopes were demonstrated, but these were always numerically subdominant.

A completely different hierarchy of response was seen in patients with chronic hepatitis B (groups B-E). In these patients minimal expansion of core 18-27-specific cells was seen in patients with moderate control of viral replication (groups B and C), and none at all in those with HBV DNA $>10^7$ copies/ml (groups D and E). Instead, CD8+ cells specific for envelope epitopes were commonly found, and envelope 183-91 was the numerically dominant CTL epitope. Envelope responses were found in 8 of the 12 chronically infected patients in whom any CTL responses to HBV peptides were found, and envelope 183-91 was the numerically dominant epitope in 5 of these 8. In patients with high levels of virus and minimal liver injury (group E), virtually no HBV-specific CD8+ cells were found at any timepoint. The relative dominance of core and envelope-specific CD8+ cell responses in resolved and chronic infection are shown in Table 4.5.

Figure 4.4 Hierarchy of HBV-specific CD8+ cell responses after *in-vitro* peptide stimulation and intracellular cytokine staining.
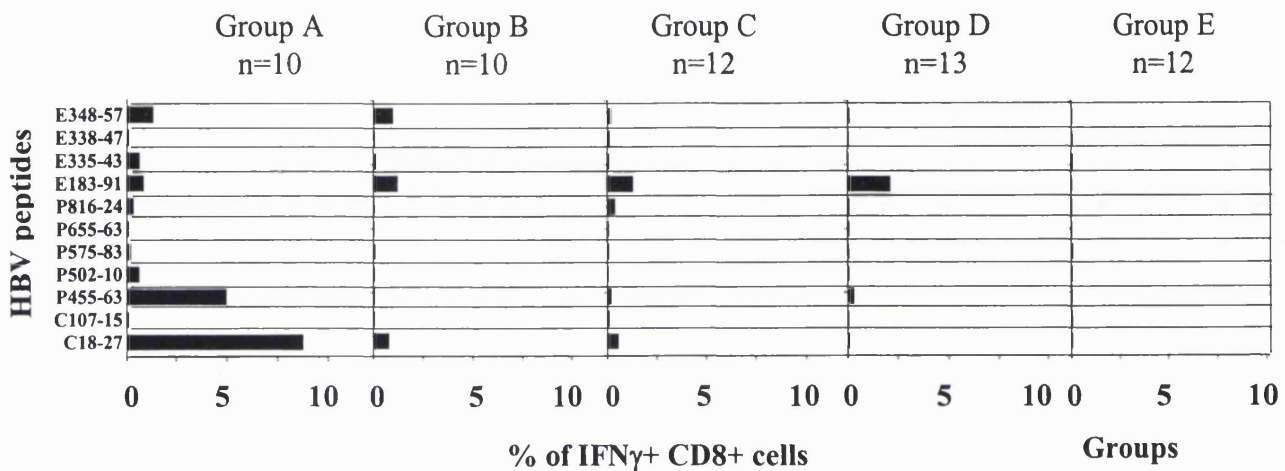
The HBV-specific CD8+ cell response was assessed at multiple time-points (n) in patients with resolved (group A) and chronic (B-E) HBV infection. The mean frequency of CD8+ cells specific for each HBV peptide in each patient group is expressed as the % of CD8+ cells which showed IFNγ+ staining after 10 days of *in-vitro* stimulation. This frequency was derived as follows: (frequency of IFNγ+ CD8+ cells out of total CD8+ cells in peptide stimulated wells) – (frequency of IFNγ+ CD8+ cells out of total CD8 cells in non-peptide stimulated (control) wells). The frequency of IFNγ+ CD8+ cells in control wells varied between individual experiments, but never exceeded 0.1%.

Groups

A: Resolved acute HBV

B: HBV DNA<10^5, ALT<40

C: HBV DNA 10^5-10^7, ALT>40

D: HBV DNA >10^7, ALT»80

E: HBV DNA >10^7, ALT<80

Table 4.5. HBV-specific CD8+ cell epitope dominance in resolved and chronic hepatitis B.

	Resolved infection (Gp A)	Chronic infection (Gps B-E)
Core (18-27)	7/9 (78%)	6/26 (23%)
Envelope (183-91, 348-57)	2/9 (22%)	20/26 (77%)

In the table, the epitope dominance of all timepoints at which any HBV-specific CD8+ cells were demonstrated after *in-vitro* expansion is shown. In those patients with resolved infection, responses were seen at every timepoint, but responses in chronically infected patients were absent at some, and occasionally all, timepoints studied. Although dominant envelope responses were usually focussed on the 183-91 epitope, envelope 348-57 dominated on two occasions in chronic infection.

A multispecific CD8+ cell response directed against a range of viral epitopes has been viewed as an important determinant of viral control after HBV infection. Chronic hepatitis B has been characterised by the CD8+ cell response being narrowly focussed against a very few epitopes (Chisari and Ferrari, 1995b). As well as defining the dominant epitope responses in the different patterns of disease, the breadth of the HBV-specific CD8+ cell response was therefore also analysed (Figure 4.5).

Figure 4.5 **Multispecificity of HBV-specific CD8+ cell response in different patterns of HBV infection.**
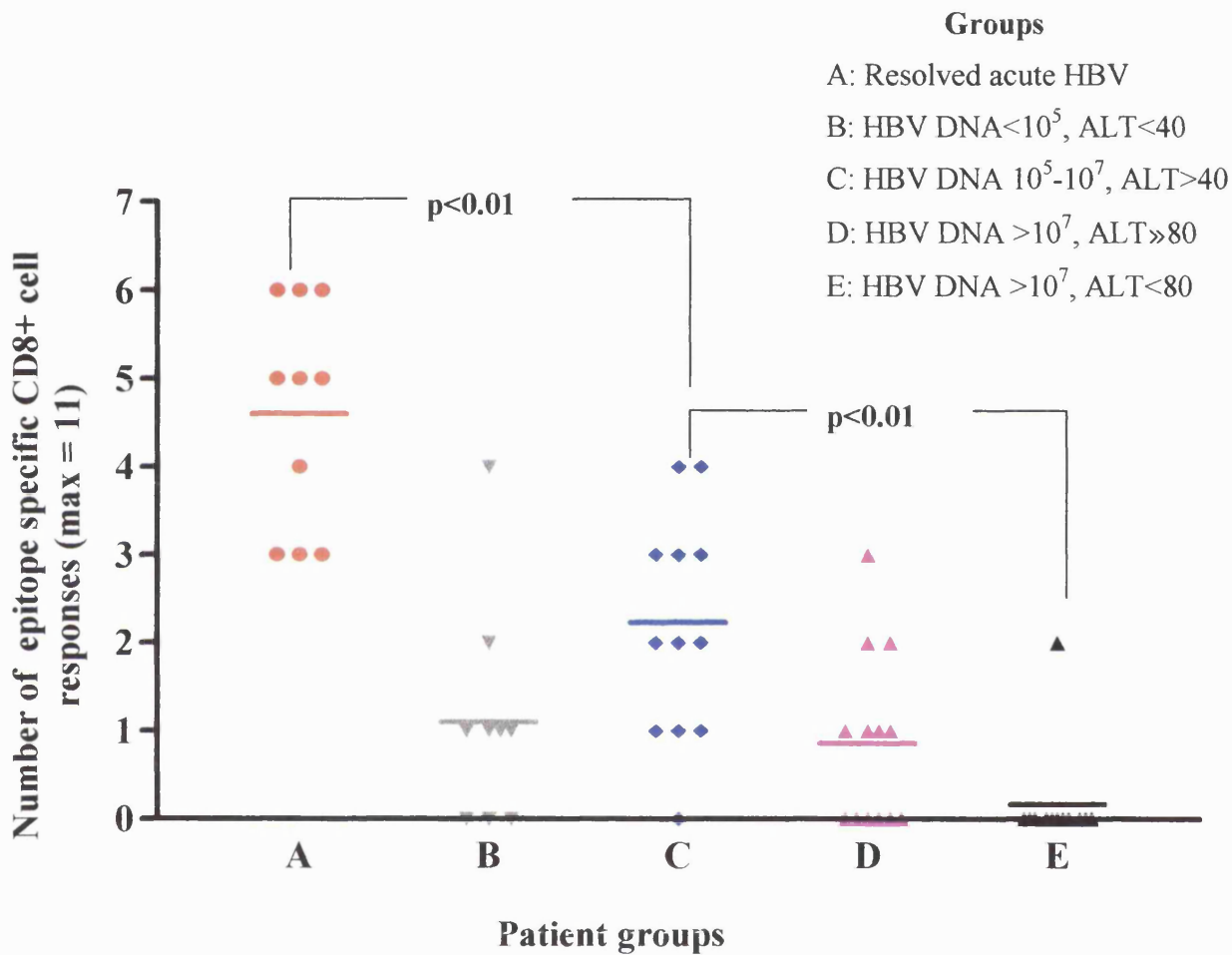
In a 96 well plate, 3×10^5 PBMC/well were stimulated for 10-12 days with one of 11 HBV peptides known to elicit CTL responses. HBV-specific CD8+ cells after expansion were identified using intracellular cytokine staining and flow cytometry. On the graph, one dot indicates the number of positive epitope-specific responses (out of maximum of 11) for an individual study timepoint. The patient groups are indicated along the bottom axis, and a key to these groups is displayed above the graph. Statistical differences in the breadth of the CTL response between study groups were analysed using the Mann Whitney U test.

Figure 4.5 shows, as expected, that a broad, multispecific CD8+ cell response against a range of HBV-peptides occurs in patients with resolved infection (Group A). Virus-specific cells directed against approximately 5 of the 11 epitopes were identified at each timepoint. In chronically infected patients a narrower response was found, with responses to more than 3 epitopes rarely demonstrated. Nevertheless, differences in the breadth of the CTL response were seen between the different groups with chronic infection. In the 7 patients with minimal liver injury and high levels of HBV DNA (group E), no HBV-specific CD8+ cell responses could be elicited at 11 of the 12 timepoints studied. In contrast, oligo-specific responses were demonstrated in patients with biochemical evidence of liver injury (groups C and E), or low levels of viral replication (group B). It is of interest that the greatest breadth of response in chronic patients was seen in those with moderately controlled viral replication ($<10^7$ copies/ml) and liver injury (group C), although only 3 patients were recruited into this group.

4.3.4 Longitudinal changes in HBV-specific CD8+ cell responses over time.

It was demonstrated that the frequency and hierarchy of peripheral HBV-specific CD8+ cell responses, both directly and after *in-vitro* expansion, differed between patients categorised into different groups on the basis of baseline characteristics. However, the phenotype of HBV infection may clearly change over the time course of chronic infection. In particular, infection characterised by high levels of viral replication and minimal liver injury (ie. group E), may evolve into a more active phase associated with flares of disease activity (group D), eventually leading to HBeAg seroconversion, low levels of viral replication, and quiescent liver disease (group B).

Using peptide-specific stimulation and intracellular cytokine staining, HBV-specific responses were therefore analysed for 6-78 weeks in 18 patients (4 in group A, 14 in groups B-E). In group E, maximum follow up was limited to 21 weeks, as these patients were

candidates for a trial of antiviral therapy, so precluding longer analysis of the natural course of their infection.

Figure 4.6, overleaf, displays the stability or instability of HBV-specific CD8+ cell responses over time, in the different patterns of infection. These changes were correlated with serum ALT (U/L) and HBV DNA (viral copies/ml). Complete longitudinal data for representative patients in each study group are shown. The time in weeks refers to the start of the study, not the duration of infection. For each timepoint, the percentage of IFNγ+ CD8+ cells (on x axis) following stimulation with specific HBV peptides (on y axis) are displayed. The x axis scale in patient 1 is set up to 20%, in view of particularly high levels of expansion of IFNγ+ CD8+ cells in this patient. For all other patients the scale is set to 6%, to allow comparison between patients and groups.

Figure 4.6 Longitudinal analysis of stability and instability of virus- specific CD8+ cell response in different patterns of HBV infection.
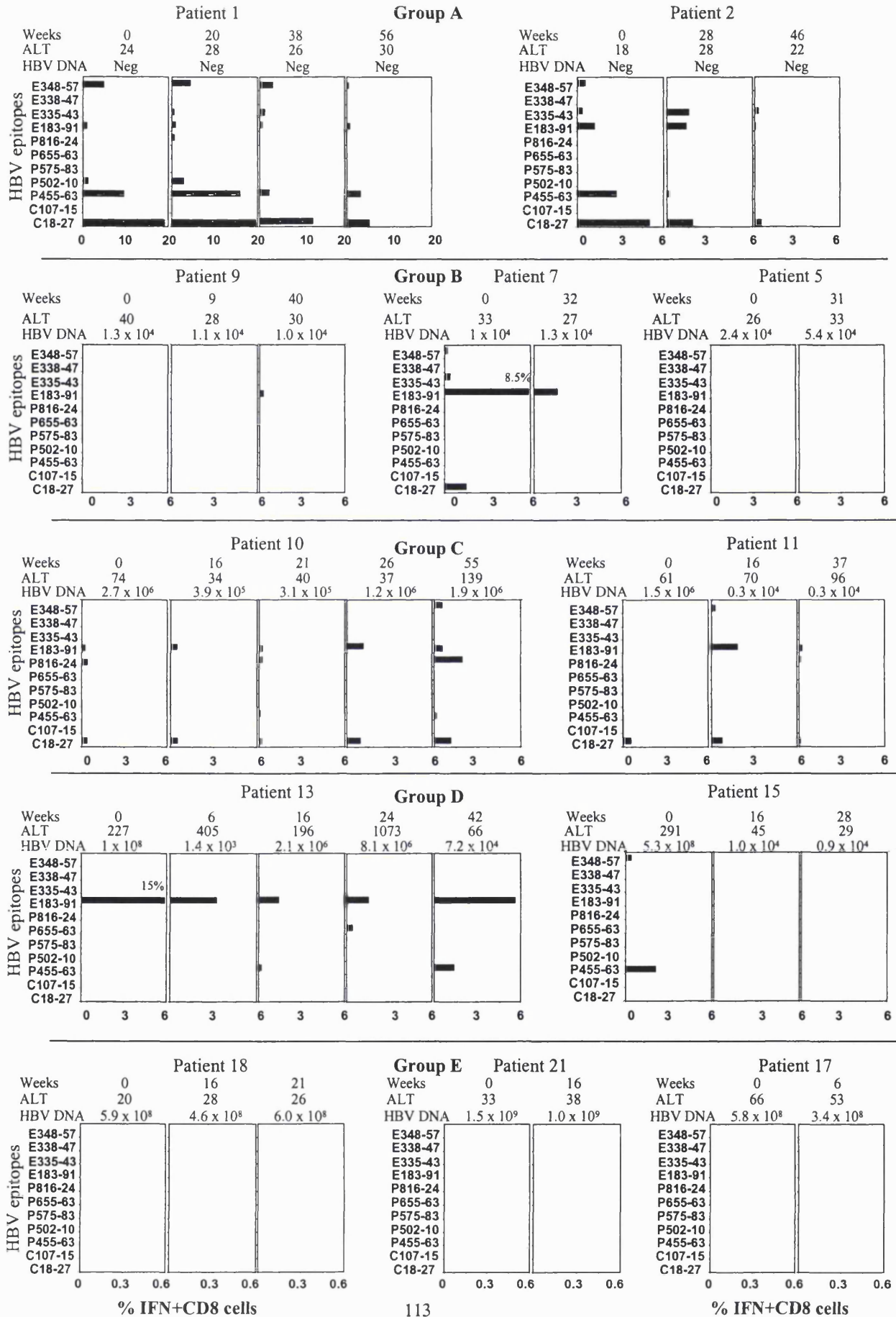
Figure 4.6 shows that in patients with resolved clinical hepatitis B (group A), a stable multispecific response persists, characterised by dominant and subdominant HBV-specific CD8+ cell responses. Significant expansion of core 18-27-specific cells was seen (up to 20% of CD8+ cells after expansion), in association with subdominant responses to envelope (particularly 183-91 and 348-57) and polymerase (455-63) epitopes. Core 18-27, envelope, and polymerase-specific CD8+ responses were elicited in every patient, at every timepoint, in this group. Patients 1 and 2 entered the study (week 0) 1 year after complete biochemical and serological resolution of acute icteric hepatitis B. It is of interest that the degree of expansion of core 18-27 cells appeared to gradually fall over the subsequent year in these patients. Nevertheless, a multispecific response, dominated by this epitope, remained present throughout the study, and was also clearly present in patient 4 (data not shown), many years after resolution of acute infection.

The pattern of CTL responses in patients with chronic hepatitis B (groups B-E) differed to that found in resolved infection. The hierarchy of epitope responses varied more than was seen in those with resolved infection, and although the time course of the study was associated with dynamic change in disease activity and viral replication, these changes could not be clearly correlated with shifts in the specificity of CD8+ cell responses. In clear contrast to the pattern found in resolved infection, the numerical dominance of core 18-27 CD8+ responses was absent, and although core 18-27-specific CD8+ cells were found in patients with relatively low levels of serum HBV DNA (groups B and C), this population expanded poorly. Nevertheless, when the frequency of core 18-27-specific CD8 + cells after in-vitro expansion was correlated with serum HBV DNA, in resolved, low, and highly replicating infection, a clear inverse relationship was seen (Figure 4.7). The low frequency of core 18-27-specific CD8 + cells in groups B and C, and absence in groups D and E, suggests that this CTL to this epitope may be more important in viral control than in liver injury.

Figure 4.7.   In-vitro expansion of core 18-27-specific CD8+ cells, related to serum levels of HBV
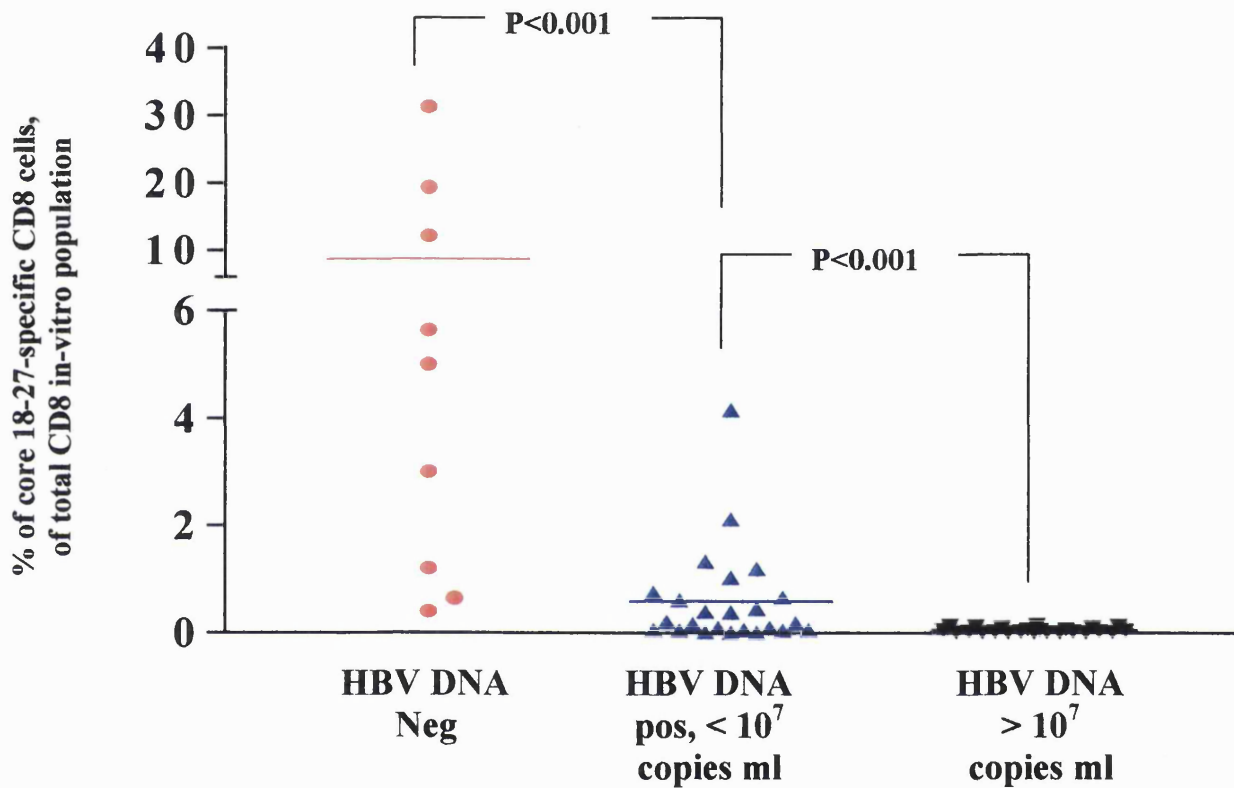
The percentage of core 18-27-specific CD8+ cells, after *in-vitro* peptide stimulation and intracellular cytokine staining, is displayed for every time-point studied. Due to the dynamic change in HBV DNA in chronically infected patients (especially in group D), data points from an individual patient could appear in more than one column. The graph demonstrates the inverse relationship between core 18-27-specific CD8+ cells and the levels of virus in the serum. Statistical analysis performed using Mann-Whitney non-parametric test.

As discussed earlier, a more restricted number of epitopes at each timepoint were seen in chronic infection (figure 4.5), with a lower frequency of HBV-specific CD8+ cell population after expansion than occurred in resolved infection. The exception to this observation was the behaviour of envelope 183-91-specific CD8+ cells, which expanded to more than 7% of total CD8+ cells in patients from groups B (patient 7), C (patient 12) and D (patient 13): a greater degree of expansion than was found for this epitope at any point in patients with resolved infection. In group E, viral load remained at very high levels, with minimal activity of liver disease, and on longitudinal analysis CTL responses were consistently absent. Two of the three patients in group D underwent spontaneous HBeAg seroconversion and subsequent near normalisation of ALT, and patients in group C also displayed fluctuations in viral load and markers of liver injury. Changes in the hierarchy of HBV-specific cells were seen, but envelope 183-91-specific cells were frequently demonstrated, both in the presence of relatively low levels of virus (HBV DNA $<10^5$ copies/ml) in patients 5 and 9, and high levels of virus (10^6-10^8 copies/ml) in patients 10 and 13.

Figure 4.8 shows a detailed display of the pattern of liver injury, HBV DNA, serology, and HBV-specific CTL responses in patient 13. The course of this patient's disease was highly dynamic, and it was of interest that he developed HBeAg seroconversion during the middle of the study, with significant reduction in viral load. This initial seroconversion was short lived, but was followed by a significant ALT flare, associated with further HBeAg seroconversion. He remained HBeAg negative, with significant biochemical resolution of his previously active liver disease. Of note, core 18-27-specific CD8+ cells were not seen, either directly or after expansion, before, during, or after seroconversion. At the beginning of the study envelope 183-91 CD8+ cells were expanded to a high frequency (>15% of total CD8), in the presence of very high levels of virus (HBV DNA 1 x 10^8 copies/ml). The 5 log reduction in viral load occurred in the setting of persistent high levels of these cells. However, these cells were not detected during the subsequent flare and seroconversion episode, becoming detectable again only following seroconversion (when HBV DNA 7.2 x 10^4 copies/ml).

Figure 4.8. Correlation between viral load, liver injury, and CD8+ cell responses during HBeAg seroconversion in a patient with chronic hepatitis B
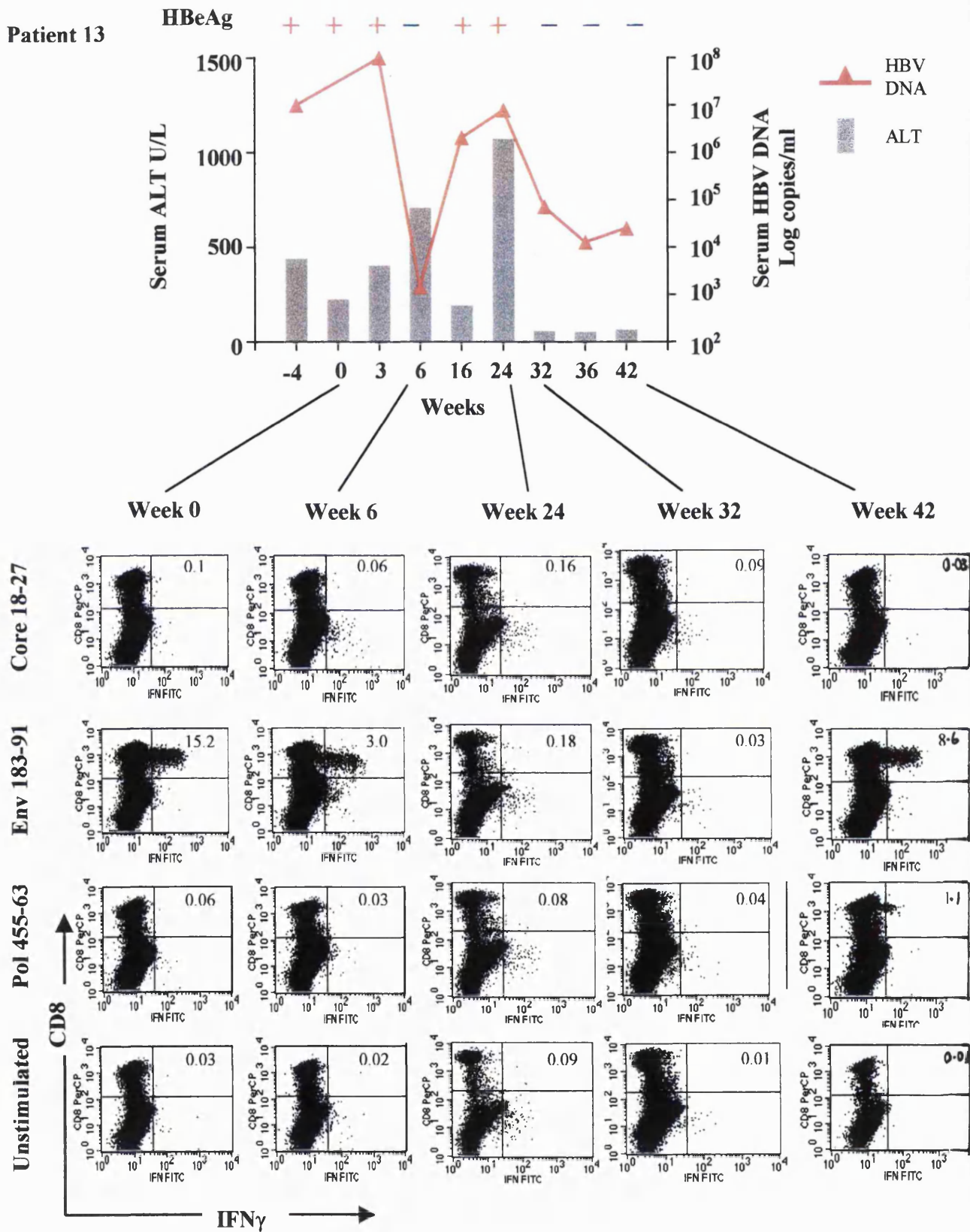
4.3.5 Viral sequencing

The persistence of high levels of HBV antigens *in-vivo* in these chronically infected patients (as implicated by measured HBV DNA levels), despite the presence of antigen-specific CD8+ cells, suggests that the virus must be escaping immunologic control by these CTL. It was wondered whether viral mutations might explain the presence of HBV-specific CD8+ cells in these persistently infected patients, as has been shown in a range of persistent viral infections, including hepatitis B (Bertoletti et al., 1994b). Table 4.6 displays the sequencing results of patients from each group with detectable HBV DNA, and those with HBV-specific CD8+ cell responses (eg patients 10 and 13).

Table 4.6   **Mutations within CTL epitopes of infecting virus in patients with chronic hepatitis B**

ID	HBV sequence	Patient 13	Patient 14	Patient 15	Patient 10	Patient 5	Patient 9	Patient 16	Patient 17
C18-27	FLPSDFFPSV	WT	WT	WT	I→V 27	ND	ND	WT	I→V 27
C107-15	CLTFGRETV	WT	WT	WT	WT	ND	ND	ND	WT
Pol 455-63	GLSRYVARL	WT	WT	WT	WT	WT	WT	WT	S→P475
Pol 502-10	KLHLYSHPI	WT	WT	WT	WT	M→L 505	M→L 505	WT	WT
Pol 575-83	FLLSLGIHL	ND	ND	WT	WT	WT	ND	WT	ND
Pol 655-63	ALMPLYACI	WT	WT	WT	WT	WT	WT	WT	ND
Pol 816-24	SLYADSPSV	WT	WT	WT	WT	WT	WT	WT	ND
Env183-91	FLLTRILTI	WT	WT	WT	WT	WT	WT	ND	ND
Env335-43	WLSLLVPFV	WT	ND	WT	WT	V→A 340	WT	ND	ND
Env338-47	LLVPFVQWFV	WT	ND	WT	WT	V→A 340 V→G 347	WT	ND	ND
Env348-57	GLSPTVWLSV	V→A 357	V→A 357	V→A 357	WT	V→A 353	V→A 357	ND	ND

Table 4.6 shows that mutations within CTL epitopes were present. Two patients (10 and 17) had mutations at position core 27. This position is one of the two major anchor residues in the core 18-27 epitope, and isoleucine at this position is a naturally occurring variant known to reduce the binding of the peptide to HLA-A2 (Bertoletti et al., 1994a; Bertoletti et al., 1994b). In a previous study of CTL responses in chronic hepatitis B using tetramers, the only patient with high viral replication and a readily expandable HBV core 18-27 cells in the circulation was infected with a virus carrying this mutation (Maini et al., 2000). It is of interest, therefore, that patient 10 was infected with this variant, as she was the only patient in our study who had HBV DNA $>10^5$ copies/ml, and marked expansion of CD8+ cells after stimulation with HBV core 18-27 peptide (up to 1.3% of CD8+ cells).

4.3.6 Direct ex-vivo quantification of intrahepatic HBV-specific CD8+ cells.

CD8+ cells specific for HBV core 18-27 were readily detected on direct analysis in the circulation of patients during the acute phase of hepatitis B ((Webster et al., 2000b; Maini et al., 1999) and chapter 3), and for years after recovery from infection, as we have shown here. Their persistence in the circulation was confirmed by significant expansion of core 18-27-specific cells after re-exposure to antigen in-vitro. In contrast, we had difficulty detecting core 18-27-specific CD8+ cells in the circulation of chronically infected patients, either directly ex-vivo, or after expansion, and using either tetramer staining or intracellular cytokine analysis. This inability to clearly identify virus-specific CD8+ cells in the circulation of chronically infected patients may be due to a number of factors. The cells may be physically eliminated by exhaustive induction or clonal deletion, as has been suggested for neonatally-transmitted non-cytopathic viral infections (Klenerman et al., 2000; Zinkernagel, 2000), such as HBV, or they may be present at a precursor frequency in the circulation too low to be differentiated from the background. Alternatively these cells might be compartmentalised in the liver, and so be undetectable in the circulation. It has previously been demonstrated that HBV-specific CD8+ cells in patients with a low level of HBV DNA and minimal liver injury (equivalent to our group B) are compartmentalised in

the liver (Maini et al., 2000). We wished to investigate whether localisation of cells to the liver, which is the site of maximal viral replication and antigen presentation, might explain the very low frequency of HBV-specific cells in the circulation found in this study.

Twelve HLA-A2+ patients with chronic hepatitis B (3 group C, 3 group D, 6 group E), who required a liver biopsy for diagnostic purposes, gave written consent for a research sample to be taken. Patients from groups A (resolved hepatitis B) and group B (low replicating chronic infection, normal ALT) were not biopsied, as is standard clinical practice. Three HLA-A2 negative patients, undergoing biopsy for the assessment of chronic hepatitis B, were studied as controls. The yield of intrahepatic lymphocytes varied between patients, and appeared influenced by a number of factors, including the size of the biopsy core, and the degree of liver inflammation. Typically $5 \times 10^4 - 5 \times 10^5$ T cells were purified from each biopsy. This low yield precluded the use of all the available HBV tetramers to quantify directly *ex-vivo* the frequency of intrahepatic HBV-specific CD8+ cells. Where a low number of T cells was isolated, tetramers specific for HBV core 18-27 were preferentially used, on the basis of our previous data showing the numerical dominance of CD8+ cells with this specificity in the circulation of patients with acute hepatitis B (Maini et al., 1999; Webster et al., 2000b), and in the livers of those with chronic infection (Maini et al., 2000).

As shown in figure 4.9, core 18-27+ CD8+ cells were found at very low frequencies (0.01-0.25% of total intrahepatic CD8+ cells) in the livers of patients with high levels of HBV DNA ($> 10^7$ copies/ml), whether with (group D), or without (group E) biochemical evidence of significant liver injury. This level was barely higher than was seen in the control group (0.02-0.04%). In contrast, a significantly higher proportion of intrahepatic CD8+ cells were specific for core 18-27 in group C, who had liver injury, but moderately controlled HBV DNA of less than 10^7 copies/ml (0.4 – 1.4% CD8 cells).

The low frequency of intrahepatic core 18-27-specific CD8+ cells, particularly in groups D and E, demonstrates that the very low circulating frequency of these cells cannot be explained by massive influx of these cells into the infected liver. Of note, sufficient

intrahepatic lymphocytes were isolated from single patients in groups C and D to allow staining with envelope 183-91 tetramers, as well as core 18-27. In both cases fewer than 0.1% of intrahepatic cells were specific for this epitope.

Figure 4.9.   Frequency of intrahepatic HBV-specific CD8+ cells in different patterns of HBV infection, analysed directly *ex-vivo* by tetramer staining and flow cytometry.
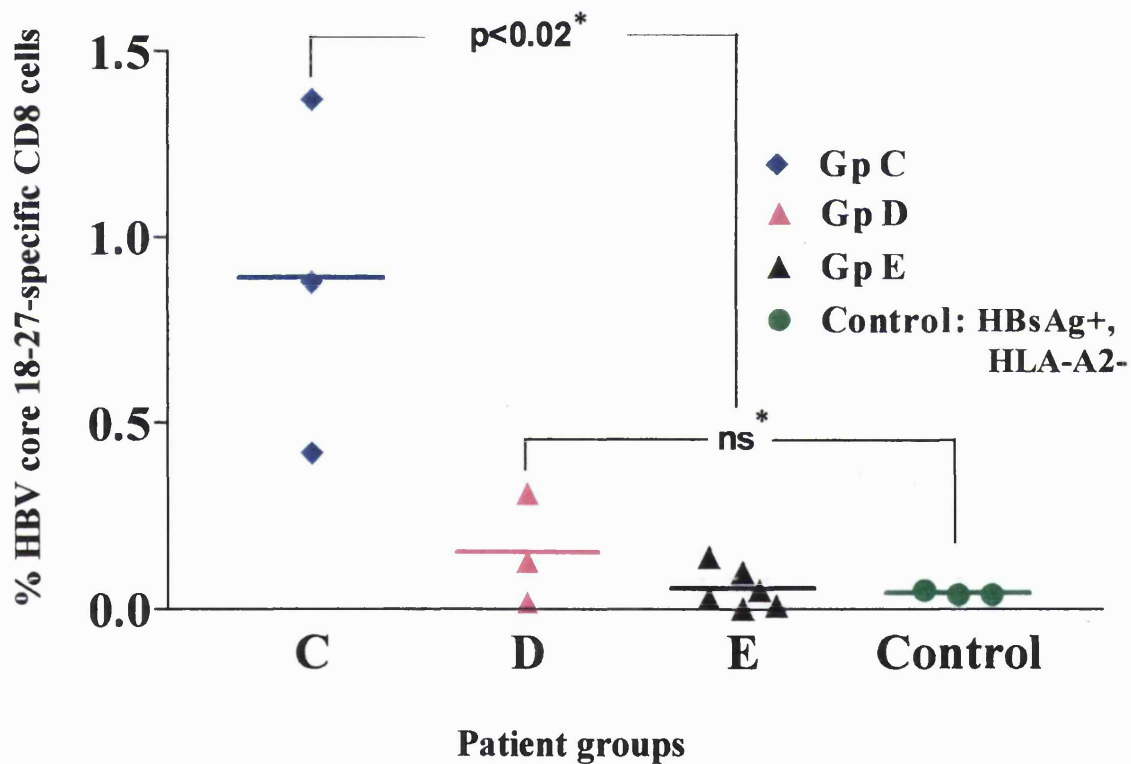
* Two-tailed Mann-Whitney test.

4.4 Discussion

Before discussing their implications in more detail, the results of this study are summarised below:

- In patients with resolved hepatitis B, HBV-specific CD8+ cells were isolated from the circulation, both directly *ex-vivo* and after *in-vitro* expansion. The response was multispecific, and was dominated by core 18-27-specific responses.

- In chronic infection the response was oligospecific, and the identification of HBV-specific cells required *in-vitro* peptide stimulation.

- HBV-specific CD8+ cell frequency appeared to correlate more closely with viral load than liver injury. An inverse correlation was seen between core 18-27-specific CD8+ cell frequency and serum HBV DNA.

- A clear difference in epitope hierarchy was seen between resolved acute and chronic hepatitis B. Epitopes which were usually subdominant in acute disease (eg envelope 183-91) were dominant in chronic infection, and core 18-27-specific cells were diminished or absent in the circulation. The absence of core 18-27-specific CD8+ cells was not explained by massive influx of these cells into the liver.

- Envelope-specific CD8+ cells persisted in chronic infection, despite the presence of high levels of non-mutated antigen.

- Analysis of acute flares of liver disease during chronic infection, associated with HBeAg seroconversion, demonstrated no pattern of HBV-specific CD8+ response similar to that seen in acute infection.

In subjects with a history of acute hepatitis B more than one year previously we directly quantified HBV-specific CD8+ cells in the circulation. HBV epitope-specific cells were found at a frequency of up to 0.32% of CD8+ cells, lower than the peak levels of more than 1% found in the acute clinical phase of infection (Webster et al., 2000b; Maini et al., 1999). Previous reports demonstrating the persistence of these cells relied on techniques dependent upon *in-vitro* stimulation, therefore precluding an analysis of the true frequency of these cells in the circulation (Penna et al., 1996). Using intracellular cytokine staining techniques to cover a broader range of epitopes, we demonstrated the presence of a multispecific response in these patients to a range of epitopes within HBV core, polymerase and envelope proteins, as has previously been reported (Chisari and Ferrari, 1995a); (Nayersina et al., 1993; Bertoletti et al., 1993). A number of studies have shown that patients who control HBV after acute infection are characterised by having clearly detectable HBV-specific CD4+ and CD8+ cell responses that persists long-term, after resolution of acute clinical hepatitis (Penna et al., 1996; Rehermann et al., 1996a). The demonstration that HBV DNA may persist for years after clearance of HBsAg from the serum (Michalak et al., 1994) has led to the argument that small quantities of virus maintain HBV-specific CTL, which in turn contribute to long-term inhibition of viral reactivation (Rehermann et al., 1996b). This hypothesis of an ongoing balance between virus and cellular immunity after apparent 'clearance' of infection, is also supported by the well documented examples of disease reactivation in patients with inactive hepatitis B who receive immunosuppressive therapy (Markovic et al., 1999). In patients with resolved acute infection the hierarchy of epitope-specific responses was stable over time, and similar to that previously shown in acute disease, with numerically dominant core 18-27 responses (Maini et al., 1999; Webster et al., 2000b). Core 18-27 CD8+ cells represent the specificity most commonly found in patients who control HBV acutely, and their presence is thought to be an important determinant of viral control or persistence (Bertoletti et al., 1997b).

In chronic hepatitis B circulating HBV-specific CD8+ cells were virtually undetectable on direct *ex-vivo* analysis, and so *in-vitro* stimulation was used to expand cell populations at low precursor frequency. This approach has been applied in the past to assess CTL responses, through the use of limiting dilution assays. In view of the maintenance of the

same hierarchy of CTL specificities both directly, and after expansion, in patients with resolved infection (in whom HBV-specific cells could be clearly quantified directly), it seems likely that the hierarchy of response in chronic patients after *in-vitro* expansion reasonably reflects the true hierarchy in the circulation. The finding that HBV-specific CD8+ cells generally expanded less well, and in response to a fewer number of peptides, in patients with chronic, compared with resolved infection, is consistent with previous reports. Nevertheless, a virus-specific CTL response to at least one epitope was demonstrated in 12 of 18 patients (66%) with chronic infection. This compares with previous studies showing a demonstrable CTL response in only 20% of untreated patients (Rehermann et al., 1996b). This difference might be explained by the refinement of methods to analyse CTL responses over the last few years, and the definition of more CTL epitopes.

A novel finding in this study was the demonstration of a different epitope hierarchy of CTL response in chronic infection, compared with resolved disease. Core and polymerase responses were subdominant, and the dominant responses were to envelope peptides, and in particular to envelope 183-91. In patients with low levels of HBV DNA and minimal liver injury (corresponding to our group B), it has been shown that core 18-27-specific cells are compartmentalised in the liver. Similarly, patients with high levels of virus and liver injury (like group D) have HBV-specific cells in the liver which are diluted within a large population of non-antigen specific CD8+ cells (Maini et al., 2000). We did not biopsy our patients in group B, but our findings in group D and E, of undetectable circulating core 18-27-specific cells directly and after *in-vitro* stimulation, and few virus-specific cells in the liver, suggests that these cells were absent from the circulation, due mainly to deletion or exhaustion, not due to massive influx into the liver. It is of interest that the patients in group C, who had the lowest level of virus of the patients biopsied in our study, had the highest frequencies of intrahepatic core 18-27 CD8+ cells, suggesting that this specificity may have a role in viral control in chronic, as well as acute, infection. It should be noted, however, that the highest frequency of core 18-27 CD8+ cells in the liver was found in the patient with a mutation within the position 27 residue, suggesting that these cells may have been exerting little viral control. The presence of HBV core 18-27-specific CD8+ cells in association with circulating virus carrying mutations within this epitope has been

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previously demonstrated, and clearly may represent a mechanism of viral escape (Maini et al., 2000). These viral variants may antagonise the action of CTL (Bertoletti et al., 1994b).

Data on the relative frequency of the different epitope-specific CD8+ cells within the liver in the different study groups is unavoidably incomplete, not least because of the unavailability of biopsy tissue from patients with resolved infection, and chronic low replicating disease with normal ALT. In view of the low yield of intrahepatic lymphocytes from needle biopsies, especially in those with minimal liver inflammation (group E), only enough cells to stain for a single epitope were often obtained. In view of previous data from the host laboratory suggesting the numerical dominance of core 18-27 CD8+ cells in acute disease (Webster et al., 2000b);(Maini et al., 1999), and of their presence in the liver in chronic infection (Maini et al., 2000), this specificity was preferentially chosen. Furthermore, the death of intrahepatic lymphocytes following peptide-specific stimulation ((Maini et al., 2000) and data not shown) precluded the expansion of a small precursor frequency of epitope-specific CD8+ cells, as was applied to peripheral lymphocytes. These differences are likely to be due to the terminal differentiation of antigen-specific cells within the liver. In view of the apparent dominance of envelope-specific CD8+ cells in the circulation in chronic infection, it would be interesting to see whether this is reflected within the liver.

During longitudinal analysis of patients with highly replicating chronic hepatitis B and liver injury (group D), we were fortunate to observe HBeAg seroconversion in two patients. This is reported to occur in 5-10% of patients each year, and may be associated with a clinical flare of liver injury indistinguishable from acute hepatitis (Hoofnagle et al., 1981; Lok et al., 1987; Liaw et al., 1983). Previous studies have shown that the exacerbation of liver injury may be accompanied by increased CD4+ cell proliferative responses (Tsai et al., 1992), suggesting that previously ineffective immune mechanisms in chronic HBV infection can be activated. Rehermann et al studied CTL responses in chronically infected patients who cleared HBsAg, and showed a similar pattern of response to that observed in resolved acute infection. However, these patients were not studied longitudinally, but underwent cross-sectional analysis an undefined time after seroconversion, which occurred

either spontaneously or in response to therapy (Rehermann et al., 1996b). In our patients no clear pattern of CTL response was seen in association with ALT flares or viral control. This might be due to a range of factors, including the principal involvement of non-HLA2-specific CTL, or other cell types, such as NK or NK-T cells.

In resolved acute hepatitis B the hierarchy of epitope specific responses was very stable over time. The same was not true in chronic disease, where epitope specific responses appeared and disappeared, and the hierarchy, as characterised by the relative quantification of epitope-specific CD8+ cells, varied at different timepoints. It is tempting to theorise that these dynamic changes might occur in response to the emergence of viral escape mutants. In fact longitudinal analysis suggests that the selection of CTL escape mutants is probably an unusual event during HBV infection (Rehermann et al., 1995c). Although our data, to date, do not include longitudinal sequence analysis, few mutations were seen within CTL epitopes at baseline, suggesting that CTL escape mutants were unlikely to have made a major contribution to long-term viral persistence in these patients. Multispecificity could have the important advantage of diminishing the likelihood of selecting CTL-escape variants (Chisari and Ferrari, 1995a).

The relevance of the differences in CTL specificity between acute and chronic infection remain uncertain. However, the quantity of particular epitope-specific CD8+ cells cannot be assumed to correlate with their functional importance. A number of recent reports have shown that CTL specific for different epitopes could have markedly different impacts on the control of virus replication (Goulder et al., 2001), and that (Allen et al., 2000)virus-specific CD8+ cells may be in different functional states in different patterns of infection (Welsh, 2001; Zajac et al., 1998; Lechner et al., 2000a; Gallimore et al., 1998b). In macaques infected with simian immunodeficiency virus (SIV), for example, while the CTL response present in the acute phase is directed against two different epitopes in the Tat and Gag regions, only the epitope in Tat is under strong immune selective pressure (Allen et al., 2000). Similarly, in chronic HIV infection CTL specific for epitopes within p17 Gag are strongly associated with relative viral control and reduced disease progression, but these CTL are not associated with the initial CTL response in acute infection (Goulder et al.,

2001). In parallel with these findings, the data presented here also suggests that the CTL response in chronic HBV infection is not simply a 'diluted' version of that present in acute disease, in that the specificity numerically dominant in chronic disease (env 183-91) seems to play relatively little role in acute infection, and core 18-27, which is dominant in acute and resolved infection, is usually subdominant in chronic infection.

These findings related to CTL epitope hierarchy may be of some relevance to the development of new therapies for hepatitis B. Present therapies for chronic hepatitis B carry a relatively low probability of initiating HBeAg seroconversion, are expensive, and carry the potential of side-effects and treatment complications (Dienstag et al., 1999; Hoofnagle et al., 1988). In recent years increasing interest has been directed towards the development of immunotherapy for chronic HBV infection, based on the concept of boosting the weak HBV-specific T cell response that is typically found in chronically infected patients (Ganem, 1998). The chances of realising the goal of this strategy have increased following the demonstration that chronically infected patients with lamivudine leads to an increase in the circulating frequency of both HBV-specific CD4+ (Boni et al., 1998) and CD8+ cells (Boni et al., 2001). This restoration of virus-specific immunity might provide the perfect platform from which to boost HBV-specific T cell responses, but the selection of ideal HBV epitopes to be considered for CTL immunotherapy remains uncertain. Specificities most likely to exert immunological pressure would clearly be favoured candidates. The dominance of a strong HBV-core CD8+ response in subjects with acute and resolved infection, its inverse correlation with HBV DNA, and its reduction/absence in chronic infection might all suggest that it would be the obvious choice. However, as CTL vaccines are intended to augment a pre-existing epitope-specific CD8+ cell response, the impaired expansion of core 18-27-specific cells in chronic HBV infection shown here points against a potential role for stimulating this response to achieve viral control. In line with this data is the report of the limited therapeutic success of the Theradigm-vaccine, based on the single HBV core 18-27 epitope (Heathcote et al., 1999). Would envelope epitopes, which have been shown to expand in chronic hepatitis B, be better candidates for inclusion in therapeutic vaccines? Perhaps, but the presence of CD8+ cells with this specificity, in the setting of high antigen load, suggests that they may exert limited viral control. Virus-

specific T cells may persist in the presence of antigen due to anergy, impaired cytolytic activity, and/or an inability to produce IFN-γ (Welsh, 2001). Experiments are underway in the host laboratory to better define the functional characteristics of these env 183-91-specific cells, which appear to bind tetramers poorly (Reignat et al., 2002). Despite possible functional defects in these cells, DNA vaccines based on envelope antigens have recently been shown to reduce levels of HBsAg in the chimpanzee (Pancholi et al., 2001) and in other animal models of hepatitis B (Shimizu et al., 1998; Mancini et al., 1996; Pol et al., 1994; Akbar et al., 1997; Rollier et al., 1999; Oka et al., 2001), providing some support for attempts to stimulate envelope-specific CTL to achieve viral control.

Once specific epitopes have been selected for inclusion within treatment vaccines, careful consideration would need to be given to the problem of balancing the stimulation of specific-immune response to the quantity of infectious virus. This is because, as discussed earlier (section 1.5.6), the exposure of virus-specific CTLs to a quantity of virus that exceeds a certain threshold may trigger a cascade of events which lead to liver damage, mainly through the action of non-antigen specific cells (Moskophidis and Kioussis, 1998). For this reason, the use of antiviral drugs to inhibit viral replication prior to an immuno-therapeutic approach may be beneficial, not only because of their role in restoring HBV-specific T cell responsiveness in chronic patients (Boni et al., 1998);(Boni et al., 2001) but also in order to reduce the quantity of viral antigen. This strategy might result in the reconstitution of HBV-specific CD8+ cells in an environment of reduce viral antigen, such that they effectively inhibit viral replication without activating pro-inflammatory pathways leading to the recruitment of large numbers of non-antigen-specific inflammatory cells and ultimately to liver damage.

CHAPTER 5

CONCLUDING REMARKS

In this thesis I have addressed a range of epidemiological, clinical, and immunological aspects of HBV infection. This opportunity was initially presented by the identification of a large and unusual outbreak of acute infection, related to an alternative therapy clinic. Effective collaboration between a number of health care teams allowed the probable mechanism and source of infection to be defined, and for infected individuals to be identified and treated, as necessary. These prompt measures probably reduced secondary spread of the virus. The use of viral sequencing and phylogenetic analysis provided an important aid to the epidemiological investigation, as it allowed infected cases to be definitively linked to the common source. In the past, linkage of cases has relied heavily on serological data and clinical information concerning infective risks. The outbreak demonstrated the risks of parenteral virus transmission through unregulated skin-piercing practices, and the public health issues surrounding hepatitis B vaccination when this is targeted only towards those perceived to be at risk, and not to the population as a whole.

The prompt testing of clinic attendees also led to the identification of individuals during the incubation phase of acute HBV infection. This provided a unique opportunity to study the pattern of dynamic change in viral level, liver injury, and host immune response, during the crucial early phase of infection. Using HLA class I tetramers, HBV-specific CD8+ cells were demonstrated in the circulation for several weeks prior to symptomatic acute hepatitis, during the period of significant reduction in viraemia, which occurred prior to maximal liver injury. Elevated levels of HBV-specific CD4+ cells and NK cells were also found at an early stage, confirming that the incubation phase of hepatitis B is not simply a period of unchecked viral replication, but represents a crucial period of host-virus interaction, which may determine viral persistence or control.

Having identified circulating HBV-specific CD8+ cells, directly *ex-vivo*, during the incubation phase of hepatitis B, and shown their temporal association with viral control and liver injury, similar techniques were then applied to the study of cellular immune responses in different patterns of resolved and chronic HBV infection, with groups characterised according to patterns of liver injury and viral replication. It was shown that the CTL response in chronic infection was not simply a weak version of the multispecific response seen in acute and resolved disease. Instead, a completely different epitope hierarchy was demonstrated, with the persistence of CD8+ cells specific for epitopes which were sub-dominant in resolved infection, and the absence of core 18-27-specific cells, which dominated in acute infection.

Although the focus of this work has been on virus-specific CD8+ cells these are, of course, only one of the cellular components likely to be important in determining viral control or liver injury. Indeed some of the findings lend further support to the argument for a revision of the traditional view of HBV-specific CD8+ cells being the direct cause of both virus control and liver injury. In particular, the inability to identify HBV core-specific CD8+ cells in active chronic hepatitis B or during acute flares suggests that other cell types may be important, and that seroconversion episodes in chronic infection, despite the clinical similarities to acute disease, may occur through entirely different immunological processes (Perrillo, 2001). Experience from adoptive transfer studies suggests that reconstitution of the whole repertoire of anti-HBV immunity leads to effective resolution of chronic hepatitis B (Lau et al., 2002). The importance of CD4+ cell responses in promoting CTL-mediated viral control has been shown for a range of infections (Zajac et al., 1998), including hepatitis B (Lau et al., 2002; Marinos et al., 1995), and the finding in this work of core-specific CD4+ cells in the incubation phase of infection supports this. The lack of correlation of CTL with acute flares and seroconversion, and the early peaks of circulating NK cells seen in acute disease, are also consistent with the emerging data on the importance of NK and NKT cells in viral control and liver injury in viral hepatitis (Kakimi et al., 2000; Baron et al., 2002; Trobonjaca et al., 2002).

Most of the experimental work here relates to cells isolated from the circulation. Yet effective viral control undoubtedly depends on more than having a significant population of functionally active antigen-specific CD8+ cells within the circulation; they also need to be 'in the right place, at the right time'. As discussed earlier, similar absolute numbers of HBV-specific CD8+ cells within the liver may be associated with very different patterns of chronic hepatitis B (Maini et al., 2000). Whether these cells promote viral control or tissue injury is likely to depend not only on their activation status, but also on their ability to specifically localise to the site of replication within the liver. This may in turn depend upon the production of a range of chemokines from target tissues, and the presence of corresponding chemokine receptors on the surface of CD8+ cells. In chronic HCV infection, IFNγ production by sinusoidal epithelium has been shown to promote the production of inducible protein-10 (IP-10) and chemokines, leading to the recruitment of chemokine receptor-CR3 (CXCR3)-expressing CD8+ cells, and subsequent lobular inflammation. In contrast, CCR5-expressing CD8+ cells appeared to be confined to the portal tracts (Shields et al., 1999). Despite the importance of defining the anatomical site of intrahepatic CD8+ cells in chronic hepatitis, and their activation characteristics, studies to date have not been able to define the exact distribution of the antigen-specific cells *in-situ*. This is of particularly relevance when considering that usually less than 1% of CD8+ cells extracted from the HBV infected liver are HBV-specific (Maini et al., 2000). Relatively large three dimensional class I tetramers appear to be poorly accessible to the lymphocytes within blocks of liver tissue. Nevertheless, the refinement of tetrameric complexes, to allow the *in-situ* staining of antigen-specific T cells in liver tissue (Skinner et al., 2000), could represent an important advance in the study of the immunopathogenesis of viral hepatitis.

The development of new tools to quantify virus-specific CD8+ cells has led, perhaps paradoxically, to an appreciation that cell function, rather than cell numbers, is likely to be the vital determinant of their anti-viral effect (Zajac et al., 1998; Webster and Bertoletti, 2001). The activation status of tetramer-positive cells may be clearly demonstrated during acute viral infection, when the direct *ex-vivo* cell frequency is at its greatest (Maini et al., 1999; Murali-Krishna et al., 1998), but is harder to define when the cell frequency is barely

above background levels, as seen in patients with chronic infection. However, it may be possible to define important differences in the *in-vivo* function of CD8+ cells, on the basis of their behaviour following peptide stimulation *in-vitro*. For example, data within the host laboratory suggests that the env 183-91-specific CD8+ cells that we have demonstrated in chronic hepatitis B may be functionally different to those found in acute infection, in particular displaying altered tetramer binding (Reignat et al., 2002). The ability to bind tetramers has recently been linked to the proper organisation of the TCR with membrane associated lipid rafts (Spencer and Braciale, 2000), suggesting that these cells might possess an alteration in the lipid raft organisation, with important implications for T cell activation (Drake, III and Braciale, 2001). Experiments will be needed to evaluate in detail the functional characteristics of these cells. It will be particularly interesting to know whether the functional characteristics of these cells change over time, either spontaneously or in response to therapy, as the reconstitution of cells with a greater 'anti-viral efficiency' might have important implications for the future treatment of chronic hepatitis B.

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