

The Cytotoxic response to HBV.

A dissertation submitted for the degree PhD.

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by

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Dedication.

To Michael and Dad.

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Abstract.

Successful elimination of hepatitis B virus (HBV) during acute infection involves raising an effective MHC class I restricted cytotoxic (CTL) response against viral antigens expressed by infected cells, particularly the nucleocapsid antigens.

In a patient with chronic HBV infection, nucleocapsid specific class I restricted CTL were detected in cytotoxicity assays following depletion of CD4+ cells from the effector lymphocyte population. Low level MHC class I restricted cell lysis was also detected during HBeAg/ HBe antibody seroconversion, but a statistical analysis of the data obtained by cytotoxicity assay on lymphocytes derived from sixty patients with chronic HBV, demonstrated that the cellular response occurring during HBeAg/anti-HBe seroconversion, although antigen specific, was not primarily MHC class I restricted. It was not possible to fully characterise the response, but it was most effectively elicited by particulate rHBcAg, and to a lesser degree by HBcAg derived peptides.

Antigen presenting cells derived from chronic carriers were demonstrated to process and present nucleocapsid antigens as efficiently as those derived from acute HBV patients when transfected with an expression vector which encoded for the HBe antigen. This contrasts to the unresponsiveness observed when exogenous HBcAg is cultured with lymphocytes derived from chronic carriers in standard lymphocyte proliferation assays, suggesting that a state of partial tolerance was overcome when HBcAg (or HBeAg) was introduced directly into the endogenous processing pathway, indicating that the mode of processing and presentation of the nucleocapsid antigens is critical in determining the course of the disease.

High level anti-HBe lysed cells which express HBeAg, in the presence of complement. This constitutes a mechanism by which tissue damage may occur during hepatitis, but no correlation between anti-HBe in serum, and antibody mediated HBeAg/anti-HBe seroconversion, could be made.

Abbreviations.

ADCC	antibody dependent cellular cytotoxicity
ALT	serum alanine transaminase
APC	antigen presenting cell
AST	serum aspartate transaminase
BuDr	5-bromo-deoxyuridine
CAH	chronic active hepatitis
CD	cluster differentiation
CD4+	CD marker of human cytotoxic T lymphocytes
CD8+	CD marker of human helper T lymphocytes
CPE	cytopathic effect
CPM	counts per minute
CPH	chronic persistent hepatitis
CR	complement receptor
CTL	cytotoxic T lymphocytes
DC	lymphoid dendritic cells
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotides
EBO	Epstein Barr virus origin of replication
EBV	Epstein Barr virus
ELISA	enzyme linked immunoassay
ER	endoplasmic reticulum
FACS	fluorescence activated cell scanner
FcR	human antibody Fc receptor
HAV	hepatitis A virus
HBV	hepatitis B virus
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HBxAg	hepatitis B X antigen
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
ICAM	intracellular adhesion molecule
IFN	interferon
IL	interleukin
Ig	immunoglobulin
kb	kilobase
kD	kilodalton
K:T	killer to target ratio (cytotoxicity assay)

LAK	lymphokine activated killer
LB	lymphoblastoid cell (EBV transformed B cell)
LFA	leucocyte function associated antigen
LCMV	lymphocytic-choriomeningitis virus
MOI	multiplicity of infection
Mϕ	macrophage
LH	lobular hepatitis
MHC	major histocompatibility complex
ml	millilitre
mRNA	messenger RNA
NK	Natural Killer cell
ORF	open reading frame
PBL	peripheral blood lymphocyte
PCR	polymerase chain reaction
pfu	plaque forming units
PHA	phytohaemagglutinin
rHBcAg	recombinant hepatitis B core antigen
RNA	ribonucleic acid
rV	recombinant vaccinia virus
TCR	T cell receptor
TNF	tumour necrosis factor
μM	micromolar
pM	picomolar
wt	wild type vaccinia virus
μg	microgram

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Chapter One. The cytotoxic immune response to viruses.

(1) Accessory cells and intercellular signalling.

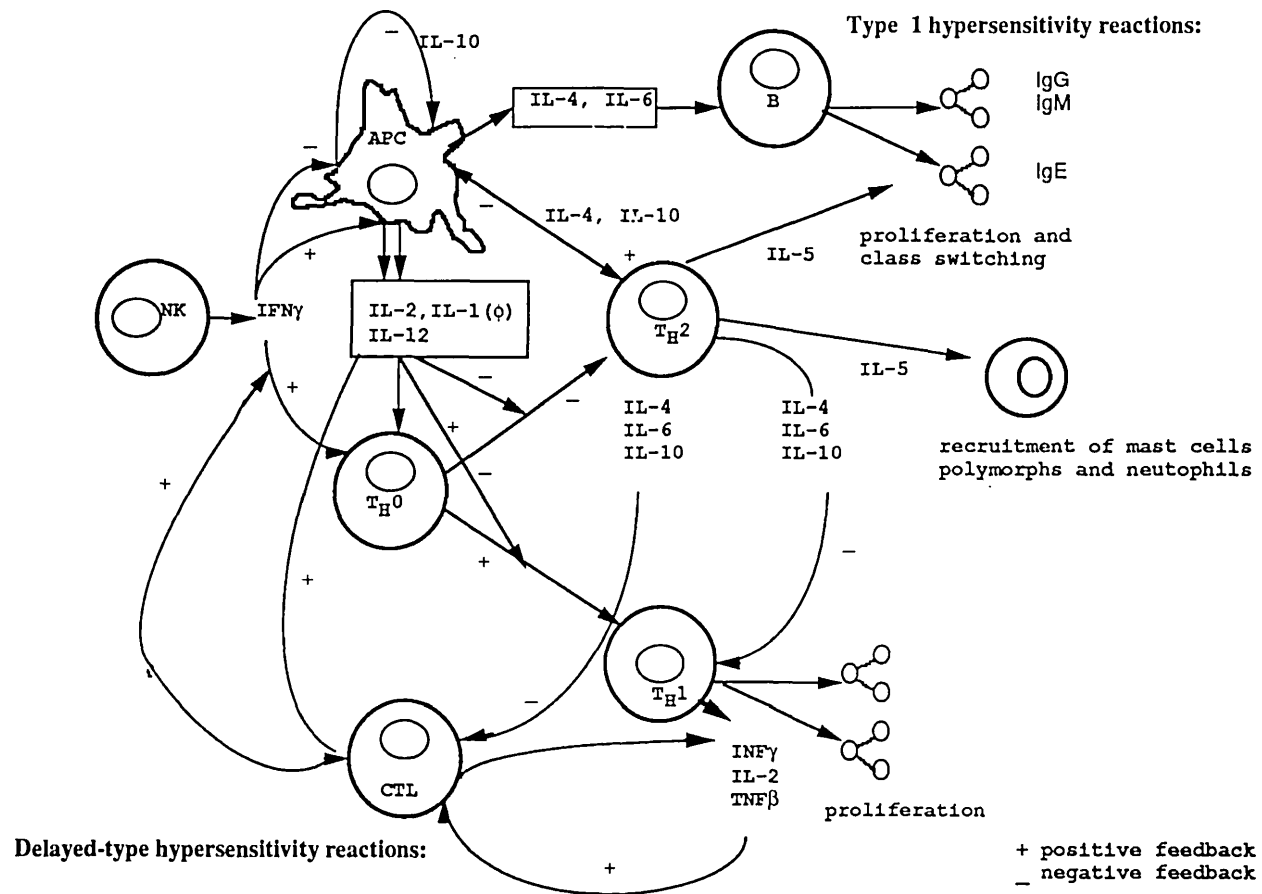
Elimination of infected cells during acute viral infection is effected by a complex array of antigen specific and non-specific immune surveillance cells, and regulatory cytokines (Fig 1). The non-antigen specific accessory cells such as macrophages and lymphoid dendritic cells (DC) (Steinman and Cohn, 1973) reviewed (Austyn, 1987), are an essential component of the response, presenting antigens to lymphocytes, and secreting regulatory cytokines and cytolytic factors. Activated macrophages present antigen in an MHC restricted manner (Unanue and Allen, 1987) and produce IL-1, a promiscuous activator originally viewed as the key immune signal, stimulating an immediate innate response, recruiting mast cells, polymorphs and neutrophils to the site of pathogen invasion.

DC can stimulate primary responses in both helper (CD4+) and cytotoxic (CD8+) T cells *in vitro* and *in vivo* (Knight et al, 1985), and can induce proliferation of CD8+ cells, even in the absence of CD4+ cells (Sprent et al, 1985, Inaba et al, 1987).

Activated DC stimulate proliferation of cells selected via antigen-specific T cell receptor engagement, and secrete interleukin 2 (IL-2), IL-4, and possibly several other cytokines. IL-2 stimulates proliferation of primed and naive helper and cytotoxic cells, maturation of natural killer (NK) cells, and induction *in vitro*, of lymphokine activated killer cells (LAK) (Grimm et al, 1982). It also stimulates B cells to produce antibodies, which, in a first line of defence against pathogens, can bind foreign antigens facilitating complement lysis and macrophage engulfment.

Activated CD4+ cells (Th0; possibly undifferentiated cells, Th1 and Th2) secrete a spectrum of cytokines which regulate activity of the immune system (reviewed Mossman and Coffman 1989), and are characterised by cytokine production. The Th1 subclass secretes cytokines associated

Fig 1. The interaction between lymphocytes and cytokines (adapted from Trincheri, 1993).



with delayed type hypersensitivity including IL-2, interferon- γ (IFN γ), and tumour necrosis factor- β (TNF β). IFN γ has direct anti-viral activity via induction of 2,5,A synthetase which degrades cellular mRNA, in addition to its immuno-regulatory functions such as stimulation of increased surface expression of MHC class I, and receptors for human antibody Fc domain (FcR). CD8+ cells produce a similar range of anti-viral cytokines as TH1 cells (IFN γ , TNF β , reviewed, Ramsay et al, 1993). The TH2 subclass produces IL-3, IL-4, IL-5, IL-6, and IL-10, cytokines associated with immediate or type 1 hypersensitivity, infiltration of mast cells and polymorphs, B cell maturation, isotype switching, production of acute phase proteins in the liver (IL-6), and inhibition of cytolytic production by the action of IL-10 on APC.

The mechanism of antigen processing in DC has not been fully elucidated. DC were originally characterised as non-phagocytic, but they can degrade whole exogenous antigen for presentation to T cells (Kaye et al, 1985) possibly through the action of surface endopeptidases. After the initial degradation, antigen appears to enter the standard (lysosomal) exogenous pathway, inhibited by chloroquine (Chain et al, 1986).

(2) MHC restriction of cytotoxic T cells.

Zinkernagel and Doherty (1974), investigating the cytotoxic reaction to murine lymphocytic choriomeningitis virus (LCMV) infection, demonstrated that lysis of target cells occurred only when target and effector were matched at common loci of the H-2 complex. The phenomenon was observed with other pathogens in mice, and subsequently humans (reviewed Zinkernagel and Doherty 1979) and termed "Major Histocompatibility Complex" (MHC) restriction. The human response was restricted by the human leucocyte antigens (HLA) A, B and C. MHC molecules are surface glycoproteins defined (in man) as MHC class I and MHC class II. The class I molecule consists of a highly polymorphic α chain noncovalently bound to β_2 microglobulin, expression

is ubiquitous, but levels vary during the course of viral infection. The class II molecule consists of two noncovalently linked polymorphic chains (α and β), and is expressed on lymphoid and antigen presenting cells.

Virally primed CTL have been isolated from the periphery during the course of a number of human viral infections. These include: influenza, (McMichael et al, 1986); respiratory syncytial virus (Bangham et al, 1986) herpes simplex (Zarling et al, 1986), HIV (Nixon et al, 1988), Epstein-Barr Virus (EBV) (Lakhadar et al 1984), cytomegalovirus (Borysiewicz et al, 1987), varicella zoster (Diaz et al, 1989), measles (Kreth et al, 1979), acute hepatitis B (HBV) (Bertoletti et al, 1989), hepatitis C virus (Shirai et al, 1992), Friend virus (Holt et al, 1984). To effect lysis of autologous cells expressing viral antigen, *in vitro* stimulation is almost always essential, and appears to be dependent on the precursor frequency of primed CTL. DC express class I and II molecules, and are an extremely potent stimulant of antigen-specific T cell responses.

(3) Cytotoxic T cells recognise degraded antigen on the surface of cells.

Townsend et al (1984, 1985), investigating the immune response to influenza, demonstrated that CTL recognised degraded fragments of protein bound to MHC molecules on the surface of cells which were not recognised by antibodies raised against proteins with conformational determinants. One of the most important tools for the study of individual viral antigens was the development of recombinant vaccinia viruses expressing foreign gene products which are synthesised in the cytosol of permissive cells and processed naturally for presentation to cytotoxic cells *in vitro* (reviewed Moss and Flexner 1987). It soon emerged that both glycosylated and non-glycosylated (including viral nucleocapsid and non-structural) proteins were displayed on the cell surface in a manner identified by CTL. Townsend et al (1986a) demonstrated the existence of

a signal-independent channel for the passage of processed peptides to the cell surface. They also demonstrated that the processing requirement antigen presentation to CD8+ cells, could be by-passed by coating target cells with peptides 9 to 25 amino acids in length (Townsend et al, 1986b).

Figure 2 summarises the known interactions between T cells and antigen-presenting or virally infected cells. Antigens presented by MHC molecules are recognised by T cell receptors (TCR) on allogeneic cells (reviewed Marrack and Kappler 1987). The stimulatory signal associated with engagement of the TCR is transmitted via the closely associated CD3 complex. CD8+ T-cells are cytotoxic effectors which, within minutes of recognition of the target through the TCR / MHC class I interaction, induce specific target cell lysis via perforin polymerisation (Marz and Benacerraf 1973).

Different processing mechanisms are utilised for class I and II presentation (Morrison et al, 1986). Inhibitors which block movement of membrane proteins through the secretory pathway from the endoplasmic reticulum (ER) to the Golgi apparatus, such as brefeldin A, block class I processing (Fujiwara et al, 1988, Cox et al, 1990). Inhibitors of protein synthesis have a similar effect. Lysosomotropic agents which block degradation of exogenously derived antigen and/or class II assembly, have no effect on the synthesis and display of class I molecules loaded with peptide. This led to the conclusion that proteins which stimulate a class I restricted response are endogenously derived, either produced by the cell itself or by an intracellular pathogen. Analysis of cell lines with defects in the class I presentation pathway revealed molecular aspects of processing. Proteinase complexes in the cytoplasm break down proteins (Rivertt et al, 1989; Orłowski et al, 1990), into short peptides which are transported to the endoplasmic reticulum (ER) in a signal-independent manner via peptide transporter proteins (reviewed Monaco, 1992), to the cell surface. The transporter protein genes are located within the MHC class II gene complex (Trowsdale et al, 1992) and have been

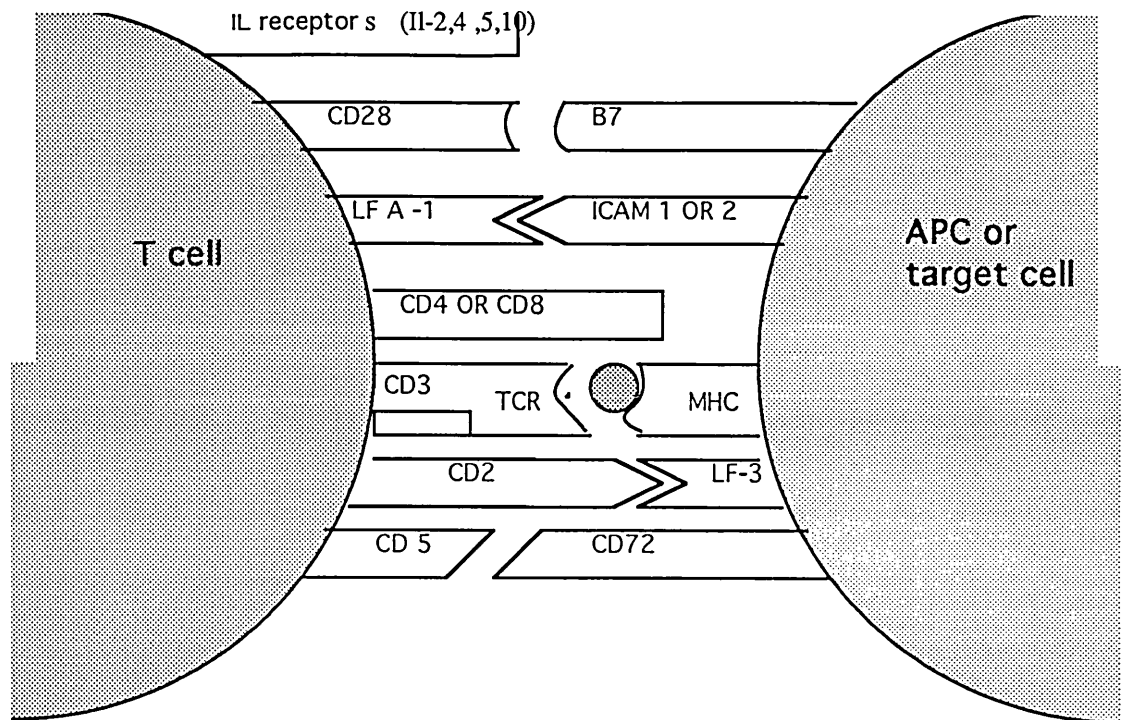
postulated to play a role in selecting the peptides presented by class I molecules.

High resolution X-ray crystallography has determined the structure of three class I molecules, HLA-A2.1, Aw68 and B27 (Bjorkman et al, 1987a, 1987b; Garret et al, 1989). A deep groove runs between the $\alpha 1$ and $\alpha 2$ domains of the α chain. The base and sides of the groove are formed by the β -pleated sheet and alpha helical regions of $\alpha 1$ and $\alpha 2$ respectively. Most of the polymorphic residues defining alleles line the bottom of the groove.

On analysis of the B27 molecule, the groove was seen to be loaded with a "self" peptide 9, plus or minus 1, amino acids in length (Madden et al, 1991). Viral peptides occupy a similar position in molecules derived from HLA A2 virally infected cells (reviewed Madden et al, 1993). Complementary studies of peptides eluted from MHC molecules immunoprecipitated from influenza A infected cells (Rotzschke et al, 1990) demonstrated "motifs" specific to each molecule. The peptides eluted from HLA A2 molecules had anchor positions at residues 2 and 9 numbering from the amino terminal (Falk et al, 1991), longer bound peptides (10-11 amino acids) bulge out of the middle of the MHC molecules (Guo et al, 1992; Parker et al, 1992). The existence of "motifs" (reviewed Stern and Wiley, 1994), suggests that MHC alleles play a major role in determining the particular viral epitopes presented.

Soluble exogenous antigens (excepting peptides) do not stimulate cytotoxic (CD8+) T cells, but particulate antigens with no known route of entry to the cytosol have been reported to do so (Gooding et al, 1980; Carbone et al, 1990; Debrick et al, 1991; Reddy et al, 1992). Peifer et al (1993) suggested an alternative method of phagocytic processing in which antigen becomes associated post-Golgi with class I MHC molecules, either by peptide exchange or "regurgitation" of peptides from class I molecules on the surface of cells.

Fig 2. Schematic representation of the interactions between the T cell receptor and target or antigen presenting cells.



Adapted from Frazer et al, 1993

(4) Natural Killer cells.

Mononuclear lymphocytes of the innate immune response mediating non-MHC restricted lysis are less well documented than the CD8+ / class I system, but evidence is accumulating that non-MHC restricted clearance of virally infected cells is significant in some viral infections, particularly before acquired immunity has developed. Lymphocytes without the $\alpha\beta$ T cell receptor include $\gamma\delta$ T cells, natural killer cells (NK) and large granular lymphocytes, the latter both have anti-tumour and anti-viral effects *in vitro*, but less well defined roles *in vivo*.

NK are a heterologous population of cells bearing FcR receptors (Perussi et al, 1989) and some specific cell surface markers which include CD11, CD16 and CD56 (reviewed Trinchieri et al, 1989). NK were originally defined as having anti-tumouricidal activity, but also lyse virally infected cells (Santoli et al, 1979; Biron et al, 1989). Lymphocytes cultured with IL-2 can mediate an analogous function (Grimm and Schaub, 1991). NK may mediate antibody dependent mechanisms of cell lysis.

(5) Antibody mediated immunopathology.

Antibody mediated immunopathology may be caused directly by immune complex deposition and complement mediated damage. Circulating immune complexes have been identified concurrent with viral infection, including HBV infection (Dienstag et al, 1981). Tissue damage may be indirect, via FcR by effector cytotoxic cells. Three distinct subsets of FcR specific for IgG have been identified, Fc γ RI (CD64), Fc γ RII (CD32), and Fc γ R III (CD16) on the surface of lymphocytes and phagocytic cells. CD64 has a sufficiently high affinity for IgG to bind monomeric antibody, the other molecules recognise complexes. Some viruses such as Sinbis, vesicular stomatitis, measles and RSV (Kaul et al, 1984) can directly activate complement via the alternative pathway. Cells particularly susceptible to lysis bear receptors for activated components of the complement cascade. EBV infection of B lymphocytes for example, produces cells that directly activate the alternative complement cascade (McConnell et al, 1978).

Activation of the classical pathway requires antibody. Most viruses, including HBV, display viral antigen on the surface of infected cells in a form detectable by immune sera, (Schlicht et al, 1990) therefore, are theoretically susceptible to lysis by activation of the classical complement pathway. HIV infected lymphocytes bind complement component C3b in the presence of anti-HIV. Lysis of infected cells may be enhanced by cells which bear

complement receptors, but evidence of direct complement mediated lysis is contradictory (Lederman et al, 1989; Spear et al, 1990).

Antibody dependent cellular cytotoxicity (ADCC) mediated by killer cells is an ill defined mechanism of cell lysis, thought to involve FcR receptor cross linking by antibody bound to cell surface expressed antigens. A postulated recognition mechanism is via CD16 on NK.

ADCC has been implicated in HIV infection reviewed by Nixon et al, (1992) and HTLV I and II viral clearance (Zhang et al, 1992).

Chapter Two. An Introduction to the Hepatitis B virus.

(1) *HBV isolation and structure.*

The first causative agent of human viral hepatitis to be identified was the Hepatitis B virus (HBV).

In 1970, Dane and co-workers isolated an infectious hepatitis B virion, 42nm in diameter. The virus particles (Dane particles) comprise an outer lipoprotein envelope of surface antigen (HBsAg) and an inner 27nm nucleocapsid of core protein (HBcAg), which was obtained by non-ionic detergent treatment of Dane particles (Almeida et al, 1971), and contained the viral DNA and polymerase (Kaplan et al, 1973). HBsAg is produced in vast excess during viral infection and self-assembles into non-infectious 22nm spherical and rod-shaped particles (Dane, 1970, Robinson et al, 1976) which can be detected in the serum of infected individuals. Four major subtypes of HBsAg adw, ayw, adr, and ayr were identified by serology (Le Bouvier, 1971; Bancroft et al, 1972), and now denote the different phenotypes of the virion.

A second antigen detected in serum during HBV infection, was identified by Magnus and Epsmark in 1972, and termed Hepatitis B "e" antigen (HBeAg). Its presence was strongly associated with the detection of infectious virions (Nordenfeld et al, 1975; El Sheik et al, 1975; Takahashi et al, 1976; Imai et al, 1976).

HBV is classified as a member of the *hepadna viridae* (Robinson 1982), a small group of enveloped DNA viruses which replicate through an RNA intermediate, and have hepatotropic sequelae. In 1975, Summers et al, established the structure of the viral genome to be a 3.2 kb DNA circle.

Molecular cloning of the viral genome in *E.coli* (Valenzuela et al, 1979; Charnay et al, 1979; Galibert et al, 1979; Sninsky et al, 1979; Ono et al, 1983) and sequence analysis (Pasek et al, 1979; Siddiqui et al, 1979; Tiollas et al, 1981) revealed a complex and compact genetic organisation. The genome is a partially double

stranded DNA circle held together by base pairing at two direct repeat areas (DR1 and DR2). The long strand, L(-), is linear and 3,200 base pairs in length. It has four major open reading frames (ORFs) in the same transcriptional orientation, which overlap but are conserved "in frame" in different HBV subtypes (reviewed Ganem and Varmus, 1987). The open reading frames are designated ORF S, ORF C, ORF X and ORF P, (following the terminology adopted by Tiollais) and code for several proteins. ORF S encodes HBsAg and two additional envelope proteins termed pre-S1 and pre-S2.

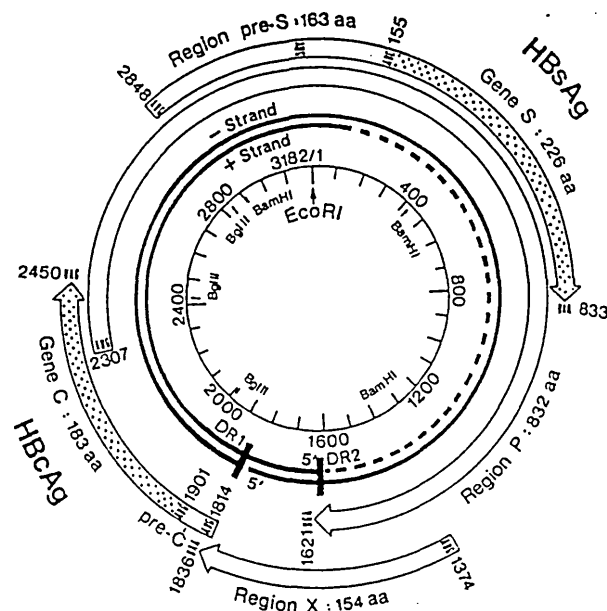
ORF C codes for HBcAg, which forms the nucleocapsid, and a short in frame sequence which when read in conjunction with the core gene produces a 25 Kd polypeptide (Pasek et al, 1979) pre-core/core, which is subsequently processed to produce HBeAg. HBcAg is a 21Kd phosphorylated protein (Gerlich et al, 1982), 183-185 amino acids long, with an arginine rich carboxy terminal essential for nucleic acid packaging (Nassal et al, 1992). HBcAg aggregates spontaneously with approximately 180 copies forming one nucleocapsid (Feitelson et al, 1982; Onodera et al, 1982). Recombinant core antigen produced in *E.coli* (Cohen and Richmond 1982, Stahl et al, 1982) aggregates to form particles morphologically identical to those isolated from serum.

ORF X encodes a nonspecific transactivator (Twu and Schloemer, 1987). ORF P codes for the viral polymerase and reverse transcriptase (Ou et al, 1990).

(2) *Transcription and replication of the viral genome.*

Viral replication takes place within the nuclei and cytoplasm of infected cells, in an extrachromosomal manner (Seeger et al, 1986). Control of replication of the genome is complex. HBV transcription is regulated by at least four promoters and two enhancers (reviewed Ganem and Varmus 1987; Gerlich 1994).

Fig 3. The Hepatitis B genome.



From Tiollais et al, (1985).

The partial restriction map and numbering corresponds to the ayw3 genome. The broad arrows represent the four large open reading frames of the (-) strand transcript.

On hepatocyte entry, the partially double stranded virus is converted to double stranded then covalently closed circular DNA (cccDNA). The cccDNA is located predominantly in the nuclei, and serves as a template for synthesis of several messenger RNAs encoding the viral proteins. The major viral transcripts are 3.5, 2.4 and 2.1 kb in length, and encode the nucleocapsid and polymerase, the pre-S1, pre-S2 and HBsAg, and the X mRNA sequences respectively. The 3.5kb has been reported as having differing 5' ends (Rall et al, 1983; Enders et al, 1985; Will et al, 1987). The shortest of the 3.5 kb transcripts is termed the pregenome, and is thought to act as a template for replication (Summers and Mason 1982), as well as coding for the core and polymerase proteins. The other two 3.5 kb transcripts encode the pre-core/core gene, as

well as the polymerase and reverse transcriptase. Two mRNA transcripts 2.4 and 2.1kb long, are responsible for the production of the pre-S1, and the pre-S2, HBsAg and X proteins respectively (Cattaneo et al, 1983, Standring et al 1984). A putative 0.7 kb X transcript has also been described, (reviewed Ganem and Varnus 1987).

Viral replication follows the packaging of pregenomic RNA into subviral cores in a process mediated by the viral polymerase. DNA synthesis is then thought to begin at a direct repeat sequence, DR1, at the 3' end of the pregenome, primed by the 5' end of the polymerase gene polyprotein. After degradation of the redundant template by RNase H, the second DNA strand is synthesised from DR2, priming with a sequence derived from the 5' end of the pregenome.

Although replicative viral forms have been identified in non-hepatic tissue such as lymphoid cells, efficient production of the 3.5kb pregenome (hence viral replication) occurs only in highly differentiated hepatic cells (Surreau et al, 1986). Production of pregenomic RNA, and the synthesis of core and polymerase gene products is under the control of the core promotor 1699-1800 (numbering from the EcoRI site) and two cis-acting enhancer elements, EhnI 1074-1234, and Ehn II 1636-1741. Activation of both the promotor and the second enhancer have found to be dependent on factors present only in liver cells (Honigowachs et al, 1989; Yee, 1989; Chiou-Hwa and Ting 1990; Tay et al, 1992). A suppressor sequence which down regulates transcription of the 3.5 kb transcripts, has recently been located within the 282bp BamHI/ HincII restriction sites 5' to the core gene, partially overlapping EhnII. Regulation of the suppressor by a viral protein has been postulated, but the precise nature of control has not been determined (Gerlach and Schloemer 1992).

The longer pair of 3.5kb transcripts initiate upstream from the pre-core ATG at nucleotides 1785-1786, and 1791-1793, and result in the production of two transcripts which contain the pre-core initiation codon (position 1816). The shorter pregenomic transcript, initiates at position 1818-1820, and codes for HBcAg and polymerase (Bruss and

Gerlich, 1988).

HBeAg shares amino acid sequence with HBcAg except that it has an additional 10 amino acids at the amino terminal, and lacks the arginine rich sequence at the carboxy terminal. Translation initiated from position 1903 results in the production of HBcAg (21-22Kd) which accumulates intracellularly, particularly localising in the cell nuclei (Ou et al, 1989; Roosnick et al, 1986; Jean-Jean et al, 1989).

The initial 19 amino acids of the 25Kd polyprotein (P25) constitute a signal sequence which directs the amino terminal of the polypeptide into the lumen of the endoplasmic reticulum (ER) (Ou et al, 1986; Standring et al, 1988) where the signal sequence is cleaved generating a 23Kd polypeptide (P23). The arginine rich region of the P23 prevents complete translocation into the lumen of the ER generating a transmembranous configuration (Bruss and Gerlich 1988) which is either processed by proteolytic cleavage of the carboxy terminal resulting in secretion of HBeAg (15-18Kd) through the Golgi apparatus, or relocates in the cytosol (Garcia et al, 1988). Murine fibroblasts transfected with vectors expressing HBV express HBeAg antigenicity on the cell surface, in the cytoplasm and nuclei (Seifer et al, 1990). HBeAg is also found on the cell surface of HepG2 cells infected with recombinant vaccinia virus expressing HBeAg (Schlicht and Schaller 1989).

(3) HBV induced pathology.

HBV is currently estimated to infect between 200 to 300 million people world-wide. HBV transmission is parenteral and in adult life infection is usually through sexual contact, transfusion of contaminated blood products or through intravenous drug abuse. Following an incubation period of three to six months, the acute disease is characterised serologically by circulating HBsAg, which lasts approximately 20 weeks, and raised serum liver enzyme levels.

HBeAg and replicative HBV can usually be detected for the first eight weeks of infection. Clinical symptoms of infection range from asymptomatic through varying states of hepatocellular injury and inflammation to fulminant disease. The intensity of lobular hepatitis increases during seroconversion from HBeAg to anti-HBe status, and is usually associated with loss of replicative HBV and normalisation of liver histology (Hoofnagle et al, 1981).

The histology of acute HBV infection is characterised by focal necrosis, lobular hepatitis and regeneration spreading throughout the lobule. A similar but more extensive pattern (confluent necrosis) is revealed *post mortem* in fulminant disease. Seroconversion in acute disease is associated with elevated serum transaminases, increased HLA class I expression on hepatocytes, and a transient increase in interferon detected in the serum (Fig 5). Anti-HBc antibodies are the first to appear, approximately two months after the initial infection. High levels of IgM anti-HBc are characteristically associated with acute HBV, but IgG anti-HBc persists throughout acute and chronic infection, irrespective of clinical disease. Anti-HBe antibodies usually develop following a rise in serum transaminase levels and loss of HBeAg. Conversion to anti-HBe status was initially used as a marker denoting loss of infectivity, but the more sensitive serum DNA measurement has superseded it.

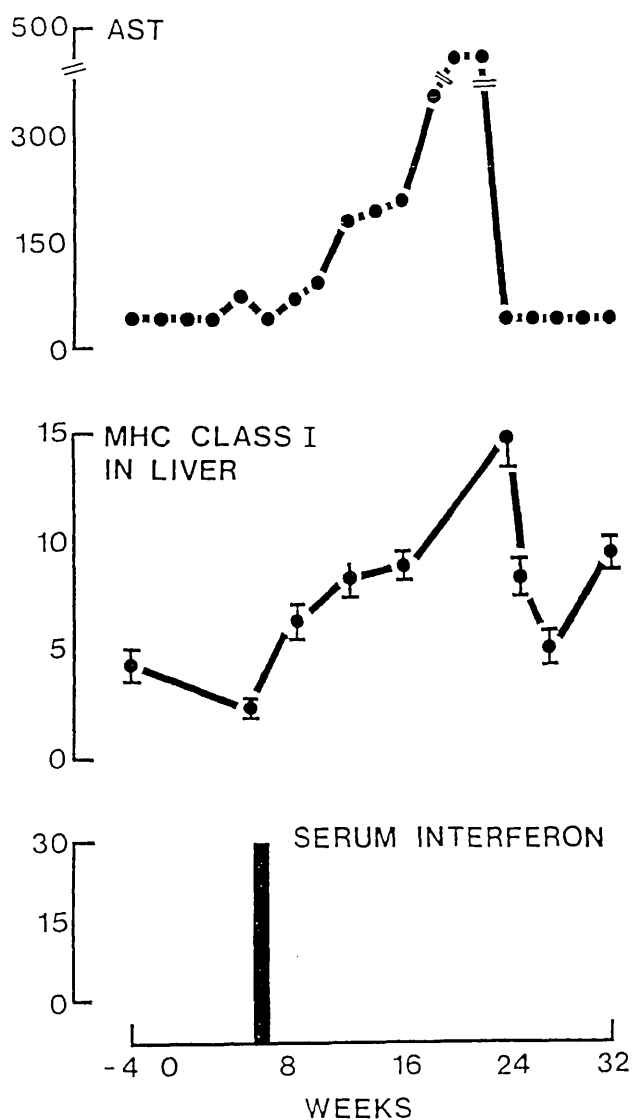
HBV is not thought to be directly cytopathic. During the clinical incubation of HBV, individuals are HBsAg positive, but there is no biochemical or histologic evidence of damage (Thomas et al, 1982). Accumulation of HBcAg in a non-secretor HepG2 clone was noted to cause a cytopathic effect, but there was no evidence that this was other than an *in vitro* phenomenon (Roingeard et al, 1990).

Clinical observations led Dudley et al, (1972) to suggest that hepatic injury associated with HBV infection was mediated by the host immune system. Several lines of evidence implicated HBcAg as a target of the cellular response during both the acute and chronic disease phases. Immunisation of chimps with recombinant HBcAg protected against or at least reduced the severity of infection upon

Fig 5. Hepatocyte expression of MHC class I increases co-incident with serum transaminases and a detectable pulse of circulating interferon during acute HBV infection.

ALPHA INTERFERON PRODUCTION DURING
EARLY HBV VIRAEamia

HBsAg	- - - - + + + + + + + - - - - -
ANTI HBC	- - - - - - - + + + + - + + + + +
ANTI HBs	- - - - - - - - - - + + + + + + + +



Liver damage (rising aspartate transaminase (AST) also coincides with the appearance of IgM anti-core. The data was collected from acute HBV infection in a chimpanzee. Reproduced with permission from H.C. Thomas (1990).

challenge with HBV (Murray et al, 1984), despite the fact that anti-HBc antibodies are not protective. Both envelope and nucleocapsid antigens are displayed by infected hepatocytes, the latter, coincident with hepatic injury (Dienstag et al, 1984), and elevated HLA expression, (Montano et al, 1983a).

Elevated CD4+ HBeAg and HBcAg responses have been observed early during acute HBV infection, and associated with recovery from chronic disease (Ferrari et al, 1990, 1991; Tsai et al, 1992). Both nucleocapsid specific CD4+ and CD8+ cells are clearly important during acute infection. A CD4+ epitope recognised on HBcAg aa57-69 (Ferrari et al, 1991) is recognised by 90% of acute HBV patients, the response to this epitope diminishes following seroconversion.

Concurrent with the study described in the following chapters, MHC restricted cytotoxic T cells specific for nucleocapsid protein were demonstrated in the peripheral blood of HBV patients during the acute phase of infection. The patients studied went on to clear HBsAg, and completely recover, suggesting that the cytotoxic response to the nucleocapsid antigens is critical in the clearance of infected hepatocytes during acute HBV infection.

Peripheral precursor cytotoxic cells appear to exist at a very low frequency, even during acute infection, and require intense stimulation to amplify and detect a response. Initially, an epitope encoded by amino acids 11-27 of the core antigen was identified and used to stimulate HLA-A2 restricted lymphocytes *in vitro* in the presence of rHBcAg and IL-2. The primed CD8+ cells recognised both HLA-A2 restricted p11-27 peptide coated targets, and (to a lesser degree) rVHBcAg infected targets, in a cytotoxicity assay (Bertoletti et al, 1991). The epitope was further defined to amino acids 18-27, which was confirmed to be the minimal epitope optimally recognised by HLA-A2 restricted acute HBV patients (Penna et al, 1991; Guihlot et al, 1991 et al). A second nucleocapsid epitope corresponding to amino acids 141-151 was subsequently described in acute HBV patients, with dual specificity for HLA-A31 and HLA-Aw68 haplotypes (Missale et al, 1993).

Anti-HBs antibodies, which confer protective immunity to HBV infection (Desmyter et al, 1975) are detected one to two months post clearance of HBsAg from serum. Serum derived (formalin inactivated) HBsAg was effective in immunising against infection (Maupas et al, 1976; Szmuness et al, 1980), and recombinant HBsAg vaccines expressed in the yeast *Saccharomyces cerevisiae* have been shown to be equally efficacious (McAleer et al, 1984; Waters et al, 1987).

Between 1 and 10% of adults infected with HBV fail to eliminate the virus and become persistently infected (Ganem 1982; Seef and Koff 1986). Patients are categorised as chronic carriers if they remain HBsAg positive for greater than 6 months (Dudley et al, 1972). Vertical transmission from HBeAg positive mother to child accounts globally for the most significant viral reservoir. In South East Asia and sub-Saharan Africa, up to 20% of women may be HBV carriers. Ninety per-cent of children infected perinatally become chronic HBV carriers. Infection *in utero* is reported to occur at a far lower frequency than neonatal or perinatal infection (Sheng et al, 1986), and the mechanism by which HBV infection persists is unclear. HBeAg has been postulated to cross the placenta, and has been implicated as a CD4+ HBeAg/HBcAg tolerogen in a HBeAg transgenic mouse model of infection (Milich et al, 1990).

Chronic carriers may be asymptomatic or suffer varying degrees of injury with persistent recurrent inflammation, which may lead to cirrhosis (Hoofnagle and Alter 1984), or hepatocellular carcinoma (Beadsley et al, 1982; Popper et al, 1986). The extent and distribution of inflammatory infiltrate and necrotic tissue characterises the different states of chronic disease, defined by de Groote et al (1968) and Dudley et al, (1972) as chronic persistent (CPH), chronic active (CAH), and lobular hepatitis. Although effectively a continuum of disease progression, CAH and CPH have differing clinical manifestations and prognosis. In CPH, necrotic tissue (if present) is limited, and restricted to areas surrounding the portal tracts. CAH is characterised by portal tract necrosis. The disease is described as lobular when an

inflammatory infiltrate is observed to extend from the portal tract towards the portal vein, and may be associated with bridging necrosis destroying the lobular architecture.

Histological examination of the inflammatory infiltrate revealed NK and CD8+ cells (Eggink et al, 1982, 1984; Montano et al, 1983b). Both CD4+ and CD8+ T-cell clones were isolated from liver biopsies of patients with chronic active disease (Hoffmann et al, 1986; Ferrari et al, 1987a, 1987b; Barnaba et al, 1989), and peripheral blood lymphocytes isolated from a chronic active carrier, were reported to lyse autologous HBV infected hepatocytes (Mondelli et al, 1982; Naumov et al, 1984).

The significance of the innate response in HBV is unclear. Although NK are present in hepatic inflammatory infiltrate, reports on peripheral detection during disease have been contradictory (Novick et al, 1986; Chemello et al, 1986). IFN α therapy has been reported to induce a decline in peripheral NK activity (interpreted to signify sequestration in the liver) concurrent with a drop in serum HBV, a rise in serum transaminases, and an altered CD4:CD8 ratio (Scully et al, 1990). The study also suggested a role for CD4 cells in IFN α induced viral clearance, initially identified by McDonald's observation (1987), that HIV infected patients fail to respond to IFN α therapy.

Patients with chronic infection typically have evidence of viral replication characterised by the detection of serum derived HBV DNA by dot blot hybridisation (Weller et al, 1982), although an aggressive, HBV DNA dot blot negative, anti-HBe positive disease has been described. (Hadziannis et al, 1983; Bonino et al, 1981; Lok et al, 1984). Mutant viruses with an untranslated pre-core gene were characterised in the majority of these patients by PCR sequencing (Carman et al, 1989, 1991; Brunetto et al, 1989; Okamoto et al, 1990). The pre-core stop codon results from a mutation (G to A) at position 1896, HBeAg is not produced *in vitro* by the mutant sequence (Tong et al, 1992). An untranslated mutation (G to A) at position 1899 was frequently found to co-exist.

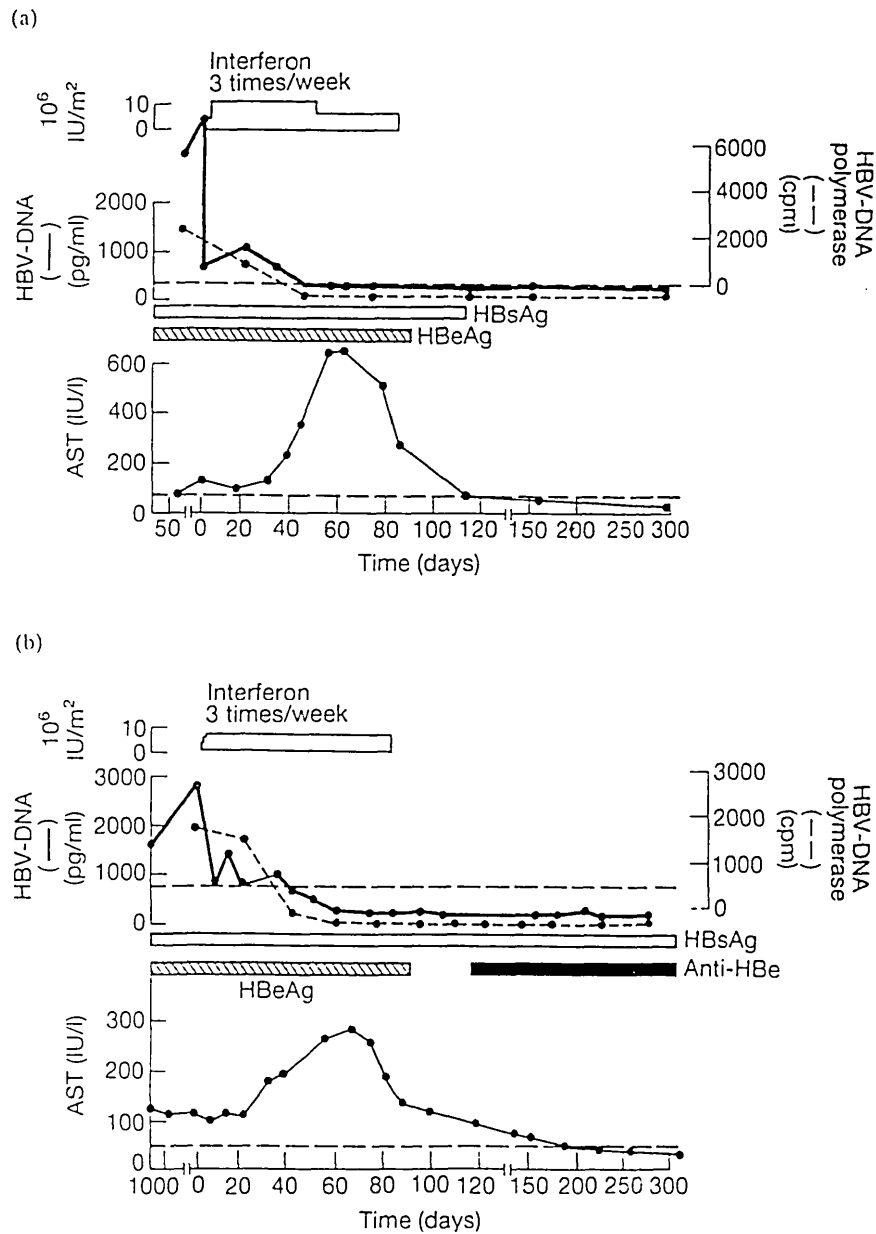
Translation of the pre-core gene is not essential for viral packaging, and the pre-core mutant viruses can

continue to replicate. There have been some suggestions that the mutant virus has lost some down regulating control and can replicate to higher levels than the wild type, detection of high levels of the pre-core mutant has also been associated with fulminant disease (Carman et al, 1990), but this is not born out in the woodchuck model (Chen et al, 1992).

PCR has revealed HBV at very low levels in serum (not detected by dot blot) up to five years after sero-conversion from HBeAg to anti-HBe during the normal course of recovery from HBV infection. A mixture of wild type and the pre-core mutant virus can commonly detected at this time, but the level of detection distinguishes it from the high levels of replication associated with continued severe disease.

Approximately 10% of chronic carriers undergo spontaneous seroconversion to HBV DNA negative, anti-HBe positive, healthy carrier status per annum. Recombinant IFN α has been demonstrated to effect seroconversion in 40% of European patients treated (Brooke et al, 1989), the course of the response is illustrated in Fig 6. HBsAg may still be detected in some of these patients indicative of viral integration, but hepatocellular damage usually ceases (Hadziannis et al, 1983). Treatment of individuals infected at birth has a reduced efficacy (Scullard et al, 1981, Lok et al, 1988). The mechanism by which seroconversion is affected by interferon treatment is unknown. IFN α has both a direct anti-viral effect via induction of 2-5-A synthetase inhibiting protein synthetase (Kimchi 1981), and immunomodulatory functions.

Antibody mediated cell lysis has been hypothesised to play a role in viral immunopathology and viral clearance. HBeAg displayed on the surface of cells was proposed as a target (Schlicht et al, 1989), and IgG1 (the commonest anti-HBe sub-type) has been found to efficiently mediate ADCC in experimental systems (Sarmay et al, 1992). HBV viral mutants which fail to express HBeAg could theoretically avoid anti-HBe

Fig 6. Responses to IFN α therapy.

(a) Complete response: inhibition of HBV replication and clearance of HBs antigen.

(b) Incomplete response: inhibition of HBV replication associated with a transient exacerbation and then normalisation of the liver function tests. The patient remains HBsAg positive, but has reduced infectivity and commonly cessation of inflammatory liver disease. Reproduced with permission from H.C. Thomas (1990).

mediated cell lysis.

Increased catabolism of complement components C1q and C3 has been reported in HBeAg positive patients with chronic disease (Potter et al, 1980; Thomas et al, 1979) suggesting a role for complement mediated cell damage. Circulating immune complexes containing HBsAg are detected early in acute infection (Pernice et al, 1979; Lambert et al, 1980). During chronic infection, circulating free anti-HBe and anti-HBs are not detected by conventional immunoassay, but more sensitive assays have demonstrated free anti-HBe antibodies in the peripheral circulation up to 10 years prior to seroconversion to HBeAg-ve status (Sallberg et al, 1991). Immune complexes which consist of the envelope antigens and antibodies, have also been detected, (Brown et al, 1983; Madalinski et al, 1991), as have HBeAg / anti-HBe complexes (Takahashi et al, 1978; Takahashi et al, 1979; Sallberg et al, 1991; Maruyama et al, 1992), as has immune complex deposition on the hepatocyte membrane (Lee and Koh, 1989). Glomerulonephritis is a relatively common complication of both acute and chronic infection, and hypergammaglobulinemia has been reported co-incident with HBV infection (Joshi et al, 1990).

(4) Further investigations into the immunopathology of HBV infection: Designing the study.

HBV is a human pathogen, so study of its natural host and host response was deemed most appropriate. The questions to be answered were posed as follows:

- (1) Are antigen specific CTL present in acute HBV?
- (2) Is chronicity due to an absence of CTL?
- (3) What happens during HBeAg/anti-HBe induced sero-conversion following INF α therapy?

(4) Is the generalised lymphocyte non-responsiveness to HBcAg reported during chronic HBV infection due to defective APC?

(5) What role do humoral processes play in acute and chronic HBeAg/ anti-HBe seroconversion. Is emergence of viruses unable to produce HBeAg(anti-HBe variants) a consequence of evasion of humoral responses?

A non-invasive protocol was developed using 200ml of peripheral blood from volunteers presenting in the outpatients clinic at St. Mary's Hospital, Paddington, with acute or chronic infection. More than forty patients on INF α therapy were studied during the course of their treatment. Some patients donated lymphocytes and serum at a single time-points, others more than once, particularly when transaminases were elevated above the patient's individual 'average' range. Co-ordination and co-operation of the medical staff within the unit and local G.P.s was organised by Dr. J.A. Waters.

HBV does not replicate in tissue culture therefore targets were prepared by infecting autologous lymphoblastoid cells with recombinant vaccinia viruses expressing HBV viral antigens, or coating them with HBV derived peptides. Experiments were designed on the premise that cytotoxic memory cells present in the periphery during HBV infection, might be stimulated *in vitro* to effect lysis in chromium release cytotoxicity assays.

Chapter Three. Materials and Methods.

(1) Extraction, purification and analysis of DNA and RNA.

Standard techniques and protocols were those recommended by Sambrook, Fritsch and Man iatis, (*Molecular Cloning a laboratory Manual*, 2nd edition, 1989). Stock solutions are detailed in Appendix I.

Large scale plasmid DNA preparation.

10ml aliquots of L. B. were inoculated with single bacterial colonies (picked from fresh agar plates) and grown at 37°C for 8 hrs. These starter cultures were used to inoculate 500 ml of pre-warmed L. B. plus selective antibiotics, and incubated in an orbital shaker (200 rev hr⁻¹) at 37°C overnight before harvesting by centrifugation at 3000g for 7 mins at 4°C, in an MSE 3000 centrifuge, and henceforth kept on ice. The cells were washed in 10ml TE (Appendix I) and resuspended in 4.2 ml of buffer (25% sucrose, 50mM Tris-HCl pH 8) before transfer into 35ml polypropylene oakridge bottles. 1 ml fresh lysosyme solution (10 mg ml⁻¹ stock) was added to dissipate clumps, followed by 2.3ml 0.25M EDTA, and the samples incubated on ice for 10 mins, the cells were lysed with 4.8 ml Triton-X lysis buffer (Appendix I).

Chromosomal DNA was pelleted by centrifugation at 20000g at 4°C in an angle head rotor (AH629, Sorvall). The supernatant was adjusted to 12.5 ml with TE, 12.5 gm ultra pure CsCl₂ (BRL) and 1ml ethidium bromide (10mg ml⁻¹ stock) were added. Protein / ethidium complexes were removed by centrifugation for 30 mins at 20000g at room temperature (RT) before transferring the solution to soft walled tubes and centrifuging for 4 hrs at 400000g, and 20°C, (Beckmann NV65 rotor, Sorvall ultracentrifuge).

Plasmid DNA, visualised by ultraviolet light, was collected in a sterile syringe and needle by piercing the side of the centrifuge tube. Ethidium was removed by three extractions with water saturated n-butanol, and the plasmid precipitated by the addition of 2 volumes of water,

followed by 2 volumes of ethanol and incubation for 2hrs at 4°C. DNA was pelleted by centrifugation at 10000g for 20 mins, washed with 70% ethanol, air dried, and resuspended in 360ul TE with 40ul of 3M sodium acetate (pH 7.0) and incubated at 4°C overnight. The DNA was further purified by extracting twice with an equal volume of phenol/chloroform (Appendix I), mixing by hand for 5 mins before briefly centrifuging at 10000g and recovering the aqueous layer which was further extracted with chloroform, and precipitated by the addition of 2 volumes ethanol and incubation at 4°C for 30mins. The DNA was pelleted by centrifugation at 10000g for 10 mins and washed three times with 70% ethanol, and air dried before resuspending in 200ul TE.

Rapid isolation of plasmid DNA.

Rapid isolation of plasmid DNA was performed by a modification of the alkaline SDS method (Birnboim and Doly 1979). 5 ml L.B. cultures containing antibiotic were inoculated with single bacterial colonies and incubated overnight at 37°C with vigorous shaking (200rev hr⁻¹). A small aliquot (0.5ml) of overnight culture was frozen at -70°C in 15 % (v/v) glycerol (30% (v/v) stock). Cells from 1.5 ml of culture were pelleted in Eppendorf tubes by centrifugation at 10000g, for 2-3 mins. Culture fluid was removed and the pellet resuspended in 100ul solution I (Appendix I) incubated for 5 mins at RT, then 200ul freshly prepared solution II was added with gentle mixing, and the extracts incubated for 5 mins at RT. Chromosomal DNA and denatured protein were precipitated by adding 150ul solution III, and after 5 mins incubation on ice, the extract was centrifuged at 10000g for 5 mins at 4°C. The supernatant was transferred to a second tube, extracted with 450ul phenol/chloroform and briefly centrifuged at 10000g. Plasmid DNA was precipitated from the aqueous layer by the addition of 900ul ethanol, incubation for 5 mins at RT, and centrifugation for 5 mins at 10000g. The pellet was washed once with 70% ethanol, air dried and resuspended in 20ul of RNase A (10ug ml⁻¹).

Extraction of vaccinia virus DNA.

Monolayers of Vero cells (1×10^7 cells) were infected with wild type or recombinant vaccinia viruses at a multiplicity of infection (moi) of 2 plaque forming units (pfu) per cell. Twenty-four hrs post infection, the monolayers were gently washed with PBS-A, and lysed by the addition of 1-2ml of 2.5% Nonidet P-40 (NP-40) in PBS-A. After incubation on ice for 5 mins, the lysed cells were centrifuged at 500g for 5 mins at 4°C, and the supernatant (cellular extract) containing the virus particles was incubated with 1mg ml⁻¹ of proteinase K (Boehringer-Mannheim), 1% SDS in 10mM Tris-HCl for 1 hour at 60°C. RNA was removed by the addition of 5ug of RNase A, and a further 1 hour incubation at 37°C. The crude DNA solution was mixed gently with an equal volume of phenol/ chloroform for 5-10 mins at room temperature, briefly centrifuged at 10000g and the aqueous layer re-extracted with an equal volume of chloroform. Following a brief centrifugation step (10000g, 3 mins) the DNA was precipitated by the addition of 1/10 volume of 3M sodium acetate pH 4.8, and an equal volume of isopropanol. The mixture was incubated at room temperature for 15 mins, then centrifuged at 10000g for 10 mins to pellet the DNA. The pellets were washed with 70% ethanol, gently air dried and redissolved in 100-200ul TE buffer.

Extraction of DNA from transfected cells.

Monolayers of HepG2 cells (1×10^7 cells) transfected with EBO constructs were washed with PBS-A, incubated for 15 mins at RT in the presence of 0.8ml Hirt solution (0.6% SDS, 0.01 M Tris-HCl pH 7.6, 0.01 M EDTA). To each culture was added 200ul 5M NaCl, and the extract transferred using a rubber policeman as a guide to an Eppendorf tube. The tubes were incubated at 70°C for 15 mins, 200ul 5M potassium acetate added, and the extracts incubated on ice for 1 hr, before pelleting chromosomal DNA and proteins (10000g, 20 mins). The supernatants were removed and aliquoted in fresh Eppendorfs at 100ul of supernatant per tube. DNA was precipitated by the addition of 300ul TE and

1ml ethanol, and incubation at -20°C , for 30 mins, and pelleted by centrifugation at 10000g for 10 mins before washing with 70% ethanol and air drying. Aliquots were then digested for Southern Blot analysis.

Extraction of HBV DNA viral particles from serum.

100ul of serum was digested with 1mg ml^{-1} proteinase K, 0.6% SDS in Summers buffer (Appendix I) for 1 hour at 60°C , then purified by phenol/chloroform and chloroform extraction. DNA was precipitated by the addition of an equal volume isopropyl alcohol with 1/10 vol sodium acetate, 10ug glycogen, incubating at -20°C for 30mins, and centrifuging at 10,000g for 10 mins. The pellet was washed X 3 with 70% ethanol, briefly dried, and dissolved in a final volume of 20ul TE.

RNA extraction from cells.

Diethyl-pyrocarbonate treated H_2O , sterile Eppendorfs, pipette tips and containers were used throughout RNA extraction and handling.

RNA was extracted by a commercial single step guanidium method (Chomczynski and Sacchi, 1987). Briefly, by treating 1×10^6 vaccinia infected or EBO transfected cells with 1ml of RNazol plus 1/10 volume chloroform. RNA was solublised by syringing the lysate through a needle (23g), and vortexed for 15 seconds before incubating on ice for 5 min. Aqueous and organic phases were separated by centrifugation at 10000g 4°C for 15 min. The aqueous phase (containing RNA) was removed and precipitated by the addition of an equal volume isopropyl alcohol incubating at 4°C for 15-30 min on ice, and pelleted by 15 min centrifugation at 10000g, 4°C . Excess salt was removed by washing the pellet with 1ml 75% ethanol, centrifuging briefly (10000g, 4°C). The pellet was vacuum dried before resuspending in 10ul DEPC treated H_2O and stored at -70°C .

Restriction Enzyme digestion.

All restriction enzymes were purchased from Boehringer Mannheim or BRL, and used in the appropriate buffers as supplied by the manufacturers. For analysis, approximately 0.5 ug of plasmid DNA was digested in a volume of 20ul with 2.5 - 5 units of enzyme for 2 hrs at 37°C. For preparative purposes, 20-30 ug DNA was digested in a volume of 100ul with 50-100 units of enzyme for 2-3 hrs at 37°C. 5-10 ug of viral DNA or Hirt extract DNA was commonly digested for Southern Blot analysis. PCR products carrying restriction sites at the ends were digested for 16-18 h using 10-20 units of enzyme for 1-2ug of DNA.

Digested DNA was purified by one of three methods depending on the size of the digest product: DNA 8-12 Kbp in size were electroeluted, 8 - 0.4 Kb were "freeze squeezed" (Tautz et al, 1983) or "Gene Cleaned" (Vogelstein and Gillespie, 1979).

Agarose gel electrophoresis.

Agarose gels for DNA electrophoresis were prepared using BRL ultra-pure agarose at 1% -0.6% (W/V) as stated, in TAE or TBE buffer (Appendix I) with ethidium bromide at 100ug ul⁻¹. Gel loading buffer (Appendix I) was added to DNA samples at a 1/10 dilution prior to loading onto the gel. Gels were electrophoresed at a constant voltage of 70-100 volts for mini (50ml) and midi (100ml) gels, in TBE or TAE buffer until the dye front had migrated approximately 80% of the gel length. Gels were viewed using an ultra violet source emitting >2500 uW cm⁻², and photographed by Polaroid camera with a Wratten 22A filter.

Purification of DNA fragments and PCR products.

(1) Freeze Squeeze.

Fragments excised from TAE / agarose gels were incubated on an orbital shaker for 15 mins at RT (in the dark as ethidium bromide is still present) in 0.3M acetate buffer, 1mM EDTA. The gel was transferred to a pierced

500ul Eppendorf plugged with sterile siliconised glass wool (covered with parafilm), and incubated in an ethanol/dry ice bath for 30 mins. After removing the parafilm, the mini-Eppendorf was placed in a 1.5ml Eppendorf (while still frozen) and centrifuged for 10 mins at 10000g. The DNA fragment spun through glass wool, was precipitated by the addition of 1/10 volume of magnesium acetate (100mM MgCl_2 , 1% acetic acid), and two volumes of ethanol, incubating in the dry ice/ ethanol bath for 1 hr. The precipitate was centrifuged for 10 mins at 10000g, washed once with 70% ethanol and air dried before dissolving in 20ul of TE.

(2) Gene Clean.

Gene Clean II (La Jolla) was used according to the manufacturers' instructions. Briefly, DNA fragments excised from TAE / agarose gels were incubated for 5 mins with three volumes sodium iodide at 55°C, 10ul glass milk was added to each sample and incubated 5 mins at RT. The DNA/glass beads were pelleted for 30 seconds at 10000g, washed three times with 500ul ice cold "new wash" pelleting and resuspending the beads between washes. The beads were finally spun for 2 mins at 10000g, resuspended in 10-20ul of H_2O and incubated at 55°C for 5 mins. DNA was recovered by centrifugation at 10000g for 2 mins and removal of the supernatant containing the pure fragment.

(3) Electroelution.

After electrophoresis of the DNA fragment on a TBE/agarose gel, a well was cut in the gel adjacent to the band, and lined with dialysis tubing (pre-prepared as described in Maniatis). The fragment was then electro into the well and collected into an Eppendorf. Eluted DNA was extracted with n-butanol, phenol/chloroform and chloroform, then precipitated with sodium acetate and ethanol before resuspending in TE.

(4) Polyethylene glycol precipitation.

PCR products for sequencing were purified rapidly by polyethylene glycol (PEG) precipitation. 0.6 volumes of PEG [20% (w/v) PEG 8,000, 2.5 M NaCl] incubating at 37°C for 10 mins, and pelleting at 10000g, for 10 mins. After a 70% ethanol wash and air drying, the DNA was resuspended in 25ul of TE and 3ul analysed on an agarose gel to estimate DNA concentration and purity.

Southern Blot Analysis.

Viral inserts were cut by digestion with the appropriate restriction enzymes, and electrophoresed on 0.6% agarose gels using TAE or TBE buffer. The gel was denaturated for one hour in 500ml of 0.5M NaOH, 1.5M NaCl, rinsed in H₂O and neutralised incubating for one hour in 500ml of 0.5M Tris-HCl pH 7.0, 3M NaCl. The gel was then transferred overnight by capillary action using 20 X SSC to a nylon filter (Amersham High-Bond N), and fixed by ultraviolet (UV) exposure.

Northern Blot.

RNA was electrophoresed in a denaturing system. 6ul (approximately 40ug) of total RNA extract was mixed with 12.5ul of formamide, 2.5ul of 10 X MOPS buffer and 4ul of formaldehyde (27%). Samples were incubated for 5 mins at 65°C to denature the RNA. After quenching on ice, 2.5ul 50% (v/v) glycerol with 0.1mg ml⁻¹ of bromophenol blue were added. The RNA was electrophoresed on 1.2% agarose gels made up in 1 X MOPS containing 6.3% formaldehyde, at 100-120 V with recirculation of the buffer (1 X MOPS). RNA was transferred to High Bond-N by capillary action using 20 X SSC) and UV fixed.

Dot Blot of viral DNA.

A dot blot manifold (Schleicher and Schuell) was used to prepare DNA filters on Amersham high-bond N nylon, analysing 50ul of crude viral stock per well. Filters were denatured by placing successively onto 3MM paper saturated with 0.5M NaOH 1.5M NaCl; 1M Tris-Cl pH 7.00 3M NaCl; and 2

X SSC, 10 mins each, and fixed by UV light exposure.

DNA Hybridisation.

HBV DNA for cDNA probes was obtained from plasmid pHBV20 (Summers et al, 1975), which contains a full length copy (3.2kb) of HBV cloned into the EcoR1 site of the plasmid pBR322. The HBV insert was cut out of the plasmid with EcoR1 and purified by electroporation or "freeze squeeze". Probes were prepared by nick translation (Rigby et al, 1977) of 100-200ng of cDNA using a commercial kit (Amersham). and alpha ^{32}P dCTP. The unincorporated label was removed on a G50 "spun column" (Maniatis et al, 1989). Filters were pre-hybridised and hybridised in 50% formamide, 5 X SSC, 5 X Denhardtts (Appendix I), 50mM Na_2PO_4 pH 6.5, and 250ug ml^{-1} of denatured single stranded herring sperm (ss) DNA for 1-3hrs, at 42°C, then hybridised in 50% formamide, 5 X SSC, 1 X Denhardtts, 100ug ml^{-1} of ssDNA and 10^6 cpm ml^{-1} of denatured probe, for 16 hrs at 42°C. Filters were washed twice with 2 X SSC, 0.1% SDS for 10 mins at RT, then twice with 250ml of 0.1 X SSC, 0.1% SDS for 30 mins at 51°C, air dried and exposed to pre-flashed X-Omat (Kodak) film for 3-24 hrs.

(2) Gene cloning.

Polymerase chain reaction (PCR).

Choice of primers.

PCR products were prepared for sequencing vaccinia recombinants, patient viral isolates, and for cloning. Oligonucleotide primers (18-30 base pairs) for sequencing were chosen with GC content > 50%, and their positions within the gene under analysis. Pairs were selected with non complementary ends. Primer sequences and positions are documented in Table 1, Primers were supplied HPLC purified by Oswell Ltd. (Glasgow University) .

PCR products were prepared following stringent precautions, and always including negative controls.

Reactions were carried out in 500ul Eppendorfs, each reaction consisting of template DNA (eg 1/10 vols HBV DNA extract), 10mM Tris-HCL pH 8.0, 50mM KCL, 1.5mM MgCl₂, 0.01% (w/v) gelatin, 0.1% Triton X-100, 0.2mM deoxynucleotides (Boeringher Mannheim), 50pmol of each primer, 0.5 U Taq. Polymerase (HT Biotechnology), in a final reaction volume of 100ul, with 30ul of mineral oil added to prevent evaporation. All conditions were determined empirically (using the 3.2kb EcoR1 insert digested from pHBV20 as template DNA)

Single PCR.

For analysis, PCR was performed using the primers M3 and C3 which amplify the pre-core and core regions (Carman et al, 1989). An initial denaturation step of 95°C for 5 mins was followed by 40 cycles of 95°C for 1 min, 53°C for 1 min and 72°C for 2 mins.

Nested PCR.

For cloning, patient sera with low levels of DNA were subjected to nested PCR (Garson et al, 1990). Primers M3 and Z0162 (outer pair) were initially used to amplify the complete pre-core / core genes plus flanking regions followed by amplification of the first PCR product with primers E1041 / E1042 (inner pair) to give the pre-core / core genes flanked by Hind III and Bam HI recognition sites. Conditions were as follows:-for amplification with the outer pair (M3 / Z0162) a 5 min 95°C denaturation step followed by 5 cycles of 95°C 1 min, 37°C 1.5 mins, 72°C 2 mins, and 35 cycles with an annealing temperature of 53°C. Five per cent of the initial product was amplified further with the inner pair (E041, E042), initially denaturing at 95°C for 5 mins, followed by 35 cycles of 95°C for 1 min, 53°C for 1 min and 72°C for 2 mins, followed by a final 10 minute incubation at 72°C.

Table 1. Primers used for PCR Cloning and sequencing.

Designation	position	sequence 5'-3'	length
Cloning primers:			
M3	1732-1757	CTGGGAGGAGTTGGGGAGGAGATT	25mer
core	1816-1836*	GCGGATCCATGGACATTGACCCTTATAAA	29mer
E041	1772-1790#	CCGGAAGCTTGTATTAGGAGGCTGTAGGC	29mer
3'core	C2439-2460	CTAACATTGAGATTCCCGAGA	21mer
Z060	C2594-2615	CTGTGAGTGGGCCTACAACTG	22mer
E042	C2470-2485*	CGGGATCCGCCCAGTAAAGTTCCCCACC	28mer
Sequencing primers:			
Pre-core	1807-1834	CCAGCACCATGCAACTTTTTCACCTCT	27mer
BC-1	C1953-1974	GGAAAGAAGTCAGAGGCAA	21mer
167P	C2278-2295	GGCTGGAGGAGTGCGATT	18mer
AA100	C481-501	CAGAGCTCGTTTAGTGAACCG	21mer
PGS62-2	-21 bps from BamH1 in PGS62	ATTGCACGGTAAGGAGAAG	17mer

Key: C, complementary; * sequence plus BamH1, # sequence plus HindIII

Preparation of PCR Products for ligation.

10ul of PCR product was analysed by agarose gel electrophoresis and the remainder purified either by electrophoresis followed by "freeze-squeeze" or "Gene Clean". Purified DNA was resuspended in 10ul of TE and digested with restriction endonucleases to prepare the appropriate sequences for ligation. The digested PCR product was ethanol precipitated, dissolved in 10ul of H₂O, and stored at -20°C. 1ul of each product was analysed by gel electrophoresis to determine the concentration.

Direct sequencing of PCR products.

The USB Taquence version 2 kit was used for cycle sequencing (Lee 1991). Each sequencing reaction used 50-200ng of PCR product and ³²P end labelled oligonucleotide primer.

Primers for sequencing are documented in Table 1. 2pmol of primer (sufficient for 10 sequencing reactions) was incubated with 50 units polynucleotide kinase (PNK, New England Biolabs) and with 0.5uCi ³²P γdATP in end labelling buffer (Appendix I), in a final volume of 10ul at 37°C for 30 min, followed by a 10 min incubation at 65°C.

The sequencing reaction mixture contained 3ul DNA (50-200ng), 1ul of end labelled primer, 2ul X 5 reaction buffer (USB) and 2 units of Taq polymerase in a final volume of 17.5 ul.

4ul of reaction mix was added to 4ul of each termination mix (dideoxy and deoxy nucleotides) and overlaid with 30ul of paraffin oil. The reaction mixes were denatured for 5 mins at 95°C, followed by 10 cycles; 95°C 1 min., 53-65°C (determined by primer composition) for 1 min, 72°C 2 min before terminating the reaction with 4ul of stop solution (USB Taquence).

Samples were denatured at 95°C for 4 mins and 2-5ul of each reaction was loaded per lane (in the order TCGA) on 5 or 6% acrylamide sequencing gels (using Sequagel, National Diagnostics, according to manufacturers' instructions) at a

constant power of 65 Watts for 2-5 hrs. Gels were dried directly (under vacuum at 80°C), and exposed to Kodak X-Omat film for 4 hrs to 48 hrs.

Construction of expression vectors.

Preparation of chimeric PGS62 vectors.

Cloning into the PGS62 plasmid a gift from Dr.G.Smith was done by Dr. M. McGarvey.

The pre-core and pre-core mutant DNA sequences (mutations at position 1896, and 1896 plus 1899) were amplified from patients' sera using the primers M3 and C3, under conditions previously described. The PCR products were subcloned by blunt ended cloning into the SmaI site in the pGEM plasmid. Digestion with BamHI and EcoRI released a fragment which was subsequently cloned into the Bam HI and EcoRI sites in PGS62. The core and X PGS62 constructs were prepared from pHBV20. The core gene was amplified by PCR using the primer ATG C (Table 1), which has artificial BamHI and NcoI sites, plus the core start codon position 1901 and primer C3. The product was cloned into the BamHI and SmaI sites of PGS62. The X gene was excised from pHBV20 by digestion with NcoI and FSp2, blunt ended and cloned blunt ended into the Sma I site of PGS62. The PGS62 chimeras were screened for insert gene orientation by Dr. McGarvey by sequencing with the primer (5'ATTGCACGGTAAGGAAG) which bound to a region in PGS62 upstream from the cloning site.

EBO vectors.

CsCl₂ gradient purified plasmid was digested with BamHI and HindIII, and treated with 1-5 U calf intestine alkaline phosphatase (Boehringer Mannheim) per ug of DNA in 50ul of CIAP buffer at 37°C for 30 mins, and immediately electrophoresed. DNA was recovered from the gel by electroelution and redissolved at 1-2ug ul⁻¹ in H₂O. Wide bore (or cut) pipette tips were used to manipulate the vector DNA in solution at all times.

500ng of vector DNA was incubated with varying amounts of insert DNA (to give vector to insert ratios of 2:1, 3:1, 4:1) in 5ul of ligase buffer and 40,000 units of T4 DNA ligase (New England Bio-Labs) at 16-18°C for 18hrs. Prior to electroporation, ligated products were precipitated by the addition of 1/10 vol 3M sodium acetate, 2 vols ethanol and 10ug of glycogen (Boehringer Mannheim) at -20°C for 30 mins. The DNA was pelleted by centrifugation at 10000g for 10 mins and washed three times with 70% ethanol at RT, air dried and redissolved in 10ul of H₂O.

Preparation of electrocompetent cells.

Escherichia coli (*E.coli*) was harvested in the exponential growth phase (OD_{600nm} 0.4-0.5) and pelleted by centrifugation at 3000g for 5mins at 4°C. The cells were washed twice with an equal volume of ice cold H₂O, and once with 250ul of ice cold 10% glycerol (v/v). The bacterial cells were then resuspended in 1.5 ml 10% glycerol and snap frozen in liquid nitrogen (120ul per 500ul Eppendorf). Cells could be stored for up to six months at -70°C, but were not freeze thawed more than once.

Transformation.

5ul (200-250ng) of ligated DNA was electroporated into 20ul of electrocompetent *E.coli* strain INVa F' (Invitrogen) for the EBO vectors, and strain HB101 for the PGS62 plasmids, in a 0.2cm gap cuvette (Bio-Rad) using a Bio-Rad cell pulser, at settings of 25KV, 25uFa, 250 Ohm) according to the manufacturers' protocol. The transformed cells were resuspended within 1 minute of transformation in 0.5ml of SOC medium (Appendix I), and incubated for 1-2 hrs at 37°C. Cells were plated out on ampicillin containing agar plates (Appendix I), and incubated at 37°C for 16-18hrs.

Analysis of clones.

For each ligation experiment, three positive clones containing inserts were selected by restriction enzyme analysis of plasmid DNA prepared by the alkaline-SDS method from individual colonies, and subjected to caesium chloride purification. In order to maximise plasmid yield, 2.5ml of chloramphenicol was added to each 500ml culture (38mg ml⁻¹ of chloramphenicol in ethanol) in the early exponential growth phase of the culture. The 5' ends of inserts plus the plasmid junction were analysed by sequencing 5-10ug of recombinant vectors by direct sequencing with primer AA100 (Table 1) for pCEP4 clones, and BC-1 (Table 1) for analysis of the pMEP4 clones.

Modifications of the expression vectors.

HBV chimeric EBO plasmids were digested with restriction enzymes EcoRV and StuI to remove the EBNA and Ori. P genes. Digested plasmid was gel purified, ligated, and used to transform competent *E.coli*. Plasmid DNA was prepared by the alkaline-SDS method from individual colonies and digested with restriction enzymes to select the new vectors lacking the ability to produce EBNA 1.

(3) Cell and viral culture.

Culture of established cell lines.

All established lines (Table 2) were cultured on tissue culture grade plastic supplied by Nunc or Cell Cult (unless otherwise stated) at 37°C in 5% CO₂ atmosphere (humidified for cultures under 1ml). Mycoplasma free foetal calf serum (FCS) was supplied by Seralab, and normal human serum from the Blood Transfusion Service. Culture media and additives (Penicillin and streptomycin [Pen/Strep], glutamine, hepes, bicarbonate and trypsin/EDTA solution) were all purchased from GIBCO and Flow. 10% FCS, 2mM glutamine, 100IU/100ug Pen/Strep in RPMI 1640, is referred to in the text as F10 medium. Cells were grown in 10 or 20ml of media either in

25 or 75cm³ bottles and subcultured at weekly intervals by gently resuspending clumps of cells in suspension, and replacing 75-90% of the spent medium and cells with fresh media. Adherent cells were subcultured when near confluent by initially rinsing the monolayer with phosphate buffered saline "A" ([PBS] Dulbecco, Appendix I) to remove traces of FCS, then incubating with Trypsin / EDTA (0.5ml of 0.25% Trypsin per 75cm³ bottle) for 1-3 mins at 37°C. Complete medium was added, and the cells resuspended 1/4 in fresh media. Cells for plaque assay, plaque purification, and analysis of protein expression were grown in smaller volumes as documented in the appropriate sections. Stocks of cell lines were stored in 20% FCS (v/v), 10% DMSO (v/v) (Sigma) in RPMI in liquid nitrogen at 1 X10⁷ cells per ml per vial.

Establishing primary lines:

Peripheral blood lymphocytes.

Local ethical committee permission was granted to obtain peripheral blood from HBV patients under the care of St. Mary's Hospital. All human tissue was handled under Category II conditions, anti-HIV positive or "high risk" individuals were excluded from the study. Serum samples from patients receiving treatment were routinely tested for blood biochemistry and viral markers in two routine laboratories at St. Mary's, and HBV DNA in the Department of Medicine. One ml of each patient's serum was stored at -20°C for further analysis. To obtain peripheral blood lymphocytes (which included other mononuclear cells such as monocytes and macrophages unless specifically removed) 200 ml of peripheral blood was collected into 2000IU preservative free heparin by venapuncture of each volunteer, and separated by density gradient centrifugation. The blood was mixed with an equal volume of PBS "A" and layered onto lymphoprep (Density 1.077g L⁻¹ Nycomed) in a 2:1 ratio of diluted blood to lymphoprep. The gradients were centrifuged at 800g for 25 mins at ambient room temperature in an MSE 3000 centrifuge (Boyum

et al, 1968). Approximately 20ml of 50% plasma was stored at -20°C . The PBL were recovered by aspiration from the interface, diluted $\times 4$ with RPMI, and centrifuged at 800g for 5 mins, washing twice more with RPMI (400g for 5 mins). The cells were counted and resuspended in RPMI at a concentration of 2×10^7 cells per ml. $3-4 \times 10^6$ lymphocytes were transformed with EBV as described below and 1×10^7 were HLA typed (St. Mary's transplant unit). The remainder were frozen at a concentration of 1×10^7 cells per ml per vial in 20% (v/v) FCS, 10% (v/v) DMSO in RPMI at minus one degree per minute, and stored in liquid nitrogen. On thawing, cells were washed three times in warm RPMI and cultured at 37°C , 5% CO_2 (in a humidified atmosphere for cultures $< 2\text{ml}$) with antigenic stimulation either in F10 or in RPMI with 10% A+ or autologous serum. IL-2 at a concentration of 10 or 20 IU ml^{-1} was added to the lymphocytes when stated.

Lymphoblastoid lines.

B-95.8 cells were grown until the medium was yellow, then were cultured in the presence of 20ng ml^{-1} TPA (Sigma) for 3 days. Cells were spun down at 400g for 5 mins, the supernatant removed and subsequently kept on ice. EBV was pelleted from the supernatant by centrifugation at 18,000g for 65mins. The EBV was resuspended at 50 X concentration in RPMI, filtered through a 0.2 micron filter and stored in liquid nitrogen. Aliquots were never refrozen.

Lymphoblastoid (LB) lines were established by EBV transformation of B cells (Crawford et al, 1981) and maintained in F10 medium. $3-4 \times 10^6$ PBL were incubated with 50ul of EBV virus for 1 hour at 37°C , and the cells diluted in 4ml F10 medium with $2\mu\text{g ml}^{-1}$ of PHA. The cells were cultured (37°C , 5% CO_2) in 2ml wells feeding with fresh medium as required for 3-4 weeks before expanding into 25cm^3 flasks. Lines were prepared from all the patients studied and maintained in F10 medium. All cell lines were regularly checked for the presence of mycoplasma (Colindale, Public Health Laboratories). Lines were

Table 2 Lines and media.

Cell lines	source	media	additives			optimal growth density
			FCS	glutamine	Pen/Strep	
TK ⁻ 143 (human)	Dr.G.Smith	GMEM/ FCS 10% (v/v) *	2mM glutamine, 100IU/100 ug ml ⁻¹ P/S			2.5-5 X 10 ⁵ ml ⁻¹
TK ⁻ Rat (fibro)	Dr.J.M.Monjardino	RPMI /FCS 10% (v/v) *	"	"	"	2.5-5 X 10 ⁵ ml ⁻¹
TK ⁻ Mouse L	ATCC	RPMI/FCS 10% (v/v)	"	"	"	2.5-5 X 10 ⁵ ml ⁻¹
Vero	Flow	RPMI/FCS 10% (v/v)	"	"	"	2.5-5 X 10 ⁵ ml ⁻¹
HepG2	Aden (1979)	MEM//FCS 10% (v/v) #	"	"	"	2.5-5 X 10 ⁵ ml ⁻¹
HEP ₂	ECACC	RPMI/FCS10% (v/v)	"	"	"	2.5-5 X 10 ⁵ ml ⁻¹
K562	ATCC	RPMI/FCS 10% (v/v)	"	"	"	1-2 X 10 ⁶ ml ⁻¹
B-95.8	Flow	RPMI/FCS 5-10% (v/v)	"	"	"	2.5-5 X 10 ⁵ ml ⁻¹
ACS	Acs (1987)	RPMI/FCS 10% (v/v)	"	"	"	2.5-5 X 10 ⁵ ml ⁻¹

* Recombinant vaccinia viruses were cultured in TK⁻ 143 cells in the presence of 25ug ml⁻¹ of 5-Bromodeoxyuridine (BuDr, Si. gma) .

Cells transfected with hygromycin resistant plasmids were maintained after selection in 0.3 ug ml⁻¹ of hygromycin B (Boehringer Mannheim) .

subcultured at twice weekly intervals to a density of approximately $3-5 \times 10^5$ cells per ml, and 24 hrs prior to use as targets in cytotoxicity assay.

Dendritic cells.

A dendritic cell enriched population was prepared by overnight culture of PBMCs at 5×10^6 ml⁻¹ in 10% A+ or autologous medium on tissue culture grade plastic, followed by separation of non-adherent PBMC over metrizamide at 600g for 10 mins at ambient room temperature. Metrizamide was prepared at 14.5% (w/v) (analytical grade Nycomed) in RPMI with 10% FCS, sterile filtered through a 0.2 micron filter and stored at 20°C (Macatonia et al, 1986). Low density cells including dendritic cells were aspirated from the interface of the gradient, the higher density lymphocytes were pelleted. The cell populations were washed twice with RPMI at 400g, and resuspended in Dutch modification of RPMI with glutamine (2mM) plus 10% A+ or autologous serum. Approximately 1/3 of the low density cells had the morphological characteristics of dendritic cells on a haemocytometer incubated at 37°C for five mins. Dendritic cells were transfected, and rested for 24 hrs at 37°C, before setting up 20 ul hanging drop cultures in Terasaki plates, 1×10^3 dendritic cells per well, at a ratio of 1:25 with peripheral blood lymphocytes (Knight et al, 1987).

Construction of recombinant vaccinia viruses:

Transformation of vaccinia infected cells using lipofectin.

Mackett Smith and Moss (1982, 1984), described a protocol for the introduction of foreign genes into the vaccinia virus genome via a vector PGS62, containing the vaccinia virus thymidine kinase (TK) gene. Spontaneous recombination occurs between the plasmid and the virus when both are introduced into permissive cells. The foreign gene is inserted at a cloning site within the TK gene in

the plasmid, so disrupts the viral TK gene upon recombination, allowing selection of recombinant viruses in the presence of a toxic thymidine analogue (BuDr). Specific conditions for viral recombination were optimised empirically for transfection with lipofectin (BRL) (Felger et al, 1987). Host cells used for viral recombination include Vero and human TK⁻ 143. Cell densities, types and growth rates were optimised by repeated transfection, plaquing, amplification and dot blot assay. Optimum conditions were as follows: a confluent monolayer of cells was obtained by culturing 1×10^6 TK⁻ human 143 cells for 24 hrs in a 35mm petri dish in 3ml of non-selective medium (F10). 5 hrs prior to infection the cells were stimulated by the addition of 3ml of fresh culture medium. The medium was removed and 0.5 ml Opti-MEM (BRL) containing 5×10^4 pfu of wild type virus (multiplicity of infection [moi] 0.05 plaque forming units [pfu] per cell) was added and allowed to adsorb for 1 hour at 37°C. Virus and traces of FCS were removed from the cell monolayer by washing with Opti-MEM, and 3ml of Opti-MEM added to each dish. Prior to the end of the incubation, the DNA/lipofectin complex was prepared. 30ug of lipofectin in 50ul of H₂O was added to 10-15ug of chimeric PGS62 plasmid in 50ul of H₂O, in a polypropylene or glass vial, and gently mixed for 15 mins at RT before dropping evenly onto the medium covering the monolayers. No antibiotics were added to the medium as the cell membrane is highly permeable at this stage. After 24 hrs humidified incubation at 37°C, 5% CO₂, the Opti-MEM was replaced with non-selective medium, and the cell monolayer left for a further 24-36hrs, dependent on the extent of viral cell destruction (cytopathic effect [CPE]). Cells, virus and supernatant were harvested (referred to as neat cell lysate), and lysed by freeze thawing before selecting recombinants.

Selection of recombinant viruses.

A 200ul aliquot of each dilution (neat cell lysate and dilutions in RPMI of 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5}) was adsorbed for two hrs at 37°C onto confluent TK⁻ 143 cells

in 35mm petri-dishes (Nunc). The monolayers were then washed with RPMI and overlaid with 2ml of 1% low gelling point agarose prepared in medium containing 25ug ml⁻¹ of BuDr. The gel was allowed to set before incubating the inverted dishes in a humidified atmosphere at 37°C (as above), for 48-72 hrs.

Monolayers were stained with 1ml of a 0.1% neutral red solution in PBS (1-2hrs at 37°C) to visualise viral plaques which were picked by removing the agarose plug above unstained areas with sterile glass pasteur pipettes. Virus was released by freeze thawing, and the plaque stocks amplified by infecting monolayers of TK⁻ cells in 2ml wells. The amplified stocks were harvested when extensive CPE was evident, by scraping the infected cells into 200ul of F10 medium, and freezing at -70°C. 50 ul aliquots were analysed by dot blot hybridisation, recombinants selected, and further purified by two more rounds of plaque purification.

Preparation of recombinant viral stocks.

Viral stocks were prepared by infecting confluent monolayers of between 0.5-1.0 X 10⁷ cells (in selective or non-selective media as appropriate) at a moi 1 pfu per cell (TK⁻ 143 or Vero) in 75 cm³ bottles, and culturing the cells under standard conditions until extensive CPE was visible. Crude stocks of virus were prepared by harvesting the monolayers in 2ml of medium per 75cm³ bottle, freeze thawing X 3 to release the virus which was stored in aliquots of 6 X 10⁶ pfu per vial at -70°C.

Plaque assay of vaccinia virus.

1 X 10⁵ cells were cultured in 2ml multiwell plates for 24 hrs prior to assay. Viral stocks were sonicated and diluted serially (with vortexing) down to 10⁻⁷. The monolayers were infected with duplicate 200ul viral dilutions in serum free medium, absorbed for two hrs before replacing the inoculum with 1ml medium. The monolayers were cultured for 48 hrs without disturbance at 37°C, then fixed for 5-15 mins at RT in 50/50 acetone/methanol prior

to staining with haematoxylin.

Plaque dilutions with 100 plaques per well were used to calculate the viral titre. Batches of viruses were titred together for direct comparability.

Transfection with EBO vectors.

EBO vectors were introduced into permissive cells by electroporation. Cells were prepared by subculturing 24 hrs prior to transfection in fresh F10 media minus antibiotics, at $3-4 \times 10^5$ cells per ml. The cells were concentrated to 32×10^6 cells per ml, and the media retained as conditioned media. HepG₂ cells were resuspended in conditioned media. LB cells were resuspended in RPMI plus 20mM Hepes, 6mM dextrose. 250ul of each cell suspension was transferred to a 0.4cm gap cuvette (Bio-Rad), 10ul of DNA ($1\mu\text{g ml}^{-1}$) was added, and the cells pulsed immediately using a Bio-Rad Gene pulser plus capacitance extension (0.25kV 960uFa, Hep G₂, 0.18kV 960uFa lymphoblastoid cells). Electroporated cells were resuspended in 10ml conditioned media, and incubated for 24 hrs at 37°C. After 24 hrs the cells were plated out in six 2ml wells (Cell Cult) and 0.5 mg ml^{-1} hygromycin B (Boehringer Mannheim) added to select permanent transfectants. The concentration of hygromycin B was dropped to 0.3ug ml^{-1} as resistant cells began to grow through. Transient transfectants were harvested at day 3, with cadmium chloride (CdCl_2) induction for 24 hrs prior to harvesting where appropriate. A minimum of 3×10^6 cells of an enriched dendritic cell population was electroporated, in 250ul of RPMI with 20mM Hepes and 6 mM dextrose at 0.1kV, 960 uFa, with 10ug of DNA. The cells were rested for 24 hrs at 37°C, (in the presence of 5uM CdCl_2 for induction of the metallothioneine promotor) in Dutch modification RPMI, with 10% A+ serum before adding to lymphocytes.

Protein expression:

Indirect immunofluorescence and flow cytometry of surface expressed proteins.

HepG2 cells infected at a moi 2 pfu per cell of recombinant vaccinia were grown for 16 hrs before staining. Transiently or permanently transfected HepG2 cells were grown for 16-24 hrs following subculture by trypsinisation, and pMEP containing vectors induced with 5uM CdCl₂ for at least 24 hrs before staining. The monolayers were rinsed with dilution buffer (Appendix I) then incubated with 0.2 - 0.5 ml of a supernatant containing RFHBe2, a monoclonal anti-HBe or a polyclonal rabbit anti-HBc (Dako) diluted 1/500, for 40 mins at 4°C. The cells were washed for 10 mins with dilution buffer, and incubated for 40 mins at 4°C with a 1/20 dilution of rabbit anti-mouse FITC conjugate, or 1/25 swine anti-rabbit FITC (DAKO). The cells were washed, then viewed under UV illumination on an inverted microscope. Photographs were taken (20S exposure) using a Nikon camera and P1600 Fujicolour film. Cells for flow cytometric analysis (FACS, Becton Dickinson) were prepared on non-tissue culture quality plastic, (Sterilin), and gently washed off with a Pasteur pipette after staining, fixed with 1% paraformaldehyde, and analysed on a Becton Dickinson FACS.

Staining of internally expressed proteins.

The monolayers under investigation were prepared as described in section 3.5.1 using 1×10^5 HepG2 cells infected with recombinant vaccinia viruses or transfected with EBO vectors in 2ml multi-wells. The monolayers were fixed with 1ml of cold, freshly prepared 50/50 acetone/methanol for 10 mins at 4°C, and air dried. Rehydrated cells were stained as described above except incubations were carried out for 30 mins at room temperature.

Methionine labelling of vaccinia proteins.

1×10^6 TK⁻ 143 cells were infected with recombinant

and control vaccinia viruses (moi 2pfu/cell, 1×10^6 cells per recombinant virus) and incubated for 16 hrs at 37°C. Viral proteins were labelled by the addition of 50uCi ^{35}S methionine (SJ 123, 17.2mCi ml⁻¹, Amersham) in 300ul of methionine free RPMI (GIBCO) with 10% FCS, 2mM glutamine for 4 hrs at 37°C, then either dissolved immediately in 200ul of Laemlli sample buffer, and electrophoresed on PAGE /SDS gel (Laemlli, 1970), or subjected to immunoprecipitation. Total extract protein gels were dried and autoradiographed with Kodak X-Omat film overnight.

Immunoprecipitation of nucleocapsid proteins.

Cell monolayers prepared in duplicate as above, were labelled for 2 hrs with 50uCi of ^{35}S methionine (SS1015, 10mCi ml⁻¹). After one wash, the cells were lysed on ice with 300ul RIPA buffer (Appendix I). Chromosomal DNA was sheared by passage of the lysate through a needle and insoluble cell debris removed by a 5 min centrifugation at 10000g, 4°C, in a microfuge. The supernatant was removed and normal mouse serum added to give a final concentration of 1/5000, the samples were pre-incubated at 4°C for 1hr with 75ul of pansorbin beads (Calbiochem) to reduce non-specific effects. The supernatant was then removed, and incubated for 2 hrs at 4°C with a 1/10000 dilution of specific antibody, before adding a further 75ul of pansorbin beads pre-washed in RIPA buffer, and incubating for 1 hr at 4°C. The beads were then washed in RIPA buffer, centrifuging briefly at 10000g. Protein was released by boiling the beads with 100ul of sample buffer with 5% β -mercaptoethanol, and analysed by SDS/PAGE (Laemlli) on a 12.5% gel at 240V. (Appendix I for Laemlli buffers).

The antibodies used were anti-HBe (RFHBe2) and an irrelevant murine monoclonal of the same subclass. The gel was soaked in Amplify (Amersham) dried, and exposed to X-Omat (Kodak) film for 1 week.

Immunoassay.

Cell extracts and supernatants from vaccinia infected

cells, cell lines and dendritic cells transfected with the EBO constructs were analysed for HBeAg production by immunoassay (Abbott) by the routine Virology Laboratory at St. Mary's.

4) Investigations into MHC restricted cytotoxicity.

***In vitro* priming of effector cells for investigation of class I restricted cytotoxicity.**

PBL isolated from chronic and acute HBV patients were cultured at a concentration of 1.5×10^6 cells per ml (in 25cm^3 bottles or 2ml wells) in RPMI supplemented with 10% FCS, AB, A+ or autologous serum (5% CO_2 , 37°C). Lines were established by culture of cells for 1-3 weeks feeding at weekly intervals with irradiated (or mitomycin c treated) P or LB cells in the ratio 1:5 feeder to effector cells, in the presence of fresh medium and antigenic stimulant. Several methods of stimulating memory cytotoxic effector cells were devised and investigated.

- (1) stimulating with 3.125 ug ml^{-1} of recombinant HBcAg (a gift from Dr. M. McGarvey, sucrose gradient purified)
- (2) stimulating with vaccinia infected PHA blasts (Dr. Borysiewicz personal communication)
- (3) stimulating with peptides (10 ug ml^{-1}) as described by Bertoletti et al, 1991
- (4) stimulating with EBO transfectants.

Stimulation was performed with (or without when stated) the addition of 20 IU per ml of recombinant IL-2 (Boehringer Mannheim) at day 4 after antigenic stimulation.

Preparation of target cells.

Target cells for use in chromium release assay were prepared by infection of autologous and HLA mismatched LB cells with recombinant vaccinia viruses (McMichael et al,

1986). LB cells were subcultured 24hrs prior use. To minimise variation between experiments, viral aliquots of 6×10^6 pfu in 300ul of RPMI were used to infect 3×10^6 cells. After absorption of virus for 1-2 hrs 2ml of F10 medium was added and the cells incubated for a further 2 or 16 hrs at 37°C. The cells were then labelled with 100 uCi of ^{51}Cr (Sodium Chromate, 1mCi/ml, CJS1, Amersham) for 40mins at 37°C, and washed with RPMI before resuspending the cells at a concentration of 2×10^5 per ml. Peptide coated targets were also prepared by coating with the 18-27 HBcAg peptide at a concentration of 10ug ml^{-1} in 200ul for 1 hr at 37°C, then labelled with ^{51}Cr (Bertoletti et al, 1991).

Cytotoxicity Assay

Standard 4 hour chromium release assays were performed (Brunner et al, 1968). Effector cells were harvested from culture by centrifugation at 400g for 5 mins and resuspended to give a known killer to target ratio (K:T) in F10 medium (optimally of 50:1, 25:1, and 12.5:1). Duplicate 100ul aliquots of effectors were plated out in doubling dilution in round bottom 96 well tissue culture plates, and 1×10^4 target cells in 50ul of medium, added per well. Four spontaneous release control wells and four 100% lysis control wells were plated out for each target. Assay plates were incubated for four hrs at 37°C, and centrifuged at 400g for 5 mins with the brake off. 80ul of supernatant was removed from each well and counted on a gamma counter (Packard). Results were used only when spontaneous release was under 25%.

Percentage lysis was calculated as:

$$\frac{\text{CPM of lysed target} - \text{CPM spontaneous release}}{\text{CPM 100\% release} - \text{CPM spontaneous release}} \times 100$$

CPM 100% release - CPM spontaneous release

(5) Investigating the use of transfected DC to process and present antigen in an MHC restricted manner.

DC isolated from HBV patients, were transfected with EBO vectors expressing HBeAg. Following transfection, DC were incubated for 24 hrs at 37°C. The plasmid was induced to express the nucleocapsid proteins with 5uM CdCl₂, and DC were cultured in RPMI plus 10% autologous serum, at a ratio of 1:25 DC to PBL (depleted of monocyte) in 20ul hanging drops on Terasaki plates. The cultures were pulsed for 8 hrs on days 3, 4, 5, and 6 with 0.5uCi ³H thymidine (TP120, Amersham). The cultures were harvested onto glassfibre filters and counted in Ecoscint on a β-counter (Packard).

Transfected DC were inhibited with chloroquine (10-100uM) for 1 hr prior to culture, or Brefeldin A (0.5ug/ml) prior to pulsing to investigate processing of the plasmid generated antigens. CD4+ and CD8+ enriched responder PBL populations were prepared using MicroCollector cell culture flasks (AIS) pre-coated with anti-CD8 and anti-CD4 antibody, according to the manufacturers' instructions. Briefly, 25cm³ pre-coated flasks were activated with PBS "A", 1mM EDTA. 2 X 10⁷ PBL were added to the flasks in 5ml of PBS "A", 1mM EDTA, 0.5% (w/v) human gamma globulin which was pre-treated at 56°C for 30 mins. After 1 hr incubation, non-adherent cells were gently harvested. Batches of cells pre and post depletion were stained on ice for 30 mins with anti-CD4/anti-CD8 Simultest (Becton Dickinson), washed with cold PBS "A" centrifuging at 700g, fixed with 1% paraformaldehyde/PBS "A", and subjected to flow cytometry. Enriched populations were then cultured in an identical manner to the PBL (total) populations, at a 1:25 DC to PBL ratio.

A proportion of the total DC/PBL hanging drop cultures (1 X 10⁶) were harvested at day 5-6 for enzyme linked quantification of antibody anti-HBe, anti-HBc and anti-HBs producing cells (ELISPOT) by Dr M.Thurz.

Normal human donor DC and PBL were set up in an identical manner, except that they were transfected with the EBNA minus pMep1.5 vector.

(6) Non-MHC restricted cytotoxicity.

Immunofluorescent staining of cells expressing HBeAg by patient derived anti-HBe.

HepG2 cells infected with recombinant vaccinia expressing the HBeAg and HBcAg (as previously described) were stained with 200ul of patient serum diluted 1/50 in dilution buffer was used to stain the cells expressing surface HBeAg for 30 mins at room temperature for cells fixed with 1% paraformaldehyde, followed by a 20-30 min RT incubation with 1/25 dilution swine anti-human FITC conjugate (DAKO) and scored for binding. Anti-HBe in the patient serum samples was titred by immunoassay (Abbott).

Antibody dependent cellular cytotoxicity (ADCC).

Although vaccinia infected LB lines were initially used as ADCC targets according to the method of Moss et al, (1988), the protocol was modified by using monolayers of HepG2 cells expressing HBeAg, HBcAg or wild type virus, at 5×10^4 cells per 200ul well, prepared by infecting the monolayers with 2pfu per cell as previously described, and labelling with ^{51}Cr . Targets were pre-incubated for 20 mins in dilutions of heat treated (56°C , 30 mins) human test serum before adding effector cells. The effectors were freshly isolated or IL-2 stimulated lymphocytes from normal donors, and were added to the target cells at a ratio of 25:1, in an otherwise standard 4 hr chromium release assay, calculating percentage lysis as described earlier.

Complement mediated lysis of cells expressing HBeAg.

In a modification of the chromium release assay, cells expressing HBeAg (Acs et al, 1987) and Hep G2 expressing HBeAg were seeded at a concentration of 5×10^4 per 200ul flat bottomed well, 16 hrs prior to labelling with ^{3}H Ci

^{51}Cr per well for 1 hr. The monolayers were then washed, and incubated with dilutions of monoclonal antibody, RFHBe2, RFHBs1 (an anti-HBsAg murine monoclonal, and an irrelevant monoclonal antibody of the same subtype (from $10^{-2.5}$ ug ml^{-1}) in 50ul of barbitone buffer at 37°C for 1 hr, then with 100 ul (1/10) dilution of guinea pig complement (Serotec) in isotonic saline for 2 hrs (37°C). Complement concentration was determined empirically by mediation of lysis of Herpes simplex type 1 infected cells, in the presence of a human polyclonal anti-HSV antibody (Betts and Schmidt, 1981). The plates were harvested by centrifugation at 400g for 5 mins, then 50ul aliquots of supernatant were harvested and counted on a gamma counter (Packard). Lysis of targets by complement and human anti-HBe antibody (heat treated serum dilutions of 1/4 and 1/10) was investigated in an identical manner.

Natural Killer assay.

3×10^6 tumour target line K562 cells were labelled with 100uCi ^{51}Cr for 1hr at 37°C and used as targets in a standard four hour chromium release assay (Pross et al, 1981) to investigate the incidence of NK cells prior to and following *in vitro* stimulation of lymphocytes isolated from the patient pool. Effector to target ratios used were in the range 50:1, 25:1, 12.5:1. Lymphoblastoid cells infected with vaccinia virus were also assessed as targets of NK activity.

Chapter Four. Results.

(1) Construction and characterisation of recombinant vaccinia viruses.

Viral recombination.

PGS62 chimeras containing HBV gene inserts were transfected into permissive cells infected with the WR strain vaccinia virus. Recombinant viruses were recovered by viral propagation in TK⁻ cells in the presence of BuDr, a thymidine analogue toxic to wild type virus. The vaccinia recombinants were termed:

- (1) rVcore, prepared from WR strain virus and a PGS62 chimera containing the core coding sequence 1901-2460.
- (2) rVpre-core/core, prepared from a PGS62 chimera with the core coding region sequence 1732-2460.
- (3) rV1896, prepared from a chimera with the 1732-2460 sequence, with a G-A mutation at position 1896.
- (4) rV1896/1899, prepared from a chimera with the 1732-2460 sequence, with G-A mutations at positions 1896 and 1899.
- (5) rVXAg

Although most mammalian cell lines are permissive for vaccinia, viral yield, plaque form and clarity varies. Three TK⁻ lines were investigated with respect to these qualities, a rat fibroblast line, mouse L cells, and human TK⁻ 143 cells. The latter gave good viral yields, and well defined viral plaques, so were chosen for subsequent manipulations.

Conditions for transfection were optimised using lipofectin, and CsCl₂ purified DNA. Optimal conditions were selected by repeated transfection into Vero and TK⁻143 cells, varying the cell density and length of incubation with the DNA/lipofectin complex. Lipofectin transfection was highly reproducible, even with rapid plasmid preparation DNA; the efficiency of recovery of viral recombinants varied.

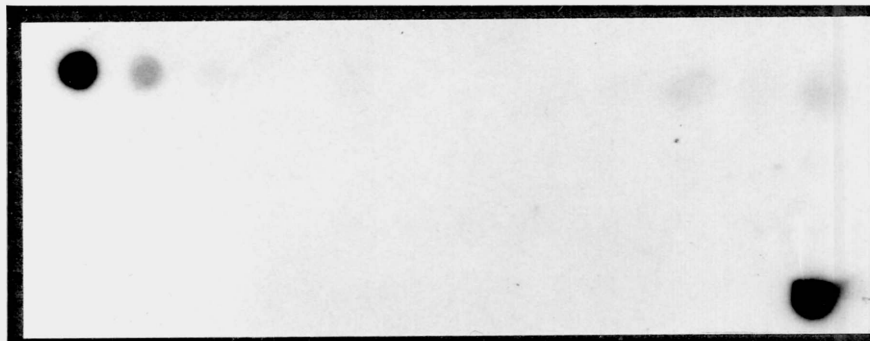
CsCl₂ purified pHB20-HBV was digested with EcoR1, the 3.2 kb band was purified by electroelution, and re-analysed on a 0.8% agarose gel to calculate the concentration.

Following viral recombination, viruses were recovered by infecting monolayers of TK⁻ cells in an agarose /selective medium overlay. Progeny from single well separated plaques were amplified, and subjected to dot blot analysis. Recombinant viruses hybridising with the ³²P labelled 3.2 kb HBV probe (specific activity approximately 1 X 10⁸ cpm/ug DNA) were subjected to a further two rounds of selection.

Purification of the rVpre-core/core recombinant is illustrated in Fig 7. Only 1/24 of progeny from the initial recombinant stock yielded virus with a HBV-DNA insert. The positive recombinant was selected, and plaqued again. By dot blot hybridisation, 13/24 of the progeny were positive, one of which was re-plaqued to yield 18/24 positive progeny. A 100% positive plaques were rarely obtained, even when more than three rounds of selection was applied, but selection of well separated, individual plaques theoretically ensured a cloned population.

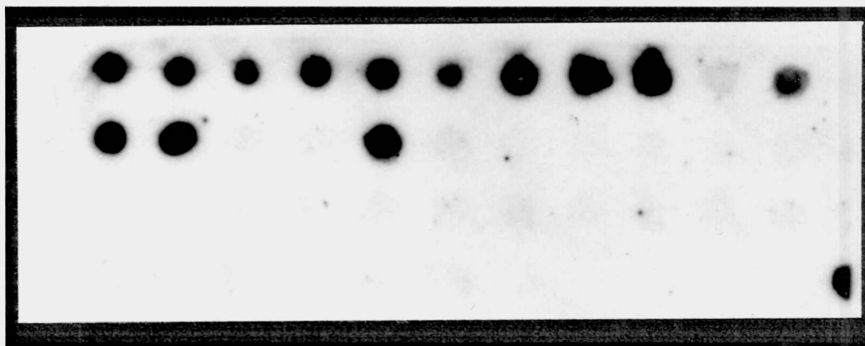
Each viral recombinant was subjected separately, to an identical procedure, and stocks of recombinant virus were titred. Fig 8 illustrates a plaque formation titration.

Fig 7. Selection of recombinant vaccinia viruses: three rounds of plaque selection, dot blot hybridisation with ^{32}P nick translated HBV digested from pHB20-HBV for rVpre-core/core.



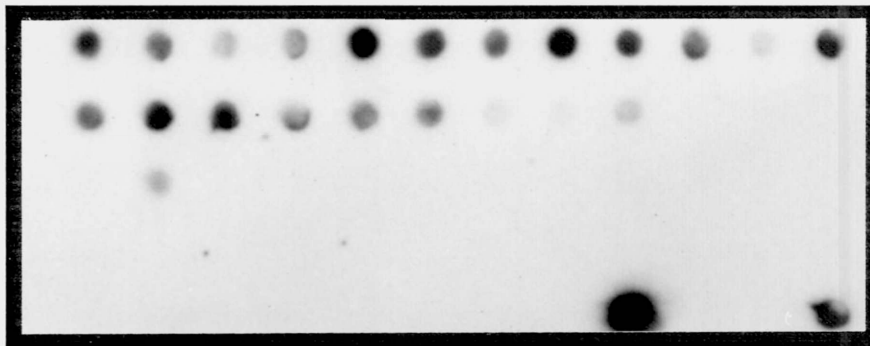
1st round
selection:
1/24 (rows
1 and 2)

++ control
rVHBSAg



2nd round
selection:
13/22 (rows
1 and 2)

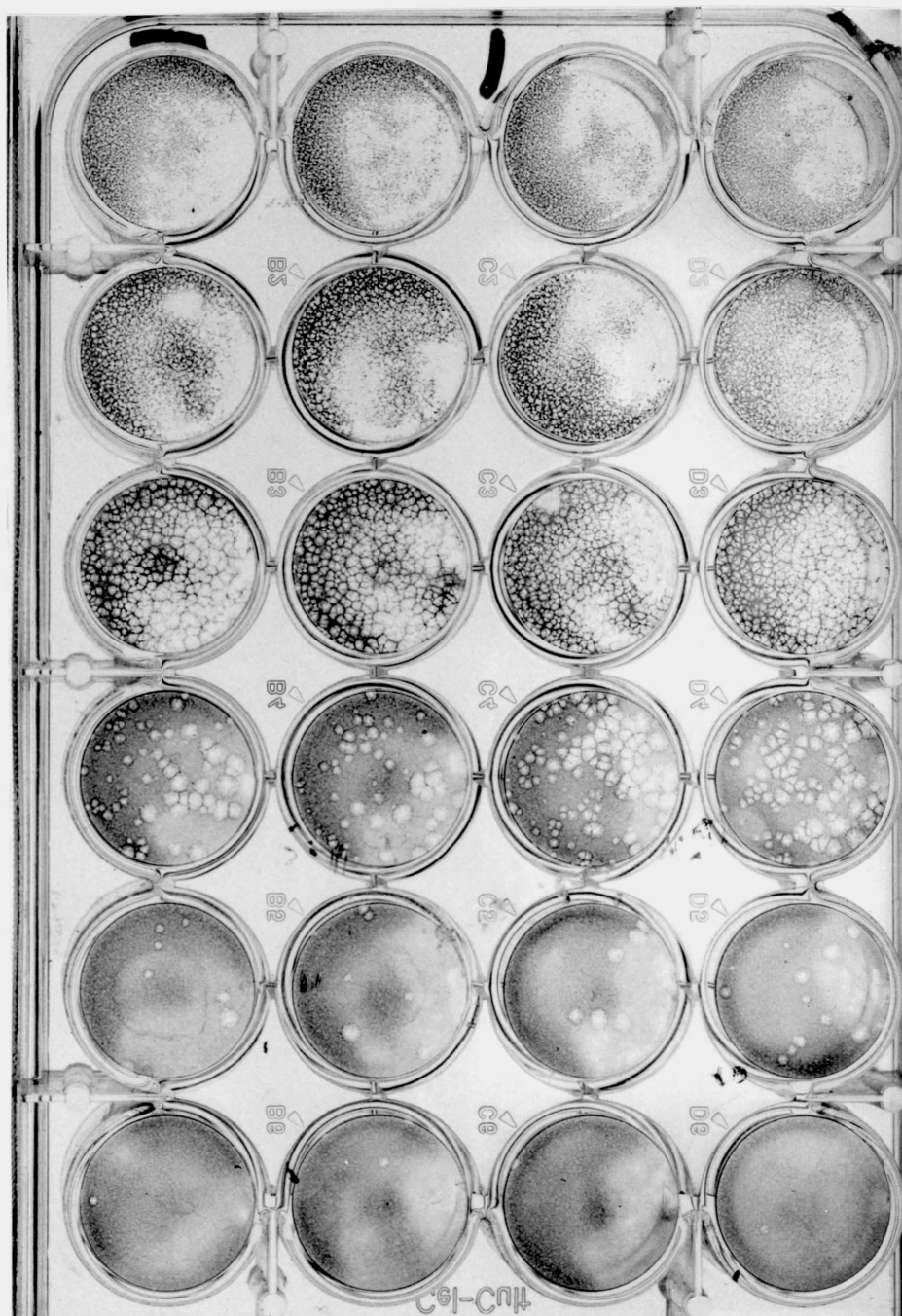
++ control
rVHBSAg



3rd round
selection:
18/24 (rows
2 and 3).

++ control
rVHBSAg

Fig 8. Titration of viral stocks by plaque assay.



Virus was diluted 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , and duplicate aliquots of 100ul and 200ul applied to monolayers (rows 1 and 2, 3 and 4 of each plate respectively), fixed plates were stored for reference and cross checking between batches of vaccinia.

Pfu/ml was calculated from the dilution resulting in 100 plaques per 100ul of diluted stock, multiplied by the dilution factor X 10.

Characterisation of the recombinant viruses.

Vaccinia virus DNA extracted from infected cells was digested with EcoRI and BamHI to release inserts, and subjected to Southern blot hybridisation with the ³²P labelled HBV-DNA probe Figs 9-10. The molecular weights of hybridising fragments were calculated from plots of log MW versus mobility, of EcoRI/HindIII digested lambda DNA electrophoresed on the same agarose gel. 730 bp fragments were released from the rVpre-core/core, rV1896, and rV1896/1899 constructs. 650 and 525 bp fragments were released from the rVcore and rVXAg constructs as predicted by the cloning strategy.

Recombinant vaccinia viruses containing nucleocapsid genes in a temperature sensitive Copenhagen strain of vaccinia were received as a gift from Dr H.J.Schlicht (Schlicht and Schaller 1989) These were :

- (a) rVHBe/c containing the whole pre-core/core gene, plus intact pre-core and core start codons,
- (b) rVHBe containing the core gene, with the core AUG codon mutated to AUA,
- (c) rVHBe containing the core gene with a deletion in the initial start codon.

Total RNA extracts of the PGS62 derived recombinants were analysed by Northern blot (Figs 11 and 12) and contained mRNA encoding HBV. The precise size of these transcripts was difficult to determine but appeared to be approximately 4.4, 2.4 and 1.4 kb in length. Transcripts appeared larger than required to code for the insert alone, probably terminating in the TK- gene.

Recombinant proteins could not be identified or separated from vaccinia proteins by gel electrophoresis (Fig 13), but immunoprecipitation of methionine labelled proteins with specific antibodies revealed single protein bands. A protein immunoprecipitated from an rVHBe/c infected cell lysate molecular weight 24kD (the predicted size of the pre-core/core gene product pre-cleavage and secretion of HBeAg) served as a positive control for analysis of the PGS62 derived constructs (Fig 14). No protein bands were detected by immunoprecipitation of

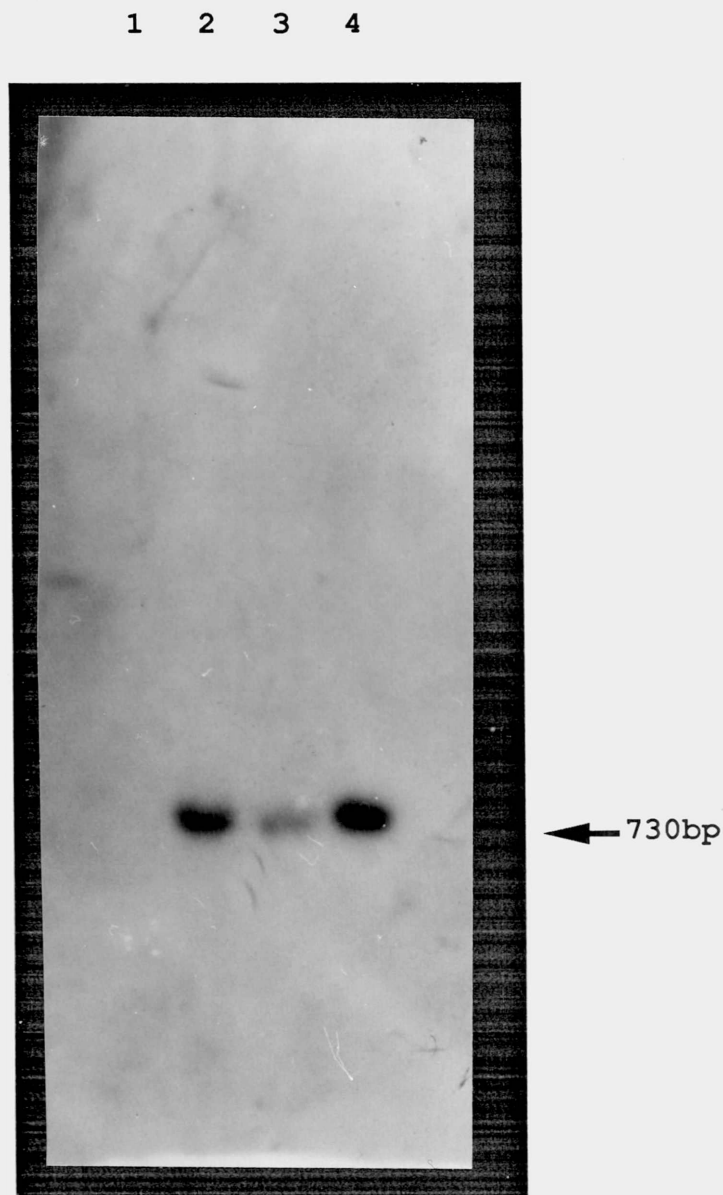
cells infected with the PGS62 derived recombinants. Standard techniques for protein detection failed to reveal protein production in any of the WR derived HBV recombinants (rVcore and rVpre-core plus rV1896 and rV1896/1899).

DNA from the rVcore and rVpre-core plus rV1896 and rV1896/1899 was subjected to direct sequencing, after amplifying the inserted fragments with the HBV primers M3 and C3. All contained the full core or pre-core genes, without deletions or rearrangements (Figs 16, 18, and Table 4). The genes inserted into PGS62 to construct the pre-core/core chimera were selected from individual patients expressing the wild type or pre-core mutations (Fig 17). Sequencing revealed all the constructs to be of the ayw subtype. Each gene was intact, but a number of independent amino acid changes occurred between each isolate (Fig 18 and Table 4) which included a mutation in the published HLA A2 epitope (S-C at position 26) in rVHB1896.

Indirect immunofluorescence, and FACScan analysis (Figs 15), and ELISA (Table 3) confirmed that the rVHBc construct produced HBcAg, and the rVHBe and rVHBe/c constructs produced HBeAg. The rVHBc/e, rVHBc and rVHBe constructs (supplied by Dr. H.J. Schlicht) had the advantage of detectable protein production, and single point mutation differences in otherwise identical genes. Although there was some indication that the WR constructs could induce a biological response (data not shown), the vaccinia recombinants which produced detectable levels of protein (rVHBc/e, rVHBc and rVHBe) were utilised exclusively in subsequent cytotoxicity studies.

The recombinant vaccinia system has some disadvantages as an expression vector. Vaccinia is cytopathic, causing membrane leakage and cell lysis. Infected targets are unsuitable for complement studies, as the virus produces a decoy C3b homologue (McKenzie et al, 1992) necessitating the production of alternate expression systems, which were further developed during the course of the study.

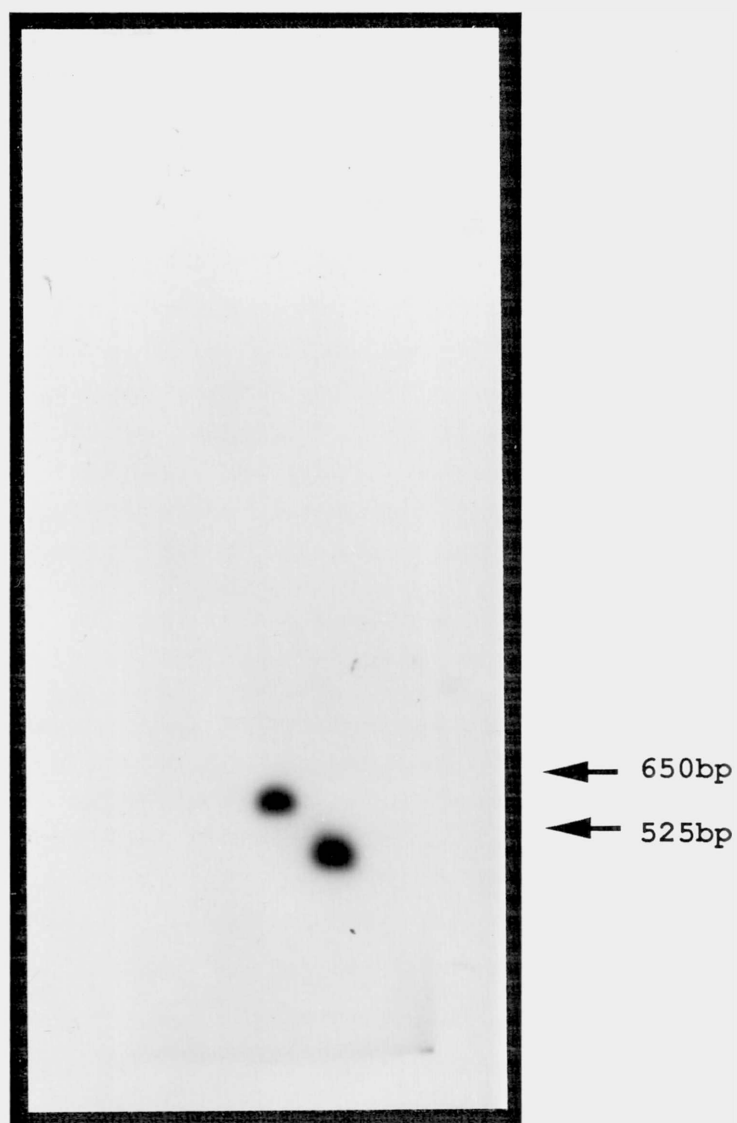
Fig 9. Southern blot of recombinant vaccinia DNA (1); extracted from cells infected with rVpre-core/core, rV1896, and rV1896/1899, hybridising with nick translated ^{32}P HBV digested from pHB20-HBV.



Lane 1: BamHI/EcoRI digest of wt viral DNA
Lane 2: BamHI/EcoRI digest of rVpre-core/core DNA
Lane 3 :BamHI/EcoRI digest of rV1896 DNA
Lane 4 :BamHI/EcoRI digest of rV1896/1899 DNA

Fig 10. Southern blot of recombinant vaccinia DNA (2); extracted from cells infected with rVcore, and rVXag hybridising with nick translated ^{32}P HBV digested from pHB20-HBV.

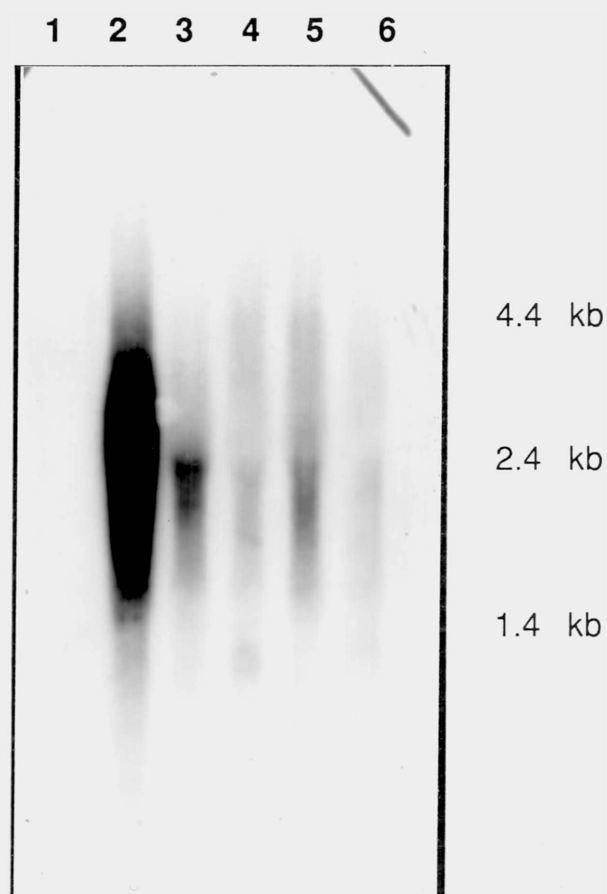
1 2



Lane 1: BamHI/EcoRI digest of rVcore DNA

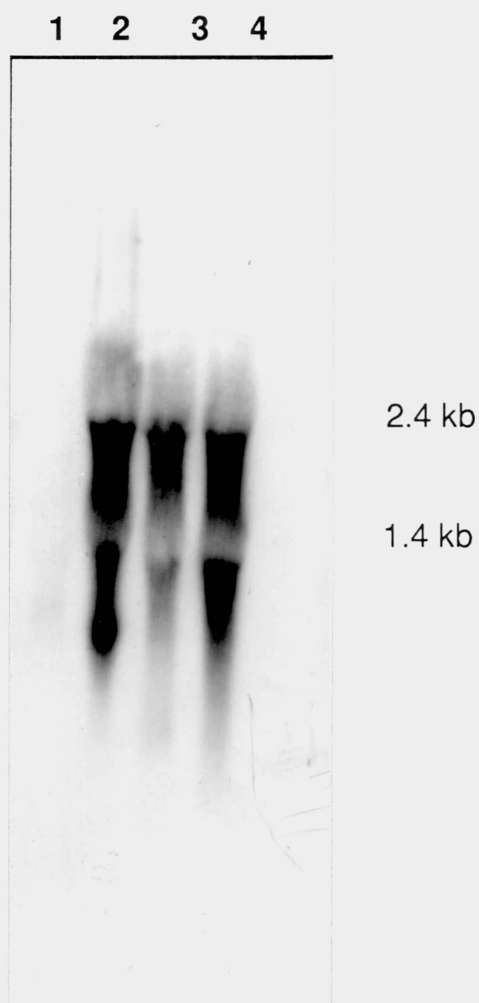
Lane 2: BamHI/EcoRI digest of rVXAg DNA

Fig 11. Northern blot of RNA extracted from cells infected with recombinant viruses, hybridising with nick translated ^{32}P HBV digested from pHB20-HBV and harvested at 24 hrs.



- Lane 1:** WR infected control, total RNA extract
Lane 2 : rVHBe/c positive control, total RNA extract
Lane 3 : rVcore ,total RNA extract
Lane 4 : rVpre-core/core, total RNA extract
Lane 5 : rV1896, total RNA extract
Lane 6 : rV1896/1899 total RNA extract

Fig 12. Northern blot of RNA extracted from cells infected with recombinant viruses, hybridising with nick translated ^{32}P HBV digested from pHB20-HBV and harvested at 6 hrs.



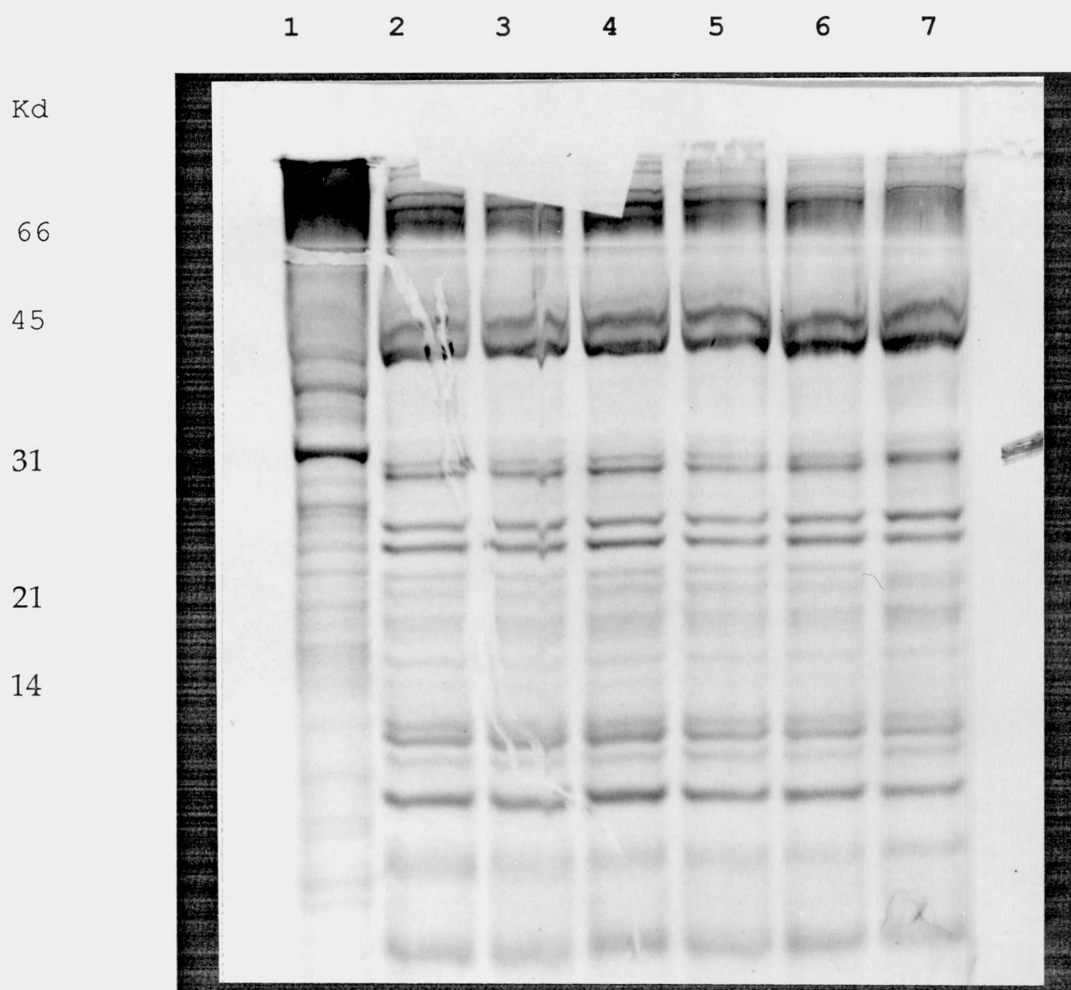
Lane 1: WR total RNA extract

Lane 2: rVpre-core/core

Lane 3 : rV1896

Lane 4: rV1896/1899

Fig 13. Methionine labelling of vaccinia viral proteins, electrophoresed on a 12.5% PAGE SDS gel.



Lane 1. Non-infected HepG2 cell lysate. **Lane 2,** WR (wild type) strain infected cell lysate, **lane 3,** rVcore, **lane 4,** rVpre-core/core, **lane 5,** rV1896, **lane 6,** rV1896/1899, and **lane 7,** lysate from HepG2 cells infected with rVHbxAg.

All the samples were electrophoresed under reducing conditions. Although the proteins labelled in vaccinia infected cells were clearly distinguishable from non-infected cells, no obvious bands of recombinant proteins were visible.

Fig 14. Immunoprecipitation of viral recombinant proteins.

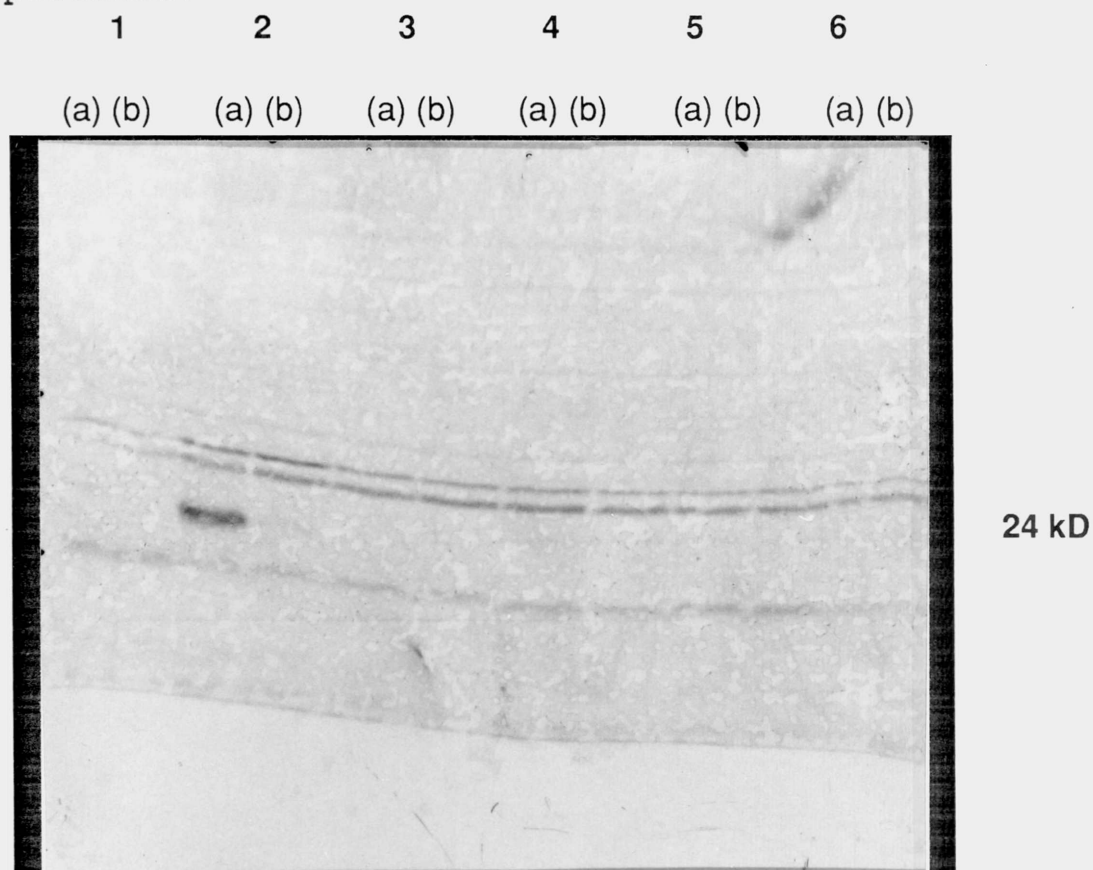


Fig 14

Lane 1 wt

Lane 2 rVHBe/c

Lane 3 rVcore

Lane 4 rVpre-core/core

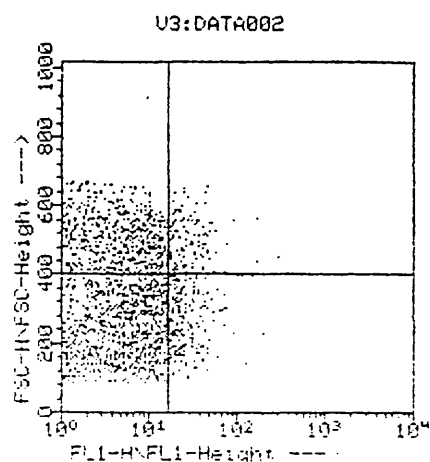
Lane 5 rV1896

Lane 6 rV1896/ 1899

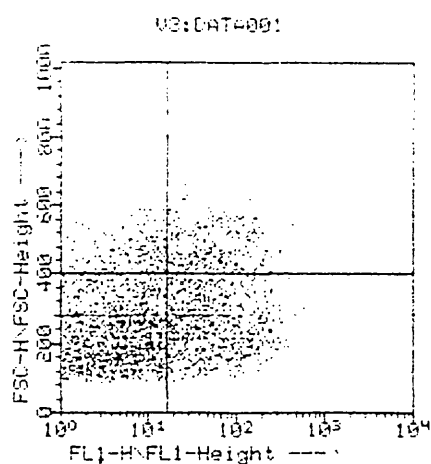
The conditions for immunoprecipitation of nucleocapsid proteins from virally infected HepG2 cells were set for the rVHBe/e construct (data not shown). Each protein extract was immunoprecipitated with RFHBe2 and an irrelevant monoclonal antibody of the same sub-class (tracks a and b respectively). A band at 24 kD was detected in the rVHBe/e construct precipitated with RFHBe2, track 2 (a), after 1 week autoradiography, but not in the rVcore, rVpre-core/core, rV1896 or rV 1896-1899.

Fig 15. FACScan of HEPG2 cells infected with rVHBc and rVHBe stained with RFHBe2, and anti-murine FITC conjugate.

(a)



(b)



The FL1 axis shows the brightness of fluorescence, FSC cell density.

(a) little or no surface fluorescence on unfixed rVHBc infected cells.

(b) brightly surface stained rVHBe infected cells

Table 3. Summary of HBeAg antigen expression as detected by ELISA.

assay of rV lysates	

Recombinant virus	HBeAg (Abbot ELISA)
1 rVHBe	positive
2 rVHBc	positive
3 rVHBe/c	positive
4 rV core	negative
5 rV pre-core/core	negative
6 rV 1896	negative
7 rV 1896/1899	negative
8 wt (WR strain)	negative

After infection with 1 pfu per cell, monolayers of 1×10^5 HepG2 were grown in 2ml wells with 2ml medium for 24 hrs. CPE was of a comparable extent in each well. 1.5ml medium was removed, and cells scraped into the remaining 500ul, and freeze thawed. The lysate was syringed through a 23g needle to disrupt chromosomal DNA, and the insoluble debris pelleted, removing the supernatant for assay. Samples 1 - 3 were control extracts from cells known to express HBeAg and HBcAg. Sample 8 was included as a negative control.

Fig 16. PCR of the pre-core/core gene from vaccinia DNA extracted from virally infected cells, primers M3 and C3.

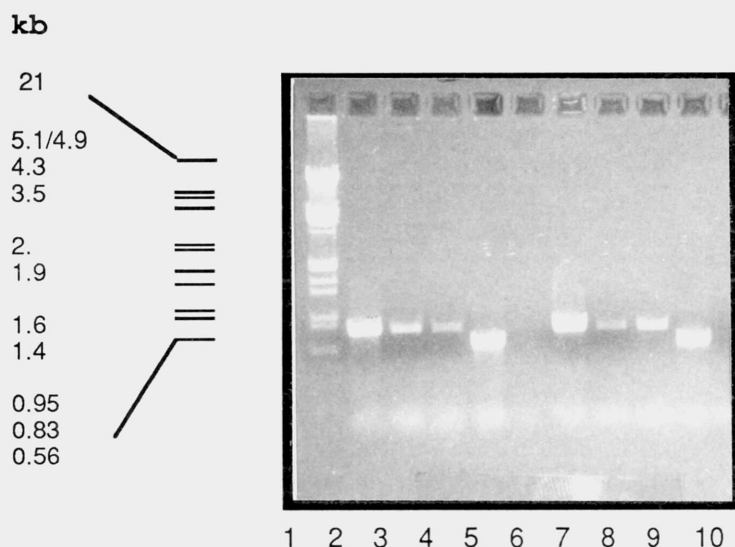
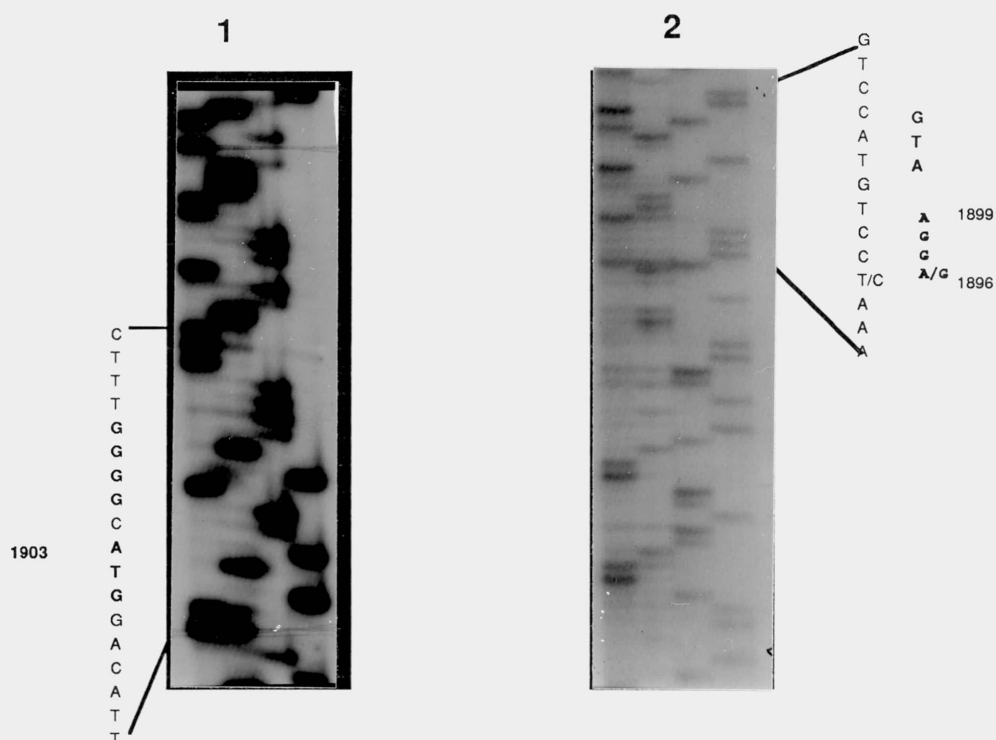


Fig 16.

Lane 1: 0.5 ug of lambda HindIII/EcoRI DNA digest
Lane 2: **Lane 7:** rVpre-core/core amplification product
Lane 3: **Lane 8:** rV1896 amplification product
Lane 4: **Lane 9:** rV1896/1899 amplification product
Lane 5: **Lane 10 :** rVcore amplification product
Lane 6: H₂O control

1/10 volume of PCR products were analysed on a 0.8% agarose gel stained with EtBr, the remainder was purified (as described in Chapter 3) and subjected to direct sequence analysis.

Fig 17 Sequencing of pre-core/core genes with BC1 and pre-core primer.



1: CW1 BC1 sequence TCGA

2: PP pre-core sequence TCGA

Two short extracts of sequence are shown for illustration. Sequence 1 was derived from patient CW (CW1) and shows the pre-core start ATG and preceding codons. Sequence 2 was derived from patient PP. The codons at position 1896 are A and / or G, but position 1899 is A (G to A mutation). Sequencing with BC1 in the opposite direction confirmed both G-A mutations.

Table 4 Identification of sequences compared in Fig 18.

sample identity	origin	code
core antigen (plus sequence predicted for pre-core/core ORF)	Galibert 1979	1
rVHBcore	pHB20-HBV (Summers)	2
rV1896	Mediterranean chronic carrier	3
rV1896/1899.	Mediterranean chronic carrier	4
rVHBpre/core/core	Mediterranean chronic carrier	5
CW(1)	Caucasian chronic carrier	6
CW(2)	CW during seroconversion	7
CW(3)	CW post seroconversion	8
CH	Caucasian acute HBV patient	9
LC	Asian acute HBV patient	10
PP	Mediterranean Chronic carrier (with anti-HBe positive disease)	11

Fig 18. Amino acid sequence comparison of HBV nucleocapsid genes used in expression vectors.

```

.....-10.....20.....-30--- ....40.....50.....60
MQLFHLCLIISCTCPTVQASKLCLGWLTGMDIDPYKEFGATVELLSFLPSDFPSVRNLLNTASALYREALSPEHCSPHHTALRQAIL
2.....A.....D..A...D.....D.....
3.....S.....*.....Q.....C..V..E..A.....
4.....*.....D.....
5.....S.....A.....RS.D.....
6.....
7.....
8.....
9.....*.....I.....D.....
10.....
11.....S.....*.....

61.-.....70..-----80-----90--.....100.....110..... 121.....130.....140.....150
CWGELMTLATWGVNLEDPASRDLVVSIVNTNMGLKFRQLLWFHISCLTFGRETVIEYLVSGVWIRTPPAYRPPNAPILSTLPETTVVR
...D.....P..Q....G.....V.....Q.....
.T.A.....G..I...T.....V.....Q.....
.....G.....Y.....
.....GH...I.....S.....I.....R.....
.....N.....I.....
.....N.....I.....
.....N.....I.....
      N.....V.....
      .....V.....
      .....S.....V.....

151..... 160.....170.....180.
RRGRSPRRRTSPRRRRSQSPRRRRSQSRESQC*
..C.....T.....*
....T.....*
.....
..DS.....

```

KEY
row 1 reference sequence.
Single letter amino acid code.
* Stop codon.
--- hypervariable region
.. identical to reference sequence.

(2) *Construction of the Epstein-Barr Origin of replication chimeras.*

HBV DNA isolates, PCR and PCR sequencing.

The patients selected as HBV DNA donors had well characterised clinical histories, and were subsequently subjected to cytotoxicity study. PCR products were prepared from serum derived HBV-DNA by single or nested PCR, with the M3/C3 primers or M3/ZO62 followed by EO41/E042. DNA was selected for cloning into expression vectors, by sequencing the least conserved regions of the pre-core /core genes. Fig 18 shows the sequence comparison.

Taq cycle sequencing at elevated temperatures gave reproducible sequences with end labelled ^{32}P primers, and was used throughout to sequence PCR products and plasmids.

Patient CW was followed by sequence analysis across the pre-core and core start codons, prior to (CW1), during (CW2) and approximately 2 months after seroconversion from HBeAg positive status to anti-HBe positive and HBV-DNA by dot blot negative status (CW3), (Fig 18). A longer term follow-up could not be undertaken as the patient failed to return. Adequate DNA for sequencing and cloning could be obtained from a single round of PCR from extracts CW1 and CW2, but nested PCR was necessary to produce products from CW3, and the other patients extracts. The HBV-DNA sequence of patient CW remained constant over the seroconversion period.

Two pre-core/core PCR products (extracted from patients CH and PP) were found to have the mutations 1896 and 1899. Patient PP had anti-HBe positive DNA dot blot negative chronic HBV, patients CH and LC had acute infection, and subsequently completely recovered. Appendix II documents the clinical observations at the time of HBV-DNA sampling.

Choice of expression vectors, cloning strategy and chimera construction.

Direct cloning of PCR product synthesised with primers

containing non-compatible restriction enzyme sites was chosen for its simplicity. The expression vectors selected pCEP4 and pMEP4 (Invitrogen) had the attributes;

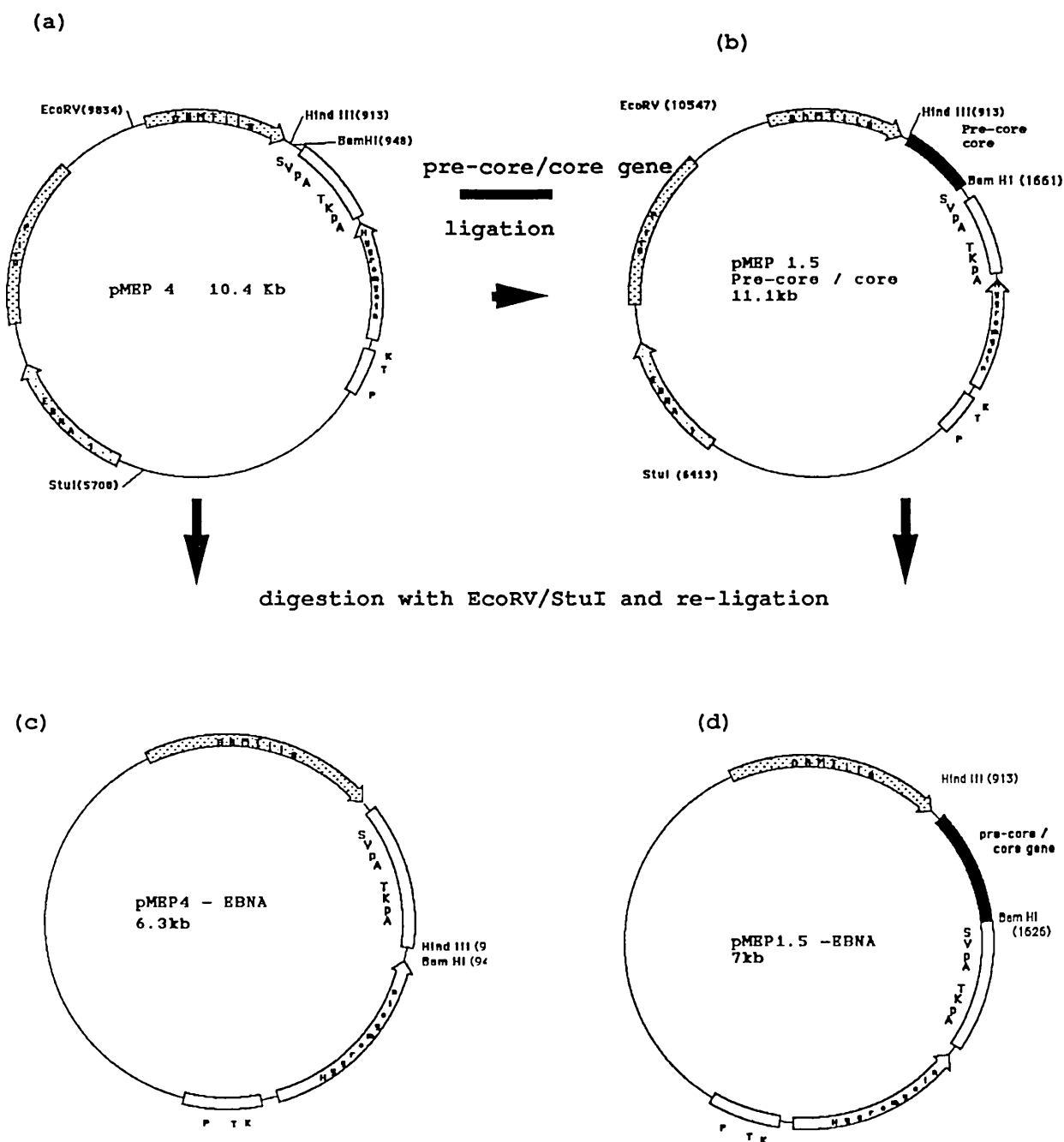
- (1) polylinker cloning site,
- (2) Epstein-Barr origin of replication and EBNA-1 gene, allowing episomal replication in a range of eukaryotic host cells,
- (3) powerful promoters (CMV, and metallothionine respectively),
- (4) hygromycin resistance for selection of permanent transfectants,
- (5) both plasmids had stabilising polyAAA tails to the insert mRNA transcripts,
- (6) the ability to grow in *E.coli* (ColE1 origin) with ampicillin selection.

Fig 19 illustrates the construction of the EBO pMEP and pMEP EBNA deleted vectors. Fig 20 shows the amplification of the pre-core gene from individual HBV-DNA extracts. Fig 21 shows pre (Fig 21a) and post (Fig 21b) ligation analysis of vector and insert. Construction of the pCEP series was performed in an identical manner.

EBO chimeras with the CW, PP and LC genes were constructed in both pCEP4 and pMEP4. Chimeric plasmids were electroporated into competent *E.coli*, (yielding at least 10^8 colonies per μg of closed circle DNA). Successful ligations contained from 10^1 to 10^4 colonies above insert negative ligase controls. The most efficient PCR product ligations were achieved when ethidium bromide was extracted from "squeeze freeze" purified DNA.

An insert (derived from CW1) identical by sequence to CW2 and CW3, was cloned into pCEP4 was termed pCEP1.6. The CW1 insert cloned into pMEP4 was termed pMEP1.5, and the pCEP4 LC derived construct, was termed pCEP2.4. Vectors constructed from the other DNA products were not used in further studies described. Colonies were analysed by restriction digestion of rapid plasmid prepared DNA. Colonies with insert were sequenced using the A100 primer

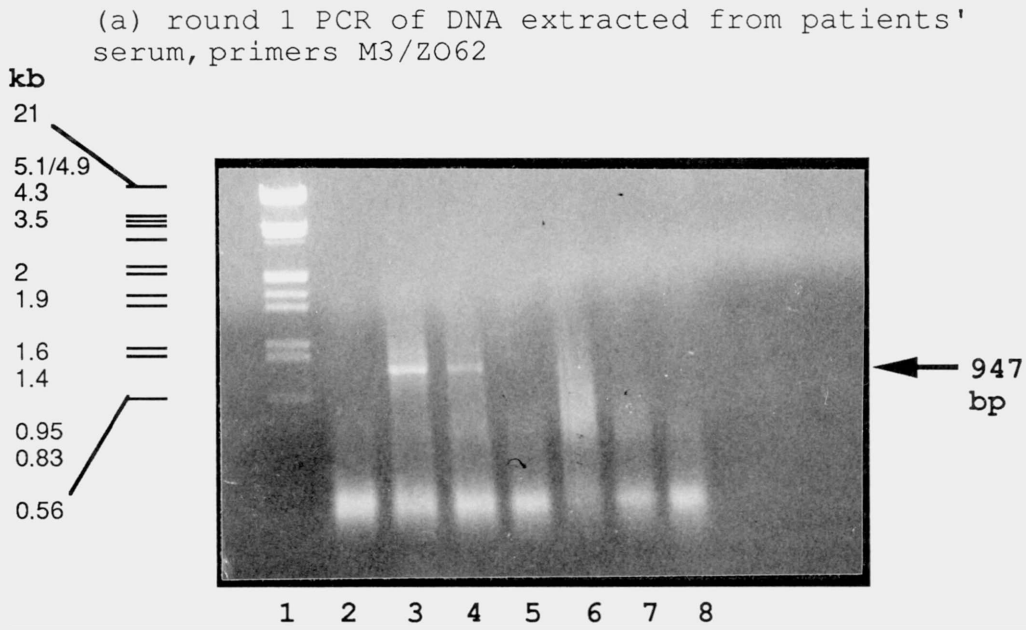
Fig 19. Construction of pMEP 1.5 pre-core / core, and pMEP1.5-EBNA vectors.



Ori P : EBV origin of replication for episomal replication
EBNA-1 : produces transactivator of *Ori P*
PhMTIIa : metallothionine promoter of inserted gene
SVpA : stabilises mRNA transcript with a poly A tail
Hygromycin : Hygromycin resistance gene, plus promoter and poly A tail.

to analyse the 5' sequence in pCEP₄ chimeras, and the BC1 primer for pMEP₄ chimeras. Fig 22 illustrates sequence data from pCEP1.6. The pre-core/core gene isolated from patient sample CW1. For stimulation of effector cells to be used in cytotoxicity assays with Epstein-Barr transformed target cells, and for the potential stimulation of HBV naive dendritic cells, a transient expression system not complicated by the expression of the EBNA-1 gene was constructed. The EBNA-1 gene and Ori-P were deleted from chimeric pMEP1.5 and pCEP1.6 (in addition to the empty vectors) by restriction enzyme digestion at the *Stu*I and *Eco*RV sites which created compatible blunt ends which were re-ligated and transformed into competent *E.coli*. Colonies were selected by restriction enzyme analysis of DNA obtained by rapid plasmid preparations. The vector sizes were confirmed by Southern blot hybridisation of digested plasmid (Fig 23).

Fig 20. PCR of HBV DNA pre-core/core gene with BamHI/HindIII restriction enzyme sites, from serum HBV extracts.



(b) second round PCR, primers EO41/EO42

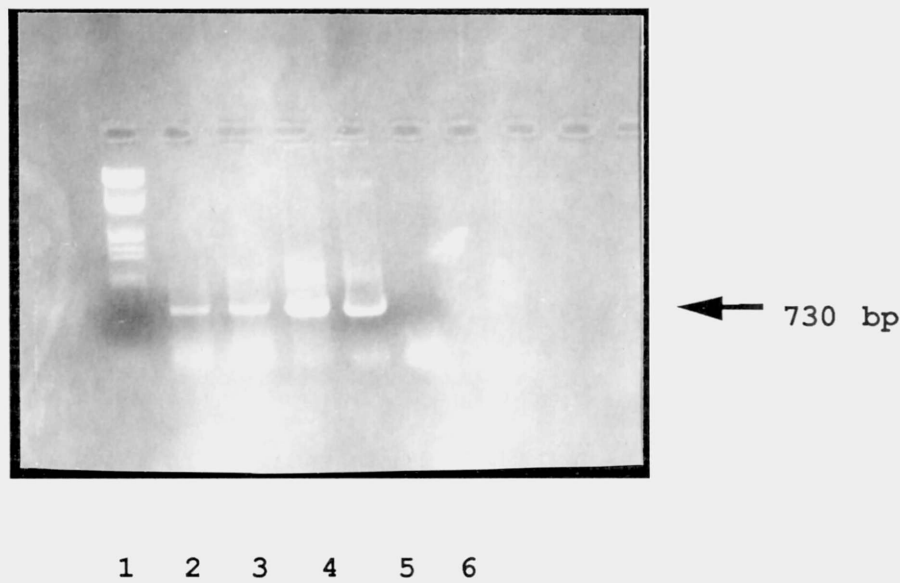
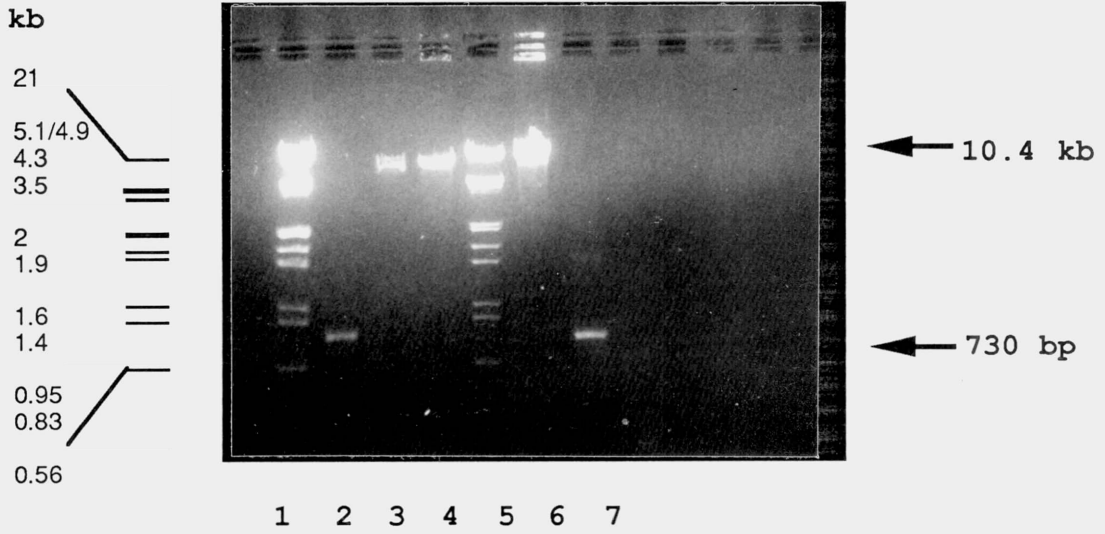


Fig 20.**(a) M3/ZO62 amplification products****Lane 1:** 0.5ug of Hind III/EcoRI lambda DNA**Lane 2:** CW (3) post seroconversion**Lane 3:** CW (2) during seroconversion**Lane 4:** CW (1) chronic carrier**Lane 5:** CH acute HBV**Lane 6:** LC acute HBV**Lane 7** PP chronic HBV**Lane 8:** H₂O control**(b) E041/E042 nested amplification of round 1 products****Lane 1:** 0.5ug of Hind III/EcoRI lambda DNA**Lane 2:** CW (3)**Lane 3:** CH**Lane 4:** LC**Lane 5:** PP**Lane 6:** H₂O

Fig 21. Cloning into pCEP4 and pMEP4.

(a) purified vectors (linearised with BamHI/HindIII) and PCR product pre-ligation.



(b) Bam HI/Hind III digestion of pMep4/CW1 ligation products.

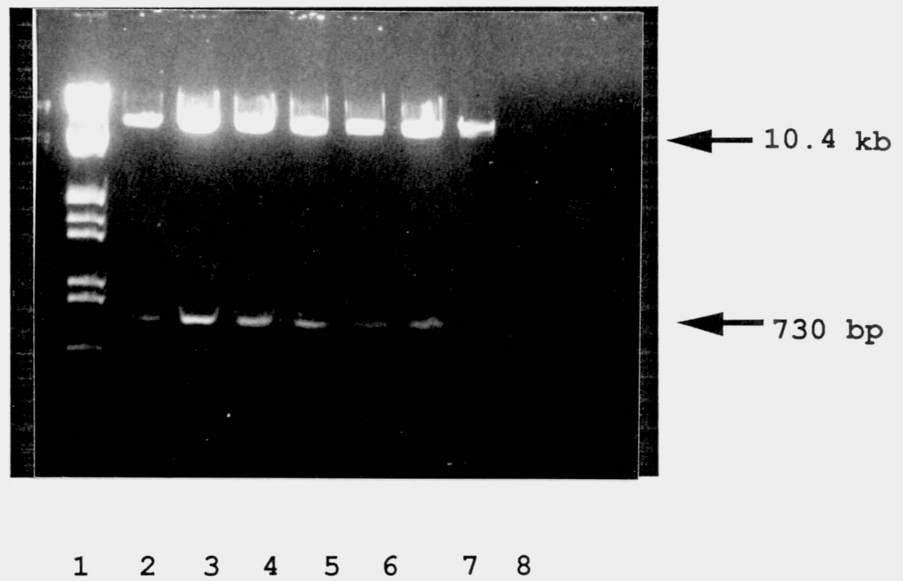
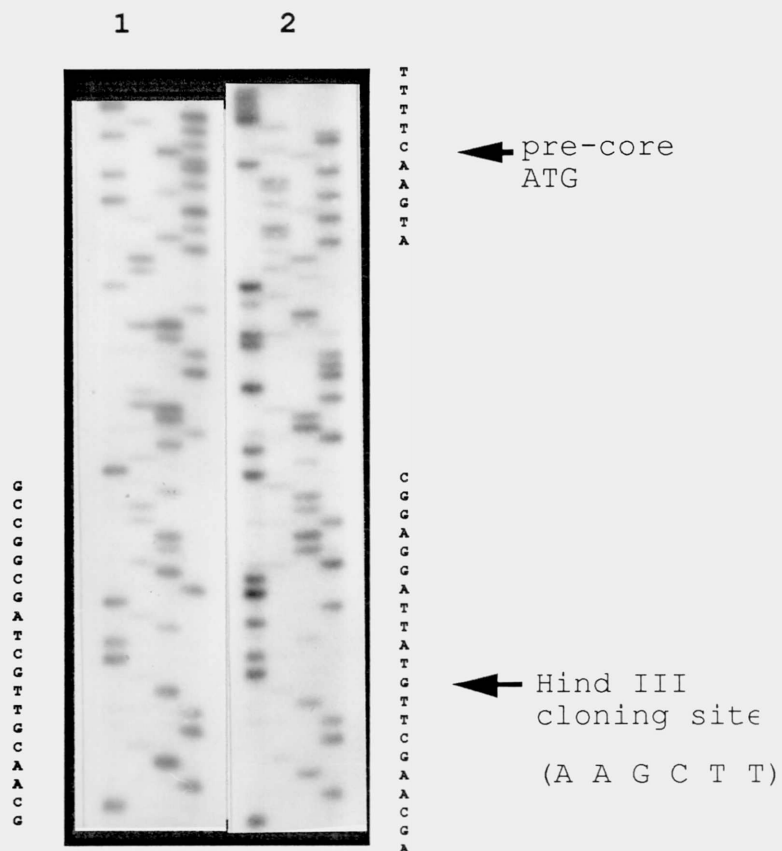


Fig 21.

0.8% agarose gels stained with EtBr of:

- (a) **Lane 1:** 0.5 ug of Hind III/EcoRI lambda DNA.
Lane 2: 1ul of Hind III/BamHI digested purified PCR product (LPC)
Lane 3: 0.5ul (400ng) of pCEP4, linearised with BamHI/HindIII and purified ready for ligation
Lane 4: as lane 3, but 1ul (800ng)
Lane 5: 0.5 ug of Hind III/EcoRI lambda DNA.
Lane 6: 1ul of pMEP4 (1000ng) of HindIII/Bam HI linearised plasmid, purified for ligation
lane 7: 1ul of CW1 insert (500ng) digested from with pCEP1.6 chimera with HindIII/Bam HI, purified for ligation.
- (b) **Lane 1:** 0.5 ug of Hind III/EcoRI lambda DNA.
Lanes 2 - 8 DNA. Rapid plasmid preparation DNA from single ampicillin resistant colonies of *E.coli* transformed with pMEP4/CW1 ligation products.
 100% of the DNA extracts prepared from vector plus DNA excised from pCEP/1.6 (containing the CW1 insert) with Bam HI/HindIII contained the 730 bp insert.

Fig 22. Sequencing the 5' end of pCEP1.6



Lane 1: pCEP4, non-digested plasmid sequenced TCGA. The HindIII cloning site is intact, and the sequence corresponds to that published.

Lane 2: pCEP1.5 sequence TCGA. The HindIII site is re-formed, (the plasmid was digested prior to ligation) and the HBV pre-core/ core derived from CW is present. The pre-core ATG is intact (as is the core ATG, not shown).

Characterisation of the gene products of the EBO vectors in eukaryotic cells.

Southern blotting of the chimeric plasmids confirmed that the inserted genes were still present after the construction of the EBNA deleted viruses.

The ^{32}P nick translated pHB20-HBV probe hybridised not only with the inserts, but also with the vector, as both carry PBR322 encoded sequences (Fig 23). The chimeric viruses contained HBV inserts and corresponded to the sizes predicted by the cloning strategy, ie. 6.5 kb for the EBNA - pCEP vector (lane 6), and 7.1kb for the pCEP1.6-EBNA, and the pMEP 1.5-EBNA (lanes 5 and 3 respectively). The pMEP1.5 and pCEP1.6 were electrophoresed and blotted concurrently as molecular markers.

Stable transfectants were raised in Akata cells, and HepG2 cells. HepG2 cells transfected with pMEP1.5 were stained by immunofluorescence for HBeAg, when the transfectants were induced with CdCl_2 (Fig 24), and the cell culture supernatants subjected to commercial ELISA assay (ABBOTT HBeAg/anti-HBe), Table 5.

Fig 23. pMEP/chimeras Southern blot of plasmids hybridising with pHB20-HBV (non-digested) ^{32}P labelled probe.

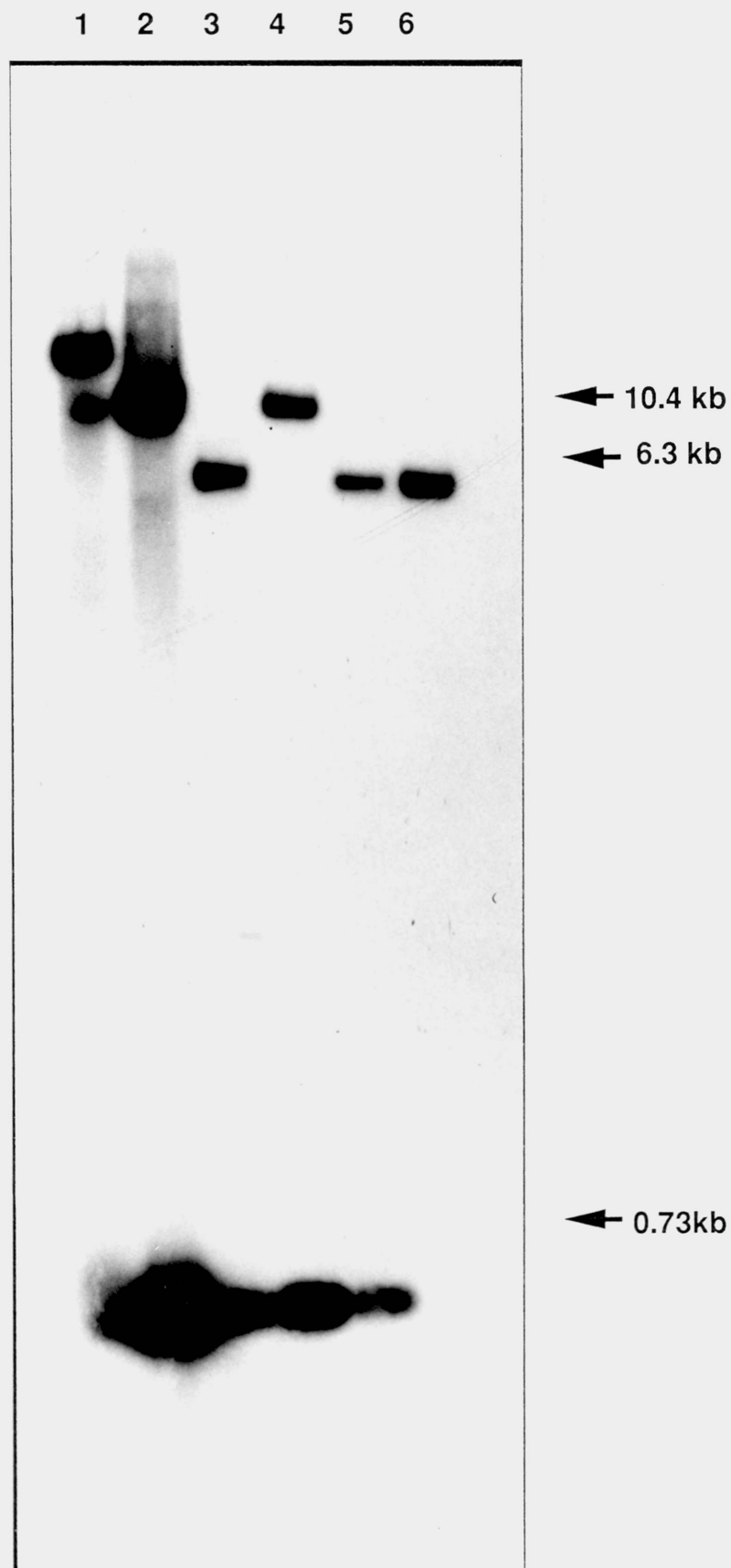


Fig 23.

Lane 1: non-digested pMEP1.5

Lane 2: pMEP1.5 digested with BamHI/HindIII

Lane 3: pMEP-EBNA digested with BamHI/HindIII

Lane 4: pCEP1.6 digested with BamHI/HindIII

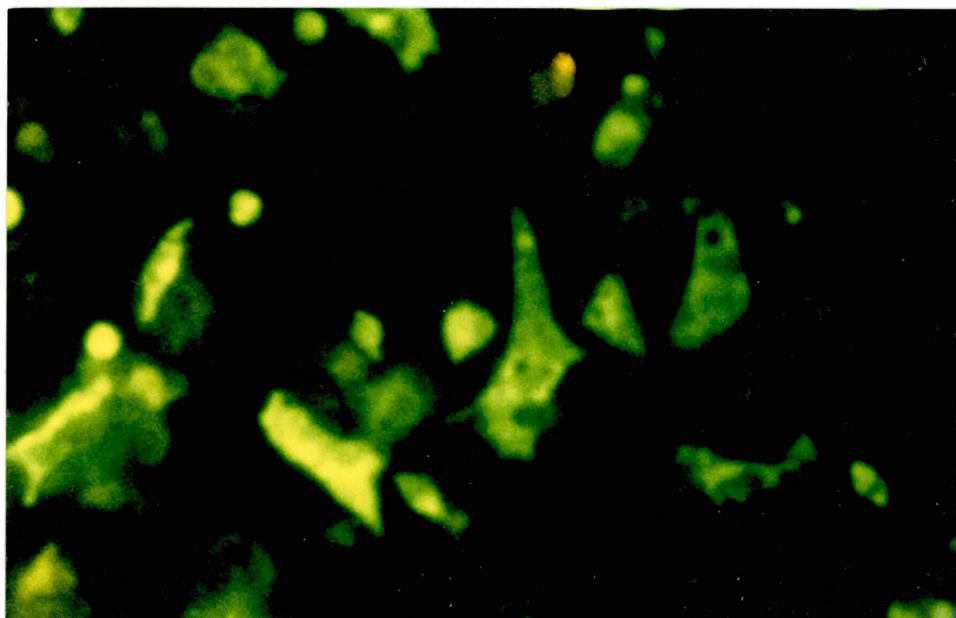
Lane 4: pCEP-EBNA digested with BamHI/HindIII

Lane 5: pCEP-EBNA digested with BamHI/HindIII

Lane 6: pCEP-EBNA digested with BamHI/HindIII

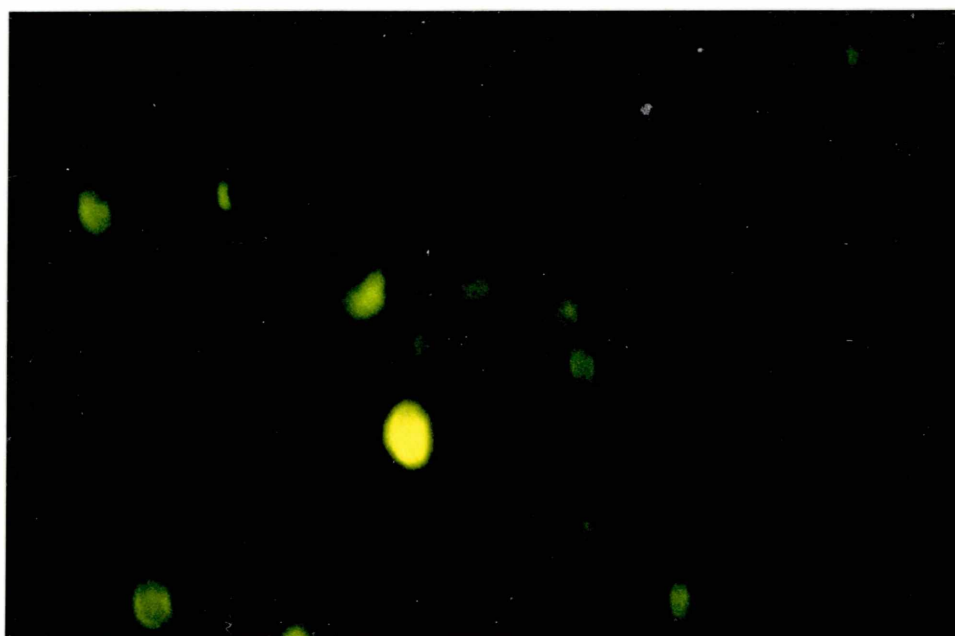
Fig 24. Indirect immunofluorescence of HepG2 cells transfected with pMEP1.5. The cells were fixed and permeabilised prior to staining.

(a)



original X 40 magnification

(b)



original X 40 magnification

(a) was induced with CdCl₂ for 24 hrs prior to staining (b) was not induced.

Table 5 Summary of HBeAg expression (detected by ELISA) in transfected cells.

supernatants from the following lines and cells were tested for HBeAg:

Line	transfected with:	HBeAg (Abbot ELISA)
1 HepG2	pCEP1.6 (P)	positive
2 HepG2	pCEP1.6 (T)	positive
3 "	pCEP2.4 (T)	positive
4 "	pMEP1.5 (T)	positive
5 "	pMEP1.5 (P)	positive
6 Akata	pCEP1.6 (P)	positive
7 LB(CW)	pCEP1.6 (T)	positive
8 HepG2	pMEP1.5-EBNA (T)	positive
9 HepG2	pCEP1.6-EBNA (T)	negative
10 DC(NHD)	pMEP1.5 (T)	positive
11 DC(NHD)	pMEP1.5-EBNA (T)	negative

T; Temporary transfectant,

P; Permanent transfectant.

NHD; normal human donor.

(3) Investigation of MHC Class I restricted cytotoxicity.

Lysis of autologous and heterologous targets expressing nucleocapsid antigens during acute HBV infection.

Clinical data on patients documented in the following figures is detailed in Appendix II. Fig 25 shows the results obtained when six acute HBV patients were studied in cytotoxicity assay. Stimulating PBL obtained from an HLA A2 restricted patient (LC) with the core peptide p18-27 in the presence of rHBcAg and IL-2, antigen specific MHC restricted CTL were detected (Fig 25 {a}). Effectors from a second HLA A2 patient, fell outside the window of detection (Fig 25{c}), and did not exhibit a significant response when stimulated with p18-27, against any target cells, possibly confirming the report that CTL can only be detected during a six week period after the onset of icterus.

Pools of overlapping 15-18 mer peptides (documented in Appendix III) were used in conjunction with rHBcAg and IL-2, to investigate the response in patients with different HLA types. Two HLA A1 restricted patients were stimulated with several pools of peptides, rHBcAg, and IL-2, and responded with high level lysis of all targets (Fig 25 {b}, {d}). Lysis of nucleocapsid expressing cells was marginally higher than wt infected cells when effectors were stimulated with peptide pool 13,14,15,16, but this was not confirmed by lysis of non-autologous HLA A1 matched cells. No response was detectable in the HLA A28 restricted patient {e} who was normalising transaminase levels, nor the HLA A24 patient {f} stimulated with DC expressing the HBeAg.

Fig 25. Acute HBV: lysis of autologous and heterologous targets; infected with rVHBe/c or wt vaccinia virus by effector cells stimulated with rHBcAg, peptides and IL-2

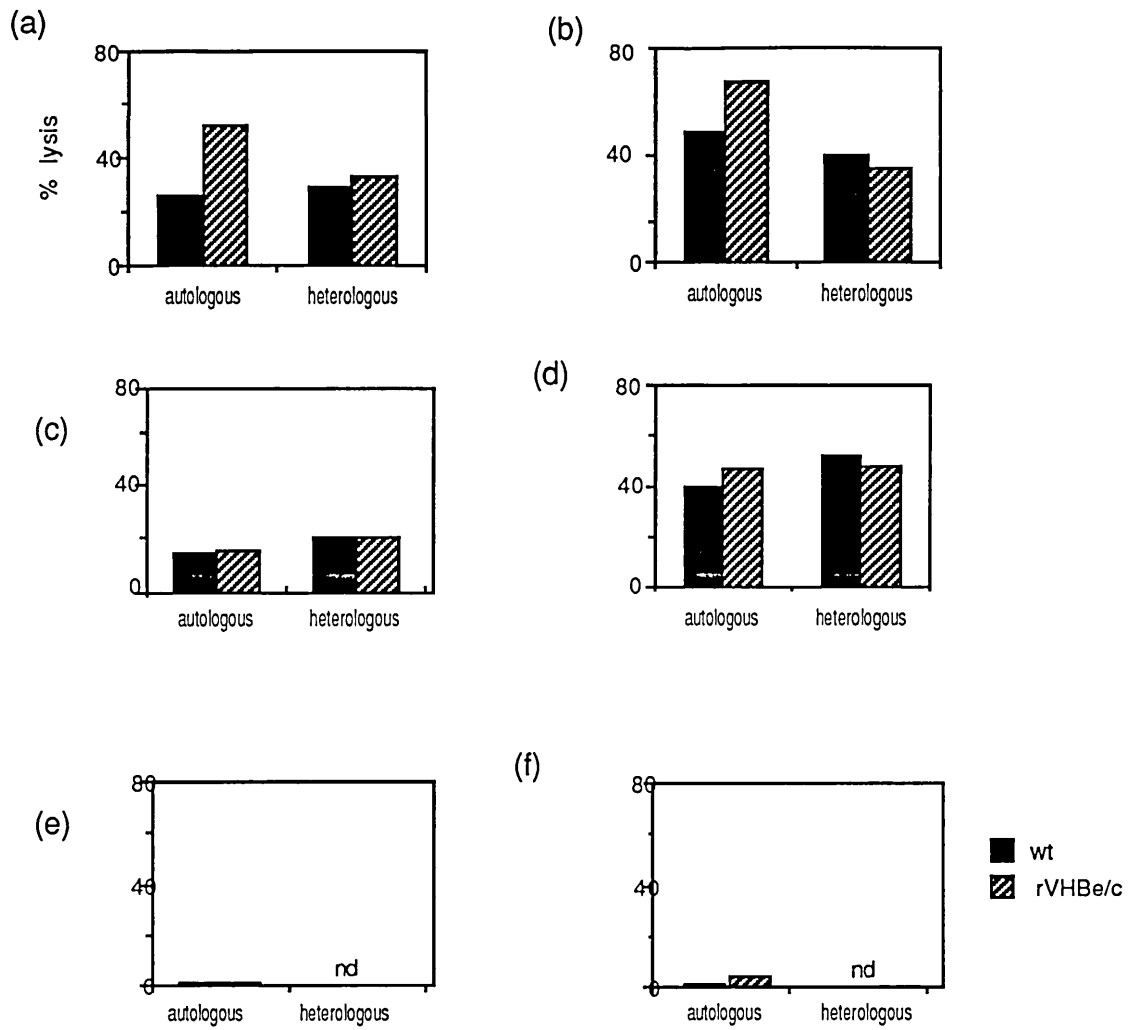


Fig 25.

(a) Patient LC: HLA A2 x, B50 x, Bw6, AST >2,500, effectors stimulated for two weeks with rHBcAg ($1\mu\text{g ml}^{-1}$), peptide 18-27 ($10\mu\text{g ml}^{-1}$) and IL-2 (10IU ml^{-1}), K:T ratio 20:1, graphs show % lysis in standard 4 hr chromium release assay with rV infected LB cell targets.

(b) Patient CH: HLA A1, x, B8, B62, CW3, CW7, BW5, AST 721, effectors stimulated for 1 week with rHBcAg ($1\mu\text{g ml}^{-1}$) peptides and IL-2 (20IU ml^{-1}), added 4 days after the initial antigenic stimulation Stimulation with peptide pool 13,14,15 ($10\mu\text{g ml}^{-1}$) shown. 50:1 K:T ratio.

(c) Patient HT: HLA A2, B44, B51, x, AST 3,000, effectors stimulated as in (a) 50:1 K:T ratio.

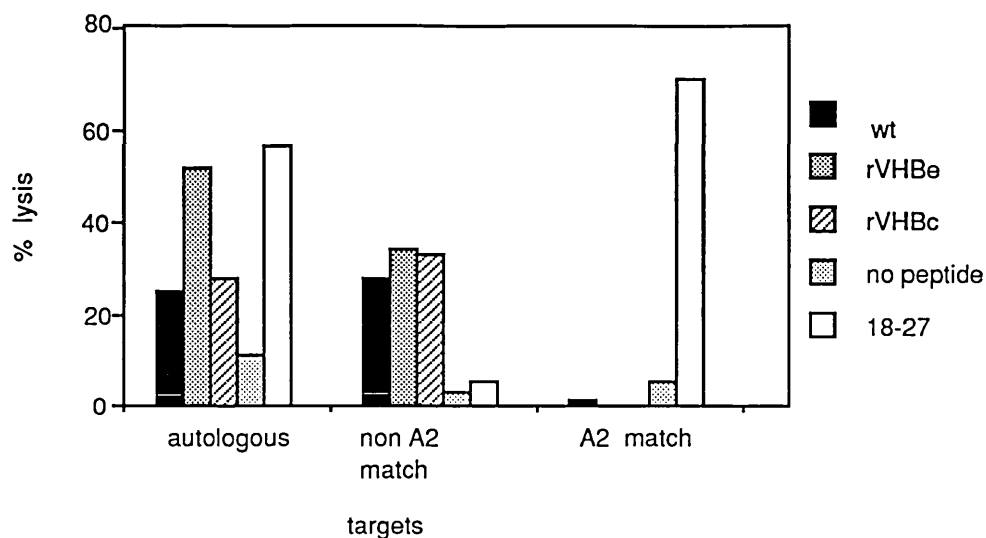
(d) Patient AA: HLA A1, A33, B49, CW2, BW4, BW6 , recovering from acute infection, effectors stimulated for 1 week with rHBcAg ($1\mu\text{g ml}^{-1}$), with pools of peptides (pool 13,14,15 16 shown) and IL-2 (20IU ml^{-1}) 50:1 K:T ratio.

(e) Patient G: HLA A28, A23, B44, B45, CW3, BW4, BW6 recovering from acute infection, effectors stimulated as in (d), p18-27 stimulation is illustrated 50:1 K:T ratio.

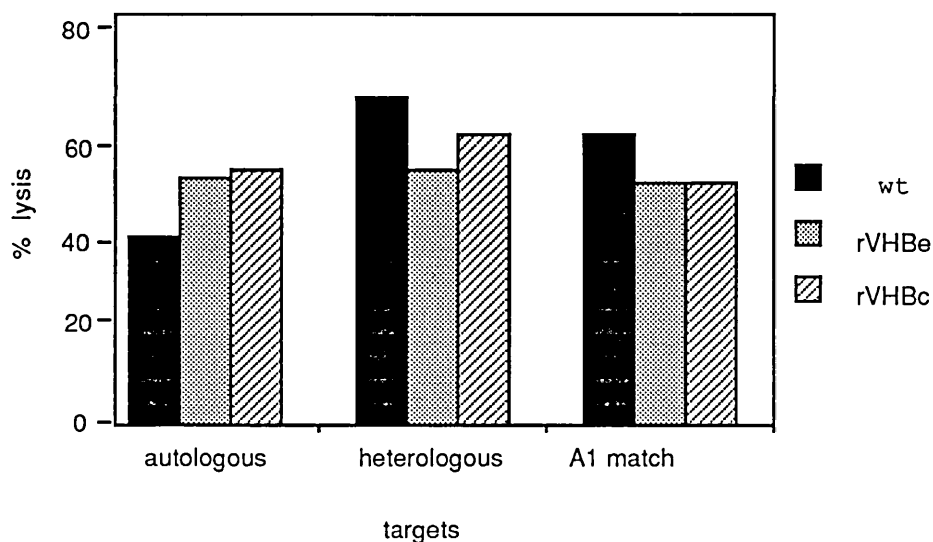
(f) Patient GJ: HLA A24, A25, B41, B62, BW6, CW3 patient, AST 1,500, effectors stimulated in hanging drop culture with EBO/pre-core/HBeAg expressing DC, 50:1 K:T ratio.

Fig 26. Acute HBV patients: lysis of autologous and heterologous targets infected with rVHBe/c or wt vaccinia virus by effector cells stimulated with rHBcAg, peptides and IL-2.

(a) acute HBV patient, HLA A2 (LC in Fig 25)



(b) acute HBV patient, HLA A1
(patient AA in Fig 25)



(a) effector cells were stimulated for two weeks with peptide 18-27 ($10\mu\text{g ml}^{-1}$), rHBcAg ($1\mu\text{g ml}^{-1}$), and 10IU ml^{-1} IL-2, feeding with autologous, irradiated PBL at the end of the first week, K:T ratio 20:1.

(b) effector cells were stimulated with pools of peptides, $1\mu\text{g ml}^{-1}$ of rHBcAg and 20 IU ml^{-1} (peptide pool 13,14,15,16 is illustrated), for 1 week, K:T ratio 50:1

**Fig 27. Chronic (HLA A2) HBV patients:
lysis of autologous and heterologous targets
expressing nucleocapsid antigens by effector cells
stimulated with rHBcAg, peptide 18-27 and IL-2, K:T
50:1 from 4 patients.**

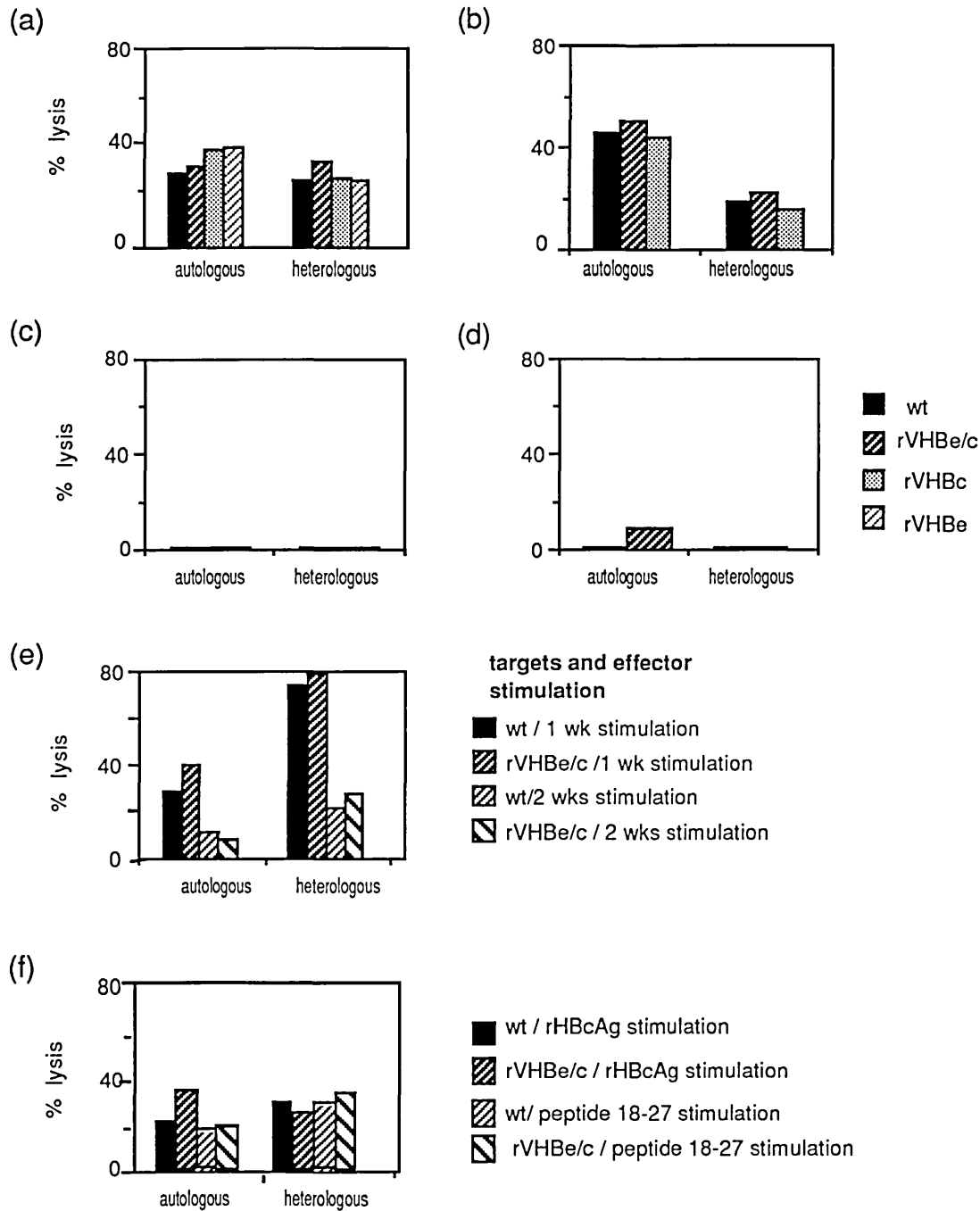


Fig 27. A 50:1 K:T ratio is illustrated.

(a) Patient CW pre-seroconversion, but elevated transaminases (2.3.92) effectors stimulated for 2 weeks ($5 \times 10^6 \text{ ml}^{-1}$) with rHBcAg ($1 \mu\text{g ml}^{-1}$), p18-27 ($10 \mu\text{g ml}^{-1}$). IL-2 (20 IU ml^{-1}) was added 4 days after the initial antigenic stimulation, and the effectors were supplemented with autologous irradiated PBL at the end of week 1, (ratio 5:1 effectors to feeders), and fresh medium. Graphs show % lysis in standard 4 hr ^{51}Cr release assay, with rV infected LB cell targets.

(b) Patient CW (29.3.92) elevated transaminases during HBeAg / anti-HBe seroconversion, effectors stimulated as (a)

(c) Patient NH (30.8.91) CAH, elevated transaminases, effectors stimulated as (a), for 1 week.

(d) Patient DH (2.4.92) grossly elevated transaminases, CAH patient. Effectors stimulated as (a), for 1 week.

(e) Patient JA (. 92) CAH patient, mildly elevated transaminases, effectors stimulated for 1 or 2 weeks as (a) feeding with irradiated LB cells at the end of week 1.

(f) Patient DH (2.4.92) CAH patient with grossly elevated transaminases, effectors stimulated for 2 weeks in culture either with peptide 18-27 $10 \mu\text{g ml}^{-1}$ (plus IL-2 20 IU ml^{-1} at day 4 or rHBcAg ($3.125 \mu\text{g ml}^{-1}$) for 1 week.

Fig 28. Seroconversion from chronic HBV:
lysis of targets expressing nucleocapsid antigens
by effector cells stimulated with rHBcAg, peptides
and IL-2, K:T 50:1.

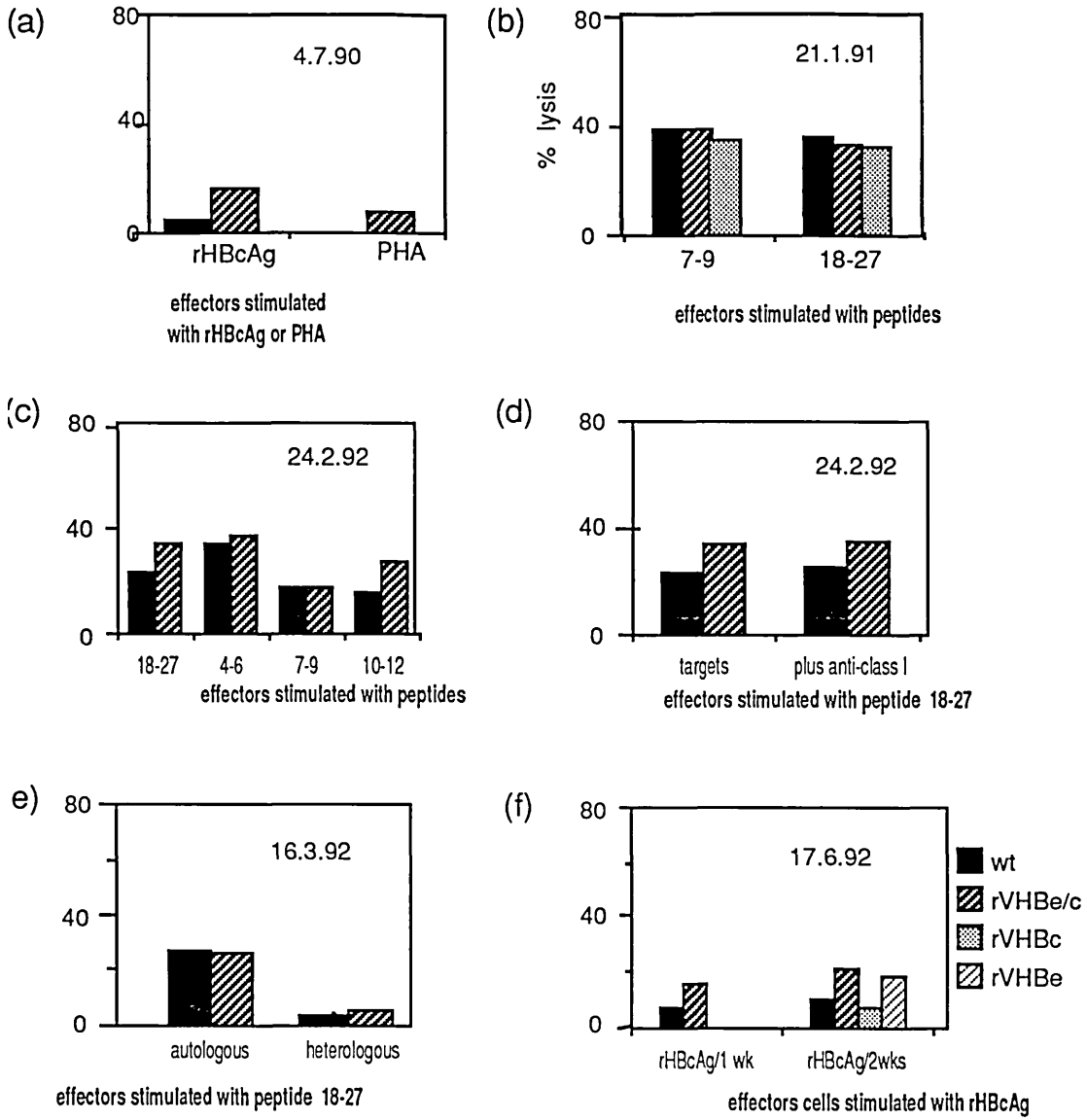


Fig 28. A 50:1 K:T ratio is illustrated.

(a) Patient AD prior to seroconversion, a comparison of PHA 1ug ml^{-1} or rHBcAg 3.125 ug ml^{-1} 1 week of effector cell stimulation.

(b) Patient AD (21.1,91) early during seroconversion, effectors stimulated in culture ($1 \times 10^6\text{ cells ml}^{-1}$) for 1 week with 10ug ml^{-1} of peptide pool 7-9, and 10ug ml^{-1} of p18-27, plus 1ug ml^{-1} of rHBcAg, adding 20IU ml^{-1} of ml^{-1} at day 4. Graphs show % lysis in standard 4 hr ^{51}Cr release assays with rV infected LB cells as targets.

(c) Patient AD during HBeAg to anti-HBe seroconversion. Effectors were stimulated ($1.5 \times 10^6\text{ ml}^{-1}$) for 1 week with pools of peptides at a concentration of 10ug/ml (4-6, 7-9, and 10-12), or with peptide 18-17; and rHBcAg, added to each culture at a concentration of 1 ug ml^{-1} , and IL-2 at day 4 (20IU ml^{-1}).

(d) Effectors stimulated with the 18-27 as (c). An anti-class I antibody (W6/32) was added to the targets during the 4 hr cytotoxicity assay.

(e) Patient AD 1 month after sample (c), still during seroconversion, effectors stimulated as in (c) but only with peptide 18-27 plus rHBcAg and IL-2.

(f) Patient AD post seroconversion, stimulating for 1 or 2 weeks with rHBcAg at a concentration of 3.125ug ml^{-1} feeding with irradiated LB cells at the end of week 1.

Fig 29. Chronic HBV: lysis of autologous and heterologous targets, plus sequential studies (c, d) and (e, f).

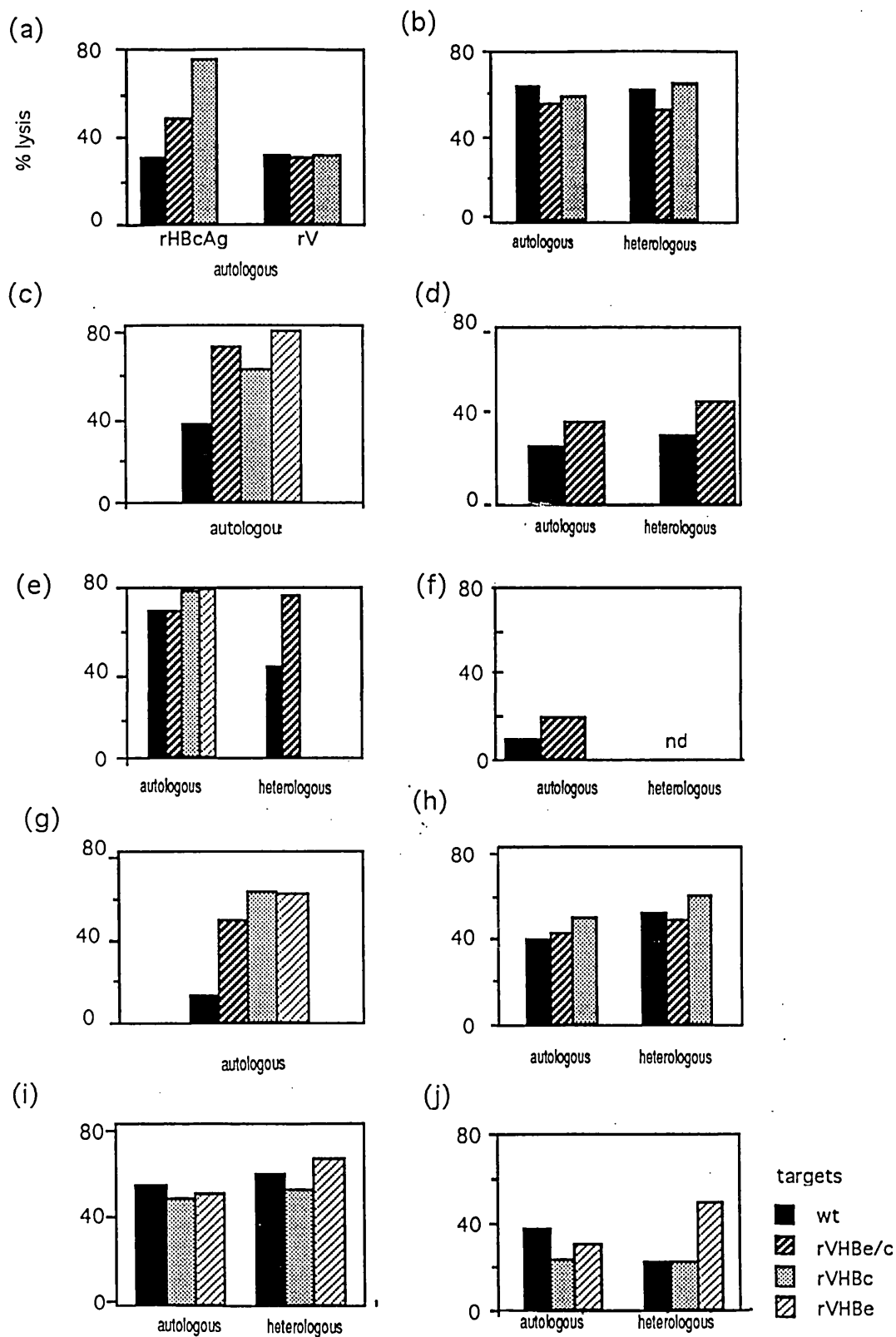


Fig 29. A K:T ratio of 50:1 is illustrated.

(a) Patient DV (1.11.90) CAH/early cirrhosis, effector cells stimulated for 1 week (1×10^6 cells/ml) with 3.125 ug ml^{-1} of rHBcAg, or with PBL (1/10 total volume) infected with rVHBe/c, washed and co-cultured with the rest of the effectors for 1 week. A standard 4 hr ^{51}Cr release assay, using vaccinia infected targets at a K:T ratio of 50:1 is shown.

(b) Patient TD (26.7.93) CPH post seroconversion, effector cells stimulated with DC transfected with rHBcAg (50:1 DC:effector ratio in culture, 1.5×10^6 cells/ml) for 1 week, and irradiated rHBcAg transfected LB cells, at a 1:5 stimulant to effector ratio.

(c) Patient LS (28.11.89) CAH seroconverting following HAV infection.

Effector cells stimulated for 1 week in culture with 3.125 ug ml^{-1} rHBcAg, at a concentration of 1.5×10^6 cells per ml.

(d) Patient LS (22.1.91) post seroconversion and normalisation of transaminases, effectors stimulated as (c).

(e) Patient MOR (11.1.90) at HBeAg / anti-HBe seroconversion, stimulating effectors for 2 weeks with 3.125 ug ml^{-1} rHBcAg, feeding at the end of week 1 with irradiated autologous LB.

(f) Patient MOR (25.1.91) anti-HBe positive, DNA -ve, HBsAg positive asymptomatic, effectors stimulated for 1 week as in (c).

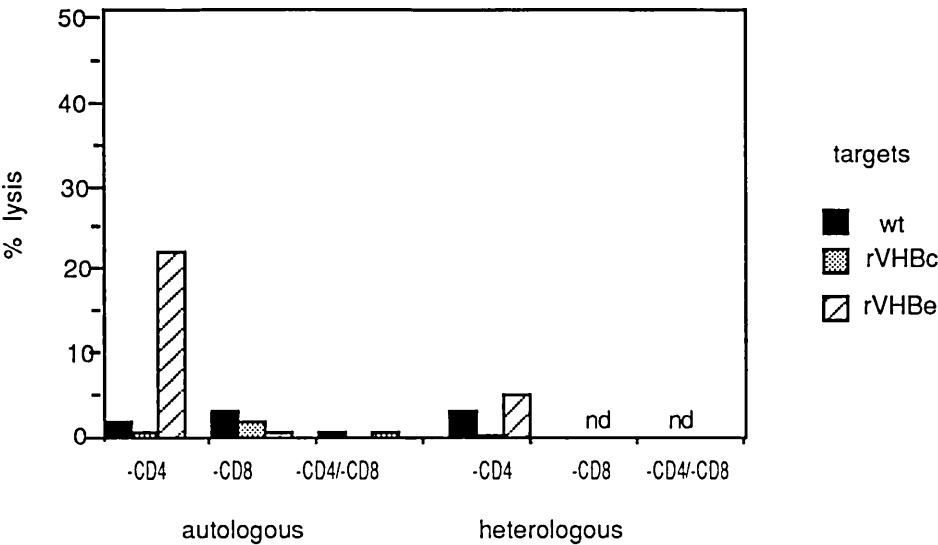
(g) Patient CG (9.3.90) CPH patient, effectors stimulated as (c).

(h) Patient DF (9.3.92) CAH patient prior to seroconversion, effectors stimulated as (c),

(i), (j) stimulating effectors with DC transfected with pCEP1.6 (i) or pCEP1.6-EBNA (j) in culture (1.5×10^6 cells ml^{-1}) at a 50:1 effector to DC ratio, feeding at the end of week 1 with autologous irradiated LB cells transfected with the appropriate vector.

Fig 30. Depletion of CD4 and CD8 cells (patient JH) .

(a) chronic HBV: depletion of CD4+ and CD8+ cells from effector population.



(b) chronic HBV: effector activity against K562 after CD4+ And CD8+ depletion.

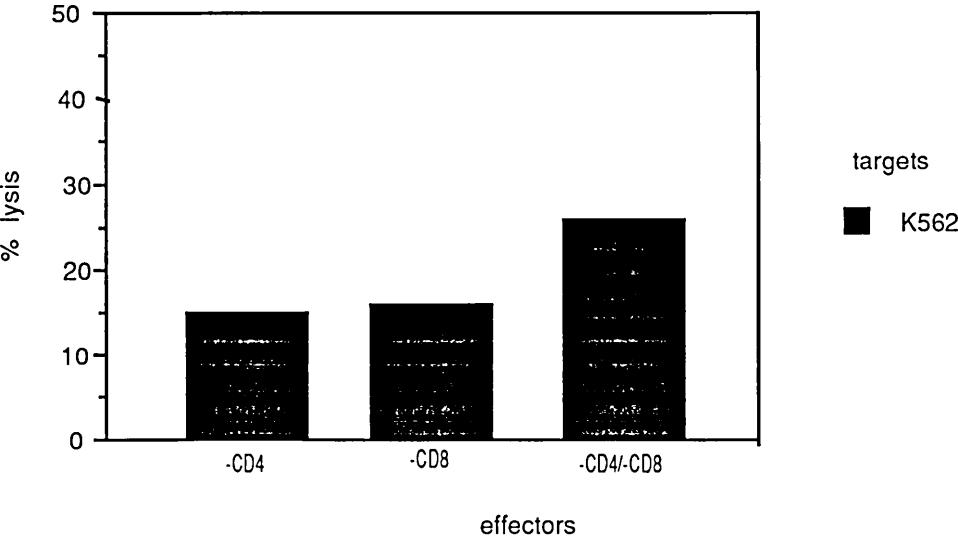


Fig 30.

(a) Patient JH (CAH). ASTs were elevated (500IU L^{-1}) but the patient did not seroconvert. Effectors were stimulated for 1 week in culture with rHBcAg ($3.125\mu\text{gml}^{-1}$), and 10IU ml^{-1} of IL-2 added at day 4. Targets were PHA blast lymphocytes infected with rVHBe/c or wt. Prior to assay, CD4+, CD8+ and both CD4+ and CD8+, were removed from the stimulated effector population. A K:T ratio of 50:1 is illustrated.

(b) Effectors stimulated and depleted as above, and set up against K562 targets in an NK assay. A K:T ratio of 50:1 is illustrated.

Two experiments are shown in more detail in Fig 26. Effectors derived from LC lysed autologous targets coated with p18-27 or expressing rVHBe, more efficiently than control wt infected and non-infected targets at K:T ratios of 10:1 (data not shown) and 20:1. Stimulation of patient AA with peptides 13-16 elicited high levels of lysis, but in a non-HLA restricted manner (Fig 26(b)).

Lysis of autologous and heterologous targets expressing nucleocapsid antigens during chronic infection.

After confirming that cytotoxic T cells could be detected during acute infection, at least in $\text{A}^{\text{w}}\text{A2}$ restricted patient, an identical stimulatory protocol was applied to effectors derived from HLA A2 restricted chronic carriers with elevated transaminases, indicative of ongoing liver cell necrosis, either during seroconversion following interferon treatment, or with ongoing chronic hepatitis without seroconversion (Fig 27).

Patient CW was followed by sequential study during a period of elevated serum transaminases, Fig 27 (a) in March 1992, and (b) one month later, which resulted in seroconversion (patient details are given in Appendix II, with dates of peripheral blood sampling for assay underlined). Effectors were stimulated for two weeks with 1ug ml^{-1} rHBcAg, 10ug ml^{-1} p18-27 and 20IU ml^{-1} IL-2 added 4 days after the initial stimulation, feeding with autologous irradiated PBL, and fresh medium at the end of week 1. At 50:1 (and 25:1 data not shown) K:T ratios, both samples exhibited an antigen specific response against autologous nucleocapsid expressing cells, and high levels of non-specific lysis. Third and fourth samples (data not shown) were taken three and six months later respectively, and all cytolytic activity had fallen to below 10% lysis of any targets. The profile of lysis appeared to change over the time between sampling, with high levels of lysis of all target cells concurrent with loss of HBV DNA.

Patients NH and DH (Fig 27 {c} and {d,f}) both had chronic active hepatitis when effectors were isolated for cytotoxicity studies. Neither patient exhibited CTL. Patient JA, had a history of chronic active hepatitis lasting over several years. Stimulating effectors for 1 or 2 weeks with peptide {e}, induced a highly activated, cytolytic cell population which lysed both autologous and heterologous targets. The level of lysis dropped after the second week, and remained marginally higher against targets expressing nucleocapsid antigens, irrespective of HLA type. Patient DH was stimulated with both rHBcAg and peptides separately, to compare the profiles of response. Stimulation with peptide alone did not induce an antigen specific response, but stimulation with rHBcAg induced a 5% increased kill of autologous rVHBe/c infected cells not present in the heterologous targets.

A sequential study of a chronic active HBV patient (AD) over seroconversion is illustrated in Fig 28. Effectors were stimulated with peptides, rHBcAg and PHA. Before seroconversion, PHA was ineffective in eliciting a cytotoxic response, but stimulation with rHBcAg resulted in lysis of autologous rVHBe/c expressing targets 10% above wt, with a low overall level of lysis (Fig 28{a}). At the beginning of seroconversion (Fig 28 {b}), one week of stimulation with peptide pool 7-9 (peptide numbering is detailed in Appendix III) and p18-27, plus rHBcAg and IL-2 (as previously reported) produced a non-specific level of lysis approximately 40% across all targets. A month later (Fig 28{c}) still during the period of seroconversion, peptide stimulation with pools of peptides elicited small antigen specific responses with pools of peptides 4-6, and 10-12 (Appendix III), peptide and peptide 18-27, but the antigen specific response elicited by p18-27 was not abolished by the addition of anti-class I antibodies (Fig 28{d}). One month later, {e}, no antigen specific response was detectable, and three months later all activity had dropped below 15-20%, a level comparable to that detected prior to the seroconversion event.

Stimulation of effectors with random pools of peptides did not elicit a clear cut MHC restricted cytotoxic

response, even during seroconversion, but a small degree of antigen specific cell lysis appeared to underlie a number of experiments, possibly indicating alternative mechanisms of cell lysis (not involving MHC class I restricted CTL) in patients with chronic active disease and during the HBe antigen / antibody seroconversion.

Alternative methods of stimulating CTL in patients with HLA phenotypes excluding HLA A2 or A31 (HLA alleles where the hepatitis derived peptide presented during acute hepatitis has been identified) were examined. Non specific stimulation with PHA, anti-CD3 plus IL-2 did not elicit a response. Neither did stimulation with rV infected antigen presenting cells or transfected DC. When compared with PHA, rVHBe/c, or peptide as a cytotoxic effector stimulant, unexpectedly, incubation of peripheral blood derived effector cells with rHBcAg, even without IL-2, appeared to induce cytotoxic cells. *In vitro* stimulation of effectors derived from chronic patients stimulated with rHBcAg or EBO transfectants is shown in Fig 29, for clarity, only a single K:T ratio is shown, but in all assays, doubling dilutions of effectors were used over the range 50:1-12.5:1 (unless otherwise indicated), and cell lysis observed correlated directly with the K:T ratio.

An antigen specific lytic response was induced by 1 week stimulation with rHBcAg (3.125 ug ml^{-1}), but not recombinant vaccinia infected LB cells in patient DV (Fig 29{a}). At time of sampling, the patient was undergoing HBeAg to anti-HBe seroconversion. Two other patients were investigated during seroconversion, patient LS Fig 29{c,d}, and patient MOR Fig 29{e,f}. During seroconversion, LS exhibited a considerable antigen specific response Fig 29{c} following 1 week of rHBcAg stimulation which fell as the patients' transaminases returned to normal {d}. Two weeks of antigenic stimulation with rHBcAg, feeding the effector population with autologous irradiated LB cells at the end of week 1, resulted in high levels of rVHBe/c infected target cell lysis using effectors derived from (MOR), irrespective of HLA type {e}. As in patient LS, lytic activity fell, 1 year after seroconversion {f}.

Patients CG {g} and DF {h} did not seroconvert during

the time course of the study, but both exhibited a degree of antigen specific cytolysis of targets, CG {g}, exhibiting considerable antigen specific lysis (25-25%) of autologous targets following rHBcAg stimulation of effector cells.

Electroporation of rHBcAg directly into the cell cytoplasm of APC, rather than simply incubating effectors with the particulate antigen, failed to elicit a significantly different response (patient TD, Fig 29{b, i, j}). EBO vectors expressing pre-core/HBeAg transfected into LB cells or DC also failed to elicit a significant cytotoxic response against rVHBe/c infected targets (Fig 29{i, j}), stimulation of the effectors with pCEP1.6 produced a higher non-specific lysis than stimulation of effectors with pCEP1.6 - EBNA.

Up to this point, there was no conclusive evidence that the rHBcAg response identified during seroconversion was MHC restricted. In order to investigate the nature of the effectors stimulated by rHBcAg, PBL from a patient (JH) with CAH and grossly elevated transaminases, were stimulated in bulk culture for 8 days with rHBcAg ($3.125\mu\text{g ml}^{-1}$) with the addition of IL-2 (20 IU ml^{-1}) at day 4. As no LB line had been established, autologous targets were prepared by infection of PHA blasts (PBL stimulated for 5 days with $10\mu\text{g ml}^{-1}$ of PHA in the presence of 20 IU ml^{-1} of IL-2) with rVHBe/c. Heterologous LB targets were prepared by infection of LB cells as previously described. Effector cell populations (post antigen stimulation) were depleted of CD4+ (-CD4+) and CD8+ (-CD8+) cells individually, and all T cells (-CD4+/CD8+) and set up in a standard 4 hr chromium release assay against the vaccinia infected targets (Fig 30{a}). An rVHBe/c specific lysis of 20% (at a K:T ratio of 40:1) was detected in the CD4+ depleted (CD8+/NK enriched) population, against autologous targets, which was not detected in the CD8+ depleted, or doubly depleted population. No or low level lysis of heterologous targets was observed. Lysis of the K562 target line by the CD4+/CD8+ depleted population was unusually low at 26% (see Fig 31), but greater than that of the CD4+ and CD8+ enriched populations.

In summary, although there was some indication that a class I MHC restricted cytotoxic response might be present in some patients during chronic active disease, other mechanisms of cell lysis appeared to be predominant during viral elimination.

Overall statistical analysis.

Samples of peripheral blood lymphocytes from sixty individual patients were analysed by ^{51}Cr release cytotoxicity assay. Samples from single patients taken at more than one time point were entered individually into the analysis. For the purpose of statistical analysis, the chronic HBV patients were divided into chronic persistent and chronic active hepatitis categories on the basis of routine biopsy reports, and seroconversion status by biochemical and virology reports. Data from individual patients illustrated in the following section are given in Appendix II.

The analysis presented in Fig 31 compares percentage lysis in standard 4 hr chromium release assays, of targets expressing the pre-core/core gene products (rVHBe/c infected cells) versus targets infected with wild type virus (wt) strain Copenhagen TS7. The graphs show the mean and standard errors calculated from the data. The numbers were analysed by a Wilcoxon Signed Rank test (non-parametric paired analysis) as the data was not normally distributed.

The numbers in each category were not pre-matched as the statistical analysis was retrospective. The number of chronically infected patients tested with autologous targets was largest; CPH $n=47$, CAH $n=21$. The number of chronically infected patients who seroconverted was 9, all of which were tested against heterologous targets. The results for the acute group must be regarded with caution as only 6 patients were investigated.

A significant difference between lysis of the two targets was defined by the null hypothesis $X_1^2 = X_2^2$, $p < 0.05$. Heterologous targets were mismatched at the major Class I loci. Data from all assays where the spontaneous

release was < 25%, irrespective of the method or duration of effector cell stimulation, was included.

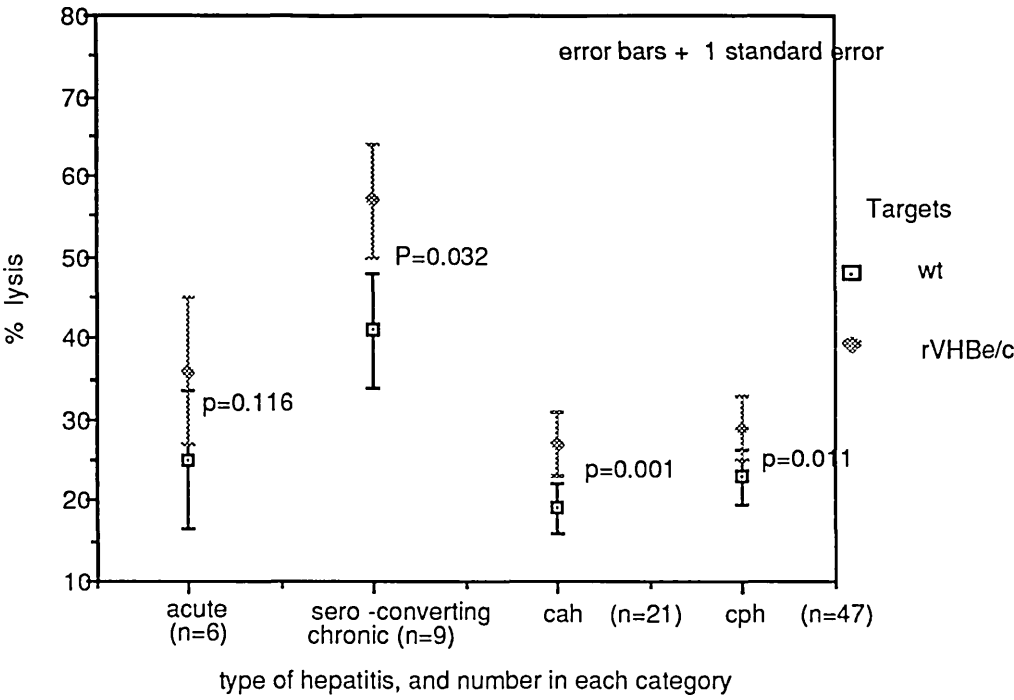
The mean levels of cytotoxicity were difficult to interpret, as the variation between individuals was large, but some trends emerged. Specific lysis of autologous rVHBe/c infected cells above wt infected cells was not significant during acute HBV infection $p=0.116$ ($n=6$), probably because of the small numbers of patients available for study during the early stage of acute hepatitis. Heterologous targets were also lysed by effector cells from these patients, but in a non-antigen specific manner; rVHBe/c above wt, $p=0.285$. If the total chronically infected patient sample is analysed, chronic persistent and chronic active hepatitis patients both had low levels of antigen specific / MHC restricted lysis; CAH rVHBe above wt $p=0.001$, CPH, $p=0.011$.

If the sample analysed is reduced to those experiments where lysis of autologous and heterologous targets were compared in one experiment then the antigen specific component of lysis is lost. CAH lysis of rVHBe/c above wt, autologous $p=0.110$, CPH $p=0.860$. Both categories lysed heterologous targets to a greater degree than autologous targets, but in a non-antigen specific manner; CAH rVHBe above wt $p=0.523$, CPH $p=0.173$.

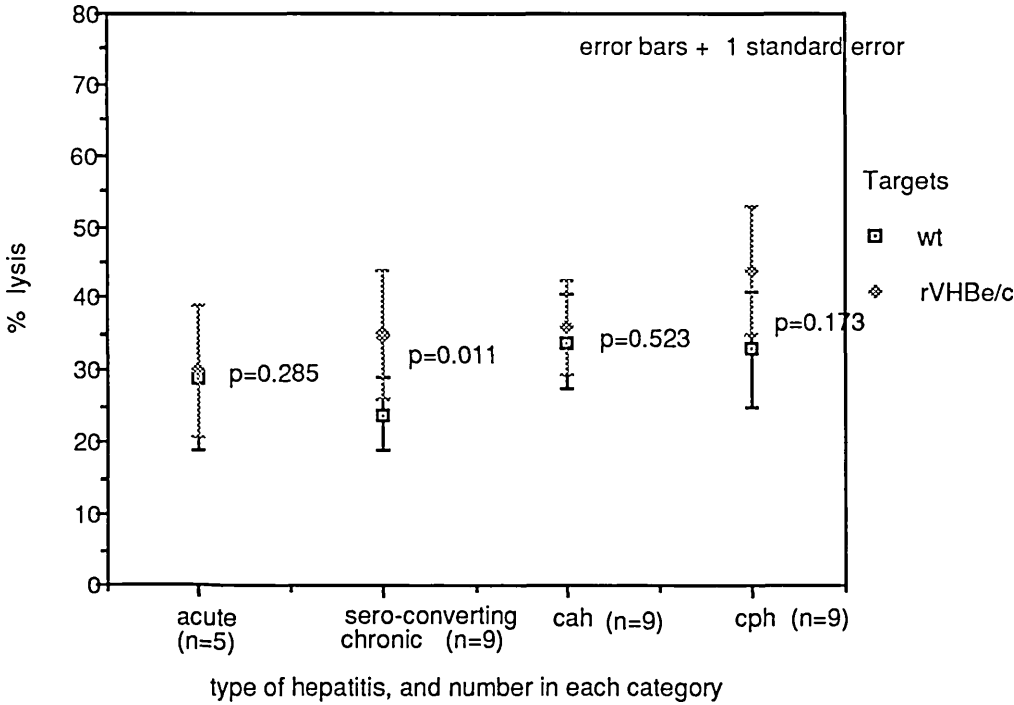
Individuals undergoing HBeAg to anti-HBe seroconversion all of whom had undergone INF α therapy were also analysed ($n=9$). Lymphocytes from these patients appeared to be highly activated when stimulated in culture with pools of peptides or rHBcAg, and set up as effector cells in cytotoxicity assays. MHC and non-MHC restricted antigen specific lysis was detectable in this group. However, the dominant cell lysis did appear to be antigen specific, but not MHC class I restricted. Both autologous and heterologous targets infected with rVHBe were lysed above background lysis of targets infected with wt virus in a statistically significant manner $p=0.032$ (autologous) targets $p=0.011$ (heterologous).

Fig 31. Lysis of targets infected with rV HBe/c and WT vaccinia during HBV infection by peripheral cytotoxic cells K: T ratio 50:1.

(a) Mean cytotoxicity against autologous targets infected with rVHBe/c or WT vaccinia virus: overall summary



(b) Mean cytotoxicity against heterologous targets infected with rVHBe/c or WT virus: overall summary



P values; wilcoxon signed rank (lysis of rVHBe/c v WT for each category)

(4) In vitro stimulation of PBL with DC expressing HBeAg.

Dendritic cells (DC) are potent antigen presenting cells. Transfection by electroporation, of an enriched dendritic population with the EBO vectors pCEP1.6 (CMV promotor) and pMEP1.5 (metallothionine inducible promotor) were found in preliminary experiments to induce a proliferative response when DC and PBL were co-incubated in hanging drop cultures. The inducible vectors pMEP1.5 and pMEP 1.5-EBNA were selected for further experiments, as cells recovered from a single transfection could be divided into non-induced control, and HBeAg expressing (induced) DC. Lymphocytes stimulated with a DC enriched population transfected with the control vectors pMEP4 and pMEP4-EBNA induced with CdCl₂, did not result in proliferation above the non-induced vectors. Cadmium used at the induction concentration (5uM) inhibited lymphocyte proliferation 5-10% in naive patients (data not shown).

Twenty-four hrs after electroporation of DC, cells were 95% viable by trypan blue exclusion, and under optimal conditions (transfecting a minimum of $8-9 \times 10^6$ enriched DC population) greater than 75% recovery of cells was obtained. pMEP1.5 transfected (naive) DC secreted HBeAg into the tissue culture supernatant. Naive donors were defined as total anti-HBc negative. 2×10^3 transfected cells (in 20ul of medium) produced sufficient HBeAg within 5 days, to detect by ELISA (Abbott). The antigen specific nature of the proliferative response was confirmed by the induction of anti-HBe and anti-HBc producing B cells from the naive donors. Both pMEP1.5 and pMEP1.5-EBNA induced antibody specific responses, but pMEP1.5-EBNA did not produce detectable levels of HBeAg in transfected DC supernatant. The pMEP1.5 vector was used to analyse the secondary response during and after resolution of acute infection, and during chronic infection / seroconversion. When a reproducible protocol for detection of proliferation was established, the effect of depletion of CD4+ and CD8+ cells on the proliferative response was analysed (Table 6).

The patients lymphocytes are described as naive (anti-HBc total negative by ELISA), acute or recovered acute, and

chronic HBV. The stimulation index is quoted as the ratio at the point of maximal stimulation when APC expressing HBeAg (induced +) as opposed to APC with non-induced vector (induced -), were mixed with PBL. Each point on the following graphs represents the mean of 6 wells, so a statistical analysis could justifiably be applied to the data. Individual data is shown in Figs 32-35, p values for comparisons at maximal stimulation are also shown. Unpaired t-test and Wilcoxon signed rank test and Mann Whitney U test, p values less than 0.05, are considered statistically significant. The effect on stimulation of PBL depleted of CD4+ or CD8+ cells, was calculated both at individual points, and over the whole course of the experiments. The effect of depletion of CD4+ or CD8+ cells from the responder population at the point of maximal response is expressed in Table 6 as significant difference in proliferation attained with or without (+/-) CD4+ or CD8+ cells. The acute and chronic patient groups' behaved in an identical manner (over the whole experiment) when CD4+ cells ($p=0.512$, Mann Whitney U test), and CD8+ cells were depleted ($p=0.827$). The stimulation index of PBL/DC (induced), that is expressing HBeAg, was significantly higher than that of PBL/DC (non-induced), ie vector control, in both acute and chronic infection, although the levels of stimulation varied between individuals irrespective of clinical status. Depletion of CD4+ cells invariably reduced the level of proliferation in both chronic and acute patients, and in a statistically significant manner in 2/4 acute patients, and 3/4 chronic patients. Depletion of CD8 cells reduced the proliferative response in 2/4 acute patients, and approached significance in a third individual ($p=0.09$).

The depletion analysis of two individuals with acute hepatitis is shown in Fig 33 {a,b and c}. Investigation of stimulation of antibody producing cells was performed by Dr M. Thurz, using the ELISPOT technique, essentially binding B cells to antigen coated plastic plates, and comparing populations pre and post stimulation for cells binding in an antigen specific manner.

Table 6.

DC: stimulation of PBL with DC transfected with pMEP1.5 pre-core/core, and pMEP 1.5-EBNA (both expressing HBeAg, but the -EBNA vector expressing no EBV proteins).

patient	vector	stimulation Index and significance		effect on stimulation of CD4/CD8 depletion		assay of antibody producing cells (elispot)			HBeAg (Abbott) in tissue culture supernatant
				CD4	CD8	anti-HBs	anti-HBe	anti-HBc	
naive									
PT	pMEP1.5	X8	p=0.008	nd	nd	nd	nd	nd	nd
JW	pMEP1.5	X7	p=0.003	nd	nd	nd	nd	nd	nd
MS	pMEP1.5	X1.2	p=0.175	nd	nd	nd	nd	nd	+
J	p1.5-EBNA	X2	p=0.675	nd	nd	nd	nd	nd	nd
JB	p1.5-EBNA	X1 (X3 PBL)	p=0.557	p=0.175	p=0.140	-	+	+	nd
MT	pMEP1.5	X1 (X3 PBL)	p=0.544	nd	nd	nd	nd	nd	+
mean (+/-sd)		X 4.0	(+/- 3.4)						
acute or recovered acute									
ET	pMEP1.5	X2.1	p=0.003	p=<0.001	p=0.09	+	-	+	+
PK	pMEP1.5	X4	p=0.002	p=0.001	p=0.001	+	-	+	nd
KT	pMEP1.5	X2.5	p=0.06	nd	nd	nd	nd	nd	nd
EA	pMEP1.5	X2.5	p=0.134	p=0.235	p=0.469	nd	nd	nd	-
BB	pMEP1.5	X7	p=0.001	nd	nd	nd	nd	nd	-
AB	pmep1.5	X4	p=<0.001	nd	nd	nd	nd	nd	-
SJ	pMEP1.5	X2.1	p=0.004	p=0.099	p=0.297	-	+	+	nd
mean (+/-sd)		X 3.5	(+/- 1.8)						
chronic HBV.									
TC	pMEP1.5	X1(X3 PBL)	p=0.23	p=0.512	p=0.461	-	+	+	-
AA	pMEP1.5	X3	p=0.004	nd	nd	-	+	+	-
RA	pMEP1.5	X7	p=0.001	p=0.012	p=0.461	-	+	+	nd
KP	pMEP1.5	X2	p=0.005	p=0.046	p=0.205	-	+	+	nd
JB	pMEP1.5	X4	p=0.399	p=<0.001	p=<0.001	-	+	+	-
mean (+/- sd)		X 4.2	(+/- 2.8)						

nd, assay not performed, AA subjected to cytotoxicity assay.

All naive patients PBL were stimulated with pMEP 1.5-EBNA or both vectors. Previously exposed patients were stimulated with the pMEP1.5 pre-core/core vector. Significance is described as p<0.05, by unpaired t-test.

Only 1/4 chronic patients exhibited a significant reduction in the proliferative response when CD8 cells were depleted, and that individual (JB) had the most severe disease of all the patients studied (CAH/cirrhosis). Pre-incubation of the transfected DC enriched population (prior to protein induction) with brefeldin A completely abolished proliferation. Chloroquine inhibited proliferation in a dose dependent manner. Low dose chloroquine (10ug ml⁻¹, 1 hr incubation) inhibits lysosomal protein degradation, hence class II processing. High dose chloroquine (50-100 ug ml⁻¹/hr) inhibits both pre-Golgi and lysosomal compartments, in a reversible manner. Inhibition of the proliferative response when DC were treated with both of these agents, confirmed that protein was being produced *de novo* inside infected cells.

Of the acute patients studied by depletion analysis, two individuals (PK and ET) had fully recovered from infection (ET several years prior to study), the third patient (AE) was in the process of seroconverting. Of the chronic patients studied JB was seroconverting, but had already developed cirrhosis following several years of active CAH. Patients RA and TC had grossly elevated transaminases, patient KP had a mild persistent disease.

Patient AA was in the process of seroconverting. Effectors derived from AA were stimulated for 4 days in hanging drop culture at a ratio 1:25 transfected DC to PBL, and investigated for lytic activity in a standard 4 hr ⁵¹Cr release assay with autologous and mismatched rVHbc/e and wt infected targets, and K562. No MHC restricted cytotoxic T cell activity could be detected, despite evidence of T cell stimulation in the proliferative assay (Fig 36{a}). Effectors from a second patient PP with elevated transaminases were investigated for cytotoxic T cell activity by chromium release assay, selecting the purified effectors prior to the assay (CD4+, CD8+, CD4/CD8 depleted. The only activity detectable was CD4 mediated non-specific cell lysis (Fig 36 {b})).

CD4+ proliferation responses were elicited during stimulation of PBL derived from both chronic and acute

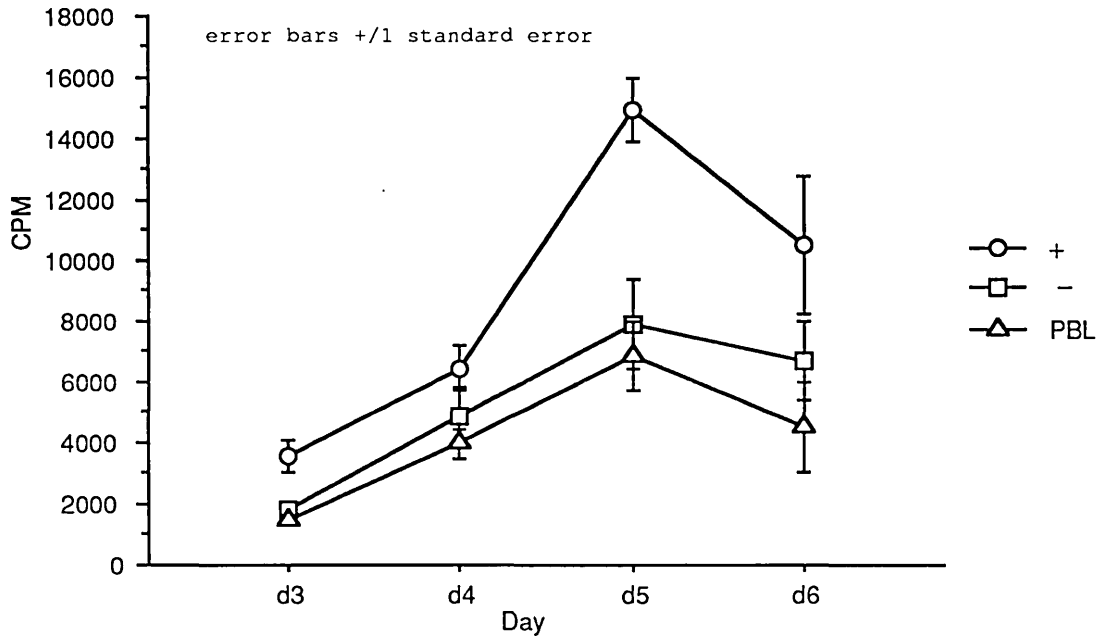
patients: both chronic and acute HBV patients clearly had the ability to produce anti-HBe and anti-HBc, even prior to conventional (ELISA) anti-HBe detection by ELISA in the patients sera.

In conclusion, CD4+ but not CD8+ responses were normal in chronic HBV patients when antigen presentation was adequate: mean SI HBV naive, $X4 \pm 3.4$; mean SI acute HBV, $X3.5 \pm 1.8$; mean SI chronic HBV $X4.2 \pm 2.8$. It might be hypothesised that deficient antigen presentation is responsible for the abnormal CD4+ responses reported in chronic HBV patients. The deficient CD8+ response was not overcome by adding antigen presenting cells expressing HBeAg, possibly indicating another abnormality in chronic HBV carriers which results in the failure or inhibition of a Th1 type response.

Fig 32. Acute HBV: analysis of the proliferative response of PBL to DC transiently expressing HBeAg.

(a) Patient ET,

DC tranfected to produce HBeAg on induction (+).



(b) Chloroquine inhibition of proliferative response.

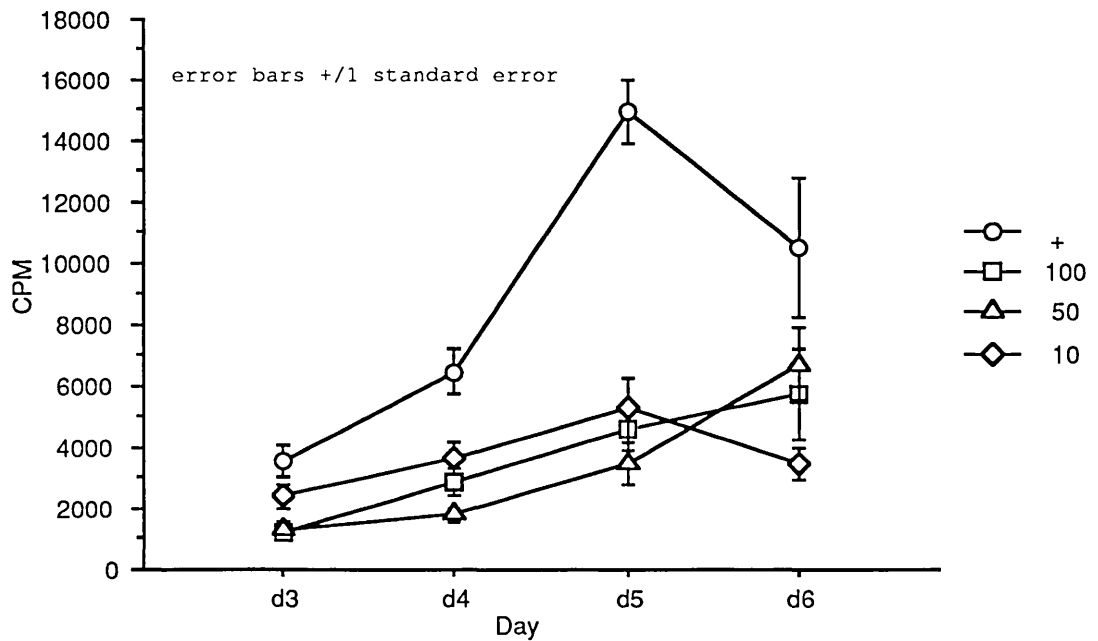


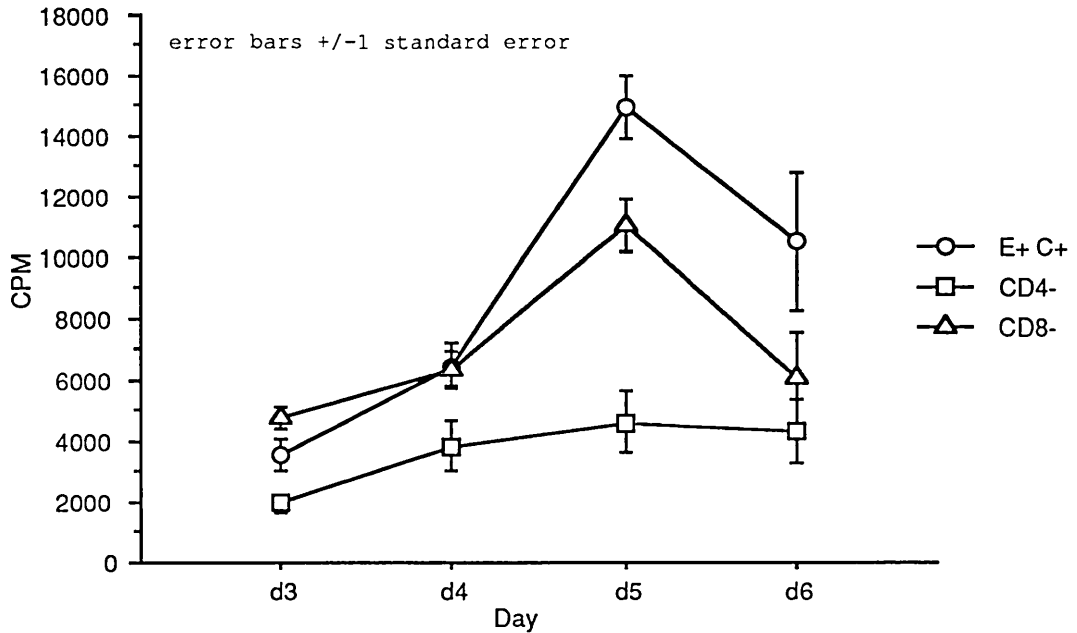
Fig 32

(a) A patient with a history of acute hepatitis (ET), > 5yrs previously. PBL stimulated in 20ul hanging drop cultures with DC expressing HBeAg (+) or not (-).

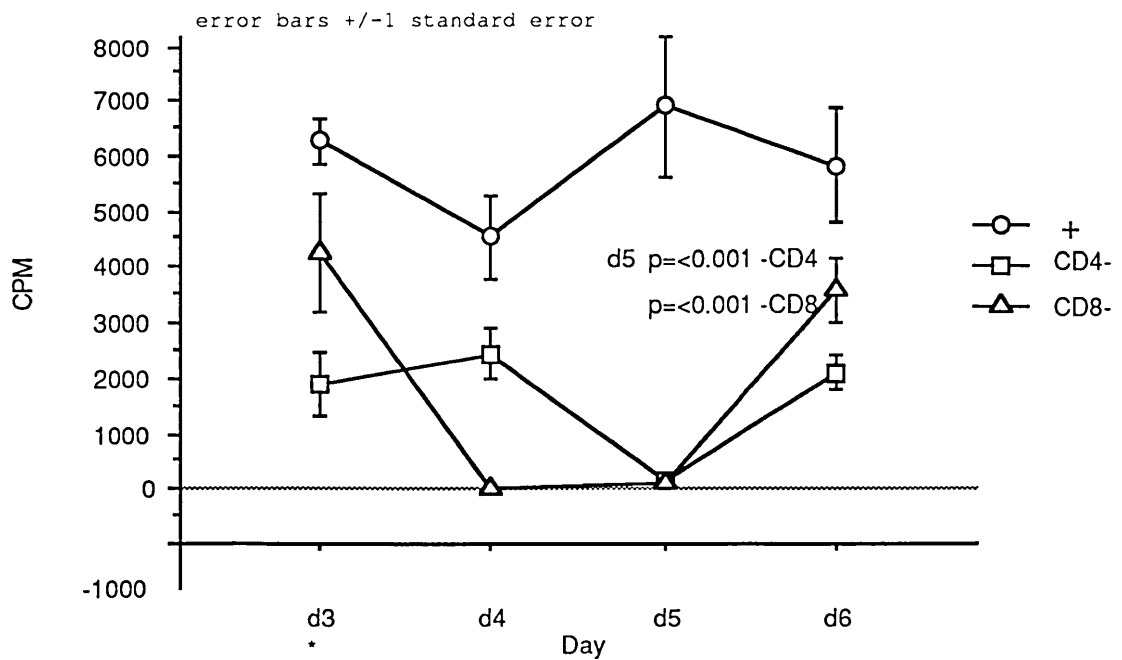
(b) Inhibition of the proliferative response of PBL from patient ET. An aliquot of DC enriched cells were cultured prior to protein induction for 1 hr with 10, 50 or 100ug ml⁻¹ of chloroquine, washed, and set up in hanging drop cultures as (a).

Fig 33. Acute HBV: analysis of the proliferative response; the effect of CD4+ and CD8+ depletion.

(a) Patient ET,
DC transfected with pMEP1.5, pre-core/core
inhibition of stimulation by depletion of CD4+ and CD8+
cells.



(b) Patient PmcK,
DC transfected with pMEP1.5, pre-core/core
inhibition of stimulation by depletion of CD4+ and CD8+
cells.



* 8 hrs ^3H incorporation (4 hrs days 4-6)

Fig 33.

(a) Patient ET, recovered from acute hepatitis; CD4+ and CD8+ cell populations were removed separately from the PBL population by incubation on antibody coated tissue culture flasks (AIS). The cells were then re-counted, and set up in an identical manner to Fig 32, at a 1:25 DC:PBL ratio.

(b) Patient PmCK, recovered from acute hepatitis; CD4+ and CD8+ cells were removed separately from the PBL population by incubation on antibody coated tissue culture flasks (AIS). The cells were then re-counted, and set up in an identical manner to (a), at a 1:25 DC:PBL ratio.

(c) FACScan analysis of CD4+/CD8+ depletion. The cells were stained pre and post depletion with Becton Dickinson Simultest:

UL CD4+ lymphocytes ,
 UR double stained cells,
 LL non-lymphocytes, unstained cells,
 LR CD8+ lymphocytes.

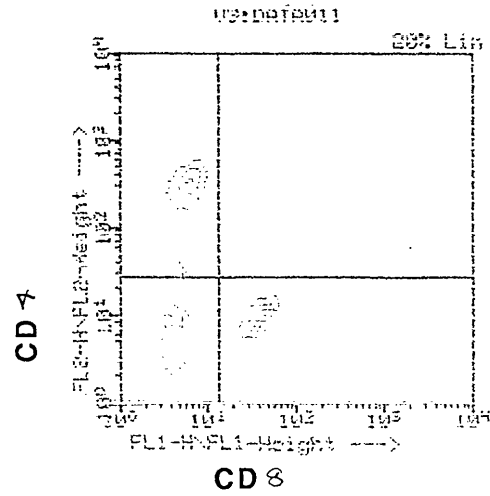
(I) PBL and monocytes pre-deletion of CD4+ and CD8+ cells.

(II) LR (CD8+) population depleted

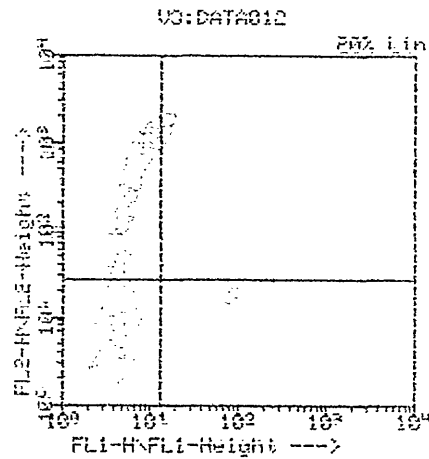
(III) UL (CD4+) population depleted

Fig 33(c). FACScan of PBL stained for CD4+ and CD8+ cells pre and post depletion with AIS plates.

(I)



(II)



(III)

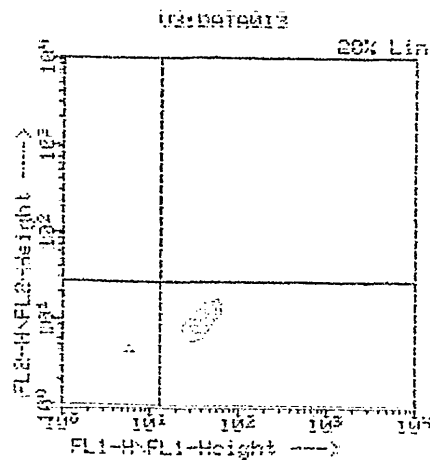
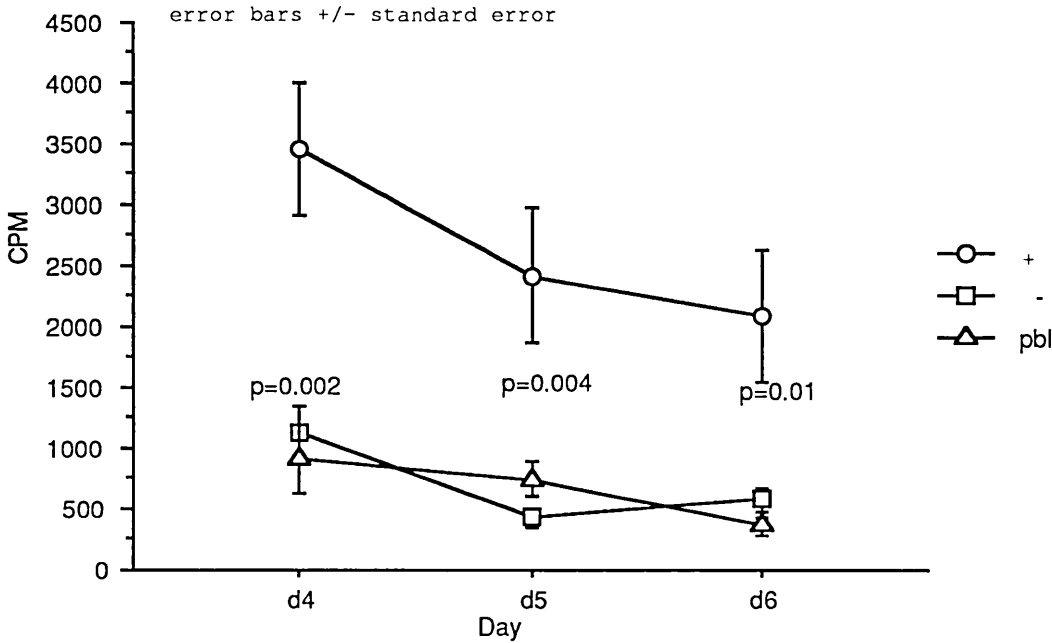


Fig 34. Chronic HBV: proliferative response to DC expressing HBeAg.

(a) Patient AA,
secondary response to DC transfected with pMEP1.5
HBeAg.



(b) Patient HA,
secondary response to DC transfected with pMEP1.5

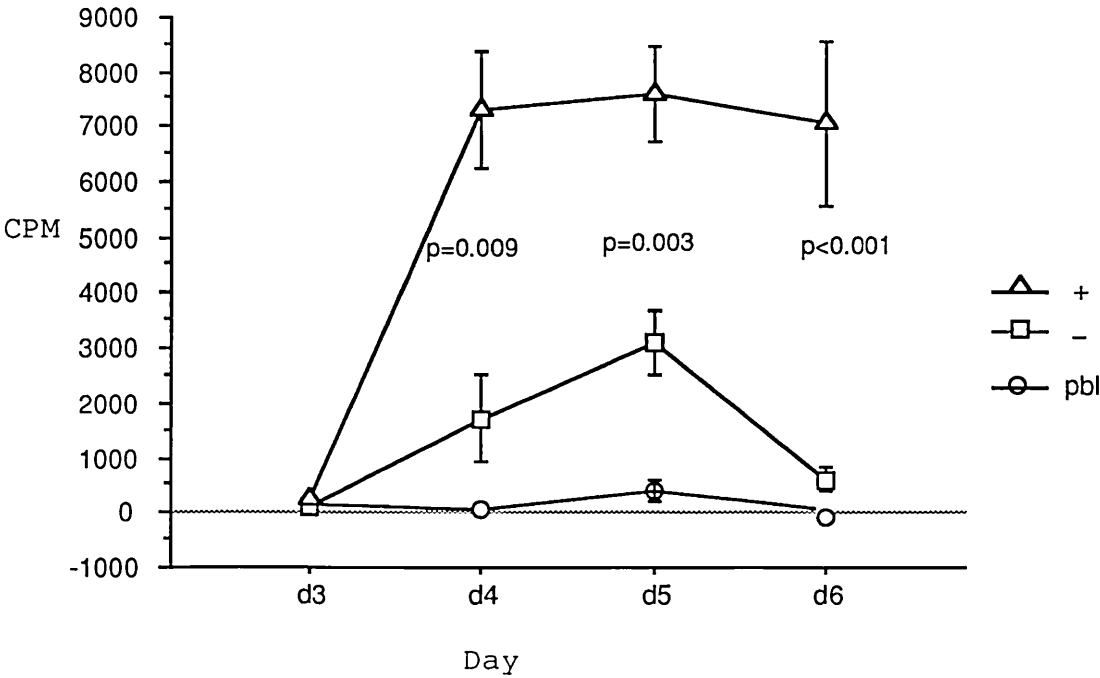


Fig 34. Chronic HBV: proliferative response to DC expressing HBeAg.

(c) Patient HA,
DC transfected with pMEP1.5, pre-core/core:
inhibition of stimulation by depletion of CD4+ and
CD8+ cells.

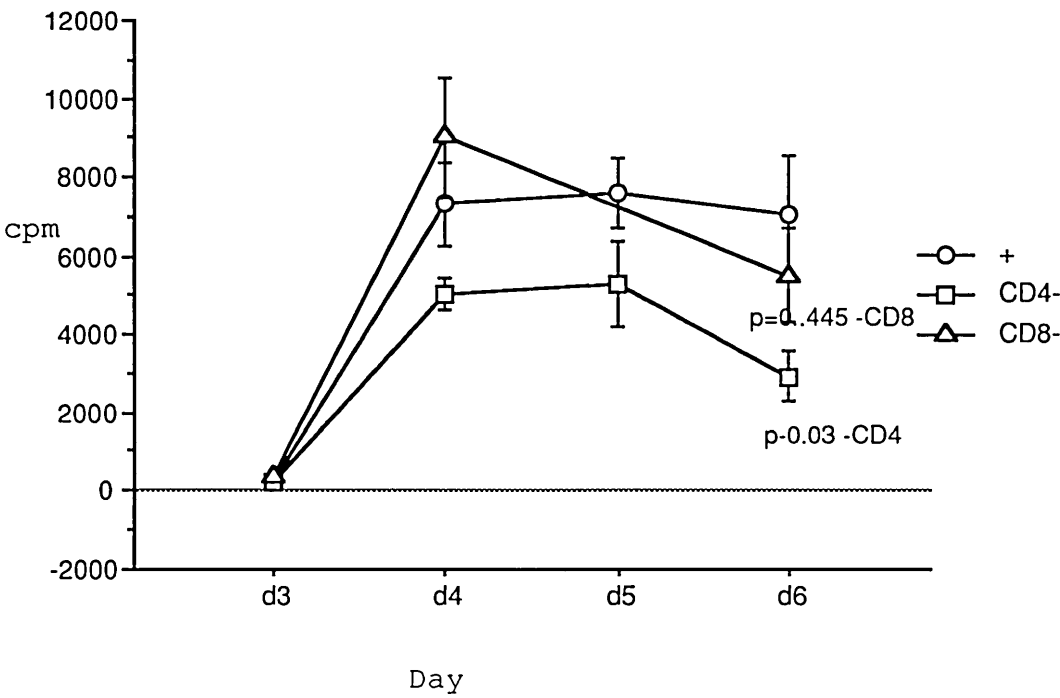


Fig 34.

(a) Patient AA, chronic HBV, during HBeAg/anti-HBe seroconversion. A dendritic cell enriched population transfected with the vector pMEP1.5 expressing pre/core/HBeAg was co-cultured in 20ul hanging drop culture at 37°C in a humidified atmosphere with autologous PBL at a ratio of 1:25, DC:PBL. The vector was either induced with 5uM CdCl₂ to express the HBeAg (+) or not (-). The cultures were pulsed for eight hours on days 4, 5 and 6 with 0.5uCi ³H tritium (Amersham TP120), harvested and counted in liquid scintillant on a β counter. Six repetitions were performed at each time point, and the data subjected to statistical analysis. Stimulated effectors were also harvested at day 4, and set up against rVHBe/c infected autologous and heterologous targets (Fig 35) in a chromium release cytotoxicity assay.

(b) Patient HA (CAH), PBL stimulated in an identical manner as (a), pulsed and harvested at days 3, 4, 5, and 6.

(c) Patient HA, inhibition of the proliferative response. CD4+ and CD8+ cell populations were removed separately from the PBL population by incubation on antibody coated tissue culture flasks (AIS). The cells were then re-counted, and set up in an identical manner to (b), at a 1:25 DC:PBL ratio, and harvested at days 4,5, and 6

Fig 35. Patients with HBV chronic patient: cytotoxicity assay using effectors stimulated with DC expressing HBeAg.

(a) Patient AA: lysis of autologous and heterologous targets, examined during seroconversion (K:T ratio 40:1)



(b) Patient PP:(cirrhotic) lysis of autologous targets by selected T cell populations, K:T 40:1.

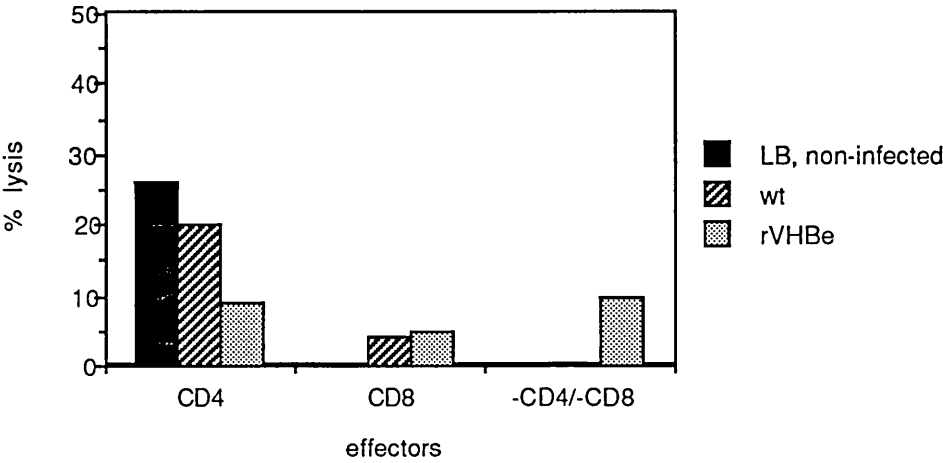


Fig 35.

(a) Patient AA. Effectors were stimulated Fig 34 (a), and a proportion of cells harvested at day 4 for analysis of CTL. The effectors were set up in duplicate against targets expressing nucleocapsid antigens (rVHBe and rVHBe) and control wt infected MHC matched and mismatched cells at K:T ratios 40:1, 20:1 and 10:1. Lysis at the 40:1 ratio is illustrated for simplicity..

(b) Patient PP (cirrhotic HBeAg patient with elevated transaminases). PBL were stimulated at a ratio of 25:1 PBL to transfected DC in hanging drop cultures, harvested at day 5, and re-stimulated with 20 IU ml⁻¹ IL-2 in fresh medium while selecting CD4 and CD8 cells on AIS plates. The purified populations were then set up against autologous targets, uninfected LB cells, LB cells infected with rVHBe or wt vaccinia. There were insufficient effectors recovered to do more than one K:T ratio (40:1 in duplicate) against autologous targets, despite initially recovering 22×10^7 PBL from 200ml of donor peripheral blood.

(5) Non MHC-restricted cytotoxicity.

Natural killer cells are a component of PBL. K562 are lysed at levels of 60-65% (50:1 K:T) in chromium release cytotoxicity assay by NK isolated from normal human donors. To investigate the possibility that NK cells were contributing to the elevated cell lysis detected after stimulating effectors with rHBcAg or peptide pools, a limited analysis of the response against K562, non-infected lymphoblastoid cells and vaccinia infected lymphoblastoid cells was undertaken. No significant difference between levels of NK in CAH or CPH patients was evident, and the level of cell lysis was not significantly different from that observed in normal donors (Fig 36).

Mean normal donor K:T 50:1 62+/-2% (chronic v normal p=0.87)

During acute HBV, lysis of all virally infected cells (greater than non-infected cells) was evident, but was not significant when an un-paired t-test was applied (patients studied {n=3})

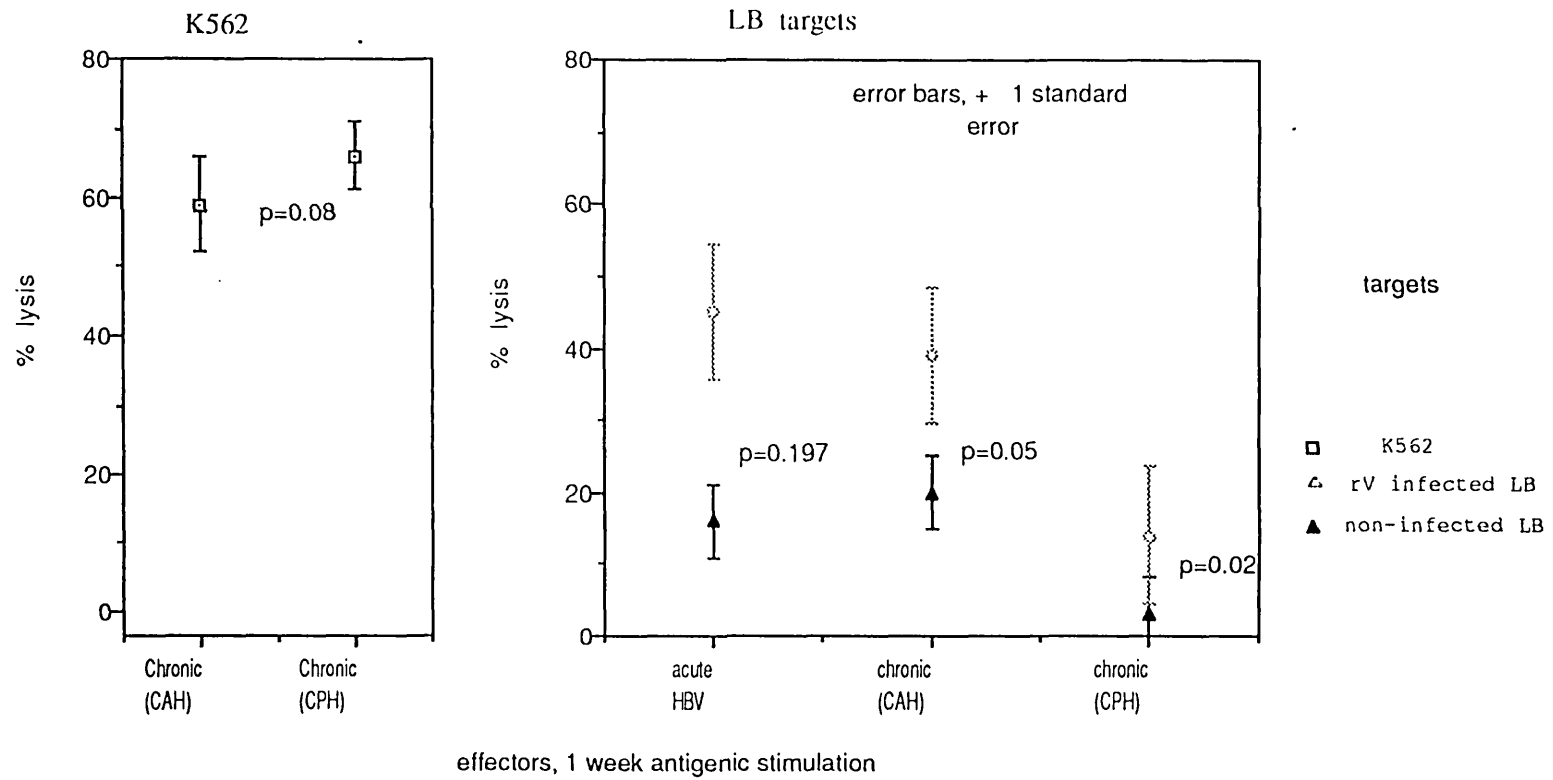
Lysis of virally infected cells did however occur in both CPH and CAH disease at significant levels (n=5 for both groups), and the mean level of lysis during CAH infection was higher than that in CPH.

CAH. Mean lysis of vaccinia infected cells 39% , non-infected cells 20% p=0.05

CPH. Mean lysis of vaccinia infected cells 14%, non-infected cells 3% p=0.02.

The effectors and targets were mismatched at the class I loci, precluding the possibility of a cytotoxic (CD8+ cell) response against vaccinia. The observation that lysis of vaccinia infected cells varied in CPH and CAH disease, but that the level of K562 lysis did not, suggested that NK cells although present, were not significant in differentiating between the two disease states, or the elevated target lysis observed in some patients in the cytotoxicity study.

Fig36. Lysis of K562 targets by effectors derived from CAH and CPH patients and lysis of vaccinia infected and non-infected LB cells



(6) Investigation of antibody mediated lysis of cells expressing HBeAg.

Anti-HBe in human serum binds to membrane displayed HBeAg.

Serum from anti-HBe +ve patients was titred by ELISA (Abbott) to cut off point by doubling dilution, all the serum samples were also investigated for anti-HBe by indirect immunofluorescence of rVHBe and rVHBc infected cells. HepG2 cells infected with rVHBe display HBeAg on the cell surface which can be detected by monoclonal anti-HBe, polyclonal anti-HBc (Dako) which has anti-HBe activity, and human anti-HBe. Cells infected with rVHBc have no plasma membrane nucleocapsid antigen expression, but HBcAg can be detected upon permeablising the cells. Human serum was investigated for staining of rVHBe infected cells with an anti-human FITC second layer. Serum staining cells above rVHBc infected cells at a dilution of 1/50 were scored as +, or ++ highly positive. Some sera stained non-specifically, and were recorded as +/- or -/+ dependent on the degree of fluorescence. Some stained cells were also subjected to FACScan, to confirm the subjective observations. Fig 37 is a FACScan of HepG2 cells infected with rVHBe or rVHBc and stained with a 1/50 dilution of human anti-HBe derived from patient (AG), anti-HBe negative by ELISA at the time of serum sampling. As can be seen from Tables 7 and 8, all patients with anti-HBe detectable by ELISA also had anti-HBe detectable by immunofluorescence.

Anti-HBe binding to membranous HBeAg was present in a large percentage of acute HBV patients, and chronic carriers, even when anti-HBe was either equivocal by ELISA or absent.

Antibody dependent cell mediated lysis.

Initially, when devising an ADCC protocol, mismatched LB cells infected with rVHBe and rVHBc were set up with effectors derived from normal humans in ⁵¹Cr release assays

in the presence of antibody at a range of dilutions. High dilutions of antibody were ineffective in mediating any target lysis. Experiments were repeated with lower dilutions of patient sera, and a K:T ratio of 25:1, but assays were irreproducible, and highly dependent on the individual effector populations. Using HepG2 targets infected with rVHBe or rVHBc, and effectors derived from normal donors, assays were reproducible, and consistently negative. No evidence of ADCC mediated cellular cytotoxicity at a dilution of 1/50 (or above) human serum, irrespective of the titre of antibody present in the serum by ELISA, or immunofluorescent assay, was detected. Freshly isolated effectors were inefficient at lysing rVHBe and rVHBc infected cells in the presence of a murine monoclonal anti-HBe. Effectors isolated from normal human donors, stimulated for 4 days in culture with 20IU ml⁻¹ of IL-2 lysed all targets irrespective of antibody or protein expression Fig 38. The high non-specific lysis elicited by IL-2 culture of effector cells suggested the possibility that ADCC effectors required lymphokine activation. Lymphokine activated killer cells have been reported to mediate ADCC. If *in vitro* lymphokine activation of cells produces high non-specific levels of cell lysis, it might be hypothesised that *in vivo*, lymphokine activated cells might have a similar role. Investigating the effect of a IL-2 /Con A *in vitro*, a minor increased non-specific cell lysis was induced by Con-A but not the lower doses of IL-2.

Complement mediated lysis of HBeAg expressing cells.

Conditions for complement mediated lysis of virally infected HepG2 cells were established using Herpes simplex type I virus infected cells, in the presence of a human anti-Herpes serum, and complement at differing concentrations. The concentration of complement effecting antigen specific lysis was utilised for further experiments

HepG2 permanently transfected with pCEP1.6, and pMEP1.5 did not express detectable surface HBeAg, but were secreting the HBeAg protein into the culture supernatant. Both these lines were lysed in a dose dependent manner by

guinea-pig complement in a ^{51}Cr release assay in the presence of monoclonal anti-HBe (RFHBe2), but not an identical subclass monoclonal and display HBe on the surface, were lysed, to a minor degree (data not shown). Lysis of HepG2/pCEP1.6 and pMEP1.5, occurred in the presence of murine monoclonal anti-HBe, but not antibody derived from acute or chronically infected HBV patients (Tables 7 and 8).

Table 7. Investigation of anti-HBe mediated cytotoxicity (1) during the course of acute HBV infection: correlation with clinical status.

PATIENT IDENTIFICATION	SEROLOGY						LIVER ENZYMES and HISTOLOGY		ANTI-HBe by immunofluorescence		ADCC		COMPLEMENT MEDIATED LYSIS
	age/sex	HBsAg	HBeAg	anti-HBs	anti-HBe titre	IgM-anti-HBc	HBV DNA+ve (DOT BLOT)	AST/ALT/ U L ⁻¹ /U L ⁻¹ /umol L ⁻¹	HepG2 rVHBc	rVHBe	HepG2 rVHBc % cell lysis	rVHBe	pMEP1.5/HepG2 HBeAg
NORMAL HUMAN DONORS													
NP	28F			+	*	-		(AST N < 40	-	-	0	0	0
RG	25F			+	*	-		ALT N< 40	-	-	0	0	0
PT	30M			+	*	-		bilirubin N< 17)	-	-	0	0	0
H	40M			-	-	-			-	-	0	0	0
SL	40F			+	*	-			-/+	-/+	0	3	0
ACUTE HBV													
AB	28F	+	+	-	-	+	nd	637/568/181	-	++	0	2	0
LC	20F	+	+	-	-	+	nd	2500/	+/-	-	0	0	0
CH	22F	+	-	-	-/+	+	-	434/721/37	-	+	3	3	5
RS	27M	+	-	-	-	+	-	nd	-	-/+	0	0	0
GS	34M	+	-	-	-	+	nd	>1000/-/88	-	++	0	0	0
RS	54M	+	-	-	1/50-1/100	-	-	<40/<40/<17	-	+	0	1	0
PmK	M	+	-	+	+	+	-	<40/<40/<17	-	-/+	1	0	0
A.AI	51M	+	-	+	+	+	-	<40/<40/<17	-	+	0	0	3
BB	30M	-	-	+	+	+	+	21/37/13	-	+	0	0	12
CJ	62M	-	-	+	+	+	-	>40/-/8	-	+	0	3	0

* vaccinated laboratory staff

Serum was used at 1/50 dilution for immunofluorescence, and ADCC AND 1/4 for complement assays.

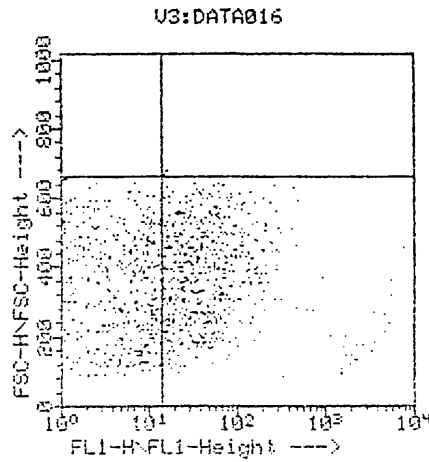
Table 8. Investigation of anti-HBe mediated cytotoxicity (2) during the course of chronic HBV infection: correlation with clinical status.

PATIENT IDENTIFICATION		SEROLOGY					LIVER ENZYMES and HISTOLOGY			ANTI-HBe by Immunofluorescence		ADCC		COMPLEMENT MEDIATED LYSIS
	age/sex	HBsAg	HBeAg	anti-HBs	anti-HBe titre	IgM-anti-HBc	HBV DNA+ve (DOT BLOT)	AST/ALT/ U L-1/U L-1/umol L-1	bilirubin L-1	HepG2 rVHBc	HepG2 rVHBc	HepG2 rVHBc	HepG2 rVHBc % cell lysis	pMEP1.5/HepG2 HBeAg
CHRONIC HBV														
ES	54F	+	+	-	-	-	+	30/28/6	CAH	-	+	nd	nd	0
NH	28M	+	+	-	-	-	+	54/37/10	CAH	-/+	-/+	0	0	0
RP	35M	+	+	-	-	-	+	43/	CPH	-	+	3	3	0
TD	36M	+	+	-	-	-	+	29/93/24	CPH	-	-	0	0	0
DF	62M	+	+	-	-	-	+	54/83/13	CPH/LH	-/+	+/-	1	2	0
CHRONIC HBV SEROCONVERTING TO ANTI-HBe FOLLOWING INTERFERON TREATMENT														
LS	36M	+	+	-	-	-	-	103/-/39	CPH	-	+	0	0	0
CW	39M	+	-	-	1/10	-	-	160/-319/12	CAH	-/+	-/+	0	0	0
AS	31F	+	+	-	-	-	+	363/-/10	CPH	-	+	0	0	0
DF	34M	+	+	-	-	-	+	136/-/10	MILD CAH	-	-	5	5	0
SD	44F	+	-	-	-	-	-	46/100/11	MILD CAH	-	+	1	0	2
CHRONIC HBV: ANTI-HBe +ve WITHOUT LIVER DISEASE														
LS	16M	+	-	-	1/1600	-	-	64/-/14	CAH	-	++	0	0	0
BS	43M	+	-	-	1/1600	-	-	46/56/9	nd	-	+/-	0	0	0
CG	30M	+	-	-	>1/200	-	-	-/29/7	MILD CPH/LH	-	-/+	0	0	0
AG	45F	+	-	-	>1/50	-	-	32/28/7	CPH/LH	-/+	+/-	0	2	0
MOR	44M	+	-	-	+	-	-	28/-/9	CPH	-	-/+	1	0	0
CHRONIC HBV: ANTI-HBe +ve WITH LIVER DISEASE														
PP	48M	+	-	-	+	-	+/-	112/125/	NO BIOPSY	-	+/-	0	0	0
PE	55M	+	-	-	+	-	+	112/208/67	CAH	-/+	-/+	0	0	0
JC	45M	+	-	-	+	-	+/-	225/526	CPH	-	+	0	0	4
PQ	60M	+	-	-	+	-	+	-/221/8	CPH/cirrhosis	-	+	0	0	0
AA	52M	+	+	-	+	-	+/-	-/168/11	CPH	-	++	nd	nd	0

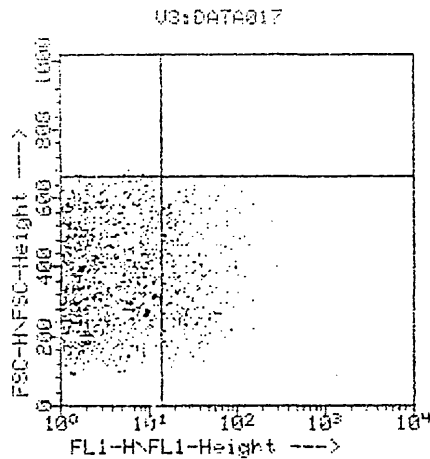
Fig 37. FACScan: HEPG2 cells infected with rVHBe (a) or rVHBc (b) stained with human anti-HBe and anti-human FITC.

FL1 axis shows level of immunofluorescence,
 LL unstained cells
 LR stained cells

(a)



(b)



The serum used at a dilution of 1/50, patient (AG) anti-HBe negative by ELISA (ABBOTT).

Fig 38. ADCC: Effectors cultured with IL-2 (20IU/ml) for 4 days prior to assay.

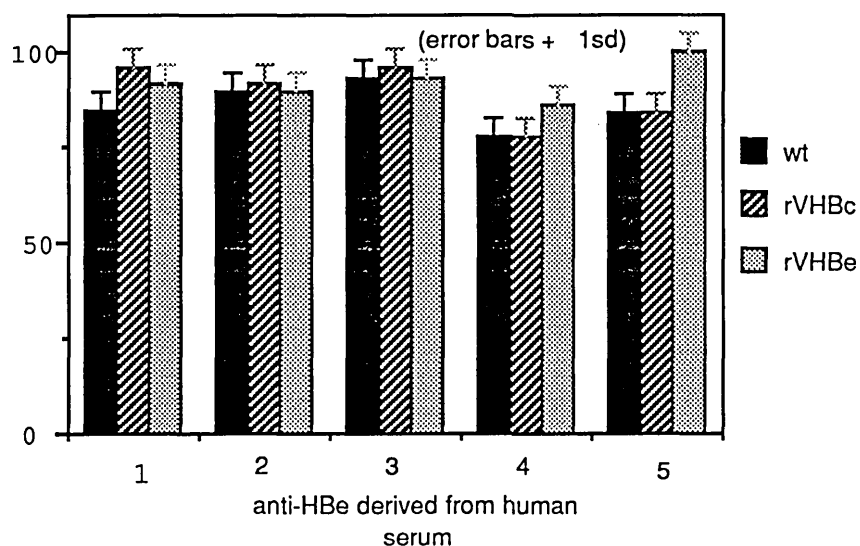


Fig 38.

Effectors were cultured with IL-2 (20 IU/ml) for 4 days prior to assay at a K:T ratio of 25:1 in the presence of anti-HBe antibody (derived from human serum, and control anti-HBe negative serum:

1 normal human serum (anti-HBe negative)

anti-HBe positive patients:

2.LS (1/1600 ELISA +ve serum)

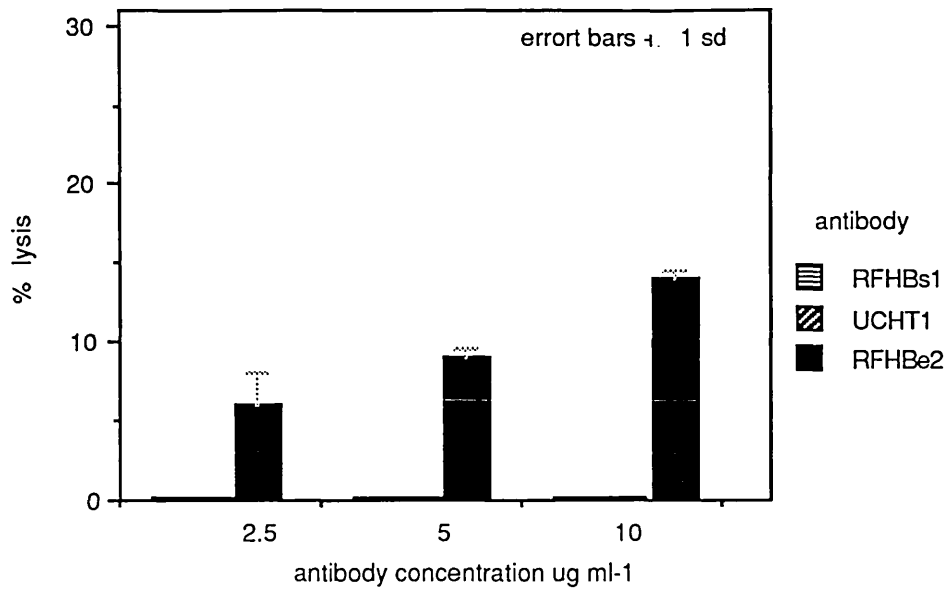
3 CJ (acute HBV)

4 RP (chronic carrier)

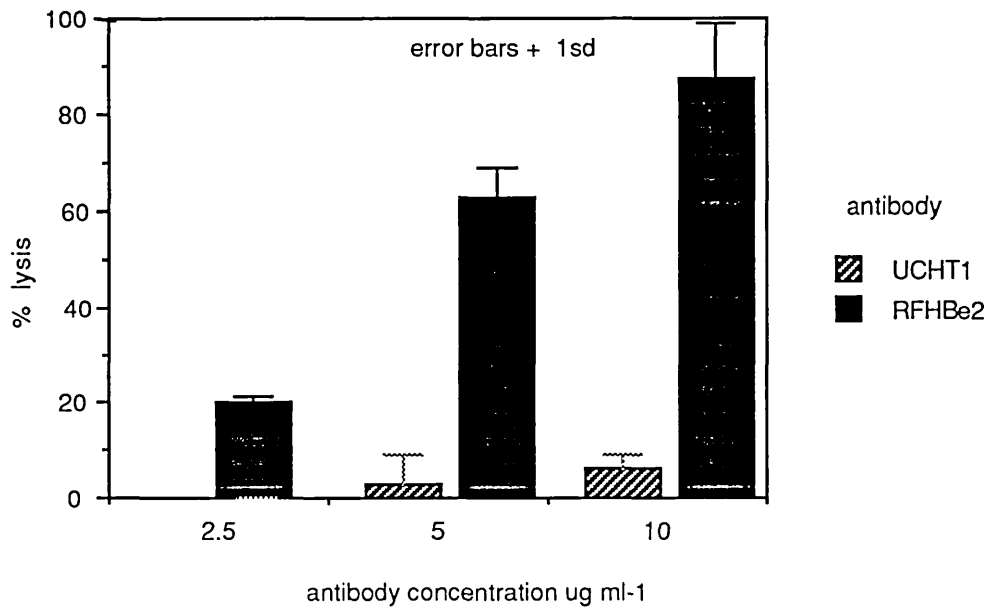
4 LC (acute HBV)

Fig 39. Lysis of HepG2 cells expressing HBeAg in the presence of anti-HBe antibody : RFHBe2

(a) complement / anti-HBe mediated lysis of HepG2/
pCEP1.6, expressing the HBeAg



(b) complement / anti-HBe mediated lysis of HepG2/
pmep1.5, expressing HBeAg



RFHBe2 : murine monoclonal anti-HBe
 UCHT1: irrelevant antibody of equivalent subclass
 RFHBS1 : anti-HBsAg murine monoclonal

Fig 39

(a) Stable HepG2 transfectants (transfected with pCEP1.6) expressing HBeAg were labelled with ^{51}Cr and incubated for 2 hours in the presence of varying titres of monoclonal antibody (anti-HBe and subtype matched controls), and complement. Percentage lysis was calculated as described for standard cytotoxicity assays.

(b) As (a) except the target line was transfected with the pMEP1.5 vector (also expressing HBeAg).

Chapter Five. Discussion.

(1) The cytotoxic response to HBV during acute and chronic infection, and during HBeAg / anti-HBe seroconversion.

The questions posed at the beginning of the study were as follows:

- (1) Are antigen specific CTL present in acute HBV?
- (2) Is chronicity due to an absence of CTL?
- (3) What happens during HBeAg/anti-HBe induced seroconversion following INF α therapy?
- (4) Is the lymphocyte proliferative non-responsiveness to HBcAg, reported during chronic HBV infection, due to defective APC?
- (5) What role do humoral (anti-HBe) mediated processes play in acute and chronic HBV during HBeAg / anti-HBe seroconversion. Is the emergence of viruses unable to produce HBeAg (HBe-ve variants) a consequence of anti-HBe selection?

There are no standard protocols for the detection of CTL against HBV infected cells, but culture systems devised and used in the study were validated by reproducing a published experiment. An HLA A2 restricted cytotoxic response was demonstrated against autologous and A2 matched targets, by stimulating effectors derived from an acute HBV patient with the HBcAg p18-27 peptide. The experiment confirmed that antigen specific CTL are present during acute infection, and that they can be detected in the peripheral blood. Random pools of overlapping peptides were not effective in generating information on other epitopes recognised during acute infection in non-HLA A2 subjects. Only six patients with acute HBV, were studied

in total, and no statistically significant CD8+ killing could be shown in the group as a whole.

After validation of the HBcAg/p18-27 HLA A2 stimulation protocol in a patient with acute HBV infection, the same protocol was used to investigate CTL derived from HLA A2 patients with chronic HBV infection. No significant response was detected using effectors derived from two patients with chronic HBV and evidence of significant hepatitis but not undergoing HBeAg/HBe antibody seroconversion. Sequential assays were performed on lymphocytes isolated from a patient with chronic HBV infection (CW), who seroconverted following interferon therapy. Despite gross transaminitis (AST 850U/L) during seroconversion, and sequencing data to confirm that the viral genome contained the appropriate DNA sequence to encode the intact p18-27 epitope, only low level (10-14%) antigen specific MHC class I restricted lysis of targets, and a similar level of antigen specific non-class I restricted response was elicited. The profile of the response altered over the course of seroconversion. Non-specific lysis of greater than 40% dropping to 20% over the course of seroconversion was observed, concurrent with the antigen specific response.

These results demonstrate that classical MHC class I restricted CTL were not present at detectable levels in patients with HBe antigen positive chronic hepatitis but were present in low numbers during seroconversion from HBeAg to anti-HBe status. In order to extend the study to patients of other HLA phenotype, alternative methods of stimulating a cytolytic response were evaluated. An HLA A28 CAH patient (AD), was studied over the course of a protracted seroconversion, following INF α therapy. Early in the seroconversion event, peptides in the presence of rHBcAg and IL-2 stimulated a non-specific response of comparable magnitude to that seen in patient CW during seroconversion, that is, approximately 40% lysis of vaccinia infected cells. A month later, overall levels of lysis had diminished, and subsequently continued to fall. The low level antigen specific lysis elicited by multiple groups of peptides was not inhibited by anti-MHC class I

antibody.

The effect of stimulation with rHBcAg and PHA was investigated in patient AD prior to seroconversion. Approximately 10% lysis of autologous infected targets was detected when effectors were stimulated with rHBcAg. The effect appeared to be antigen dependent rather than mitogenic. Purified rHBcAg was not mitogenic for naive lymphocytes in transformation assays (data not shown), and PHA alone failed to produce any cytolytic stimulation.

The response elicited by rHBcAg appeared to be marginally higher than that obtained by peptide stimulation alone, frequently an antigen specific element of 20-30% during seroconversion. The mechanism by which particulate antigen may induce a class I restricted response has not been fully elucidated, but has been previously described (Peifer et al, 1993). The interaction between cells in the effector population is clearly complex, but a detailed breakdown of effectors stimulated with rHBcAg into CD4+ CD8+ enriched populations revealed a substantial (26% specific lysis) CD8+ mediated response in a patient with chronic active hepatitis.

Although the study was not designed for statistical analysis, the methodology was a useful tool to analyse data which varied markedly between patients. Sixty patients were available for the analysis, and some general trends began to emerge about the immunopathology of chronic disease, and the events during HBeAg/anti-HBe seroconversion.

Patients with chronic HBV infection were categorised by clinical status into chronic persistent and chronic active hepatitis. Analysis revealed a small but significant class I restricted antigen specific response in both groups: lysis of autologous targets rVHBc/e above wt $p=0.001$ CAH; $p=0.011$ CPH. The number of mismatched samples was considerably smaller than the matched group, and did not approach significance; CAH: mismatched rVHBc/e above wt, $p=0.523$, CPH; $p=0.173$ (Wilcoxon Signed Rank Test).

During seroconversion (IFN α induced or natural), the magnitude of all responses was elevated. The predominant response appeared to be nucleocapsid specific but not MHC

restricted: a significant lysis of rVHBc/e infected autologous targets above wt infected targets $p=0.032$, and heterologous targets, $p=0.011$, was detected (Wilcoxon Signed Rank Test). NK or lymphokine activated cells potentially contributed to the high level of non-specific lysis of vaccinia infected cells which invariably occurred concurrent with seroconversion.

Tentative conclusions which may be drawn from the study, and which will be developed at a latter stage in the discussion are:

(1) An MHC restricted cytolytic response occurs during acute infection, but it is short lived, and effectors require intensive *in vitro* antigen stimulation and IL-2 to generate lytic potential.

(2) A low level CD8+ MHC restricted response appears to be detectable during the course of chronic infection, but upon seroconversion, the differentiation between lysis of autologous and heterologous targets disappears. Antigen specific lysis of both autologous and heterologous targets occurs. It appears unlikely that cytolytic activity by CD8+ cells is the major effector of viral elimination during HBeAg /anti-HBe seroconversion in chronic disease,

(3) There is a high level of non-specific lysis among cell populations (PBL/monocyte) derived from chronic HBV carriers during seroconversion.

(4) Non-MHC class I mediated cell lysis can be demonstrated *in vitro* and may contribute to the necrosis evident around the portal tracts during active HBV infection. Cells expressing HBeAg on the cell surface can be lysed by anti-HBe in the presence of complement. Anti-HBe is detectable in the peripheral circulation during the course of chronic HBV infection, even prior to HBeAg/ anti-HBe seroconversion, but the concentration does not appear to be high enough to mediate C1q binding, and subsequent cell lysis. The level of antibody in the portal vein flowing

directly from the spleen, is likely to be considerably higher, and potentially may mediate hepatocyte lysis.

(2) Dendritic cell function in acute and chronic HBV infection.

During acute HBV^{infection}, CTL can only be detected after intensive antigenic stimulation in the presence of IL-2. CTL although detectable in chronic carriers at low levels, do not effect seroconversion. Development of an effective CD8+ response is thought to require engagement of the TCR, the CD8 and MHC class I molecules, and co-stimulatory ligands present on "professional" antigen presenting cells, such as the B7 molecule (reviewed Schwartz, 1992), to promote a stimulatory rather than a tolerogenic response (Townsend and Allison, 1993). Macrophages, DC and activated B cells express the B7 ligand, but DC are the central antigen presenting cells involved in the primary response against invading pathogens. Understanding the interaction between DC and HBV, may elucidate the mechanisms by which chronicity develops, and is reversed.

Lymphoid DC recirculate, but after antigenic priming become targeted predominantly to the spleen and liver. DC are inefficient at phagocytosis, but have been demonstrated to present whole unfragmented bacteria to purified T cells (Kaye et al, 1985), and theoretically might present HBV antigens derived from Dane particles. Phagocytosis or receptor mediated endocytosis generally results in class II response priming, unless the invading pathogen can escape the endosomal degradation. There is no evidence for HBV having such an evasive mechanism.

B cells can internalise HBsAg, and act as antigen presenting cells to pre-primed T cells, but recent evidence suggests that antigen presenting B cells induce tolerance upon exposure to virgin T cells (Fuchs and Matzinger, 1992).

The question of HBV replication in lymphoid tissue is complex and contradictory. Lymphoid cell subsets have not been examined in detail for the presence or absence of HBV replicative intermediates. HBV has been reported to be

detected in lymphocytes by PCR (Pasquinelli et al, 1990), during acute and chronic infection, and by Southern blot analysis in chimpanzee lymphocytes (Korba et al, 1986). In direct contradiction, a body of evidence exists that HBV only replicates to high level in hepatocytes under the control of the EhII enhancer, and liver specific transcriptional activators. Production of the surface antigens is not under such a rigorous control. B7 has not been detected on the hepatocyte membrane, so, in theory, an initial CD8+ / MHC class I antigenic interaction on a hepatocyte, not complemented by a B7/CD28 interaction may be hypothesised to induce anergy, rather than an effective response, and anergy so induced is thought to be reversible in the presence of IL-2.

The transfection experiments reported indicate that the pre-core/core gene can be transcribed and translated under the control of foreign promoters in lymphoid cells. When an enriched DC population was transfected with pre-core/core EBO expression vectors, HBeAg was secreted. Lymphocytes derived from anti-HBc negative normal human donors were stimulated to produce anti-HBc and anti-HBe specific B cells after 5 days stimulation with the transfected cells, indicating that primary immune stimulatory cells (DC) had been successfully transfected. A quantitative increase in antibody producing B cells was similarly detected in both acute and chronic patients groups. The amplitude of the proliferative response elicited in lymphocytes isolated from the acute and chronic patients was almost identical. The mean stimulation indexes of PBL derived from the naive, acute and chronic patient groups respectively were $X4 \pm 3.4$ (sd), 3.5 ± 1.8 , and 4.2 ± 2.8 . Depletion of CD4+ cells invariably resulted in a significant reduction in the magnitude of the proliferation observed, when the initial response itself reached statistical significance. Depletion of CD8+ cells resulted in a significant reduction in the proliferative response in 1 out of 4 acute patients studied, and approached significance in a second. Both of these individuals completely resolved infection. Although the

CD8+ population could not be demonstrated to be cytolytic, the results supported the observation that HBcAg/HBeAg specific CD8+ cells are induced during acute HBV infection. Depletion of the CD8+ cells from chronic responder populations did not significantly reduce the proliferative response in any of the patients in which the initial antigen stimulation was statistically significant. No correlation between the detection of CD8+ cells, serum transaminases or disease state could be made among the chronic patients.

In spite of a high level of proliferation induced by HBeAg in lymphocytes isolated from a seroconverting individual (AA) (stimulation index X3), no CD8+ cytolytic response could be detected in assay. Selecting pure CD4+ and CD8+ populations of a second chronic HBV patient with severe CAH/cirrhosis, the only cytolytic population detectable in bioassay were CD4+ cells which lysed in a non-specific manner. Stimulating effectors for two weeks initially with transfected DC, then feeding with irradiated transfected LB cells, a high non-specific lysis was elicited. When the response elicited by the EBNA plus and minus vectors was analysed, it appeared that an EBV but not HBcAg response had been raised.

DC derived from chronic carriers were competent at stimulating antibody production, and lymphocyte proliferation when transfected with an expression vector which secreted HBeAg, suggesting that defective processing or presentation by APC, is not responsible for the general cellular "unresponsiveness" observed during chronic infection. Electroporation of antigen presenting cells with a whole copy of HBV DNA within a plasmid, but under the control of endogenous promoters might be successfully employed to investigate the transcriptional and translational competence of professional antigen presenting cells with respect to HBV.

(3) Non-specific lysis of virally infected cells.

The ability of effectors to lyse vaccinia infected targets by greater than 40%, demonstrated by PBL from some

patients with acute or chronic active hepatitis, diminished upon recovery. Vaccinia infected targets, but not transformed lymphoblastoid cells, were susceptible to non MHC class I restricted lysis by both primed, and freshly isolated lymphocytes. Vaccinia infected LB cells were initially used as ADCC targets, but the normal effector populations lysed these cells in a variable non-specific manner, suggesting *in vivo* activation of effectors. Normal donor K562 lysis remained constant, suggesting that the effectors lysing vaccinia infected cells were lymphokine activated killers. Lysis of vaccinia infected LB cells in a non-specific manner was elevated during seroconversion, and coincided with peak transaminase activity.

The effector cells, or lymphokines responsible for lysis of vaccinia infected LB cells, were not definitively defined, but preliminary depletion experiments implicated CD4+ cell lymphokine activated cells.

(4) What role do humoral (anti-HBe) immune responses play in lysis of infected cells in acute and chronic HBV infection during HBeAg/anti-HBe seroconversion. Is the emergence of viruses unable to produce HBeAg (HBe negative variants) a consequence of humoral immune pressure ?

Viral CTL escape mutation appears to be a rare phenomenon, documented to occur in LCMV and HIV infection (Salvato et al, 1991; Pircher et al, 1990; Phillips et al, 1991). A recent publication has described antagonist peptide sequences (detectable in two chronic HBV HLA A2 patients) which appear to be able to inhibit CTL recognition of target cells *in vitro*, during chronic HBV infection (Bertoletti et al, 1994). The report confirms the existence of CTL during chronic HBV infection in some patients who fail to seroconvert. The hypothesis that the pre-core mutant viruses were CTL escape mutants was not investigated further than some initial cytotoxic T cell studies. In a patient identified by sequence analysis (PP) to have the pre-core mutant virus, no peripheral CTL

response was detected. Class I restricted CTL were however detected against the rV1896 recombinant virus in an individual with chronic active disease, during INF α induced seroconversion, but it was not possible to reproduce the response or further characterise it in other individuals.

The humoral response is a potentially important lytic system. Using indirect immunofluorescence, anti-HBe was detected during acute infection. In order to investigate the possibility that anti-HBe has a function in the clearance of infected cells expressing HBeAg through ADCC, or complement mediated lysis, serum samples from patients with differing clinical disease states were selected. Effectors derived from normal donors did not mediate HBeAg/anti-HBe ADCC. If the effectors were cultured with a high dose of IL-2 for 4 days prior to assay, lymphokine activated cells lysed targets, irrespective of antibody concentration or target cell antigen expression. It was not possible to reproduce an environment *in vitro* in which effector cells could be induced to lyse targets in an antigen specific manner.

NK cells, primed *in vivo* with antibody via the CD16 receptor, which lyse targets in an antigen specific manner, have been described during HIV infection (Riviere et al., 1989). Existence of similar cells during HBV infection remains to be investigated.

Complement mediated lysis of targets secreting HBeAg could be detected in a dose dependent manner in the presence of high dose murine monoclonal anti-HBe (RFHBe2), but human serum containing anti-HBe caused only minor levels of target lysis. Both HBeAg and complement have been detected on hepatocyte membranes. It is in theory plausible that the damage associated with the particular pattern of necrosis around the portal tracts (peripheral necrosis) identified during chronic infection is complement mediated damage. Peripheral levels of anti-HBe may be far lower than those present in the liver. Antibody derived from splenic plasma cells will flow into the portal tracts and bind to periportal infected hepatocytes expressing high levels of HBeAg, activating the complement cascade. In the absence of lytic cellular processes, as seen in the

majority of patients with chronic HBV, (including those with HBeAg +ve and HBeAg -ve viraemia) a virus which does not produce HBeAg may be positively selected by complement mediated lysis of cells with replicating HBeAg positive but not HBeAg negative virus.

(5) A potential role for the cytokine network in maintenance of chronic HBV infection.

Reviewing the experimental data derived from the cytotoxicity studies, and published observations on lymphocyte response during HBV infection, it might be postulated that chronic HBV infection is associated with induction of a $T_H2 > T_H1$ response to HBcAg. T_H2 and T_H1 cells have different actions. A shift in the balance between induction of a predominant T_H2 to a T_H1 type response in the liver, would result in development of a local cytotoxic environment, that is induction of IFN γ , and TGF β , and recruitment of CD8+ and NK cells, which might terminate the persistent infection.

There is a body of evidence that an increased T_H2 , and a reduced T_H1 response to HBcAg may predominate during chronic HBV infection.

The T_H0 and T_H2 populations are most efficient at providing B cell help. High level production of anti-HBc, is widely reported during HBV infection. The predominant anti-HBe and anti-HBc subtype detected during chronic infection, free and complexed, is IgG $_1$ (Sallberg et al, 1991): production of this subtype is controlled by the T_H2 population. A T_H2 induced eosinophilic component of the inflammatory infiltrate has been identified by histology in association with CAH and cirrhosis (unpublished observation). If CD4+ T_H2 cells were to predominate during chronic infection, cytokines inhibitory of a cytolytic response, such as IL-10 and TGF β , might prevent clearance of infected cells. There is evidence that the T_H1 response is inhibited during chronic infection. MHC class I restricted cells were virtually undetectable during the persistent stage of chronic disease, becoming detectable (at low levels) during HBeAg/ anti-HBe seroconversion.

Persistence of HBV is associated with a decline in IL-2 production, (Saxena et al, 1985; Anastassakos et al, 1987), suggestive of a reduced T_H1 response. The increase in expression of lymphocyte activation markers detected during chronic disease, does not correlate with detection of high affinity IL-2 receptors (Vegnente et al, 1991), indicating that the activated cells are not producing IL-2. Cloned intrahepatic HBcAg specific T lines derived from patients with chronic active hepatitis (Ferrari et al 1987) did not produce detectable levels of IL-2, despite exhibiting HBcAg induced proliferation, and IFN γ production.

Mononuclear phagocytes and Kupffer cells produce TGF β (Assoian et al, 1987; Matsuoko et al 1990) which in turn induces collagen production from hepatic lipocytes, and fibrosis. A second action of TGF β is inhibition of the T_H1 and CD8+ populations.

Therapy of chronic HBV carriers may produce switching of the CD4+ T_H1/T_H2 ratio to effect a predominant T_H1 response. IFN α may mimic the action of IFN γ switching the intrahepatic CD4+ population to effect a delayed type hypersensitivity reaction. Although serum HBV DNA falls in responders over the course of IFN α treatment, seroconversion and viral elimination usually occurs some time after completion of therapy. T_H1 CD4+ cells secreting IL-2 and IFN γ promote recruitment, stimulation and proliferation of CD8+ cells, NK cells, T_H1 cells and macrophages. The combination of lytic cytokines produced by CD4+, NK and CD8+ cells (IFN γ and TNF β) and macrophages (TNF α) are known to be effective in direct elimination of virally infected cells (reviewed Ramsay and Ranshaw 1993) and might produce a response similar to that observed in the cytotoxicity assays during seroconversion.

If the CD4+ response (T_H1 v T_H2) induced to HBcAg can be influenced by the route of antigen administration to dendritic cells, that is so presentation is via endogenous rather than exogenous pathways, alternative therapy may be devised which may be effective in chronic carriers born to HBcAg positive mothers as well as individuals who developed chronic infection in adult life.

EBV produces an IL-10 analogue, potentially important in the persistence of viral infection (Moore et al, 1990). No HBV analogue was detectable by sequence comparison using the EMBL database for the IL-10 sequence. A recent study of the HLA types associated with viral elimination and chronicity has revealed a Class II MHC haplotype associated with viral clearance (M.Thurz, personal communication), adding further weight to the hypothesis that CD4+ cells and their cytokines determine the outcome of HBV infection.

An alternative view of seroconversion during chronic infection might be that CD8+ cells are involved as the initiating effectors, but are present at a level that is difficult to detect *in vitro* against the high activity of cytokine activated CD4+ and other cell types. The precursor frequency or degree of activation appeared to be far lower than that detected during acute HBV infection. Low numbers of class I restricted CTL have been demonstrated to be capable of causing elimination of virally infected cells *in vivo* at a K:T ratio lower than that at which cytolysis is affected *in vitro*. The effector mechanism was defined in an HBsAg transgenic mouse model of fulminant HBV infection, as direct lysis of cells in the vicinity of the effectors by IFN γ . Recombinant vaccinia viruses expressing IL-2 and IFN γ have been demonstrated to be effective in athymic mice at clearing viral infection, but the uncontrolled reaction is excessively severe. Targeting via the CD8+/MHC interaction increases the tissue specificity and intensity of the local reaction (Ando et al, 1993).

The observation that antigen specific lysis of autologous but not heterologous cells occurs during CPH and CAH, but at a low level, indicates the presence of a low precursor frequency of MHC restricted cytotoxic cells during these phases of disease. If small numbers of specific CTL may effect viral elimination, the question of how chronicity ever develops must be raised.

Chronicity occurs either when the virus is successful in evading immune clearance, or the immune system fails. Chronic carriers rarely produce the neutralising anti-HBs

in infection rather than effective CTL might be examined in future studies. Furthermore, it is possible that amplification of the CTL response by T_H1 cells is deficient: examination of the cytokine profile of intrahepatic inflammatory cells was outside the scope of the study described, but will be important in shedding light on this aspect of persistent infection.

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Appendix I

STOCK SOLUTIONS

(unless otherwise stated all chemicals from BDH or Sigma
Alcohols and Acids from BDH. H₂O Millipore purified)

solution	method of preparation
20 X SSC	3M NaCl . 0.3 M sodium citrate,
100 X Denhardt's solution	2% (V/V) bovine serum albumin (BSA) 2% (W/V) Ficoll 2% (W/V) polyvinylpyrrolidone (PVP)
Phenol/Chloroform 50:50 (W/V)	100g ultra pure phenol (BRL) 100ml of chloroform
Rnase A stock. (10mg/ml)	10mg per ml H ₂ O boiled 10 min then stored at -20°C
50 X TE	500mM Tris-HCL pH 7.4 50mM EDTA
TNE	10mM Tris-HCL pH 8.0 100mM NaCl 1mM EDTA (pH 8.0)
50 X Tris-Acetate (TAE)	2M Tris-acetate 0.1M EDTA
50 X Tris-Borate (TBE)	4.45M Tris -borate 0.4M EDTA
Triton-X lysis buffer:	2 % (W/V) Triton X-100 72.5mM EDTA 50mM Tris-HCl.pH 8.0
Alkaline lysis buffers	
Solution I	50mM glucose (sterile filtered) 50mM Tris-HCL pH 8.0 10mM EDTA pH 8.0
Solution II	1% SDS in 0.2M NaOH (freshly prepared)
Solution III	5M Potassium acetate.
10 X MOPS Buffer	0.2M 3-[N-morpholino]-propane- sulphonic acid 0.05M Na acetate pH 7.0 0.01M EDTA
Media for bacterial	culture.
L-Broth	1% (W/V) Bacto-tryptone 1% (W/V) NaCl 0.5% (W/V) Bacto-yeast

L-Agar	5% bacto-agar in L-Broth
SOC	2% (W/V) Bacto tryptone 0.5% (W/V) Yeast 10mM NaCl 2.5mM KCl 10mM MgCl ₂ 10mM MgSO ₄ 20mM glucose

Buffers used in cellular techniques.

Phosphate buffered saline (PBS)	0.14M NaCl 7mM KCl
"A" Dulbecco)	1.5mM KH ₂ PO ₄ 8.1 mM Na ₂ HPO ₄
5 X Barbitone buffer	mM NaCl ₂ mM CaCl ₂ mM MgCl ₂ mM barbitone buffer pH 7.6
dilution buffer (immunofluorescent staining)	1% W/V BSA (Sigma) 5% (V/V) normal human serum (NHS) 0.02% (W/V) sodium azide 0.01% (V/V) Tween-80 made up in PBS "A"
RIPA buffer:	150mM. NaCl, 1% (V/V) NP-40 0.5% (W/V) DOC 0.1% (W/V) SDS 50mM Tris-HCl pH 7.5

Buffers for protein gel electrophoresis (Laemlli):

10 X Sample Buffer	mM Tris-HCl pH 6.82. % SDS (V/V) % glycerol
Acrylamide stock	30% acrylamide (W/V), 0.8% bis-acrylamide (W/V) in 100ml H ₂ O
cross linking agents	10% ammonium persulphate ((W/V) freshly prepared every week TEMED (Sigma)
12.5% separating gel	11.2 mls of Tris-HCl pH 8.8. 3 mls 10% (W/V) SDS 12.5 mls acrylamide stock 6.5mls H ₂ O 100ul ammonium persulphate, 50ul TEMED
5% stacking gel (10 ml)	1.26 mls Tris-HCl pH 6.8 100ul 10% SDS 1.67ml acrylamide

	6.97 ml H ₂ O
	50ul Ammonium persulphate
	10ul of TEMED
Cathode buffer	mM Tris-Glycine pH
Anode buffer	10-25mM Tris-HCl pH 8.8
Protein Blot Transfer buffer	0.025 M Tris-HCl pH 8.3 0.15M glycine 20 % (V/V) methanol

DNA sequencing Gel; Sequagel (National Diagnostics)

8.3M Urea, 1 X TBE;	
100 ml 5% gel 80-200 nucleotides	20ml sequagel concentrate 66ml sequagel diluent 10 ml sequagel buffer
100 ml 6% gel 60-150 nucleotides	24ml sequagel concentrate 66ml sequagel diluent 10 ml sequagel buffer
	gels crosslinked with 0.8% (V/V) ammonium persulphate
	0.04% (V/V) TEMED

Restriction enzyme and modifying buffers:

Eco RI, Bam HI, Stu I, EcoRV all digest well in buffer B
Sma I buffer H supplied by Boehringer Mannheim.

CIAP buffer	50mM Tris-HCl pH 9, 1mM MgCl ₂ 0.1mM ZnCl ₂ 1mM spermidine
Ligase buffer	50mM Tris-HCl pH 7.5 1mM MgCl ₂ 1mM DTT 1mM ATP
End labelling buffer	50mM Tris-HCl 10 mM MgCl ₂ 5mM DTT 0.01 mM ATP

Appendix II.

SEROLOGY AND LIVER FUNCTION TESTS OF PATIENTS USED IN ASSAYS ILLUSTRATED.

Chronic HBV: clinical data from patient's cytotoxicity assays, and DC transfection studies, illustrated.

PATIENT JA (M 50 , mild CAH LH) HLA A2, A24, B44, B61, CW25

DATE	HBV-DNA	HBsAg	HBeAg	anti-HBs	anti-HBe	AST U/L	ALT U/L	bilirubin mmol/L
6.1.92	+	+	+	-	-	53	nd	11
<u>10.1.92</u>	+	+	+	-	-	50	35	13
19/11/92	+	+	+	-	-	48	67	10
17/3/93	+	+	+	-	-	nd	215	15
18/10/93	+	+	+	-	-	28	35	15

PATIENT DH (M 33yrs , CPH with focal lobular inflammation) HLA A2, A29, B44, B60, CW3, W5, BW4, BW6

DATE	HBV-DNA	HBsAg	HBeAg	anti-HBs	anti-HBe	AST U/L	ALT U/L	bilirubin mmol/L
2/8/91	+	+	+	-	-	47	77	10
16/8/91	+	+	+	-	-	53	67	9
17/1/92	+	+	+	-	-	34	66	14
<u>2/4/92</u>	+	+	+	-	+	62	125	11
6/5/93	+	+	+	-	-	217	506	11
18/5/93	+	+	+	-	-	518	670	18

PATIENT CG (M 30CPH) A34, B35, B53, CW3

DATE	HBV-DNA	HBsAg	HBeAg	anti-HBs	anti-HBe	AST U/L	ALT U/L	bilirubin mmol/L
8/1/90	+	+	+	-	-	85	nd	4
<u>9/10/90</u>	+	+	+	-	-	88	108	10
29/1/91	+	+	+	-	-	nd	60	2
7/5/91	+	+	+	-	-	nd	73	3

PATIENT MOR (M 44 , CPH, seroconversion following interferon treatment.) HLA A3, A10,B7, B13, Bw4, Bw6, CW2.

DATE	HBV-DNA	HBsAg	HBeAg	anti-HBs	anti-HBe	AST U/L	ALT U/L	bilirubin mmol/L
31/7/89	+	+	+	-	-	155	45	5
14/8/89	+	+	-	-	-	162	40	9
21/8/89	-	+	-	-	-	157	32	17
<u>11/1/90</u>	-	+	-	-	+	31	nd	9
24/3/90	-	+	-	-	+	<40	<40	<17

PATIENT LS (M , 16, CAH , natural seroconverter following HAV infection in October 1989) HLA A2 B7,B13,BW4,BW6,CW6.

DATE	HBV-DNA	HBsAg	HBeAg	anti-HBs	anti-HBe	AST U/L	ALT U/L	bilirubin mmol/L
26/10/89	+	+	-	-	+	935	1875	23
1/11/89	+	+	-	-	+	1360	2935	45
14/11/89	+	+	-	-	+	120	nd	19
<u>28/11/89</u>	+	+	-	-	+	64	nd	14
21/12/89	+	+	-	-	+	68	nd	10
10/7/90	+	+	-	-	+	35	nd	14
<u>22/1/91</u>	-	+	-	-	+	<40	<40	<7
11/8/92	-	+	-	-	+	23	15	12
4/8/93	-	+	-	-	+	24	19	14

PATIENT TD (M 36, CPH, seroconverted following interferon treatment).
HLA A3,B14,B35,CW3,CW8.

	HBV-DNA	HBsAg	HBeAg	anti-HBs	anti-HBe	AST U/L	ALT U/L	bilirubin mmol/L
DATE								
28/10/91	+	+	+	-	-	48	97	30
9/3/92	+	+	+	-	-	29	93	24
15/6/92	+	+	+	-	-	29	64	19
15/6/93	+	+	+	-	-	33	60	20
14/1/93	+	+	+	-	-	67	206	23
2/4/93	+/-	+	+	-	+	121	327	25
<u>26/7/93</u>	-	+	-	-	+	25	31	33

PATIENT CW (M, 39, CAH, seroconverting following interferon treatment).
HLA , A2, A24, B35, B44, BW4, BW6, CW4, CW5.

	HBV-DNA	HBsAg	HBeAg	anti-HBs	anti-HBe	AST U/L	ALT U/L	bilirubin mmol/L
DATE								
5/12/89	+	+	+	-	-	495	nd	13
17/9/90	+	+	+	-	-	89	nd	19
22/4/91	+	+	+	-	-	41	38	7
2/12/91	+	+	+	-	-	64	122	15
<u>2/3/92</u>	+	+	+	-	-	800	nd	13
<u>129/3/92</u>	+	+	+	-	-	nd	635	13
5/6/92	+	+	+	-	-	160	160	12
<u>22/6/92</u>	+	+	+	-	-	103	387	14
29/6/92	+	+	+	-	-	290	508	13
30/7/92	-/+	+	+	-	+	338	171	12
24/8/92	-	+	-	-	+	57	106	12

PATIENT DF (M 33, mild CAH /LH) HLA A A3, B7, B51, CW7

	HBV-DNA	HBsAg	HBeAg	anti-HBs	anti-HBe	AST U/L	ALT U/L	bilirubin mmol/L
DATE								
29/10/90	+	+	+	-	-	135	198	12
4/12/90	+	+	+	-	-	96	115	10
31/1/91	+	+	+	-	-	209	369	8
2/1/91	+	+	+	-	-	nd	181	8
30/1/92	+	+	+	-	-	96	122	11
<u>9/3/92</u>	+	+	+	-	-	150	296	8
13/11/92	+	+	+	-	-	88	113	9
1/5/93	-	+	+	-	-	126	246	11

PATIENT AD (M 36 , mild CAH LH, seroconverted following interferon therapy) HLA
A28,A29,B14, B44.

	HBV-DNA	HBsAg	HBeAg	anti-HBs	anti-HBe	AST U/L	ALT U/L	bilirubin mmol/L
DATE								
16/7/90	+	+	+	-	-	58	nd	6
17/12/90	+	+	+	-	-	47	47	47
<u>20/2/91</u>	nd	+	+	-	-	112	155	7
<u>20/5/91</u>	-	+	+	-	-	84	115	10
2/12/91	-	+	+	-	+	57	90	8
<u>24/2/92</u>	+	+	-	-	-	nd	51	4
<u>12/10/92</u>	+/-	+	-	-	+	36	54	7
5/1/93	-	+	-	-	+	nd	38	7
27/9/93	-	+	-	-	+	nd	47	7

PATIENT JH (M , 18 CAH.) HLA A1,A2,B8,B60,CW3,CW7

	HBV-DNA	HBsAg	HBeAg	anti-HBs	anti-HBe	AST U/L	ALT U/L	bilirubin mmol/L
DATE								
/5/92	+	+	+	-	-	95	nd	8
7/6/93	+	+	+	-	-	nd	162	9
16/9/93	+	+	+	-	-	53	134	17
27/10/93	+	+	+	-	-	nd	431	6
<u>29/11/93</u>	+	+	+	-	-	72	224	7

PATIENT NH (M 30 , CAH) HLA , A2, A3, B35, B60, CW3, BW6.

	HBV-DNA	HBsAg	HBeAg	anti-HBs	anti-HBe	AST U/L	ALT U/L	bilirubin mmol/L
DATE								
4/7/91	+		+	-	-	57	34	
	+	+	+	-	-	179	179	6
<u>30/8/91</u>	+	+	+	-	-	64	69	5
14/2/92	+	+	+	-	-	99	107	6
6/11/92	-	+	+	-	-	63	64	9
4/3/93	+	+	+	-	-	nd	80	7
19/7/93	+	+	+	-	-	99	107	16
8/11/93	+	+	+	-	-	89	86	9
22/11/93	+	+	+	-	-	76	71	11
6/12/93		+	+	-				7

PATIENT (DV M 25 CAH, early cirrhosis) HLA ND.

	HBV-DNA	HBsAg	HBeAg	anti-HBs	anti-HBe	AST U/L	ALT U/L	bilirubin mmol/L
DATE								
8/8/90	+		+	-	-	127	nd	18
<u>1/11/90</u>	-	+	+	-	-	108	169	14
7/10/91	-	+	+	-	+	nd	48	14
3/1/92	+	+	+	-	+	nd	70	12
16/3/92	+	+	+	-	+	nd	36	14
3/4/92	-	+	+	-	+	37	48	17
7/5/92		+	+	-	+	35	48	14

PATIENT TC (M 35, CPH,) HLA A3, A30, B14, B50, CW6, BW4, BW6.

	HBV-DNA	HBsAg	HBeAg	anti-HBs	anti-HBe	AST U/L	ALT U/L	bilirubin mmol/L
DATE								
<u>20/5/91</u>	+	+	+	-	-	nd	121	18
11/12/91	+	+	+	-	-	51	105	7
22/6/92	+	+	+	-	-	45	97	13
25/11/92	+	+	+	-	-	35	72	12
19/1/93	+	+	+	-	-	64	142	18
19/4/93	+	+	+	-	-	52	140	15
14/5/93	+	+	+	-	-	51	117	17

PATIENT JB (F, 55, CAH , cirrhosis) Not HLA typed

	HBV-DNA	HBsAg	HBeAg	anti-HBs	anti-HBe	AST U/L	ALT U/L	bilirubin mmol/L
DATE								
18/2/93	+	+	+	-	-	52	77	9
15/3/93	+	+	+	-	-	87	55	13
28/4/93	+	+	+	-	+	55	82	11
23/6/93	+	+	-	-	+	85	149	15
23/8/93	+	+	+	-	+	67	115	15
<u>15/11/93</u>	+	+	-	-	+	27	27	10
29/11/93	+	+	-	-	+	nd	nd	nd

PATIENT AA (M 52, MILD CAH, IgM anti-HAV positive for > 6mths) HLA A3,A24,B18,B511.

TE	HBV-DNA	HBsAg	HBeAg	anti-HBe	anti-HAV IgM	AST U/L	ALT U/L	bilirubin mmol/L
-/12/92	-	+	-	+	+/-	185	279	11
11/1/93	-	+	-	+	+	182	270	10
25/5/93	-	+	-	+	+	139	196	11
25/10/93	-	+	+	+	+	nd	nd	nd
<u>8/2/93</u>	-	+	-	-	+	177	266	10
20/9/93	-	+	-	-	+	nd	118	8
2/11/93	-	+	-	-	+	nd	nd	nd

PATIENT HA (M, 49, CPH/) HLA A2, A3, B7, B35, CW4, CW7.

DATE	HBV-DNA	HBsAg	HBeAg	anti-HBs	anti-HBe	AST U/L	ALT U/L	bilirubin mmol/L
14/1/92	+	-	+	-	-	62	95	11
3/3/92	+	-	+	-	-	62	85	6
9/2/93	+	-	+	-	-	115	189	10
19/4/93	+	-	+	-	-	91	115	8
28/6/93	+	-	-	-	-	102	145	13
<u>6/12/93</u>	+	-	-	-	-	182	218	17

PATIENT KP (32 F, mild CPH/LH) HLA ND.

DATE	HBV-DNA	HBsAg	HBeAg	anti-HBs	anti-HBe	AST U/L	ALT U/L	bilirubin mmol/L
26/10/92	+	-	+	-	-	nd	50	10
22/7/93	+	-	+	-	-	44	64	16
27/9/93	+	-	+	-	-	39	54	21
<u>20.10.93</u>		-	+	-				17

Dates underlined, date at which peripheral blood sample was taken for cytotoxicity assay.

TE HBV, LIVER FUNCTION TESTS AND SEROLOGY AT THE POINT USED IN CYTOTOXICITY ASSAY, OR FOR STIMULATION WITH DC/ PRE-CORE TRANSFECTANTS .

Acute HBV. Clinical data at the time peripheral blood was taken.

PATIENT SAMPLE	HBV-DNA	HBsAg	HBeAg	anti-HBs	anti-HBc IgM	AST U/L	ALT U/L	bilirubin
LC (20)F	nd	+	+	-	+	2.500	nd	nd
CH (22F)	+	+	-	-	+	434	721	31
HT (35 M)	nd	+	+	-	+	3080	nd	128
A.A1 (51M)	+	+	-	-	+	<40	<40	<17
G (35)	nd	-	+	+	+	nd	nd	nd
JG (35 M)	nd	+	+	-	+	3000	nd	nd
AB (F28F)	nd	+	+	-	+	887	588	181
EA (60M)	nd	+	+	-	+	84	88	148
BB (30M)	+	-	-	+	+	28	16	13
PK (M)	nd	-	-	+	(total	<40	<40	<17
ET (25F)	nd	-	-	+	antiHBc +)	<40	<40	<17

* previous history of acute HBV infection, totally resolved. Patient AB subsequently totally resolved infection.

Appendix III

Peptides

1 D I D P Y K E F G A T V E L L	15MER
2 T V E L L S F L P S D F F P S	15MER
3 D F F P S V R D L L D T A S A L	16MER
4 D T A S A L Y R E A L E S P E	15MER
5 L E S P E H C S P H H T A L R	15MER
6 H T A L R Q A I L C W G E L M	15MER
7 W G E L M T L A T W V G V N L	15MER
8 V G V N L E D P A S R D L V V	15MER
9 R D L V V S Y V N T N M G L K	15MER
10 N M G L K F R Q L L W F H I S	15MER
11 W F H I S C L T F G R E T V I	15MER
12 R E T V I E Y L V S F G V W I	15MER
13 M D I D P Y K E F G A T V E L L S F	18MER
14 T V E L L S F L P S D F F P S V	15MER
15 F F P S V R D L L D T A S A L	15MER
16 D T A S A L Y R E A L E S P E	15MER
17 L E S P E H C S P H H T A L R	15MER
18 P H H T A L R Q A I L C W G E L M T L A	20MER
19 E L M T L A T W V G V N L E D P A S R D	20MER
20 D P A S R D L V V S Y V N T N M G L	18MER
21 T N M G L K F R Q L L W F H I S C L	18MER
22 I S C L T F G R E T V I E Y V S F G	19MER
23 V S F G V W I R T P P A Y R P P N A P I L	21MER
PRE-CORE 1-19	
24 M Q F H L C L I I S C T C P T V Q A	18MER
P 18-27	
F L P S D F F P S V	9MER