# Development of Novel Vaccine Strategies for Duck Hepatitis B Virus Infection

A thesis submitted for the degree of

## Doctor of Philosophy

as a portfolio of publications

by


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January, 2008

# Abstract

Hepatitis B virus (HBV) is a life-threatening pathogen with major economic significance. Acute infection in adults is common, albeit usually self-limiting. Importantly, infection in infants typically results in chronic infection and increased incidence of hepatocellular carcinoma (HCC). Furthermore, the infectious carrier state is perpetuated in chronically infected individuals. Successful immuno-therapeutic vaccination would reduce the incidence of chronic infection and of HCC as well as reduce transmission of the disease.

Recovery from acute and chronic HBV infection typically occurs in the presence of robust antigen-specific humoral and cellular immune responses (CMI), whereas these responses are low or absent in chronically HBV-infected individuals. Therefore, it was hypothesised that effective stimulation of both humoral and CMI responses, in conjunction with currently available antiviral therapies, may contribute significantly to development of vaccines for treatment of chronic HBV infection.

The duck hepatitis B virus (DHBV) model of HBV infection was used to test novel vaccine strategies that could complement existing antiviral therapeutic approaches to treat chronically HBV-infected humans. To this end, three separate vaccine studies were conducted to investigate potential therapeutic regimes.

Methods to assess the efficacies of the vaccine strategies included immunoperoxidase detection of viral antigen and immune cell markers within the liver and development of sensitive assays to monitor levels of DHBV DNA, duck hepatitis B virus surface antigen (DHBsAg), antibodies to duck hepatitis B core (anti-DHBc) and surface antigens (anti-DHBs) in serum were developed and validated which allowed monitoring

of the kinetics of the humoral immune response following vaccination and the course and outcome of experimental DHBV infection.

The first vaccine study tested the protective efficacy of DNA vaccines encoding either the small form of DHBsAg (DHBs) protein or the larger antigen (DHBpre-S/S), These were administered to ducks at day 4 and 14 of age. On the same day as the second vaccination, ducks were challenged intravenously with DHBV. Immunoperoxidase staining of biopsy tissue collected at day 4 p.i. showed significant decreases in the number of DHBV infected hepatocytes in ducks receiving the DNA vaccines compared to the mock-vaccinated control ducks. Significant protection against development of chronic DHBV infection was observed in ducks vaccinated with DNA vaccines expressing either pre-S/S or S protein. Although anti-DHBs antibodies were not detected prior to DHBV challenge, the decrease in the percentages of DHBV-infected hepatocytes at day 4 p.i is suggestive that neutralisation of the inoculum by low-level anti-DHBs antibodies in cohort with CMI responses induced by vaccination were the most probable mechanisms of action.

The second vaccine study examined the protective efficacy of a novel whole-cell vaccine that expressed the DHBV core antigen (DHBcAg). Ducks were vaccinated on day 4 and 14 of age and DHBV challenge was administered 4 days later. Detectable anti-DHBc antibodies were generated as soon as 4 days after the initial vaccination suggesting that this regimen elicited increased immunogenicity than vaccination with DNA vaccines alone. In contrast to the first vaccine study with DNA vaccines expressing DHBsAg, no significant differences in the percentage of DHBV-infected hepatocytes were observed in biopsy tissue collected at day 4 p.i. This finding is confirmation that anti-DHBc antibodies were not neutralising to the initial DHBV

inoculum. However, significant protection against development of chronic DHBV infection was observed in the whole-cell vaccinated ducks suggesting that the mechanism of protection was consistent immune-mediated killing of DHBV-infected hepatocytes following CMI responses to determinants of DHBcAg.

The final vaccine study involved a combination strategy of antiviral drug Entecavir (ETV) and prime-boost vaccination with DNA vaccines and recombinant fowlpoxvirus (rFPV) expressing DHBV antigens. Immediately following DHBV infection, ducks were dosed by oral gavage with the antiviral drug Entecavir (ETV) and at the same time received the priming DNA vaccines encoding DHBV antigens. Seven days later the boosting vaccination consisting of recombinant fowlpox viruses (rFPV) also expressing DHBV antigens was administered. Extraordinary protection was observed, with 100% of ducks given combination therapy rapidly resolved their DHBV infection while 100% of non-treated ducks developed chronic infection. It was concluded that protection resulted from a combination of at least three factors. First, reduction and control of DHBV levels with the aid of ETV; secondly, stimulation of surface antigen-specific humoral immune responses resulting in neutralisation of newly produced virions; and finally, the combined up-regulation of CMI responses against DHBV core and surface antigens, resulting in elimination of infected hepatocytes.

The four manuscripts that comprise this thesis provide insights into the viral kinetics and immune responses that follow DHBV infection and/or vaccination of ducks. The results provide new directions for future vaccine studies aimed at developing effective treatments for chronic HBV infection.

# Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Darren Scott Miller

# Acknowledgements

First and foremost I would like to thank my supervisor Dr. Allison Jilbert for your support, guidance and encouragement to embark on a research career. You have taught me many things over the years for which I am forever grateful. My co-supervisors, Professor Christopher Burrell and Professor Ieva Kotlarski; to have you both, with Allison in the same room during lab meetings was sometimes frightening but always inspirational. I also thank Associate Professor Graham Mayrhofer for co-supervision in the later stages of my candidature and also for editing final versions of the immunology sections of the thesis.

I also wish to thank our collaborators who helped to make this work possible. Dr. Michael Halpern for innovative ideas about the whole cell vaccines, Dr. Richard Colonno and Bristol-Myers Squib for generously providing the Entecavir, Dr. David Boyle and Dr. Barbara Coupar for the derivation of recombinant fowl pox viruses and Dr. Bill Mason for critical review of the manuscripts.

Whilst conducting these studies I was supported by a Royal Adelaide Hospital Dawes Scholarship for funding for which I am grateful. I also thank the School of Molecular and Biomedical Science and the Institute of Medical and Veterinary Science (IMVS) for other resources relating to my project.

To all past and present members of the Hepatitis Laboratory and the Discipline of Microbiology and Immunology who made research both interesting and fun, thank you. In particular, I thank Ed Bertram for his input with the manuscript in Chapter 2 of this thesis and Cathy Scougall for re-counting hundreds of stained slides and editing the final version of the thesis.

Finally, an extra special thanks to Georget Reaiche for understanding, love and support during the final stages of my candidature and also my daughters Jacqui and Elise for the real pleasures in life.

# Publications and patent arising

## Publications

I. Darren S. Miller, Edward M. Bertram, Catherine A. Scougall, Ieva Kotlarski, and Allison R. Jilbert. Studying host immune responses against duck hepatitis B virus infection. *Methods Mol Med* 2004 96:3-26.

II. Darren S. Miller, Ieva Kotlarski and Allison R. Jilbert. Vaccination of ducks with a whole-cell vaccine expressing duck hepatitis B virus core antigen elicits antiviral immune responses that enable rapid resolution of *de novo* infection. *Virology* 2006 348:297-308.

III. Darren S. Miller, Michael Halpern, Ieva Kotlarski and Allison R. Jilbert. DNA vaccines expressing the duck hepatitis B virus surface proteins lead to reduced numbers of infected hepatocytes and protect ducks against the development of chronic infection in a virus dose-dependent manner. *Virology* 2006 351:159-69.

IV. Darren S. Miller, David Boyle, Feng Feng, Georget Y. Reaiche, Ieva Kotlarski, Richard Colonno and Allison R. Jilbert. Antiviral therapy with Entecavir combined with post-exposure "prime-boost" vaccination eliminates duck hepatitis B virus infected hepatocytes and prevents the development of persistent infection. *Virology*, 2008 373:329-341.

## Patent

I. Miller D. S., Kotlarski I., Burrell C. J., and Jilbert A. R. **"Combination Treatment". *PCT/AU2006/000828***

# Conference presentations and abstracts arising

Miller D. S., Kotlarski I., Jilbert A. R. Development of a novel whole-cell DNA vaccine for duck hepatitis B virus (DHBV) infection. 2004 Australian Centre for Hepatitis Virology & HIV Virology, National Scientific Workshop, Barossa Valley 25th-27th June, 2004

Miller D. S., Kotlarski I., Jilbert A. R. Development of a novel whole-cell-DNA vaccine for duck hepatitis B virus (DHBV) infection: The Molecular Biology of Hepatitis B Viruses, Marine Biological Laboratory Woods Hole, Massachusetts USA. 24-27th October, 2004.

Miller D. S., Kotlarski I., Jilbert A. R. Development of a novel whole-cell-DNA vaccine for duck hepatitis B virus (DHBV) infection. Australian Society of Immunology: Student Meeting, Adelaide, South Australia. October, 2004.[*1]

Miller D. S., Kotlarski I., Jilbert A. R. Development of a novel whole-cell-DNA vaccine for duck hepatitis B virus (DHBV) infection. The Australian Society for Medical Research, South Australian Annual Meeting. June, 2005.[*2]

Miller D. S., Kotlarski I., Colonno R., Boyle D., Jilbert A. R. Entecavir and prime-boost vaccination strategies for hepatitis B virus infection. The Molecular Biology of Hepatitis B Viruses. University of Heidelberg, Germany. September, 2005.

Miller D. S., Kotlarski I., Colonno R., Boyle D., Jilbert A. R. Entecavir and prime-boost vaccination strategies for hepatitis B virus infection. Poster presentation. 3rd Australian Virology Group Meeting, Phillip Island, Australia. December, 2005.[*3]

Miller D. S., Kotlarski I., Colonno R., Boyle D., Jilbert A. R. Entecavir and prime-boost vaccination strategies for hepatitis B virus infection. Australian Society of Immunology, Student meeting. Adelaide, December, 2005.[*4]

Miller D. S., Kotlarski I., Colonno R., Boyle D., Jilbert A. R Entecavir and prime-boost vaccination strategies for hepatitis B virus infection. 1st Australian Vaccines & Immunotherapeutics Development, Melbourne, Australia. May, 2006.

# Awards arising

*[1] Best Oral Presentation. Australian Society of Immunology: Student Meeting, Adelaide, South Australia. October, 2004

*[2] Best Oral Presentation. Student Biotechnology Award. The Australian Society for Medical Research, South Australian Meeting. June, 2005.

*[3] Best Poster Presentation. 3rd Australian Virology Group Meeting, Phillip Island, Australia. December, 2005.

*[4] Runner-up, Best Student Oral Presentation. Australian Society of Immunology, Student meeting. December, 2005.

# Chapter 1: Introduction

## 1.1 Hepatitis B Virus

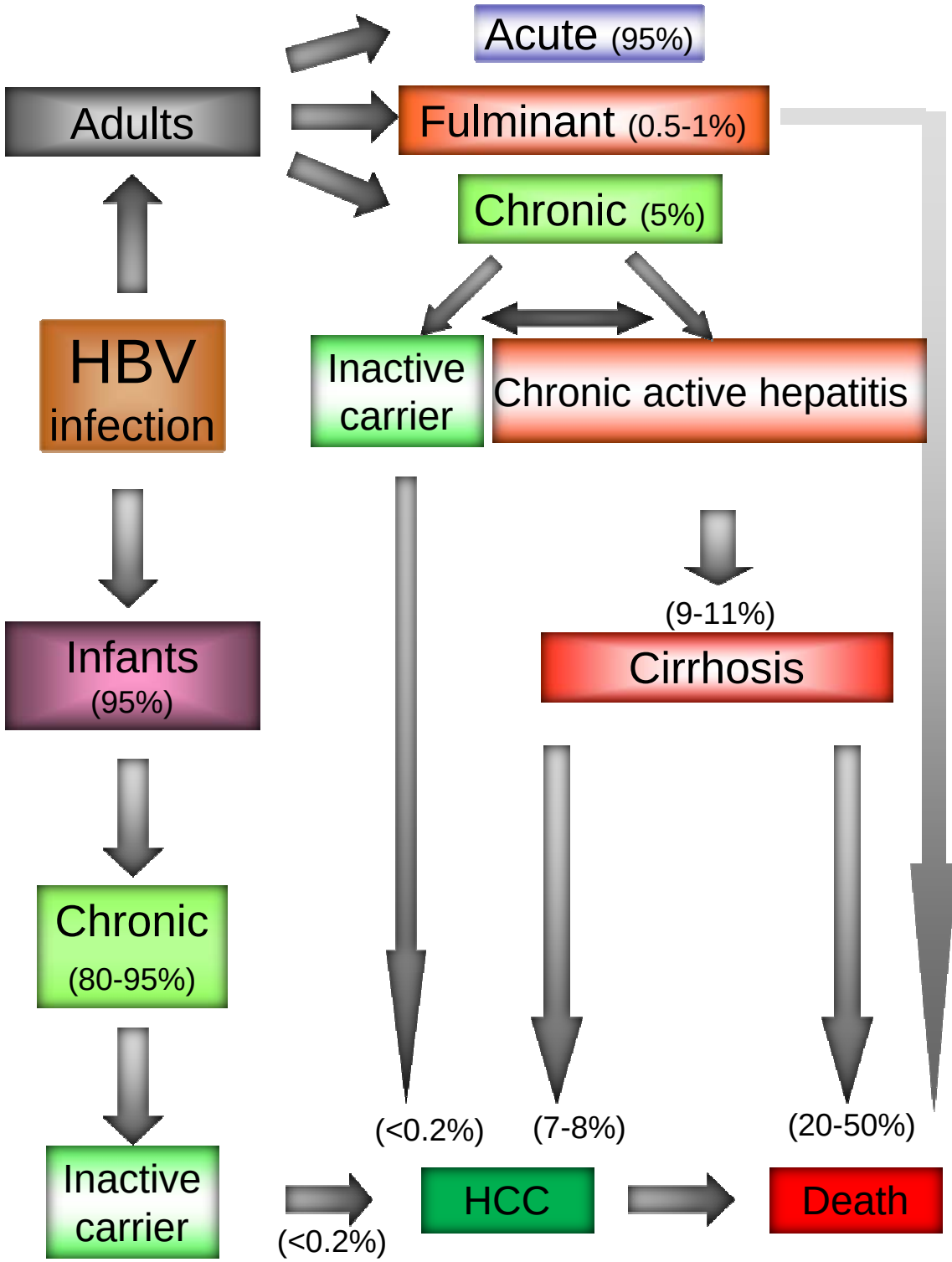
Hepatitis B virus (HBV) is the prototype member of the family Hepadnaviridae. It has been estimated that more than 2000 million individuals alive today have been exposed to HBV. Of these, approximately 350 million people remain chronically infected, which in many cases results in chronic liver disease, cirrhosis, or progression to hepatocellular carcinoma (HCC) (Lavanchy 2004; Hoofnagle 2006). As will be discussed in more detail below, exposure to HBV results in transient infection in 90-95% of non-vaccinated immune-competent adults. In contrast, 30% of children infected under five years of age develop chronic HBV infections, and this number increases to 90-95% of unvaccinated infants exposed to HBV during childbirth (Bertoletti and Ferrari 2003) (Figure 1).

HBV particles were first discovered in the sera of Australian aborigines in 1965 (Blumberg, Alter *et al.* 1965). The finding that blood containing these particles could transmit hepatitis with the appearance of similar particles in the bloodstream of infected individuals, positively identified the infectious agent of hepatitis B. Further research by Dane and his colleagues provided a detailed description of the infectious agent now known as HBV (Dane, Cameron *et al.* 1970). HBV virions are 42nm in diameter and circulate in the bloodstream at titres as high as $10^{10}$/ml (Whalley, Murray *et al.* 2001). Non-infectious "empty" particles composed only of viral surface proteins are also produced (Figure 2). These are observed as 22nm pleomorphic spheres or filaments and can be present in ~500-fold excess relative to the numbers of infectious particles (Sakamoto, Yamada *et al.* 1983; Hourioux, Touze *et al.* 2000; Huang, Lin *et al.* 2006). All hepadnaviruses are enveloped and share a similar virion structure consisting of a

**Figure 1. Outcomes of HBV infection.**

Following HBV exposure, acute infection is observed in 95% of non-vaccinated, immuno-competent adults but only 5% of exposed neonates. Approximately 1% of these acutely infected individuals die from fulminant hepatitis. The remaining 5% of adults and 95% of neonates develop chronic HBV infection.

Chronic HBV infection in most neonates, and some adults, results in an inactive carrier state associated with minimal disease. Less than 0.2% of these individuals develop HCC. In contrast, for cases where chronic active hepatitis arises, cirrhosis (11%) and HCC (8%) are observed (Buendia 1998; Yim and Lok 2006).



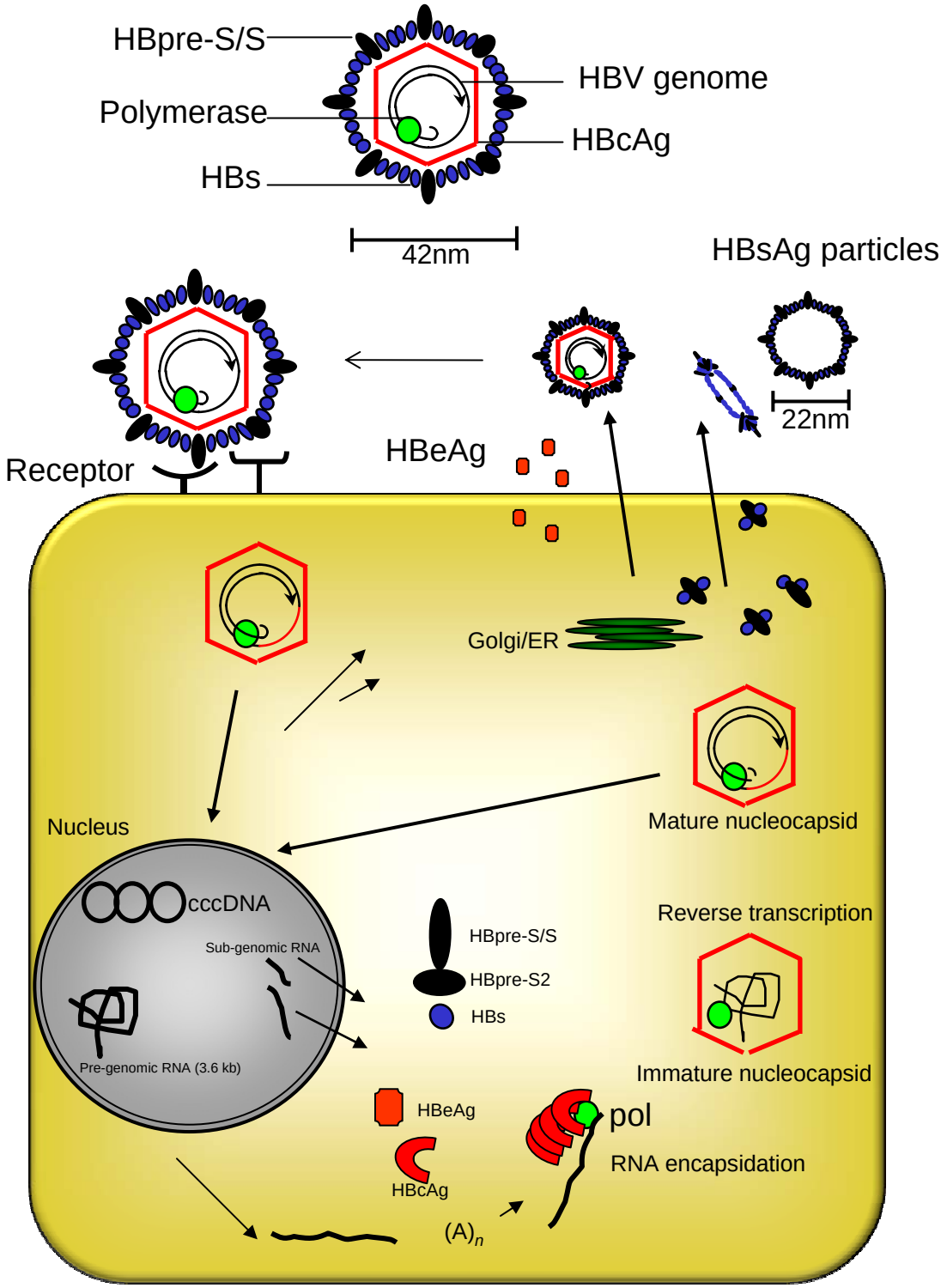

Adapted from Buendia, 1998; Yim, 2006

**Figure 2. HBV morphology and life-cycle**.

HBV virions, found in the blood of infected individuals, are 42nm in diameter. They contain a partially double-stranded DNA genome, covalently attached to the viral polymerase, which is encapsidated within the HBcAg (Galibert, Mandart et al. 1979). The envelope of the virion is comprised of HBsAg and HBpre-S/S antigens and host-cell derived lipid. Non-infectious 22nm pleomorphic particles composed only of viral surface proteins can be also be found circulating in the blood in ~500-fold excess over the number of genome containing infectious virions (Whalley, Murray et al. 2001).

Attachment of virions to the hepatocyte surface is followed by receptor mediated endocytosis (Treichel, Meyer zum Buschenfelde et al. 1997). The virions are uncoated, releasing the viral DNA-containing nucleocapsid into the cytoplasm. The partially double stranded genome is then released into the nucleus and converted to cccDNA, which is the template for HBV mRNA expression (Summers and Mason 1982; Spangenberg, Thimme et al. 2004). Transcription of the HBV RNA produces 5 distinct viral mRNA species, which encode for the pol, HBcAg, HBe, HBpre-S1, HBpre-S2, HBs and HBx proteins (Locarnini 2004).

In the cytoplasm of the infected hepatocyte, the polymerase protein binds to the encapsidation signal on the pre-genomic RNA molecule. The resultant poly-RNA complex is then encapsidated by dimers of HBcAg. Within capsids, virally encoded reverse transcription occurs to synthesise the viral genome (Summers and Mason 1982). These nucleocapsids containing the partially double-stranded genome are either recycled to the nucleus to expand the pool of cccDNA, or enveloped with HBV surface antigens plus host-derived lipid and released from the hepatocyte (Tuttleman, Pourcel et al. 1986; Spangenberg, Thimme et al. 2004).


HBpre-S/S
Polymerase
HBs
HBV genome
HBcAg
42nm
HBsAg particles
22nm
Receptor
HBeAg
Golgi/ER
Mature nucleocapsid
Nucleus
cccDNA
Sub-genomic RNA
Pre-genomic RNA (3.6 kb)
HBpre-S/S
HBpre-S2
HBs
Reverse transcription
Immature nucleocapsid
HBeAg
HBcAg
pol
RNA encapsidation
(A)n

Hepatocyte

partially double-stranded DNA genome ranging in size from 3.0-3.3kb. The virus replicates through an RNA intermediate using the reverse transcriptase activity of the virally encoded polymerase (pol) (Summers and Mason 1982; Nassal and Schaller 1993; Locarnini 2004; Locarnini 2005).

### 1.1.1 HBV genome organisation

The HBV genome is a partially double-stranded DNA molecule approximately 3200 base pairs in length (Galibert, Mandart *et al.* 1979). The positive strand of the genome is incomplete. The molecule is held in a circular configuration by cohesive overlaps of the 5' ends of the positive and negative strands. The 5' end of the negative strand is covalently attached to the viral pol (Tuttleman, Pourcel *et al.* 1986; Newbold, Xin *et al.* 1995) (Figure 3A). Two direct repeat sequences, DR1 and DR2, consist of 11 nt and are involved in HBV replication. DR1 is responsible for strand transfer of the pol, thus allowing synthesis of the negative DNA strand. DR2 is located on the negative DNA strand and it is the initiation site of positive strand DNA synthesis (Nassal and Schaller 1996) (Figure 3A).

HBV genomes contain four distinct overlapping open reading frames (ORF) that codes for seven proteins named pol, HBpre-S/S, HBpre-S2, HBsAg, HBcore, HBpre-C/C and HBx proteins (Galibert, Mandart *et al.* 1979) (Figure 3A). The pol gene encodes for the viral polymerase responsible for synthesis of the positive and negative strands of viral DNA. The HBpre-S1 gene contains three internal translation initiation codons and therefore encodes three different proteins. The HBpre-S1 gene encodes the largest of the viral surface proteins, HBpre-S/S. The HBpre-S2 ORF encodes the HBpre-S2 protein, and the HBs reading frame encodes the smallest of the viral surface antigens, HBsAg (Kann, Lu *et al.* 1995) (Figure 3A). These viral surface proteins are thought to be the

ligands required for viral attachment to specific cellular receptors, leading to infection. These HBV surface antigen particles are produced in excess of infectious virions and can be readily detected in the sera of infected individuals (Bocher, Herzog-Hauff *et al.* 1999; Bertoletti and Naoumov 2003).

The HBc gene encodes two different proteins. The first is HBcAg which is involved in the encapsidation of the viral pre-genome. The second, secreted form of core protein is often referred to as HBe or eAg. The role of HBe has not been defined, but it is also thought to act as an "immune decoy" (Kann, Lu *et al.* 1995; Nassal and Schaller 1996; Locarnini 2004). Finally, the HBx gene in mammalian hepadnaviruses encodes the HBx protein (Figure 3A). A definitive role for HBx has not been clearly defined, but trans-activation properties have been described (Arbuthnot, Capovilla *et al.* 2000). Additionally, humans, woodchucks and transgenic mice expressing HBx have an increased susceptibility to developing HCC whereas avian species which lack the x gene do not (Chisari, Klopchin *et al.* 1989; Tennant, Toshkov *et al.* 2004) suggesting that the HBx protein plays a role in the development of liver cancer. The pre-core/core region of the HBc gene also contains a U5-like retrovirus long terminal repeat, which has been reported to enhance *in vitro* recombination in the presence of extracts from actively dividing cells. This repeat is also thought to increase the probability of carcinogenesis (Kajino, Hotta *et al.* 1994).

Two enhancer elements encoded within the HBV genome are responsible for the tight control and regulation of transcription of the HBV RNA species (Nassal and Schaller 1996; Locarnini 2004) (Figure 3A). Additional control of transcription is provided by the glucocorticoid responsive element (GRE), which recognises a specific nucleotide

sequence in HBV DNA, thereby causing increased activity by the HBV enhancers (Figure 3A) (Tur-Kaspa, Shaul *et al.* 1988).

### 1.1.2 The HBV Receptor

The cellular receptors by which HBV binds to cells have not been characterised, although several putative HBV receptors have been described in the literature. Polymerised human albumin was proposed in 1985 (Okamoto, Imai *et al.* 1985), while fibronectin, (Budkowska, Bedossa *et al.* 1995), the transferrin receptor (Franco, Paroli *et al.* 1992) and human glyceraldehyde-3-phosphate dehydrogenase (Duclos-Vallee, Capel *et al.* 1998) have all been shown to bind to HBV via HBpre-S/S protein. Molecules that bind to HBs include asialoglycoprotein and Annexin V (Treichel, Meyer zum Buschenfelde *et al.* 1994). Controversially, it has been demonstrated that transfection of HBV-refractive rat hepatotoma cell lines with plasmids that express the Annexin V gene allows productive infection with HBV (Hertogs, Leenders *et al.* 1993; Hertogs, Depla *et al.* 1994; Gong, De Meyer *et al.* 1999). However, the role of Annexin V in the binding of HBV to normal cells remains to be established.

### 1.1.3 The life cycle of hepadnaviruses

Hepadnaviruses are primarily hepatotrophic, although extrahepatic sites of replication such as the pancreas, kidney, spleen and macrophages have been described (Halpern, England *et al.* 1983; Halpern, England *et al.* 1984; Lieberman, Tung *et al.* 1987; Korba, Gowans *et al.* 1988; Ogston, Schechter *et al.* 1989; Hosoda, Omata *et al.* 1990; Pontisso, Morsica *et al.* 1991; Walter, Teubner *et al.* 1991; Chen, Chen *et al.* 1992; Jilbert, Wu *et al.* 1992; Lanford, Michaels *et al.* 1995; Meier, Scougall *et al.* 2003; Umeda, Marusawa *et al.* 2005). As previously discussed, cellular attachment of virions requires binding between specific receptors on the hepatocyte surface to large and/or

**Figure 3A. HBV genome organisation.**

HBV contains four distinct overlapping ORF that encode for seven viral proteins; pol, HBpre-S/S, HBpre-S2, HBsAg, HBcore, HBpre-C/C and HBx. The pol gene encodes the viral polymerase responsible for synthesis of the positive and negative strands of viral DNA. The HBpre-S/S gene contains three internal translation initiation codons, producing three different proteins. The HBpre-S/S gene encodes the largest of the viral surface proteins, HBpre-S1. The HBpre-S2 open reading frame encodes HBpreS-2, while the HBs reading frame encodes the smallest of the viral surface antigens, HBsAg . The HBc gene encodes two different proteins: HBcAg is involved in the encapsidation of the viral pre-genome, the second is the secreted form of the core, HBe. The HBx gene in mammalian hepadnaviruses encodes HBx (Locarnini 2004).

**Figure 3B. DHBV genome organisation.**

The genome consists of four distinct overlapping ORF that encode for five separate proteins. The pol gene encodes the polymerase protein. The DHB S gene encodes the DHBpres-S/S and DHBsAg, the later of which is initiated by an internal translation initiation codon within the ORF (Mason, Seal et al. 1980). The core gene also contains an internal translation initiation codon and hence encodes the viral nucleocapsid, DHBcAg and the secreted, DHBe (Triyatni, Ey et al. 2001). It is controversial whether the X gene exists in DHBV (Chang, Netter et al. 2001; Meier, Scougall et al. 2003).

NOTE: This figure is included in the print copy of the thesis held in the University of Adelaide Library

(Adapted from http//www.gsbs.utmb.edu/microbook/ch047.htm)

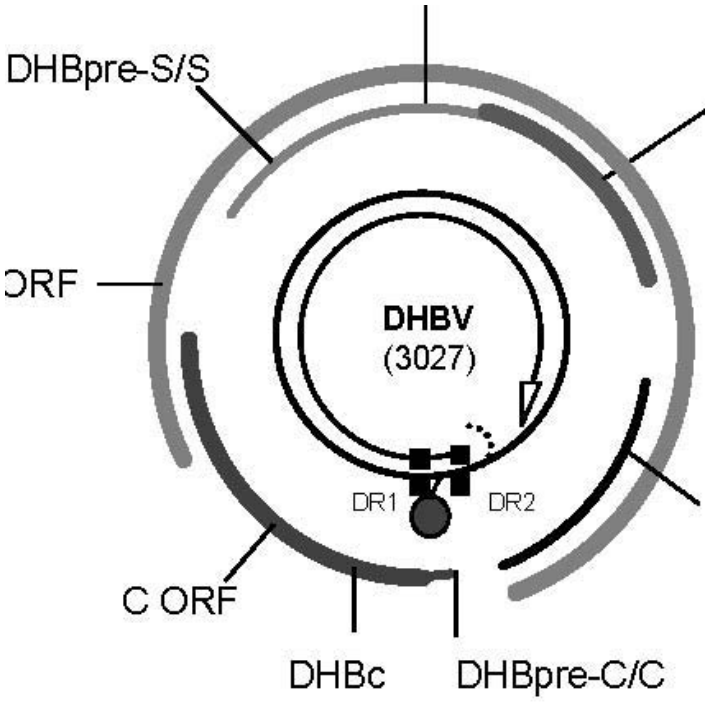
SORF

B)

DHBs

P ORF

X-like ORF




(Adapted from Jilbert and Kotlarski, 2000)

small forms of the viral surface antigens (Figure 2). Binding is followed by internalisation via receptor mediated endocytosis (Treichel, Meyer zum Buschenfelde *et al.* 1997). The surface proteins of the virus are removed and the viral DNA-containing nucleocapsid is transported to the nuclear membrane (Guo, Jiang *et al.* 2007) (Figure 2). Fusion with the nuclear membrane results in release of the partially double-stranded genome into the nucleus and conversion (by either host-derived or viral polymerases) to covalently closed circular DNA (cccDNA) (Summers and Mason 1982; Spangenberg, Thimme *et al.* 2004) (Figure 2). CccDNA is the nuclear template for the synthesis of the viral RNA species that code for the viral antigens (Figure 2). Infected cells contain between 10 and 50 copies of cccDNA, which are highly stable and long-lived (Jun-Bin, Zhi *et al.* 2003; Singh, Dicaire *et al.* 2004; Wong, Yuen *et al.* 2004). Therefore, elimination of cells containing cccDNA is a requirement for successful treatment of chronically infected individuals. Unfortunately, antiviral drug treatment alone has not been found to significantly reduce the pool of intra-nuclear cccDNA (Zoulim 2006).

Transcription of HBV RNA occurs in the nucleus of infected cells via host-derived RNA polymerase II (Nishizono, Maeno *et al.* 1991). Five distinct viral mRNA species are produced. The pregenome transcript is a greater than genome length bi-cistronic mRNA that encodes for the polymerase and HBcAg as well as being the template for synthesis of the viral DNA genome. HBeAg is also encoded by the greater than genome length precore mRNA. Distinct subgenomic length mRNA species encode for HBpre-S1, HBpre-S2, HBsAg and the HBx protein (Nassal and Schaller 1996; Locarnini 2004) (Figure 2).

In the cytoplasm of the infected hepatocyte an encapsidation signal, epsilon (ε), on the pre-genomic RNA molecule folds to form a stem loop structure (Kidd and Kidd-

Ljunggren 1996). The pol protein binds to the ε/stem-loop structure and this pol-RNA complex is encapsidated by HBc dimers to form an immature nucleocapsid (Nassal and Schaller 1996; Locarnini 2004) (Figure 2). Priming for DNA synthesis occurs following interaction with the bulge region of ε as a template and the hydroxyl group of a tyrosine residue located at the amino terminus of pol. This priming is also unique in that the first three or four nucleotides are template directed, using a stem-loop structure within the pol mRNA. The resulting primer subsequently translocates to another portion of the genome to initiate full-length first-strand DNA synthesis. RNase H activity degrades the template RNA to a terminal segment of ~20 nucleotides, which itself is translocated to another region of homology to serve as the primer for second-strand DNA synthesis. This polymerase activity occurs within a cytoplasmic nucleocapsid particle assembled from HBV core proteins, into which pol directs the inclusion of itself and its template. The final product is a partially double-stranded gapped DNA which is released in mature virions (Locarnini 2004; Guo, Jiang *et al.* 2007).

Within the capsid, virally encoded polymerase begins reverse transcription of the negative and positive DNA strands of the virus. These partially double-stranded genome-containing nucleocapsids are either recycled to the nucleus to expand the pool of cccDNA or are enveloped with HBV surface antigens and host-derived lipid (Locarnini 2004). The encapsidated virus is then released from the hepatocyte (Figure 2).

### 1.1.4 HBV transmission and clinical outcomes

HBV is highly infectious and is readily transmitted by various routes, including blood transfusion, needle-stick injury, intravenous drug use, contaminated medical and dental instruments, sexual contact or exchange of body fluids. The most common means of

transmission is perinatal infection, occurring during birth or the immediate post-natal period (Bertoletti and Naoumov 2003; Hoofnagle 2006). As discussed above, infection with HBV usually results in one of two outcomes. In 95% of immuno-competent individuals an acute infection is observed. The remaining 5% of adults and 95% of neonates develop a chronic infection (Buendia 1998; Bertoletti and Ferrari 2003; Bertoletti and Naoumov 2003; Hoofnagle 2006; Yim and Lok 2006) (Figure 1). The hallmarks of HBV infection are the presence of serum HBV DNA, HBsAg, HBe protein and flares of liver-derived aminotransferases, in particular alanine aminotransferase (ALT), a marker of hepatocyte death (Buendia 1998; Bertoletti and Ferrari 2003) (Figure 4A).

## 1.2 Patterns of HBV Infection

### 1.2.1 Acute HBV infection

Recovery from acute HBV infection occurs in approximately 95% of adults (Figure 1). HBV DNA, HBe protein and HBsAg are detectable in the serum of infected individuals between 21 and 40 days after infection, and are followed by an increase in the levels of serum transaminase. HBsAg and HBV DNA titres then fall below detectable levels and transaminase levels return to normal. The most reliable marker of resolution of HBV infection is the presence of neutralising anti-HBs antibodies, which confer lifelong protection against re-infection (Bertoletti and Ferrari 2003). Anti-HBc antibodies (anti-HBcore) are also detected shortly after infection and persist for many years following acute and chronic infection (Figure 4A). Acute infection is asymptomatic in many cases, but jaundice, myalgia, fever and nausea can occur. Less than 1% of patients acutely infected with HBV die from fulminant hepatitis (Buendia 1998; Bertoletti and Naoumov 2003; Hoofnagle 2006; Yim and Lok 2006) (Figure 1).

### 1.2.2 Chronic HBV infection

Chronic infection is characterised by the presence of HBV DNA and HBsAg in the blood for a period of more than six months (Bertoletti and Ferrari 2003) (Figure 4 B).

As further discussed in section 1.10, individuals who develop chronic HBV infection, either as neonates or adults, generally have low or undetectable T-cell responses to HBV antigens by *in vitro* assays (Penna, Chisari *et al.* 1991; Nayersina, Fowler *et al.* 1993; Rehermann, Fowler *et al.* 1995; Bertoletti and Ferrari 2003; Thimme, Wieland *et al.* 2003; Hoofnagle 2006). However, they appear to have sufficient reactivity *in vivo* to cause progressive liver damage. Chronic HBV infection is usually delineated into four stages, namely (1) immune-tolerant, (2) immune clearance, (3) inactive carrier state, and (4) reactivation. Not all patients experience all of the phases (Yim and Lok 2006) (Figure 1).

Immune-tolerance (Stage 1) occurs most commonly in individuals infected as neonates. Conversely, those who are infected during adulthood are rarely HBV immune-tolerant. This phase is associated with high levels of HBV DNA and HBsAg in serum, without immune-mediated flares in serum transaminase levels and with normal liver histology on biopsy (Figure 4B). Cirrhosis and HCC are uncommon during this phase (Chu, Karayiannis *et al.* 1985) (Figure 1). This clinical outcome is presumed to be due to clonal deletion of HBV-specific T-cells during early development and hence the absence of immune-mediated hepatocyte killing.

Immune clearance (Stage 2), refers to the transition from HBe-positive to HBe-negative status with detectable levels of anti-HBe antibodies in the serum (Figure 4B). Fluctuating HBV DNA levels are commonly observed, but sustained reductions of viral DNA rarely occur. During the immune clearance stage, there are elevations of serum

transaminases and enhanced T-cell responses against HBcAg and HBe can be measured (Tsai, Chen *et al.* 1992). These flares of immune-reactivity correlate well with an increased incidence of cirrhosis and HCC (Chu, Karayiannis *et al.* 1985) (Figure 1).

Inactive carriers (Stage 3), are HBV-infected patients who, over the long-term are HBV DNA, HBe and HBsAg positive, anti-HBe negative, but have stable low levels of transaminase (Figure 4B) and whose liver biopsies show only slight inflammation. This stage can persist for the duration of the individual's life, but can also spontaneously erupt into Stage 2 of chronic HBV infection, often following liver transplantation or during immunosuppression (Buendia 1998; Lavanchy 2004; Hoofnagle 2006; Boni, Fisicaro *et al.* 2007).

Re-activation (Stage 4) of HBV infection is observed where a chronically-infected individual is HBV DNA positive, HBe negative and positive for anti-HBe antibodies (Figure 4B), with necro-inflammatory changes in the liver. The individuals in Stage 4 with re-activated disease are typically older. It has been proposed that long-term immune pressure in these carriers, due to circulating anti-HBe antibodies, selects for HBe antigen negative mutants. Sequence analysis of HBV genomes found in the serum of these patients has shown that mutation of a single nucleotide (nt) base encoding a premature stop within the HBe gene, or in the HBpre-core promotor region, stops HBe being produced (Okamoto, Tsuda *et al.* 1994).

As discussed above, the most reliable marker of spontaneous resolution of HBV infection is the appearance of anti-HBs antibodies in blood. Sero-conversion to anti-HBs usually coincides with loss of HBV DNA and HBsAg in serum. This is estimated to occur in approximately 1% of HBV carriers each year and is associated with

improved liver histology and a decreased risk of HCC (Manno, Camma *et al.* 2004). This observation provides a strong theoretical basis for attempts to increase the rate of resolution of chronic HBV infection by therapeutic vaccination.

## 1.3 Overview of animal models of hepadnavirus infection

Since the initial discovery of HBV numerous other members of the hepadnavirus family have been identified. These can be divided into two distinct host-specific genera. The orthohepadnaviruses that infect mammalian species, including chimpanzees (HBV) woodchucks (WHV), woolly monkeys (WMHV), arctic squirrels (ASHV) and ground squirrels (GSHV) (Summers, Smolec *et al.* 1978; Marion, Oshiro *et al.* 1980; Testut, Renard *et al.* 1996; Lanford, Chavez *et al.* 1998) and the avihepadnaviruses that infect avian species, including ducks (DHBV), herons (HHBV), Ross' goose (RGHV), snow goose (SGHV) and white stork (STHBV); (Mason, Seal *et al.* 1980; Sprengel, Kaleta *et al.* 1988; Chang, Netter *et al.* 1999; Pult, Netter *et al.* 2001). Other related avihepadnaviruses have also been identified in wild mallard ducks and exotic anseriformes maintained in captivity (Lambert, Cova *et al.* 1991; Guo, Mason *et al.* 2005).

Some studies have been performed using chimpanzees but the high costs and ethical issues associated with using higher primates prohibits their use (Pancholi, Lee *et al.* 2001; Prince 2001; Thimme, Wieland *et al.* 2003; Gagneux and Muchmore 2004; Murray, Wieland *et al.* 2005; Komiya, Katayama *et al.* 2007). WHV infection in woodchucks has also been studied extensively (Summers, Smolec *et al.* 1978; Robinson 1980; Wong, Shih *et al.* 1982; Lindberg, Pichoud *et al.* 1985; Roos, Fuchs *et al.* 1989; Schodel, Neckermann *et al.* 1993; Mason, Cullen *et al.* 1998; Lu, Hilken *et al.* 1999; Guo, Zhou *et al.* 2000; Colonno, Genovesi *et al.* 2001; Siegel, Lu *et al.* 2001; Churchill

and Michalak 2004; Tennant, Toshkov *et al.* 2004; Lu, Isogawa *et al.* 2005). Transgenic mouse models have also been developed (Akbar and Onji 1998; Guidotti, Morris *et al.* 2002; Chen, Sun *et al.* 2007). In these mice integrated HBV transgenes encoding HBV antigens mimic HBV infection. However, this model does not accurately depict true HBV infection as covalently closed circular DNA (cccDNA) is not formed and the transgene is present in all cells of the mouse.

### 1.3.1 DHBV

Several groups have also used the duck model to study hepadnaviral infection (Summers and Mason 1982; Halpern, England *et al.* 1983; Halpern, England *et al.* 1984; Tuttleman, Pourcel *et al.* 1986; Marion, Cullen *et al.* 1987; Schlicht, Salfeld *et al.* 1987; Hosoda, Omata *et al.* 1990; Lambert, Cova *et al.* 1991; Jilbert, Wu *et al.* 1992; Newbold, Xin *et al.* 1995; Chang, Netter *et al.* 2001; Havert, Ji *et al.* 2002; Marion, Salazar *et al.* 2002; Foster, Miller *et al.* 2003; Schultz, Grgacic *et al.* 2004; Urban 2004; Foster, Miller *et al.* 2005). Within Australia, ducks are the only animal model available to study hepadnavirus infection. As discussed in more detail below, both HBV and DHBV share similar genomic and antigenic structures. Moreover, acute and/or chronic outcomes of infection are observed in both host-species. The duck model therefore, provides a good model for studying vaccines and developing protocols aimed at controlling HBV infection in humans.

### 1.3.2 DHBV virion and genome organisation

DHBV virions are 40nm in size and different strains contain a partially double-stranded DNA genome ranging from 3021-3027 nt. (Mason, Seal *et al.* 1980; Triyatni, Ey *et al.* 2001). The DHBV genome consists of four distinct ORF that encode five separate proteins (Jilbert and Kotlarski 2000; Triyatni, Ey *et al.* 2001; Schultz, Grgacic *et al.*

**Figure 4A. Acute HBV infection.**

HBV DNA, HBsAg and HBe are detected in the serum between 21 and 40 days after infection and are accompanied by increases in serum transaminase levels. Recovery from HBV infection results in falls in levels of HBsAg and HBV DNA to below detectable and transaminase levels return to normal. The appearance of anti-HBs antibodies confers lifelong protection against re-infection. Anti-HBc antibodies are also detected shortly following acute infection and persist for many years (Buendia 1998; Bertoletti and Naoumov 2003; Lavanchy 2004; Hoofnagle 2006).

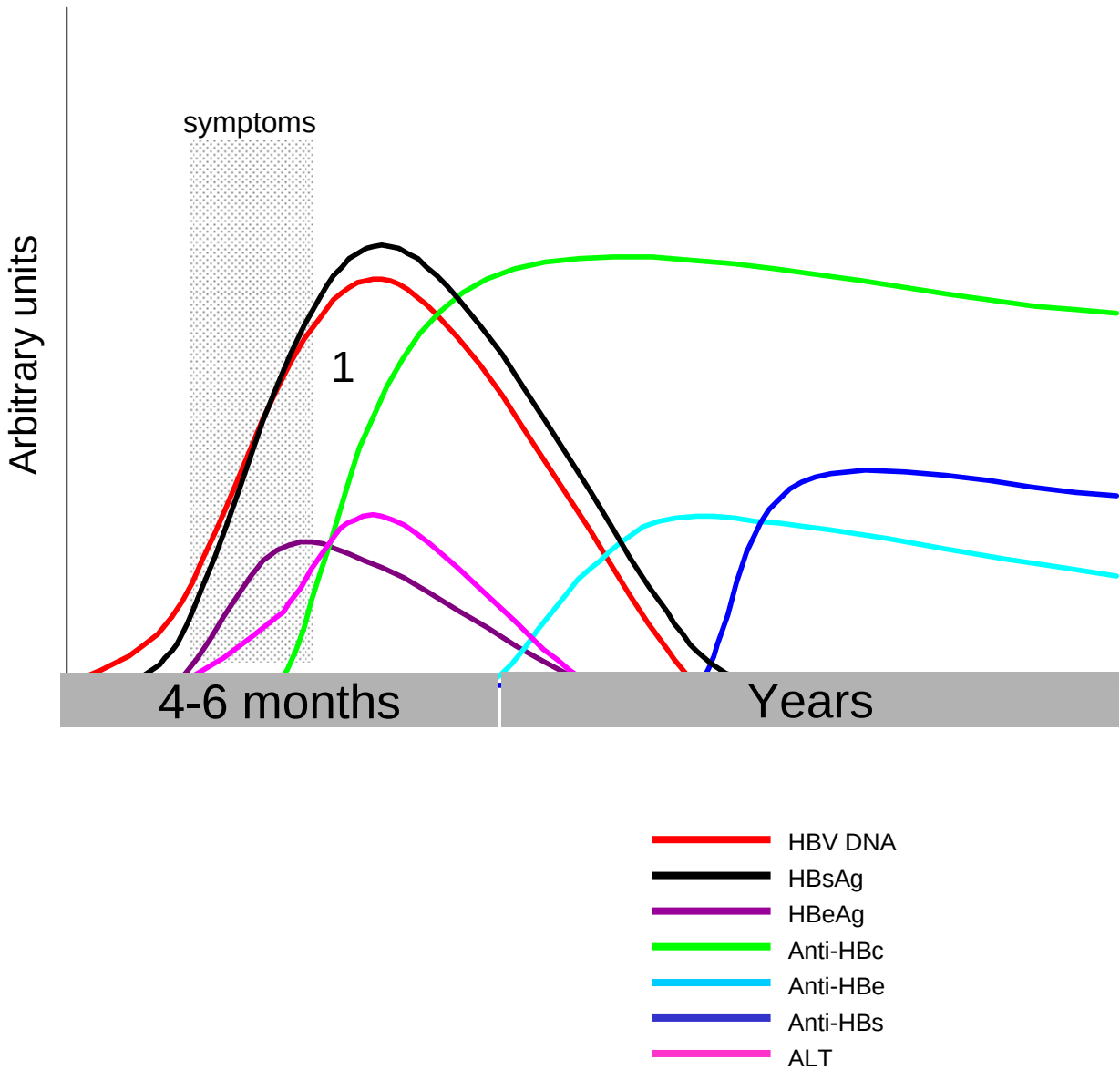

symptoms
Arbitrary units
1
4-6 months
Years
HBV DNA
HBsAg
HBeAg
Anti-HBc
Anti-HBe
Anti-HBs
ALT

**Figure 4B. The four stages of chronic HBV infection.**

**Stage 1. HBV immune-tolerance**

Immune-tolerance is most commonly observed in individuals infected as neonates. High-levels of HBV DNA and HBsAg are found in serum. Levels of serum transaminase and liver biopsy histology generally remain normal (Buendia 1998; Bertoletti and Naoumov 2003; Lavanchy 2004; Hoofnagle 2006).

**Stage 2. Immune clearance.**

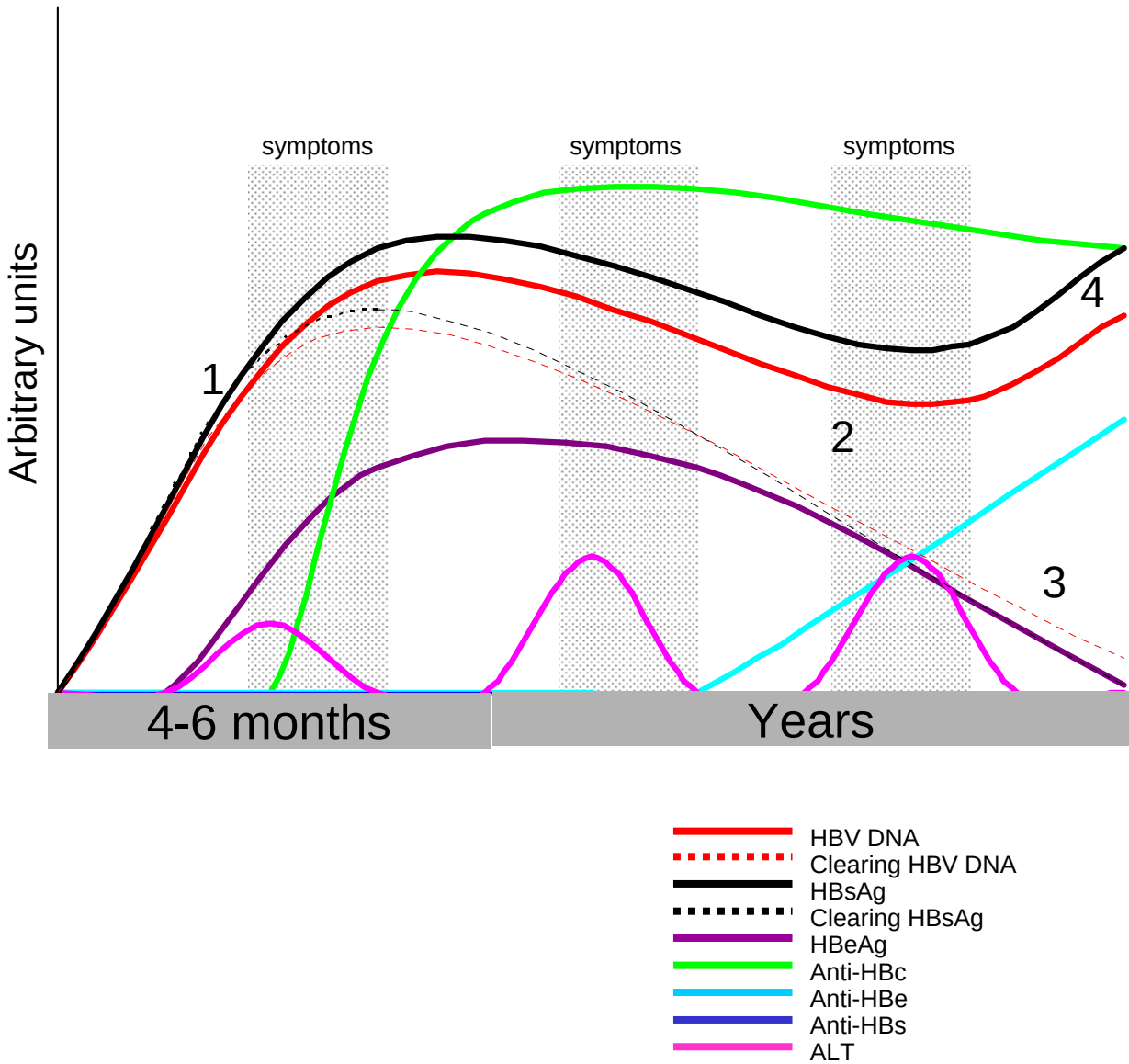
The immune clearance stage is associated with transition from HBe positive to HBe negative, and seroconversion to anti-HBe antibodies. Fluctuating serum transaminase and HBV DNA levels and immunoreactivity are also often observed but rarely result in sustained reductions in HBV DNA (Buendia 1998; Bertoletti and Naoumov 2003; Lavanchy 2004; Hoofnagle 2006).

**Stage 3. Inactive carrier.**

These individuals are HBV DNA, HBe and HBsAg positive, anti-HBe negative with stable transaminase levels. This stage can be life-long or can spontaneously erupt into stage 2 of the disease, particularly under immune-suppression (Buendia 1998; Bertoletti and Naoumov 2003; Lavanchy 2004; Hoofnagle 2006).

**Stage 4. Re-activation (out-growth of HBe mutants)**

The reactivation stage is most commonly observed in older individuals. They are HBV DNA positive, HBe negative, positive for anti-HBe antibodies with necro-inflammatory changes in the liver. It has been proposed that long-term immune pressure in these carriers, in the form of anti-HBe antibody responses, selects for HBV mutants that do not express HBe (Okamoto, Tsuda et al. 1994; Buendia 1998; Bertoletti and Naoumov 2003; Lavanchy 2004; Hoofnagle 2006).


Arbitrary units
symptoms
symptoms
symptoms
1
2
3
4
4-6 months
Years
HBV DNA
Clearing HBV DNA
HBsAg
Clearing HBsAg
HBeAg
Anti-HBc
Anti-HBe
Anti-HBs
ALT

2004) (Figure 3B). The nomenclature of the DHBV proteins is similar to that of their mammalian counterparts. The pol gene encodes the polymerase protein. The S gene encodes two separate proteins, the second initiated by a translation initiation codon within the ORF (Triyatni, Ey *et al.* 2001). The large pre-S/S is encoded by the DHBpres-S/S gene, and the small S protein is encoded by the S gene (Triyatni, Ey *et al.* 2001). The core gene also contains an internal translation initiation codon, and hence encodes the viral nucleocapsid, DHBcAg and the secreted form, DHBe (Triyatni, Ey *et al.* 2001). DHBe is singly or doubly glycosylated in the lumen of the endoplasmic reticulum of hepatocytes, and truncated by C-terminal processing prior to secretion into the blood of infected ducks (Schlicht, Salfeld *et al.* 1987) (Figure 3B).

The relaxed circular DHBV DNA molecule is also held in a circular configuration by cohesive overlaps of the 5' ends of the positive and negative strands. The 5' end of the negative strand is covalently attached to the viral pol. In DHBV, DR1 and DR2 are direct repeat sequences that are 12 nt in length and they are also involved in replication of the viral genome (Summers and Mason 1982; Jilbert and Kotlarski 2000; Havert, Ji *et al.* 2002) (Figure 3B).

There is controversy as to whether an DHBx gene exists in DHBV (Chang, Netter *et al.* 2001; Meier, Scougall *et al.* 2003). The absence of a DHBx gene in DHBV could explain the scarcity of evidence for HCC in chronically DHBV-infected ducks not exposed to hepato-toxic compounds such as aflatoxins (Uchida, Suzuki *et al.* 1988).

### 1.3.3 The DHBV receptor

The search for the DHBV receptor has been less intensive that of HBV. Early reports identified glycine decarboxylase (p120) as a putative DHBV receptor (Li, Tong *et al.*

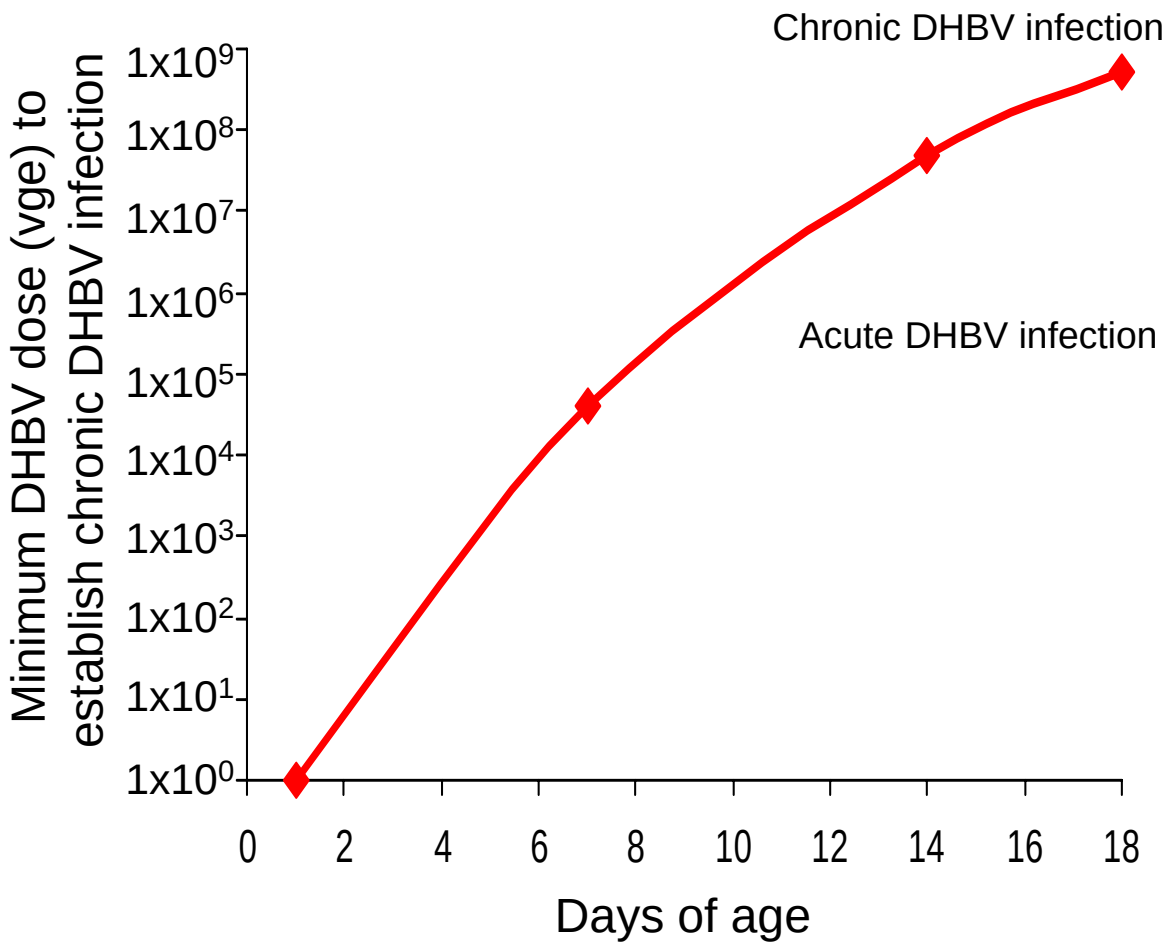
1996; Li, Tong *et al.* 1999). Other studies included epitope mapping with DHBV-specific monoclonal antibodies have shown that DHBpre-S/S and DHBs envelope proteins contain the epitopes involved in neutralisation of infectivity (Cheung, Robinson *et al.* 1989; Pugh, Di *et al.* 1995). To date, carboxypeptidase D is the best candidate as the putative DHBV receptor, as it has been shown to bind to the DHBpre-S/S protein (Kuroki, Cheung *et al.* 1994; Kuroki, Eng *et al.* 1995; Breiner, Urban *et al.* 1998; Urban, Schwarz *et al.* 2000; Breiner, Urban *et al.* 2001; Urban 2004). However, it is noteworthy that plasmid DNA expressing carboxypeptidase D does not confer infectivity to otherwise refractory cell types (Breiner, Urban *et al.* 1998). It is, therefore, reasonable to assume that other cell surface receptors may also be involved in binding, internalisation and infection of hepatocytes with DHBV.

## 1.4 Patterns of DHBV infection

DHBV infection is vertically transmitted through the egg and causes natural chronic viral infection in Pekin Aylesbury ducks (*Anas domesticus platyrhynchos*), (Mason, Seal *et al.* 1980; Marion, Cullen *et al.* 1987). Replication is detectable within the embryonic liver as early as 12 days post-fertilisation (O'Connell, Urban *et al.* 1983). Similar to human infants, young ducks are also highly susceptible to development of chronic DHBV infection. For example, it has been shown that a single DHBV virion is capable of establishing chronic infection in 2-3 day-old-ducklings (Jilbert, Miller *et al.* 1996). Again, comparable to HBV infection of humans, susceptibility to development of chronic DHBV infection decreases rapidly with age (Jilbert, Botten *et al.* 1998; Foster, Miller *et al.* 2005) (Figure 5).

**Figure 5. Age/dose response in ducks.**

An age/dose response curve in ducks of various ages was constructed to establish the minimum dose of DHBV that established chronic infection. DHBV challenge of ducks with doses of above the line resulted in chronic DHBV infection whereas doses below the line resulted in resolution of infection.


Chronic DHBV infection
Acute DHBV infection
Minimum DHBV dose (vge) to establish chronic DHBV infection
1x10^9
1x10^8
1x10^7
1x10^6
1x10^5
1x10^4
1x10^3
1x10^2
1x10^1
1x10^0
0
2
4
6
8
10
12
14
16
18
Days of age

### 1.4.1 Acute DHBV infection

As in adult humans, 90% of adult ducks are able to resolve high-dose DHBV infections, while approximately 5 to 10% of ducks develop chronic infections (Jilbert, Botten *et al.* 1998; personal observation-unpublished data). Perhaps due to more rapid development of the immune responses and the shorter lifespan of ducks, the time-course of DHBV infection is much faster than that of HBV, resolving in weeks rather than months (Jilbert, Wu *et al.* 1992; Jilbert, Miller *et al.* 1996; Jilbert and Kotlarski 2000; Foster, Miller *et al.* 2005; Miller, Halpern *et al.* 2006; Miller, Kotlarski *et al.* 2006). Recovery from acute DHBV infection is associated with a loss of DHBV DNA and DHBsAg in liver and serum, and is accompanied by lifelong anti-DHBc antibodies and anti-DHBs antibodies that protect against re-infection (Jilbert, Miller *et al.* 1996; Jilbert, Botten *et al.* 1998; Jilbert and Kotlarski 2000; Foster, Miller *et al.* 2003; Miller, Bertram *et al.* 2004; Foster, Miller *et al.* 2005; Miller, Halpern *et al.* 2006; Miller, Kotlarski *et al.* 2006) (Figure 6A).

### 1.4.2 Chronic DHBV infection

Chronic DHBV infection is associated with high-titres of DHBV DNA and DHBsAg in the blood and with more than 95% of hepatocytes within the liver of ducks are DHBsAg-positive. If widespread infection of the liver has been established for periods greater than two weeks, lifelong infection appears to be inevitable (personal observation). Only mild to moderate inflammatory changes are detected within the liver (Foster, Miller *et al.* 2005; Miller, Halpern *et al.* 2006; Miller, Kotlarski *et al.* 2006), while significant increases in serum transaminase levels have only been detected in ducks experimentally infected with a cytopathic DHBV mutant (Meier, Scougall *et al.* 2003). HCC has been reported in ducks that have consumed aflatoxin contaminated grains, but there are no reports of cirrhosis or HCC in aflatoxin-free, experimentally

DHBV-infected ducks (Uchida, Suzuki *et al.* 1988). It is hypothesised that the general low immunoreactivity observed in the liver following DHBV infection, the relatively short lifespan of experimental ducks and the absence of the DHBx gene could all be reasons for the low incidence of HCC in ducks. Anti-DHBc antibodies are readily detected in the serum of chronically infected ducks (Jilbert, Miller *et al.* 1996; Jilbert, Botten *et al.* 1998; Triyatni, Jilbert *et al.* 1998; Jilbert and Kotlarski 2000; Foster, Miller *et al.* 2003; Miller, Bertram *et al.* 2004; Foster, Miller *et al.* 2005; Le Mire, Miller *et al.* 2005; Miller, Halpern *et al.* 2006; Miller, Kotlarski *et al.* 2006). Anti-DHBs antibodies are commonly detected in the serum by ELISA but at low levels as circulating DHBsAg mask their detection (Foster, Miller *et al.* 2005; Miller, Halpern *et al.* 2006; Miller, Kotlarski *et al.* 2006) (Figure 6B).

## 1.5 The duck immune system

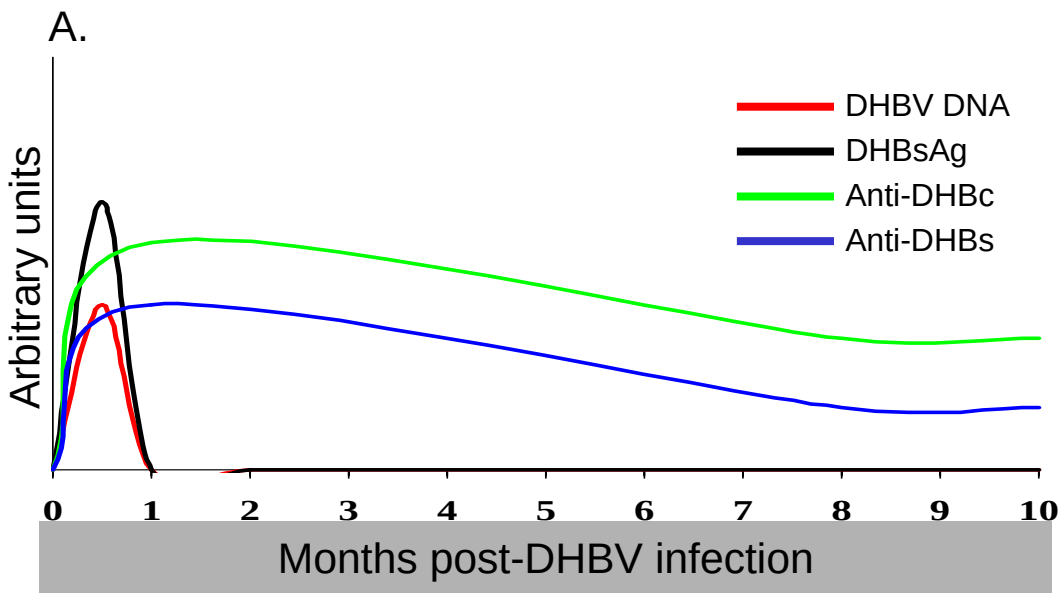
Immune responses in humans and common laboratory animals (e.g., mice) have been extensively studied. It is necessary to extrapolate from human and mouse data when considering other animal models because the immune responses in these species are less well described. As in other higher vertebrates, the thymus is the main site of T-cell maturation in ducks. It is at its largest during the first few weeks of life and decreases in size as the duck matures, becoming vestigial by six weeks of age (Higgins and Chung 1986). Unlike mammals, where B-cells are derived from bone marrow, the bursa of fabricius is the site of B-cell maturation in ducks as it is in chickens (Papermaster and Good 1962). Pioneering work by Higgins and co-workers in 1992 detected the presence of three unique isoforms of duck immunoglobulin in serum. Duck immunoglobulin M (IgM) is similar in structure to other vertebrate IgM. The polymeric immunoglobulin has a molecular weight molecule of approximately 900 000 kDa. Duck IgY performs the role of mammalian IgG and exists as two isoforms; IgY of 180 000 kDa and IgY

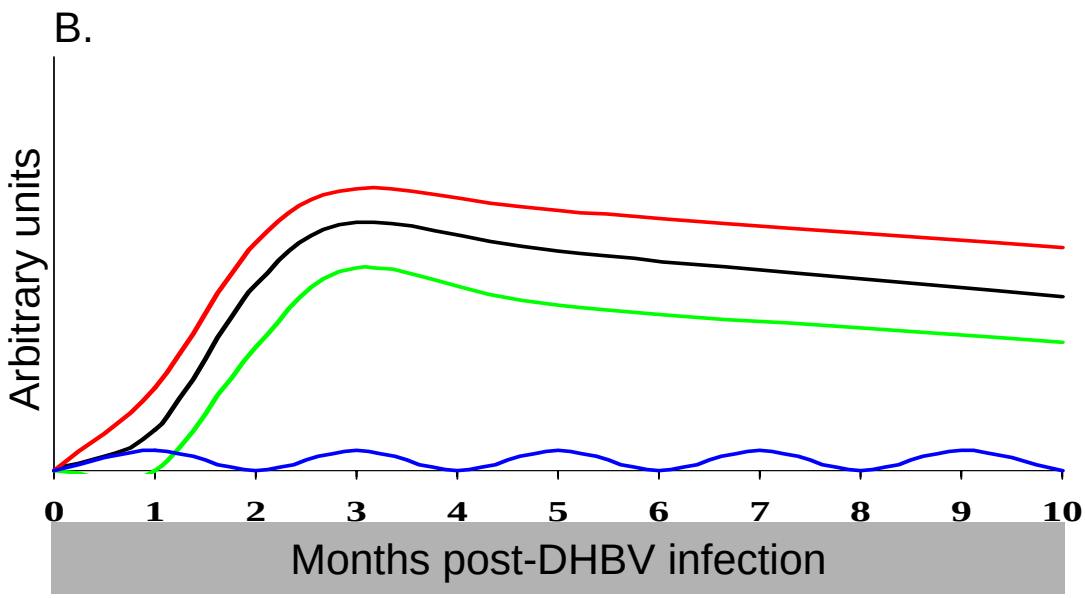
**Figure 6A. Acute DHBV infection.**

The time-course of DHBV infection is considerably faster than HBV infection, resolving in weeks rather than months. Acute DHBV is characterised by a loss of DHBV DNA and DHBsAg in liver and serum, accompanied by lifelong anti-DHBc antibodies and anti-DHBs antibodies that protect against re-infection (Jilbert, Wu et al. 1992; Jilbert, Miller et al. 1996; Jilbert, Botten et al. 1998; Jilbert and Kotlarski 2000; Miller, Bertram et al. 2004; Miller, Halpern et al. 2006; Miller, Kotlarski et al. 2006).

**Figure 6B. Chronic DHBV infection.**

Chronic DHBV infection is characterised by high-titres of DHBV DNA and DHBsAg in the bloodstream and sustained infection of 95% of hepatocytes. Anti-DHBc antibodies are readily detected in the serum of chronically infected ducks. Anti-DHBs antibodies are commonly detected in the serum by ELISA, but they are at fluctuating low levels (Jilbert, Wu et al. 1992; Jilbert, Miller et al. 1996; Jilbert, Botten et al. 1998; Jilbert and Kotlarski 2000; Foster, Miller et al. 2003; Miller, Bertram et al. 2004; Foster, Miller et al. 2005; Miller, Halpern et al. 2006; Miller, Kotlarski et al. 2006).


A.
Arbitrary units
DHBV DNA
DHBsAg
Anti-DHBc
Anti-DHBs
0
1
2
3
4
5
6
7
8
9
10
Months post-DHBV infection


B.
Arbitrary units
0
1
2
3
4
5
6
7
8
9
10
Months post-DHBV infection

(ΔFC) of 120 000 kDa respectively (Magor, Higgins *et al.* 1994). Functionally the two IgY species are distinct. IgY antibodies occur early in immune responses, whilst IgY (ΔFC) is produced later. IgY fixes complement and is the major species present in egg-derived maternal antibodies. IgY (ΔFC) is effective where no secondary effector function is required, such as in virus neutralisation (Magor, Warr *et al.* 1992). This difference in function is due to the absence of the Fc region of the IgY (ΔFC) isoform.

## 1.6 Development of assays to analyse outcomes of DHBV infection

A necessity to be able to carry out the studies described in this thesis was the development of assays that specifically and accurately monitor humoral immune responses in ducks following vaccination and experimental DHBV infection**.** To address this issue, assays that reproducibly detect specific duck immunoglobulins were developed and described in the first manuscript of this thesis (Miller, Bertram *et al.* 2004). More specifically, development of ELISA assays for detection of DHBsAg, anti-DHBs and anti-DHBc antibodies was the first step in initiating a detailed investigation of the antibody responses to DHBsAg and DHBc antigens during DHBV infection and following vaccination of ducks. In addition to these assays, other methods were also developed to inexpensively purify DHBV from serum thus enabling rapid and sensitive PCR detection of circulating DHBV DNA. Moreover, sensitive immunoperoxidase methods for detection of DHBV antigens in hepatocytes and Kupffer cells were also established. All of these assays provided the tools for determining dose-response curves during DHBV infection (Figure 5), and proved essential for monitoring the efficacy of three novel vaccine strategies (Miller, Halpern *et al.* 2006; Miller, Kotlarski *et al.* 2006; Miller, Boyle *et al.* 2008). These methodologies were also adopted for use in other studies within the Hepatitis B Research Laboratory (Jilbert, Miller *et al.* 1996; Jilbert,

Botten *et al.* 1998; Triyatni, Jilbert *et al.* 1998; Jilbert and Kotlarski 2000; Foster, Miller *et al.* 2003; Foster, Miller *et al.* 2005; Le Mire, Miller *et al.* 2005).

Few studies have reported success in detecting CMI responses in ducks. Proliferation has been reported following stimulation of duck peripheral blood lymphoid cells with phytohaemagglutinin (Jilbert and Kotlarski 2000; Miller, Bertram *et al.* 2004). However, attempts to detect antigen-specific responses to DHBV antigen following experimental DHBV infection or vaccination of ducks have not been successful. Recently, monoclonal antibodies have been produced against duck $CD4^+$ and $CD8^+$ T-cells (Kothlow, Mannes *et al.* 2005). These monoclonal antibodies will hopefully provide future tools for dissection of duck T-cell responses to vaccination and infection.

## 1.7 Overview of immunity to viral infection

As already alluded to, the duck immune system is poorly characterised. Therefore, extrapolation from rodent and human studies is required when describing the mechanisms operating in ducks following DHBV infection and vaccination. Immune responses in general can be divided into the innate and adaptive immune responses. There is considerable interplay between these modes of response and it is vital to consider them as interdependent during the induction of immunity and the establishment of immune tolerance.

### 1.7.1 Innate immune responses

Innate immune responses, were first described in 1884 by Metchnikoff (Revillard 2002). In these early studies he discovered that phagocytic cells of the water flea *Daphnia magna* were attracted to and engulfed a yeast-like fungus, resulting in killing of the fungus. It was not for another 100 years before studies in the fruit fly, *Drosophila*

*melanogaster* revealed the mechanism by which cells of the innate immune system recognised invaders. In these studies it was shown that a human homologue of the *Drosophila* Toll protein, Toll-like receptor-4, (TLR-4) could signal activation of the adaptive immune response (Medzhitov, Preston-Hurlburt *et al.* 1997). It has since been demonstrated that activation is the result of recognition of pathogen-associated molecular patterns (PAMPs) present on or within invading organisms by various Toll-like Receptors (TLR). Some of the better characterised TLR are TLR-2 which recognises bacterial lipopolysaccharides, TLR-9 which is stimulated by unmethylated CpG motifs present in bacterial and viral DNA, TLR-3 which is activated by double-stranded RNA molecules, and TLR-7 and TLR-8 by virus derived single-stranded RNA molecules (Beutler 2000; Kaisho and Akira 2000; Hacker, Redecke *et al.* 2002; Ulevitch, Mathison *et al.* 2004). Although beyond the scope of this thesis, understanding the roles played by innate immune responses may provide insights into immunity and immune tolerance. Of particular interest are the effects of activation via PAMP on the outcomes of antigen presentation by macrophages and dendritic cells at the interface between the innate and adaptive immune responses.

### 1.7.2 Adaptive immune responses

#### 1.7.2.1 Humoral immune responses

Humoral immune responses following vaccination (or infection) result in the production of specific antibodies, some of which have protective neutralising functions (Milich, Peterson *et al.* 1985). This response is generated primarily by the uptake of antigen from the extracellular milieu (exogenous antigen) by professional antigen presenting cells (APC) such as dendritic cells, macrophages, Kupffer cells of the liver and Langerhans cells of the skin, and B cells (Haque and Blum 2005; Rodriguez-Pinto 2005). Uptake occurs by fluid phase pinocytosis or is augmented by receptor-mediated

endocytosis or antibody-antigen complexes binding to Fc-receptors (Figure 7). The antibodies involved can be either natural cross-reactive antibody produced during primary exposure to antigen, or those resulting after immunisation or infection.

#### 1.7.2.2 Cellular immune responses

Cellular immune (CMI) responses provide no protection against initial viral infection in the naive host, but they play an important role in the destruction of virus infected cells. APCs can direct the immune bias towards CMI, probably in response to signals induced by the PAMP associated with individual infectious agents.

## 1.8 Major histocompatability complexes (MHC)

Important determinants of which epitopes are recognised in adaptive immune responses are the gene products of the MHC. These proteins are expressed and displayed on the surface of cells of most vertebrates and they play key roles in immune recognition of "self" or "non-self". MHC proteins can be divided into two classes, MHC class I (MHC I) and MHC class II (MHC II).

### 1.8.1 MHC I

A small portion of all proteins synthesised within cells of the body (endogenously) are "labelled" with the cellular protein ubiquitin. Following ubiquitination, these proteins are directed to the proteosome, where they are degraded to peptides (Figure 7). The resulting peptides are then transported to the ER by the cellular transporter associated presentation (TAP) apparatus, where a fraction of them are bound by nascent MHC-I proteins. These MHC-I/peptide complexes are then transported to the surface of the cell (Rudolph, Stanfield *et al.* 2006) (Figure 7). Foreign peptides presented on the cell surface in context with MHC-I proteins are "recognised" by T-cells bearing the CD-8

marker, enabling immune recognition of intracellular pathogens as discussed in more detail below (Figure 7). It is important to note that MHC-I proteins are only expressed on the surface of nucleated cells (Dyer and Martin 1991).

### 1.8.2 MHC II

Internalised exogenous antigens are transported to the endosome, where proteases degrade them to small peptides (Rudolph, Stanfield *et al.* 2006). MHC II proteins are assembled in the endoplasmic reticulum of APC as heterodimers, which consist of the MHC II alpha and beta sub-units. Within the endoplasmic reticulum, the nascent MHC II form complexes with a glycoprotein termed the invariant chain, whose function is to block the peptide-binding groove (Harding 1996) (Figure 7). In the endosome, the invariant chain is released and selected peptide fragments are loaded into the vacated binding groove of the MHC II proteins, the resultant MHCII/peptide complex is trafficked to the cell surface where it is available for presentation to CD4+ T-cells ($T_H$) (Figure 7).

## 1.9 Cell types involved in induction of adaptive immune responses

### 1.9.1 Dendritic cells

Most dendritic cells arise from the myeloid hematopoetic lineage and they are described as the sentinels of the immune system (Spits, Blom *et al.* 1998; Vandenabeele and Wu 1999; Liu 2001). Several different lineages of dendritic cells exist, presumably reflecting their functions at different sites of antigen contact. Langerhans cells of the skin, interstitial dendritic cells of the major organs, interdigitating dendritic cells found in T-cell areas of lymph nodes, and circulating dendritic cells, all express high-levels of MHC II and other co-stimulatory molecules such as B7 on their surfaces. They are

therefore able to present antigen to naive $T_H$ cells (Spits, Blom *et al.* 1998; Vandenabeele and Wu 1999; Liu 2001). Follicular dendritic cells, on the other hand, are thought to be non-myeloid in origin and do not express MHC II. Dendritic cells, particularly those residing in secondary lymphoid tissues, also have the ability to cross-present antigens. Thus they can present both exogenous and endogenous antigens to effector cells in the context of either MHC I or MHC II proteins (Carbone, Hosken *et al.* 1989; Kundig, Bachmann *et al.* 1995; Carbone and Heath 2003) (Figure 7). Follicular dendritic cells express high-levels of receptors for antibodies and complement, and they are thought to be involved in organisation of B cell follicles, maintenance of immunological memory and selection of B cells in affinity maturation in germinal centres (Spits, Blom *et al.* 1998; Vandenabeele and Wu 1999; Liu 2001).

### 1.9.2 B cells

In mammals, B cells are generated in the bone marrow. At this stage of B cell development, rearrangement of the immunoglobulin genes occurs which results in surface expression of membrane-bound immunoglobulin with a single idiotype and antigenic specificity (Warner 1974; Honjo, Nakai *et al.* 1981). It is important to note that this process occurs independent of an antigenic stimulus. It has been estimated that only 10% of the B cells produced daily are released into circulation, as those that express self-reactive membrane-associated immunoglobulin die by apoptosis (Liu, Joshua *et al.* 1989). The surviving naive B cells that leave the bone marrow re-circulate through secondary lymphoid tissues such as the spleen, lymph nodes and Peyer's patches. Activation of naive B cells by protein antigen requires capture of the antigen by membrane-bound immunoglobulin and processing and presentation via MHC II molecules. After activation and proliferation, the activated B cells differentiate into either antibody secreting plasma cells or memory B cells (Kishimoto, Yoshizaki *et al.*

1984). Those naive B cells that do not encounter specific antigens in the periphery die by apoptosis within two weeks after release from the bone marrow (Melchers 2005).

The pathway of B cell activation depends on whether a cell is stimulated by thymus-dependant or thymus-independent antigen (Paul and Benacerraf 1977). As described above, activation by a thymus-dependant antigen requires direct contact with cognate $T_H$ cells, whereas thymus-independent antigens can activate B cells without requirement for $T_H$ cells (Kishimoto, Yoshizaki *et al.* 1984). Examples of thymus-independent antigens in mice are bacterial lipopolysaccharides and bacterial flagellin (Bitter-Suermann, Hadding *et al.* 1975). The mechanisms by which they induce their downstream effects are not well understood but it has been demonstrated that activation can occur via direct cross-linking of membrane immunoglobulin by antigen with repeating identical epitopes (Stackpole, De Milio *et al.* 1974).

As discussed below, $CD4^+$ T-helper 2 ($T_H2$) cells play an integral role in generation of humoral responses following activation with thymus-dependent antigens (Mosmann and Coffman 1989). In a primary immune response, $T_H$ cells are activated by interaction with processed antigen presented by dendritic cells. The activated T cells express CD40L (CD154), an important co-stimulatory molecule for activation of B cells. Once activated, MHC II molecules and co-stimulatory molecules such as B7 are upregulated (Greenwald, Freeman *et al.* 2005). B cells can activate T cells independently of other professional APC as antigenic peptide fragments presented on the surface of the B-cell in the context of MHC II molecules leads to recruitment of antigen-specific $CD4^+$ $T_H$ cells. B cells constitutively express the co-stimulatory membrane glycoprotein CD40 and they receive co-stimulation via CD40L on the activated $T_H$ cells (Greenwald, Freeman *et al.* 2005) (Figure 7). Activated B cells express cytokine receptors for IL-2,

IL-4 and IL-5 and they respond to the respective cytokines released by the activated $T_H$ cells (Figure 7). IL-4 produced by $T_H2$ cells enhances proliferation of B cells (Reiner and Seder 1995). Cytokines produced by $T_H1$ and $T_H2$ cells have differential effects on isotype switching and differentiation into plasma cells is promoted by IL-6 (DeKruyff, Rizzo *et al.* 1993).

### 1.9.3 T lymphocytes (T cells)

T cells arise from committed lymphoid bone marrow precursors and mature in the thymus. T cells are characterised by expression of a T cell receptor (TCR) (Janeway 1980). The complete TCR consists of a heterodimeric molecule composed of $\alpha$ plus $\beta$ chains ( $\alpha\beta$ TCR ) or $\gamma$ plus $\delta$ chains ($\gamma\delta$ TCR), associated with the CD3 complex (Rudolph, Stanfield *et al.* 2006) (Figure 7). The TCR does not bind soluble antigen. Instead, antigen recognition requires interaction of the TCR with peptides presented in association with MHC molecules (Marrack, Endres *et al.* 1983). CD3 does not influence interaction of antigen with the TCR but it is vital for signal transduction. T cells can be divided into two main subsets by expression of the CD4 or CD8 co-receptors (Janeway 1992).

### 1.9.4 $CD4^+$ T cells

The CD4 protein expressed on the surface of $CD4^+$ T cells binds to the MHC II complex present on the surface of APCs, increasing the strength of interaction between the TCR and the foreign peptide fragments in the MHC II binding groove (Janeway, Carding *et al.* 1988) (Figure 7). Furthermore, it is involved in recruiting the tyrosine kinase *lck* into the receptor complex (Zamoyska, Basson *et al.* 2003). This interaction induces antigen-specific stimulation of $CD4^+$ T-cells, most importantly by inducing expression of the genes encoding IL-2 and components of the IL-2 receptor. Depending on cytokines produced by the APC or by other cells in the vicinity of the interaction, $T_H0$ cells may

become biased in the cytokines that they produce, resulting in two partially over-lapping cellular lineages (Bendelac and Schwartz 1991). $T_H1$ cells secrete cytokines such as IL-2, IFN-γ, and tumour necrosis factor beta (TNF-β), which all promote effector functions of the sort involved in delayed-type hypersensitivity (DTH) (Plate, Lukaszewska *et al.* 1988) (Figure 7), but also class switching to the IgG2a isotype in mice (Snapper and Mond 1993). $T_H2$ subsets secrete cytokines such as interleukin 4 (IL-4) and interleukin 5 (IL-5), which are involved in proliferation of B cells, leading to the production of antibody-producing plasma cells (Castellino and Germain 2006; Rudolph, Stanfield *et al.* 2006) (Figure 7). IL-4 production by $T_H2$ cells is important in biasing immune responses towards antibody production, while IFN-γ and IL-23 produced by activated $T_H1$ cells biases responses towards CMI (Langrish, McKenzie *et al.* 2004).

### 1.9.5 $CD8^+$ T cells

$CD8^+$ T cells are the major effector cells in non-lytic viral infections and they are known as CTL due to their ability to lyse foreign antigen-expressing cells *in-vitro* (Kaufmann 1988). Activation of CTL is required for effector function. Prior to activation, $CD8^+$ T cells do not express IL-2 or IL-2 receptors or exhibit effector function (French and Plate 1985; Stohl, Elliott *et al.* 1994). Activation is initiated by engagement of the TCR with cognate antigenic peptide presented by an APC in association with MHC II molecules (de Vries, Yssel *et al.* 1989). Appropriate co-stimulatory signals via interaction of T cell CD28 with B7 on the APC (McAdam, Schweitzer *et al.* 1998) and recruitment of *lck* to the immunological synapse by interaction between the CD8 co-receptor and the MHC I peptide complex activates the T cell (Zamoyska, Basson *et al.* 2003). Activation can be separated into two main phases. Following TCR engagement, the $CD8^+$ T cell initiates *de novo* expression of the IL-2 receptor and may also express IL-2 (McAdam, Schweitzer *et al.* 1998). Although

autocrine stimulation via endogenously produced IL-2 probably occurs, the main source of IL-2 in some CTL responses is paracrine and derived from neighbouring $TH_1$ cells (Figure 7). Once activated, $CD8^+$ effector CTL can engage with viral antigen presented by target cells, without further co-stimulation (Khanolkar, Badovinac *et al.* 2007). Following elimination of the virus infected target cells, IL-2 secretion decreases, resulting in apoptosis of the activated CTL, therefore minimising the effects of ongoing immune reactivity (Minami, Kono *et al.* 1993; Gomez, Gonzalez *et al.* 1998).

Activation of $CD8^+$ T cells also results in secretion of type I IFNs such as IFN-α and IFN-γ, ensuring up-regulation of MHC I molecules in neighbouring cells, activation of natural killer T-cells (NK) and apoptosis of virus infected cells by activating degradation of viral and cellular nucleic acid (Zuniga, Hahm *et al.* 2007) (Figure 7). IFN-γ can also initiate a positive feedback loop by stimulating secretion of IL-12, a key molecule that drives the immune response towards CMI, and in turn this up-regulates additional synthesis and secretion of IFN-γ (Stohl, Elliott *et al.* 1994) (Figure 7). Infected target cells are killed by CTL via a number of mechanisms. When activated antigen-specific CTL encounter an infected cell, they form a CTL-target cell conjugate. Shortly after conjugate formation, cytoplasmic granules containing perforin and granzymes are relocated and become polarised to the area of contact with the target cell. Activated CTL then release perforin, a pore-forming protein, into the target cell contact zone (Berke 1995). The resultant pores are then utilised by the CTL to deposit granzyme into the cytoplasm of the target cell. These enzymes activate apoptotic pathways within the target cells, resulting in their death (Berke 1995). The activated CTL can then recycle to resume surveillance for other infected cells.

### 1.9.6 NK cells

NK cells are a discrete sub-set of non-T, non-B cells that comprise 5-10% of the total circulating lymphocytes. They are bone marrow-derived cells that appear to be related to T cells but they do not mature in the thymus, do not express either TCR or CD3 and they do not interact via antigen specific receptors or require MHC-peptide complexes for effector function (MHC unrestricted) (Figure 7) (Rees 1990; Kumar and McNerney 2005). The exact mechanisms by which NK cells mediate cytotoxicity against some potential target cells, or conversely fail to attack other cells remain areas of intense interest. Nevertheless, there is a general consensus that "missing self" on the target cell, in the form of MHC class I molecules, is an important condition that permits killing (Ljunggren and Karre 1990; Karre 1993; Karre 2002). NK cells express a number of activating molecules, including the CD16 receptor for the Fc region of IgG utilised in antibody-dependant cell-mediated cytotoxic (ADCC) killing interactions (Santoli, Trinchieri *et al.* 1978; Trinchieri and Valiante 1993) and CD28, the receptor for the co-stimulatory molecule B7 (Galea-Lauri, Darling *et al.* 1999). Inhibition of killing involves killing inhibitory receptors (KIRs), which are members of the C-type-lectin-inhibitory receptors in mice, and the Ig-superfamily-inhibitory receptors in humans (Boyington and Sun 2002; Sun 2003). Absence or reduction of MHC I expression on target cells, as occurs in some tumours and infection with certain viruses, stimulates killing by removing the inhibitory effects of KIRs (Kirwan, Merriam *et al.* 2006). As in CTL, the NK cells have cytoplasmic granules that contain perforin and granzymes and these are released following interaction with susceptible target cells. NK cells also secrete cytokines such as TNF-$\alpha$ and IFN-$\gamma$ and their activity is up-regulated by IFN-$\alpha$, IFN-$\beta$, IFN-$\gamma$ and IL-12 (Young and Ortaldo 2006). NK cells do not exhibit immunological memory (Kirwan and Burshtyn 2007).

### 1.9.7 Natural Killer T cells (NKT)

NKT cells are a distinct subset of lymphocytes that share some characteristics of both T cells and NK cells and exhibit innate-like antimicrobial functions. In mice, ~0.5% of all peripheral blood T cells, 2.5% of T cells in spleen and up to 30% of the T cells within the liver are NKT cells (Bendelac, Rivera *et al.* 1997; Crough, Nieda *et al.* 2004; Bendelac, Savage *et al.* 2007). Several lineages of NKT have been described but those expressing the semi-invariant TCR Vα14-Jα18/Vβ8, Vβ7 or Vβ2 constitute more than 80% of the population (Bendelac, Savage *et al.* 2007). NKT cell activation is dependent on recognition of lipid or glycolipid antigen presented via the non-classical MHC I–like molecule CD1d which is expressed on dendritic cells, B cells, T cells, and macrophages as well as hepatocytes (Bendelac, Rivera *et al.* 1997; Brossay, Jullien *et al.* 1997). Although the naturally occurring ligands are poorly described it is clear that NKT cells recognise marine sponge-derived and synthetic forms of α-galactosylceramide (α-GalCer) and other microbial glycosphingolipid ligands presented on CD1d (Tupin and Kronenberg 2006; Bendelac, Savage *et al.* 2007; Fujii, Shimizu *et al.* 2007; Iwamura and Nakayama 2007; Onoe, Yanagawa *et al.* 2007). This interaction results in a "double-edged sword" response stimulating up-regulation of both $T_H1$ and $T_H2$ cytokines such as IFN-γ and IL-4 (Au-Yeung and Fowell 2007). Enormous interest in the potential of α-GalCer for treatment of infectious disease and tumour has therfore been shown. For example, α–GalCer combined with influenza vaccine administered to mice via the intranasal route protected against lethal influenza A infection (Ko, Ko *et al.* 2005; Youn, Ko *et al.* 2007). Additionally, transgenic mice deficient in CD1d or NKT cells expressing the semivariant Vα14-Jα18 TCR had impaired clearance of herpes simplex virus type 1 (Grubor-Bauk, Simmons *et al.* 2003). α-GalCer was also tested in a HBV transgenic mouse model (Kakimi, Guidotti *et al.* 2000). In these studies a single injection of α-GalCer induced increases in levels of IFN-γ and IFN-α in the liver and

HBV replication was abolished. It was concluded that α-GalCer inhibited HBV replication by directly activating NKT cells and by secondary activation of NK cells to secrete antiviral cytokines in the liver. It was proposed that activation of NKT cells with α-GalCer may provide beneficial effects for the treatment of chronic HBV infection.

### 1.9.8 Regulatory T cells ($T_{reg}$)

Unregulated immune responses to infections would be potentially devastating by leading to immune-mediated destruction of affected tissues. Feedback mechanisms that modulate immune responses are essential. Regulatory T cells ($T_{reg}$) may play such a role. $T_{reg}$ cells can be divided into those which are produced in the thymus during T cell maturation (natural $T_{reg}$) and those that are produced in the periphery by conversion of naïve conventional T cells (induced $T_{reg}$) (Raimondi, Turner *et al.* 2007). Natural $T_{reg}$ cells in mice express the cellular markers CD4, CD25, the T cell inhibitory receptor CTLA-4 and the glucocorticoid-inducible tumour necrosis factor receptor (GITR) constitutively (Yi, Zhen *et al.* 2006) but the most reliable $T_{reg}$-specific marker at the present time is the transcription factor FOXP3 (Pallandre, Brillard *et al.* 2007). The lineages of induced $T_{reg}$ in response to infection in humans are not as clearly defined (Pillai and Karandikar 2007). Importantly, TGF-β secreted by $T_{reg}$ exerts negative regulation on both $T_H1$ and $T_H2$ T cells, effectively dampening immune responses (Figure 7). It is this activity that suggests that $T_{reg}$ may play an important role in the development of chronic disease such as HBV (Manigold and Racanelli 2007; Peng, Li *et al.* 2007).

## 1.10 Immunity to HBV infection

Individuals who resolve a HBV infection exhibit evidence of strong humoral and cell mediated immune responses directed against multiple epitopes of viral proteins. These

individuals have CMI responses to the virus that include HBV-specific $CD8^+$ CTL and $CD4^+$ $T_H$ lymphocytes capable of providing life-long immunity against re-infection (Thimme, Wieland *et al.* 2003; Hoofnagle 2006). Intriguingly, the CMI responses, in combination with the associated humoral immunity, provide protection that is superior to that of the mainly humoral response to current vaccines (Kedzierska, La Gruta *et al.* 2006). This suggests that vaccine regimes designed to generate both humoral and CMI responses should provide improved immunity against infection.

Those individuals who develop chronic HBV infections, HBV-specific T-cell responses are either low or undetectable. The mechanism(s) responsible for this state of specific T cell unresponsiveness or "immunological tolerance" to HBV antigens are poorly understood. However, they are most likely the result of functional and/or clonal deletion of reactive T-cells, either by T-cell anergy or by the action of $T_{reg}$ cells (Boni, Fisicaro *et al.* 2007; Rehermann 2007). Furthermore, "clonal exhaustion" could result from chronic stimulation by the excessive amounts of empty HBsAg particles and HBe circulating in the bloodstream of chronically HBV-infected individuals (Milich, Peterson *et al.* 1985; Zhou, Ou *et al.* 2004; Chen, Sallberg *et al.* 2005). However, the presence of anti-HBc antibodies in chronically HBV-infected patients suggests that functional $CD4^+$ $T_H2$ cells reactive against this antigen are still active (Figure 4B, Figure 7). If it is the case that deletion or anergy of reactive T cells is the primary cause of chronic HBV infection, development of successful therapeutic vaccination can be expected to be difficult. Interestingly, individuals with chronic HBV have greater numbers of HBcAg specific $T_{reg}$ cells compared with those able to resolve their infection (Boni, Fisicaro *et al.* 2007; Rehermann 2007). Therefore, if the immuno-modulatory effects of HBcAg-specific $T_{reg}$ can be over-come or modified, this might

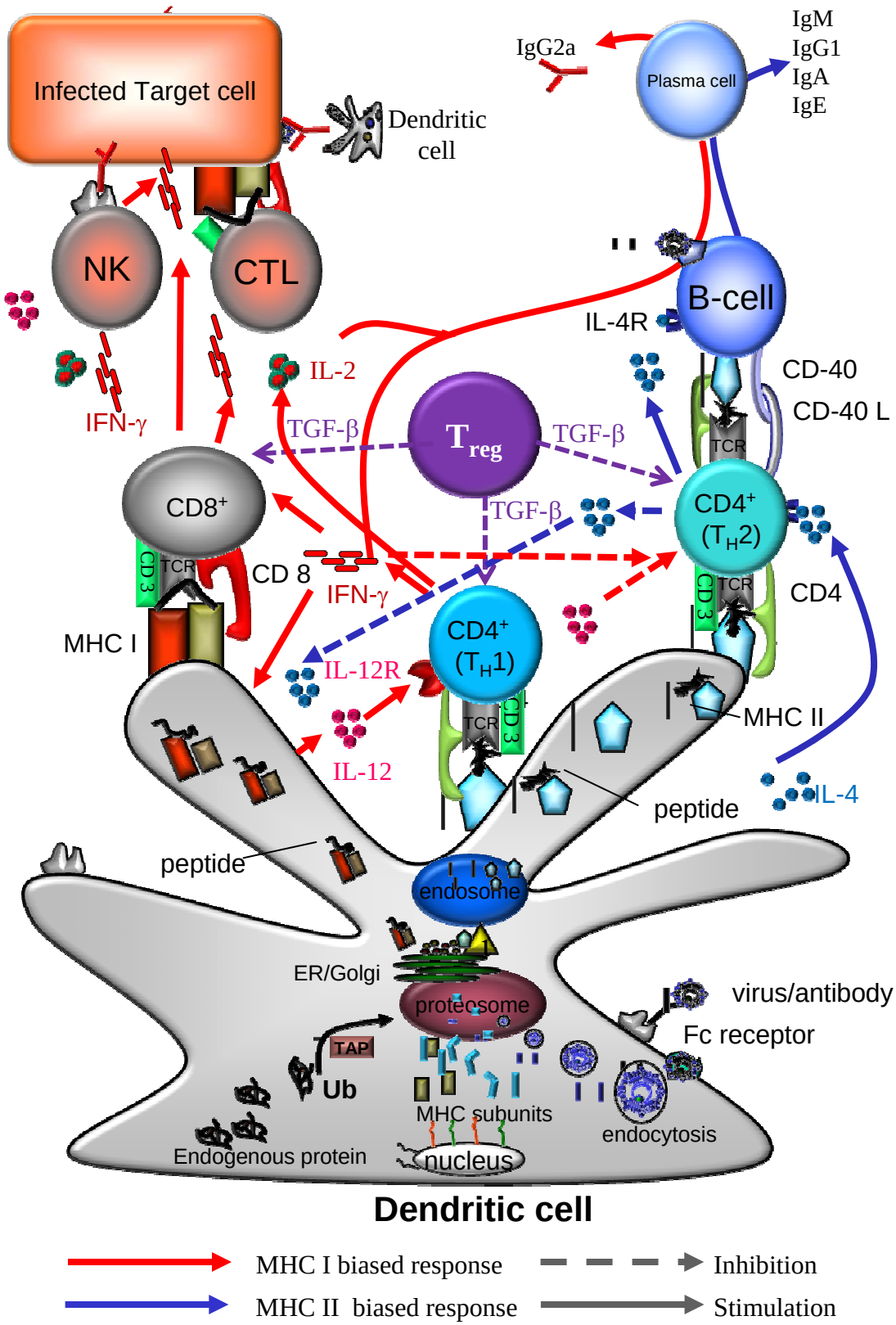



Figure 7. MHC Class I and Class II antigen presentation and resultant immune responses: (as described in text)

allow deviation of the antibody-mediated $T_H2$ immune response towards a cell-mediated $T_H1$/CTL response, with resulting improvement in clearance of infected hepatocytes.

## 1.11 Hepadnaviral vaccines

### 1.11.1 The current HBV vaccine

An effective vaccine protecting against HBV infection has been available for a considerable time (Maugh 1981). The prototype vaccine was prepared by purifying 22nm sub-viral particles directly from the serum of infected patients. These HBsAg particles were then chemically inactivated to destroy any infectious HBV virions that might have contaminated the preparation. This vaccine induces high-titres of neutralising antibodies against HBV, thus protecting against HBV infection. The mode of protection has been shown to be the direct binding of antibodies to an epitope within the HBsAg, known as the "a" determinant. This results in opsonisation or blocking of the virus/receptor interaction and thus prevents viral entry. The "a" determinant is common to all subtypes of HBV, and the vaccine therefore provides cross-protection against all HBV subtypes (Szmuness 1979).

The use of the "non-infectious" HBV vaccine preparations derived directly from HBV-infected humans was questioned for reasons of safety. More recently, the advent of recombinant DNA technology has resulted in the production of a recombinant HBsAg sub-unit vaccine produced in yeast. This vaccine does not carry the risk of HBV infection or other potential contaminants from human products (Galibert, Mandart *et al.* 1979; Charnay, Gervais *et al.* 1980). Recombinant HBsAg is prepared by transfecting a plasmid that contains the HBsAg gene into *Saccharomyces cerevisiae*. Recombinant HBsAg particles (rHBsAg) are purified from large-scale cultures. The vaccine consists of HBsAg plus aluminium hydroxide, an adjuvant that stimulates $T_H2$ biased immune response (Toth 1972; Sanchez, Ionescu-Matiu *et al.* 1980).

Three doses of the HBV vaccine are administered over approximately six months. In children and young adults, the recommended regimen is that the first and second doses of vaccine (10 μg/dose) are administered one month apart and third dose five months later. In adults, again the first two doses (20 μg/dose) are administered one month apart with the third dose around five months. The protective levels of HBsAg specific antibodies (10 mIU/ml) are generated in 90-95% of vaccinated recipients (Lin, Chang *et al.* 1991). A booster immunisation is recommended ten years after the initial vaccination. Whether the 5-10% of recipients who are "non-responders" have immunity to HBV infection remains unresolved. To address this issue, newer versions of HBs vaccines that include the addition of the large HBpre-S/S protein are being developed and tested. The efficacy of the newer pre-S/S vaccines is still under investigation (Rendi-Wagner, Shouval *et al.* 2006). Finally, it is important to note that vaccination with either human or yeast-derived HBsAg fails to induce significant levels of HBV-specific $CD8^+$ CTL (Bocher, Herzog-Hauff *et al.* 1999; Bertoletti and Ferrari 2003; Bertoletti and Naoumov 2003) and thus provides no therapeutic effect against chronic HBV infection, either alone or in combination with existing drug therapies.

As mentioned previously, newborn infants are the population that is most susceptible to development of chronic HBV infection, the extremely high transmission rates in neonates poses the greatest obstacle to the eradication of HBV infection worldwide (Figure 1). Currently, babies born to HBsAg-positive mothers are vaccinated with a paediatric dose of sub-unit recombinant HBsAg vaccine (10 μg) soon after birth and they simultaneously receive passive immunisation with anti-HBV antibodies (100 IU by intramuscular injection) derived from pooled human gamma-globulin (Lo, Tsai *et al.* 1985). Although effective in preventing approximately 80% of vertically transmitted

HBV infections, this regimen is expensive. It is, therefore, unlikely to be implemented extensively in developing countries. Therefore, vaccines designed to elicit protective immune responses in newborn individuals, and therapeutic vaccines designed to cure chronic HBV infection would reduce human suffering and substantially lower the financial burden of chronic HBV infection.

### 1.11.2 Novel hepadnaviral vaccine studies

As discussed above, the current HBV vaccine does not elicit therapeutic immune responses when used in patients with chronic HBV infection. The scientific literature also suggests that success in generating therapeutic immune responses will be difficult to achieve. Several novel combinations of vaccine regimes have been assessed to examine whether they provide improved protection compared with the current HBV vaccine, with the ultimate goal of inducing therapeutic outcomes for individuals with chronic HBV infection.

### 1.11.3 DNA vaccines

DNA vaccines are produced by cloning the DNA coding sequence of a protein of interest into an expression plasmid immediately downstream of a strong promoter that operates in mammalian species. Following inoculation, DNA constructs are taken up by a wide variety of cell types such as muscle cells, Langerhans cells (Babiuk, Baca-Estrada *et al.* 2003) and dendritic cells (Akbari, Panjwani *et al.* 1999; Sbai, Schneider *et al.* 2002). Antigen expression in these cells results in its presentation to cells of the immune system. Whereas, conventional sub-unit vaccines have been found to typically generate strong humoral immunity but weak CMI (Sanchez, Ionescu-Matiu *et al.* 1980; Wolkers, Brouwenstijn *et al.* 2004), DNA vaccines elicit both humoral and CMI responses, including the induction of antigen-specific CTL (Davis, Brazolot Millan *et*

*al.* 1997). Although poorly understood, it is thought that CMI to DNA vaccines results from the association of peptide fragments derived from the processing of endogenously synthesised antigen, and presentation of the peptide-MHC I complex at the cell surface (Davis, Weeratna *et al.* 1998). Alternatively, the expressed antigen may be released into the extracellular spaces, endocytosed by professional APC and presented either directly in association with MHC II or by cross-presentation in association with MHC I. Strong responses associated with DNA vaccination may also be due to immuno-stimulatory effects of non-methylated CpG motifs in bacterial and viral DNA (Davis, Weeratna *et al.* 1998; Krieg, Yi *et al.* 1998; Davis 2000). Recognition of CpG by Toll-like receptor-9 (TLR-9) leads to activation of intra-cellular signalling cascades in B ells, macrophages and dendritic cells. This process leads to the production of both $T_H1$ and $T_H2$ cytokines, which are involved in the generation of humoral and CMI respectively (Davis, Weeratna *et al.* 1998; Krieg, Yi *et al.* 1998; Davis 2000; Mancini-Bourgine, Fontaine *et al.* 2004). Furthermore, production of IL-2 by $T_H1$cells may potentiate the generation of specific CTL (de Vries, Yssel *et al.* 1989; DeKruyff, Rizzo *et al.* 1993; Reiner and Seder 1995; Castellino and Germain 2006).

Numerous studies have demonstrated the protective efficacy of DNA vaccines expressing HBsAg, WHsAg, or DHBsAg (Prince, Whalen *et al.* 1997; Triyatni, Jilbert *et al.* 1998; Lu, Hilken *et al.* 1999; Rollier, Sunyach *et al.* 1999). A recent phase I clinical trial was conducted to test therapeutic efficacy of injected DNA vaccines in humans. In this study, HBV carriers were vaccinated intra-muscularly with repeated doses of vaccines, each containing 0.5mg of a DNA construct expressing HBpre-S/S (Mancini-Bourgine, Fontaine *et al.* 2005). Vaccination reduced levels of HBV DNA, and led to transient boosting of HBV-specific $CD8^+$ T cells resulting in production of IFN-γ. These findings demonstrate that DNA vaccines can activate T-cell responses in

chronic HBV carriers. Unfortunately, HBV infection was not resolved in any of the vaccinated patients.

Epitope mapping studies with DHBV-specific monoclonal antibodies have shown that DHBpre-S/S and DHBs envelope proteins contain neutralisation epitopes involved in infectivity (Cheung, Robinson *et al.* 1989; Pugh, Di *et al.* 1995). In accord with these findings, DNA vaccines expressing DHBV surface envelope proteins have been shown to induce protective immune responses in older ducks (Triyatni, Jilbert *et al*. 1998; Rollier, Sunyach *et al*. 1999; Rollier, Charollois *et al*. 2000; Rollier, Charollois *et al*. 2000). Given the potential for HBV to establish chronic infection during childhood, it was surprising there were no reports examining the protective or therapeutic efficacy of DNA vaccines expressing hepadnaviral antigens in young ducks. In one study, DNA vaccination of 3-day-old ducks with DHBpre-S/S DNA vaccines was found to elicit antibody responses, albeit after further boosting vaccinations at weeks 4 and 15 (Rollier, Charollois *et al*. 2000). None of the ducks in this study were challenged with DHBV to test the protective efficacy of the vaccines.

### 1.11.4 Development of DNA vaccines against DHBV infection

Therefore, in order to further underpin the potential of naked DNA vaccines to provide protection against DHBV infection in young ducks, vaccine experiments were performed as presented in Chapter III of this thesis (Miller, Kotlarski *et al.* 2006). In these studies, three separate vaccine studies were conducted to test the protective efficacy of DNA vaccines expressing either DHBs or DHBpre-S/S antigens. Ducks were vaccinated on days 4 and 14 of age, and then challenged on day 14 with either $5x10^7$ or $5x10^8$ DHBV genomes. These doses are respectively 50 and 500 times greater than that required to establish chronic DHBV infection. All vector vaccinated ducks

(18/18) challenged with DHBV developed chronic DHBV infection. In contrast, ducks vaccinated with either the pre-S/S or S DNA and then challenged with $5x10^7$ or $5x10^8$ DHBV genomes had reduced numbers of DHBV-positive hepatocytes on day 4 days post-challenge (p.c.). In total, 10 of the 18 vaccinated ducks challenged with $5x10^7$ genomes and 4 of the 18 vaccinated ducks challenged with $5x10^8$ genomes developed transient infections. There was no significant difference in the efficacy of the DHBV pre-S/S and S DNA vaccines. Whilst virus-specific antibodies were measured, the lack of specific assays to measure CMI in ducks prevented assessment of CMI responses. However, the protection observed was consistent with neutralisation of the challenge inoculum, probably by the induction of low-levels of anti-DHBs antibodies following vaccination (Miller, Kotlarski *et al.* 2006).

Although DNA vaccines have been shown to elicit robust immune responses, improvements were obviously needed. Limiting factors in the immunogenicity of DNA vaccines may include low-level of transfection *in vivo*, and sub-optimal antigen expression and/or antigen uptake by professional APC such as dendritic cells. For this reason several different modes for DNA vaccine delivery have been tested by others. Intramuscular injection of DNA vaccines has been widely used, with variable results (Davis, Brazolot Millan *et al.* 1997; Prince, Whalen *et al.* 1997; Davis, Weeratna *et al.* 1998; Davis 2000). Gene gun transfer of DNA vaccines in which DNA vaccine-coated gold particles are administered by helium-driven bombardment of skin has been tested. This method targets the epidermal layers of the skin, with its rich complement of Langerhans cells (Vandenabeele and Wu 1999). Gene gun delivery is attractive as it requires only micrograms of DNA and if approved clinically, would alleviate the requirement for syringes and needles. Unfortunately, this mode of vaccination has also had variable efficacy (Lin, Pulkkinen *et al.* 2000; Peachman, Rao *et al.* 2003). HBsAg

expressing DNA vaccines injected into the muscle simultaneously with a small electrical charge (*in vivo* electroporation) has been reported to induce increased CMI responses in mice as compared with vaccination with the same DNA construct without electroporation. (Luxembourg, *et al.* 2006). In similar studies, *in vivo* transfection of pigs and sheep with HBsAg expressing DNA constructs elicited higher CMI responses and longer duration antibody responses when compared to conventional injection of the same DNA construct (Babiuk, Baca-Estrada *et al.* 2002; Babiuk, Tsang *et al.* 2007).

### 1.11.5 Hepadnaviral nucleocapsid-containing vaccines

Another approach to potentiate therapeutic effects is to use vaccines containing antigens other than those found on the surface of hepadnaviruses. One such protein is the nucleocapsid or core antigen. Vaccines containing HBcAg are theoretically valid, given that approximately 5% of vaccinated individuals are non-responders to the current HBsAg vaccine (and hence are possibly not protected against HBV). Inclusion of HBcAg thus may logically be considered as a means of inducing immunity in this minority subgroup. Secondly, individuals chronically infected with HBV usually have high titres of anti-HBc antibodies, indicating that they are able to respond to this antigen with a humoral response. Accordingly, it might be possible to modulate this response to also induce a strong CTL response against HBcAg, which may result in therapeutic benefits in chronically infected individuals. Finally, as virus particles contain both core and surface proteins, priming of the response to the core proteins (e.g., activation of core specific $CD4^+$ $T_H$) might also accelerate activation of the humoral response to surface protein antigens, leading to a faster termination of cell-to-cell spread of the virus (Paul and Benacerraf 1977; Schodel, Neckermann *et al.* 1993) (Figure 7).

Consistent with this theory, vaccination of chimpanzees and woodchucks with sodium dodecyl sulphate (SDS)-treated recombinant HBcAg (rHBcAg) or recombinant WHV core antigen (rWHcAg) has been shown to provide protection against transient HBV and WHV infection respectively (Murray, Bruce *et al.* 1987; Roos, Fuchs *et al.* 1989). Therefore, vaccines expressing hepadnaviral core antigen may have therapeutic potential.

There is also evidence that the immunogenicity of DNA vaccines can be increased by vaccinating with other hepadnaviral antigens. Vaccination of woodchucks with DNA vaccines expressing WHcAg protected adult woodchucks against WHV infection (Lu, Hilken *et al.* 1999; Siegel, Lu *et al.* 2001; Lu, Isogawa *et al.* 2005). However, improved strategies aimed at increasing expression and uptake of vaccine antigens are needed to induce greater protection if they are to be useful for preventative vaccination of the young and/or therapeutic vaccination purposes. It was, therefore, hypothesised that whole cell vaccines are candidates for these purposes.

### 1.11.6 Whole cell vaccines

It has been shown that fibroblasts can present antigen efficiently in lymphoid organs (Kundig, Bachmann *et al.* 1995). In these studies, a fibrosarcoma cell line was transfected *in vitro* with the glycoprotein of the lymphocytic choriomeningitis virus (LCMV). Vaccination of mice via the intraperitoneal route induced $CD8^+$ T-cell responses capable of protecting against LCMV infection. It was proposed that the protection was the result of transfected fibroblasts migrating to draining lymphoid organs. This finding was further supported by the observation that homogenised transfected fibroblasts did not provide protection against LCMV challenge, whereas small numbers of transfected fibroblasts injected directly into the spleens of mice were

potent antigen-presenting cells and provided good protection. It was hypothesised that intact transfected fibroblasts presented antigenic peptides in the context of MHC I. It appears likely that the antigen produced by the fibroblasts was cross-presented by professional APC such as dendritic cells as they are believed to be uniquely able to cross-present antigens as well as provide co-stimulation for the production of effector T cells (Carbone and Heath 2003). In contrast with these studies, it was also shown that cells used to present the antigen do not have to be viable to cross-present antigens (Harshyne, Watkins *et al.* 2001; Schulz and Reis e Sousa 2002). The same authors also concluded that major MHC compatibility between the cell donor and the vaccinee is not required for cross-presentation to occur.

### 1.11.7 Development of whole-cell DNA vaccines expressing DHBcAg

In theory therefore, vaccination regimes using primary fibroblasts transfected with DNA constructs expressing DHBV antigens could be optimised to maximise delivery of vaccine antigens to the cell type(s) most likely to potentiate robust humoral and or CMI immune responses. Based on the above research and conclusions, it was hypothesised that vaccination of ducks with primary duck embryonic fibroblasts (PDEF), previously transfected with DHBcAg encoding DNA constructs, would result in enhanced immune responses. Chapter 4 of this thesis describes investigation of the protection provided to young ducks by whole-cell vaccines expressing DHBcAg (Miller, Halpern *et al.* 2006).

In these studies, ducks were vaccinated with PDEF that had been previously transfected *in vitro* with DNA constructs encoding DHBcAg. Groups of ducks were vaccinated with either PDEF expressing DHBcAg or PDEF alone on days 4 and 14 of age, and challenged on day 18 of age with $5x10^8$ DHBV genomes. Liver tissue collected on day 4 p.c. revealed that vaccination did not prevent initial infection of the liver, as similar

numbers of DHBsAg-positive hepatocytes were detected in all vaccinated and control ducks. Conversely, analysis of autopsy liver tissue revealed that 17 of 22 of the ducks vaccinated with the whole-cell DHBc-expressing PDEF resolved the DHBV infection, whereas only 1 out of 11 of the control ducks resolved its DHBV infection. It was hypothesised that this mode of delivery ensured efficient supply of endogenously expressed antigen for sampling and cross-presentation by dendritic cells. It was concluded that the net result of this vaccine regimen activated a robust CMI response to the vaccine encoded DHBcAg. Although significant protection was observed with this particular vaccine formulation, it was believed that additional immunity could be elicited if DHBV vaccine antigens were delivered with the aid of viral vectors.

## 1.12 Family Poxviridae

A milestone in vaccine development occurred in 1796, when Edward Jenner observed that milkmaids who contracted cowpox were protected from smallpox infection. In an experiment (that would have been considered unethical today) Jenner inoculated an 8-year-old boy with puss from a cowpox lesion isolated from an infected milkmaid. After two to three weeks he then intentionally infected (“variolated”) the boy with smallpox. Fortunately, the boy did not develop any symptoms of smallpox. Jenner termed the methodology “vaccination”, derived from “vaca”, the Latin word for cow. Improvements to the vaccine were quickly achieved, including the inoculation of cow pox into the flank of calves and communal sharing of the exudate. More sophisticated approaches such as lymphatic inoculation of calves to produce high titres of the virus and, more recently, production of the virus in cell culture have also been implemented. Worldwide eradication of smallpox occurred in 1977 (Fenner 1977).

Poxviruses consist of a large and diverse group of viruses which infect numerous species. The sub-family Chordopoxvirinae consists of the orthopox viruses, primarily infecting mammalian species. The genus includes variola virus (or smallpox) which is human specific, camelpox virus, cowpox virus, ectromelia virus (or mouse pox virus), monkey pox virus, vaccinia virus (VV), buffalo pox virus, rabbit pox virus, the capripoxviruses (also known as sheep pox virus), leporipoxvirus (or the myxoma virus of rabbits) and suipoxvirus (swine pox) (Fenner 1979; Fenner 2000). Avipoxviruses replicate productively only in avian species. They include fowlpox (FPV) (Boyle and Coupar 1988), canarypox (Lancet 1992), Quailpox, Turkeypox (Davies and Mungai 1978), and pigeonpox viruses (Winterfield and Reed 1985).

Poxviruses are enveloped viruses. They vary considerably in size, ranging from 140-260nm in diameter to 220-450nm long (Fenner 1979; Fenner 2000) (Figure 8). They are the largest known viruses and they can be visualised at the limit of high-quality light microscopes. They have an external coat composed of host cell lipid, which encloses two lateral bodies and a large double-stranded DNA genome which is encapsulated within the viral core (Fenner 1979; Field 1982). The receptor(s) for viral attachment to cells are not known (Figure 8). The diverse range of cell types that can become infected with poxviruses suggests that either several different receptors are utilised, or that the specific receptor is expressed ubiquitously on many cell types. Poxviruses notably contain all of the genetic material required for replication to occur in the cytoplasm of infected cells (McFadden 2005; Moss 2006) (Figure 8).

The poxvirus is uncoated after entering a cell (Moss 2006). Approximately 50% of the genome is expressed, under the control of the poxvirus early gene promoters. The viral genome is then replicated in the form of linear, double-stranded DNA, concatamers

(Boyle and Coupar 1988; Paoletti 1996; Boyle, Anderson *et al.* 2004). This is followed by expression of intermediate and late genes which enable assembly and maturation of virions. Envelopment occurs prior to release from the cell. Both enveloped (EEV) and intracellular non-enveloped (INV) forms of poxviruses have been described. It is thought that INV are involved in cell-to-cell transmission and therefore do not require envelopment. The mechanism responsible for species-specificity of these viruses has not been defined, but it is thought to occur either in the intermediate or late gene expression phase of the poxvirus lifecycle (Boyle and Coupar 1988; Paoletti 1996; Boyle, Anderson *et al.* 2004) (Figure 8).

### 1.12.1 Poxvirus Vaccines

The advent of molecular biological tools, particularly the ability to introduce foreign genes into viral genomes, has opened up many opportunities to develop and test novel vaccine strategies. Early work by Boyle and colleagues described the construction and the efficacy of some of the first recombinant FPV (rFPV) vectors for delivery of vaccine antigens (Boyle and Coupar 1986; Boyle, Coupar *et al.* 1987; Boyle and Coupar 1988). In these studies, DNA sequences encoding influenza A haemagglutinin were inserted with the aid of “shuttle vectors” into the non-essential thymidine kinase gene of the mild vaccine strain of FPV (FPV-M3) (Boyle and Coupar 1986; Boyle, Coupar *et al.* 1987; Boyle and Coupar 1988). Chickens vaccinated with this rFPV developed peak serum anti-haemagglutinin antibodies 30 days after inoculation. These early rFPV studies opened up an exciting new field of vaccinology. This interest is due to the broad range of cell types that are able to be infected, the reliable expression of early poxvirus promoters (to which foreign DNA sequences of a gene of interest can be inserted), and non-productive nature of the infection in mammals including humans. The latter ensures the safety of recombinant poxviruses as vehicles for delivery of recombinant antigen (Roizman and Jenkins 1985; Boyle and Coupar 1986; Boyle, Coupar *et al.* 1987; Boyle

**Figure 8. Poxvirus lifecycle**

Poxviruses attach to the cell via receptor (s) unknown. Following entry to a cell, the virus is uncoated and approximately 50% of the genome is expressed, under the control of the poxvirus early gene promotors prior to replication of the viral genome. The viral genome is then replicated as linear, double-stranded DNA concatamers. Intermediate and late gene expression allows assembly and maturation of virions prior to envelopment and release from the cell. Both enveloped (EEV) and intracellular non-enveloped (INV) forms of poxviruses have been described. It is thought that INV are involved in direct cell-to-cell transmission.

Adapted from http://www.micro.msb.le.ac.uk/3035/Poxviruses.html

and Coupar 1988; Moss 1991; Boyle 1992; Boyle and Heine 1993; Heine and Boyle 1993; Boyle and Heine 1994; Heine, Hyatt *et al.* 1994; Boyle, Pye *et al.* 1997; Heine, Foord *et al.* 1997; Hertig, Coupar *et al.* 1997; Boyle, Anderson *et al.* 2004).

### 1.12.2 Fowlpox virus

FPV is the type member of the Avipoxvirus genus. It causes infection of chickens (*Gallus domesticus*) and there are no reports of natural FPV infection in ducks. FPV virions are large, enveloped particles containing a linear double-stranded DNA genome of approximately 300kb. When used for vaccination, rFPV is usually injected via intradermal or intramuscular routes which results in expression of the gene(s) of interest under the transcriptional control of early FPV promoters (Fenner 1979; Boyle and Coupar 1986; Boyle, Coupar *et al.* 1987; Boyle, Anderson *et al.* 2004; Coupar, Purcell *et al.* 2005). Transient infection ensures high-level antigen expression and robust $CD4^+$ and $CD8^+$ T-cell responses (Ramsay, Kent *et al.* 1999; Boyle, Anderson *et al.* 2004; Coupar, Purcell *et al.* 2006; Dale, Thomson *et al.* 2006). These responses are presumably due to the intracellular expression of viral antigens possibly in local APC and presentation to T cells in the context of MHC I and/or MHC II proteins. rFPV have been used for vaccine antigen delivery in a number of studies (Heine and Boyle 1993; Heine, Hyatt *et al.* 1994; Heine, Foord *et al.* 1997; Hertig, Coupar *et al.* 1997; Kent, Zhao *et al.* 1998; Dale, Zhao *et al.* 2000; Kent, Zhao *et al.* 2000; Boyle, Anderson *et al.* 2004; Dale, De Rose *et al.* 2004). Importantly, different rFPV vaccines, expressing vaccine antigens from diverse infectious agents such as HIV and malaria, have now been produced and approved for use in humans (Moorthy, Imoukhuede *et al.* 2004; Hutchings, Gilbert *et al.* 2005; Coupar, Purcell *et al.* 2006; Dale, Thomson *et al.* 2006).

### 1.12.3 Poxvirus vaccines developed to treat hepadnavirus infection

An early study tested the ability of the related vaccinia virus to deliver DHBV antigens. In these experiments, the pre-S/S gene of DHBV was cloned behind early vaccinia virus promoters. The recombinant vaccinia virus (rVV) was then used as a therapeutic vaccine to immunise ducks chronically DHBV-infected. In this study, immunisation only resulted in transient decreases in levels of serum DHBsAg and no ducks resolved their DHBV infection (Ma, Kong *et al.* 1996). It would have been interesting to see the outcome of these experiments if ducks had been treated with antiviral drugs prior to rVV vaccination.

Using a similar strategy, encouraging results were obtained in a study involving one chimpanzee chronically infected with HBV. In this study, two priming doses (2mg each) of DNA vaccines expressing HBpre-S1 and HBsAg were given, separated by 4 weeks. This was followed by two boosting doses of recombinant canarypox expressing HBpre-S1, HBpre-S/2 and HBsAg at weeks 15 and 28. Remarkably, this vaccine regime lowered the levels of serum HBV DNA to undetectable rates for at least 212 weeks. The loss of HBV DNA coincided with a reduction in liver transaminase flares, decreases in cccDNA detected in the liver, and increases in levels of IFN-γ secreting cells (Pancholi, Lee *et al.* 2001).

Based on these results, a similar study was conducted with a second chimpanzee that had a high viral load (8.6 $\log_{10}$ DNA copies/ml) (Shata, Pfahler *et al.* 2006). In the first phase for the study, 1mg of plasmid DNA encoding HBsAg and Pre-S2 was administered three times over a period of three months. Twenty-four weeks after the last DNA vaccination, $5x10^8$ plaque forming units (pfu) of recombinant canarypox virus expressing HBsAg, Pre-S1, Pre-S2 and HBcAg was administered. No decrease in HBV

viral load was observed following this multi-pronged vaccine strategy. The same chimpanzee then entered phase 2 of the study. In this phase, it was treated with the antiviral drug lamivudine for eight weeks, resulting in a 3 $\log_{10}$ decline in HBV load. Following cessation of lamivudine treatment, the chimpanzee was immunised with 4 mg of a DNA vaccine encoding HBsAg, Pre-S2 and HBcAg. Viral loads returned to pre-treatment levels within one week after lamivudine was withdrawn. In the third and final phase of the study, lamivudine treatment was recommenced for 18 weeks, which resulted in a 4.3 $\log_{10}$ drop in viral load. The chimpanzee was then vaccinated with rVV vectors expressing HBsAg, Pre-S1, Pre-S2, and HBcAg. Lamivudine treatment continued for eight weeks after vaccination. Because lamivudine was ceased inadvertently for a period of nine days, the animal received another booster immunisation with the same rVV vectors, administered under the cover of lamivudine for a further four weeks. Although this combination vaccine regime did induce detectable CMI in the form of increases in $CD8^+$ and $CD4^+$ $T_H1$ responses against the vaccine antigens, it was disappointing as a strategy for therapeutic HBV immunisation as the levels of HBV DNA rapidly returned to pre-treatment levels after withdrawal of lamivudine. It was concluded that the high viral load present in the second chimpanzee prior to treatment may have been responsible for the poor therapeutic response that was observed. This suggests that if another antiviral drug, such as Entecavir, which has been shown to reduce the levels of HBV DNA by 2 $\log_{10}$ lower than lamivudine, a more favourable outcome may have been achieved (Lai, Rosmawati *et al.* 2002; Chang, Gish *et al.* 2006; Lai, Shouval *et al.* 2006). It has also been shown that dosage rates of both the DNA vaccine and the poxvirus are critical for optimal immune responses. For example, the doses of DNA vaccines and rFPV that encode HIV antigens that were immunogenic in macaques were found not to be immunogenic in humans (De Rose, Sullivan *et al.* 2006). Additionally, responses by humans to the rFPV-HIV antigen

constructs were greater when the doses of DNA vaccines and rFPV were increased (De Rose, Sullivan *et al.* 2006). This research concluded that future vaccine trials assessing DNA prime and rFPV boost regimes should be based on estimated body surface area (EBSA), as is used in pharmokinetic and drug dosing studies (Mosteller 1987).

## 1.13 Antiviral treatment of chronic HBV infection

Antiviral drugs specifically inhibit one or more steps of virus replication without causing unacceptable side effects. The key benefit afforded by currently approved anti-HBV drugs is their ability to reduce viral loads which ultimately results in reduction in the number and severity of elevations of serum aminotransferases or histologically active disease and therefore, reducing the incidence of cirrhosis and HCC (Hoofnagle 1998; Yim and Lok 2006)**.**

Current therapeutic interventions against chronic HBV infection employ nucleoside/nucleotide analogues or IFN-α. However, the efficacy of these treatments is low, with sustained responses and seroconversion from HBe-positive to HBe-negative plus anti-HBe antibody status occurring in only 10 to 30% of patients (van Zonneveld, Honkoop *et al.* 2004; Wright 2004). It is assumed that those chronically infected patients who respond to nucleoside analogues and IFN-α therapy do so because they have some immunity to the virus, compared to non-responders. Evidence for this is the presence of more active liver disease in the responding patients at the initiation of therapy and in some cases HBV-specific $CD8^+$ CTL as assessed by *in vitro* assays (van Zonneveld, Honkoop *et al.* 2004; Wright 2004). Increases in transaminase activity during effective IFN-α therapy for chronic HBV infection suggests that an acute hepatitis occurs which eliminates the infection in a similar manner to the clearance of a transient HBV infection, that is, through the production of inflammatory cytokines to

block virus replication, induce antigen-specific CTL to kill infected hepatocytes and neutralizing antibodies to prevent reinfection of cells preventing virus rebound (Hoofnagle 1998; van Zonneveld, Honkoop *et al.* 2004).

Another class of widely used anti-HBV inhibitors are the nucleoside and nucleotide analogues which are functional derivatives of naturally occurring deoxyribonucleotide precursors. They include Lamivudine (deoxycytidine analogue developed by GlaxoSmithKline), Adefovir dipivoxil (deoxyadenosine analogue developed by Gilead) and Entecavir (ETV) (a deoxyguanosine analogue developed by Bristol-Myers Squibb). Once nucleoside analogues enter a cell, they require cellular enzymes to convert them to their active triphosphate forms. In this active form they are able to be incorporated into nascent DNA chains resulting in premature termination of DNA synthesis (Pastor-Anglada, Felipe *et al.* 1998) and thus, inhibiting hepadnaviral replication and suppressing HBV viral load in humans (Locarnini and Mason 2006), woodchucks (Mason, Cullen *et al.* 1998; Cullen, Li *et al.* 2001) and ducks (Colledge, Locarnini *et al.* 1997; Nicoll, Colledge *et al.* 1998).

As previously mentioned one of the newer generation nucleoside analogues used to treat chronic HBV infection is ETV. Several modes of action against HBV have been reported including inhibition of the priming activity of the hepadnaviral polymerase, reverse transcription, and positive strand DNA synthesis. These features, in combination with the weak inhibition of normal cellular and mitochondrial DNA polymerases (Seifer, Hamatake *et al*. 1998, the efficient intracellular conversion to its triphosphate form {Yamanaka, 1999 #828; Levine, Hernandez *et al.* 2002), the low therapeutic doses required (0.5 to 1.0 mg in humans) and the exceptional effectiveness in animal models (Colonno, Genovesi *et al.* 2001; Marion, Salazar *et al.* 2002), cell culture (Innaimo, Seifer *et al.* 1997; Ono, Kato *et al.* 2001; Villeneuve, Durantel *et al.* 2003) and in

clinical studies (Chang, Gish *et al.* 2006; Lai, Shouval *et al.* 2006) made it the drug of choice to test against DHBV in the Hepatitis B Virus Research Laboratory. For example, treatment of chronically infected ducks with ETV resulted in clearance of DHBV DNA and DHBsAg from the bloodstream and a 4-log decrease in the levels of DHBV DNA from the liver (Foster, Miller *et al.* 2003). In a more recent study, administration of ETV to ducks at the time of high-dose DHBV challenge resulted in a 50% decrease in the number of ducks developing chronic DHBV infection (Foster, Miller *et al.* 2005), suggesting that ETV could provide additional protection against HBV infection if incorporated into existing post-exposure treatments.

Although antiviral drugs provide significant beneficial effects against chronic HBV infection, sustained reductions of HBV levels are rarely maintained as in most cases the serum levels of HBV DNA and HBsAg return to pre-treatment levels shortly after the drug is withdrawn (Lavanchy 2004). The other ongoing challenge in the use of anti-viral drugs is the limited effective therapeutic duration due to the emergence of drug-resistant mutants (Locarnini 2005; Yuan and Lee 2007). Such mutants arise from the inherent error rate associated with reverse transcription and from the high replication rate of HBV in humans. For example, if it is assumed that the error rate of the HBV reverse transcription is similar to that estimated for human immunodeficiency virus (HIV) ($1x10^{6}$ base substitutions per nt per replication cycle) (Roberts, Bebenek *et al.* 1988), and that $10^{13}$ HBV virions are produced daily (Perelson 1999), it can be calculated that all single, (and most double) mutations could be created within the time-frame of one day (Whalley, Murray *et al.* 2001). This also suggests that these drug-resistant mutants may already be present at low-levels within some HBV-infected individuals. The mutants would naturally then be selected by their greater relative fitness allowing for their preferential growth over wild-type HBV. Based on these

observations, it is most likely treatments based on the use of a single antiviral drug will fail. Therefore, the most promising avenue for a cure for chronic HBV infection is multiple combinations of antiviral drugs, including IFN-α in combination with novel vaccine regimens.

## 1.14 Development of a combination treatment against DHBV infection

As the next step towards development of a therapeutic DHBV vaccine, a combination approach was tested as presented in Chapter 5 of this thesis (Miller, Boyle *et al.* 2008). In these studies, ducks were challenged with $5x10^8$ DHBV genomes on day 14 of age and then immediately treated with ETV and vaccinated intramuscularly with the priming DNA vaccines encoding DHBpre-S/S, DHBs and DHBcAg. Seven days later ducks were boosted intramuscularly with two different rFPV vaccines encoding DHBpre-S/S and DHBcAg. DHBV infection was observed in the liver of all ducks at day 4 p.i., with reduced numbers of infected cells in all ETV-treated ducks. Analysis of biopsy and autopsy tissues collected at days 14 p.i. and 67 respectively revealed that all 10/10 ducks that received ETV in combination with prime-boost vaccination had resolved their DHBV infection. In concordance with previously published results, ~50% of ducks treated with ETV and the vaccine vectors also resolved their infection whilst the remaining 50% developed chronic DHBV infection (Foster, Miller *et al.* 2005). Prime-boost vaccination alone (without ETV) also protected 1 in 5 ducks. Conversely, 5 of 5 of non-ETV treated, non-vaccinated controls developed chronic infection.

Although the mechanism of protection was not successfully elucidated primarily due to the lack of sensitive assays to measure CMI in ducks, it was assumed again that the protection was the combined result of reduction of circulating and liver-associated

DHBV by ETV, neutralisation of DHBV virions in the bloodstream by anti-DHBs antibodies, and finally the targeting and killing of infected hepatoctyes by DHBpre-S/S and DHBc-specific CTL.

This study demonstrated for the first time that combined post-exposure treatment with ETV and prime-boost vaccination has the potential to induce immune responses that prevent establishment of chronic infection with DHBV in ducks. Further studies are being conducted to assess the therapeutic efficacy of the ETV and prime-boost vaccination protocols in chronically DHBV-infected ducks to determine if this regimen could provide therapeutic responses in chronically infected humans.

# Chapter 2

# Studying host immune responses against duck hepatitis B virus infection.

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***Methods in Molecular Medicine*** 96, 3-26 (2004)

Miller, D.S., Bertram, E.M., Kotlarski1, I. and Jilbert, A.R. (2004) Studying host immune responses against duck hepatitis B virus infection.
*Methods in Molecular Medicine v. 96, pp. 3-26, 2004*

# Chapter 3

# DNA vaccines expressing the duck hepatitis B virus surface proteins lead to reduced numbers of infected hepatocytes and protect ducks against the development of chronic infection in a virus dose-dependent manner.

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***Virology*** 351,159-69 (2006)

Miller, D.S., Kotlarski1, I. and Jilbert, A.R. (2006) DNA vaccines expressing the duck hepatitis B virus surface proteins lead to reduced numbers of infected hepatocytes and protect ducks against the development of chronic infection in a virus dose-dependent manner
*Virology, v. 351 (1), pp. 159-169, 2006*

# Chapter 4:

# Vaccination of ducks with a whole-cell vaccine expressing duck hepatitis B virus core antigen elicits antiviral immune responses that enable rapid resolution of *de novo* infection.

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***Virology*** 348, 297-308 (2006).

Miller, D.S., Halpern, M., Kotlarski1, I. and Jilbert, A.R. (2006) Vaccination of ducks with a whole-cell vaccine expressing duck hepatitis B virus core antigen elicits antiviral immune responses that enable rapid resolution of de novo infection. *Virology, v. 348 (2), pp. 297-308, May 2006*

# Chapter 5:

# Antiviral therapy with Entecavir combined with post-exposure "prime-boost" vaccination eliminates duck hepatitis B virus infected hepatocytes and prevents the development of persistent infection.

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*Virology* 373:329-341 (2008).

Miller, D.S., Boyle, D., Feng, F., Reaiche, G.Y., Kotlarski1, I., Colonno, R. and Jilbert, A.R. (2008) Antiviral therapy with entecavir combined with post-exposure "prime-boost" vaccination eliminates duck hepatitis B virus-infected hepatocytes and prevents the development of persistent infection
*Virology, v. 373 (2), pp. 329-341, April 2008*

# Chapter 6: Discussion

The primary aim of this thesis was to test novel vaccination regimes designed to stimulate resolution of chronic DHBV infection. A vital tool to achieving this goal was the development of assays with which to measure the spread of DHBV through the liver and bloodstream, and to assess the rapidity and magnitude of humoral immune responses to both DHBV infection and vaccination with DHBV antigens.

## 6.1 Assay development

Chapter 2 of this thesis described and evaluated ELISA, PCR and immuno-histochemical assays for monitoring the kinetics of viral infection and the humoral immune responses activated following DHBV infection. These assays are reliable and sensitive and can usually be performed within 2-3 hours. The techniques have since been used extensively within the Hepatitis B Research Laboratory, and they have made a significant contribution to several publications, in addition to those in this thesis (Jilbert, Miller *et al.* 1996; Jilbert and Kotlarski 2000; Jilbert, Botten *et al.* 1998; Foster, Miller *et al.* 2005; Foster, Miller *et al.* 2003; Triyatni, Jilbert *et al* 1998; Meier, Scougall *et al.* 2003; Le Mire, Miller *et al.* 2005). Although development of ELISA assays to detect DHBsAg were successful, attempts to develop an ELISA that could detect DHBe and anti-DHBe antibodies in the serum of DHBV-infected ducks were not successful. Further research focusing on the development and validation of assays related to DHBe should provide additional information about the kinetics and immune responses to DHBV infection.

Assays to measure humoral immune responses in ducks are now available; it is also imperative to develop reliable and sensitive assays to measure cellular immune

responses against DHBV and following vaccination. Established techniques, such as proliferation and CTL assays, ELISPOT and intracellular cytokine staining, have been successful in measuring mitogen-induced duck T-cell responses (Jilbert and Kotlarski, 2000; Miller, Bertram *et al.* 2004; unpublished data, Stephen Blake Honours thesis 2004). However, measurement of vaccine-induced and DHBV-specific CMI responses has remained elusive, despite a number of attempts by several researchers from the Hepatitis B Research Laboratory. Real-time reverse transcription PCR assays specific for the duck cytokines, IFN-γ, IFN-α and TNF-α, for T-cell specific markers including CD4, CD8, CD3, MHC I, MHC II and for markers of the innate immune response including TLR-2, TLR-4 and TLR-7 have been developed recently within the laboratory (Reaiche, manuscript in preparation). These molecular tools, combined with monoclonal antibodies specific for Kupffer cells within the liver as described in Chapter 2 of this thesis (2E12, John Pugh unpublished data) and the recent addition of monoclonal antibodies specific for duck $CD4^+$ and $CD8^+$ T-cells (Kothlow, Mannes *et al.* 2005), should enable further insights into the immune mechanism(s) operating after vaccination and DHBV challenge.

A method for precipitation of DHBV from serum using polyethylene glycol was developed as also described in Chapter 2. It is a simple and inexpensive method to concentrate DHBV and remove most inhibitors of PCR. This extraction method enables sensitive, semi-quantitative PCR detection, with a limit of sensitivity of 500 copies/ml of DHBV DNA. The recent emergence of commercially available extraction kits and real-time PCR has delivered expensive but quantitative PCR assays, with sensitivities down to 100 copies/ml of DHBV DNA in serum using a High-Pure kit (#236-125) manufactured by Roche (Foster, Miller *et al.* 2005) and 10 DHBV DNA copies in 52, 000 hepatocytes using a DNeasy kit (#69505) from Qiagen as described in Chapter 5

(Miller, Boyle *et al.* 2008). However, a major drawback to using highly-sensitive PCR assays to detect low-levels of DHBV DNA in samples is the real possibility of contamination. To minimise this, all low-level DNA containing samples must be extracted in a different physical location to where high-titre samples are being processed or similar PCR reactions are being analysed and known-negative samples must also be employed at all times at several stages of the extraction process to exclude any false-positives.

Of the assays developed during this research, detection of DHBsAg-positive cells in the liver proved to be the most reliable and sensitive marker of DHBV infection. This technique is easy and robust to perform. It does not require expensive and specialised reagents or equipment and does not suffer from the possibility of low-level contamination. When used at optimised concentrations, the non-specific or background staining of the 1H.1 monoclonal antibody (Pugh, Di *et al.* 1995) is negligible. Furthermore, immunoreactive-cells are not detected in DHBV-negative liver tissue, indicating that the assay has an extremely low false-positive percentage. The physical size of biopsy and autopsy tissues analysed significantly contributes to the sensitivity of this assay. For example, biopsy samples of approximately 5mm x 5mm x 3mm in size corresponding to approximately 300,000 cells in each 6 μm thick section are analysed. Use of these large tissue samples and the high-specificity of the 1H.1 monoclonal antibody allows for precise visualisation and enumeration of small groups and even single DHBV-infected hepatocytes within the liver.

Optimisation of the challenge dose is essential if the outcomes of vaccine studies are to be consistent. Therefore, the establishment of the optimal dose that reproducibly resulted in chronic DHBV infection was an essential precursor to the vaccine studies

(Figure 5). By allowing quantitative determination of comparative vaccine efficacy, candidate vaccines could be selected for expensive, longer-term studies. Using these pre-determined challenge doses, three novel vaccine strategies were tested.

## 6.2 DNA Vaccine studies

Early reports indicated that naked DNA vaccination would be the “silver bullet” of vaccine technology. Unfortunately, although successful in generating impressive immune responses in mice, this technique does not appear to be as robust in larger animals, including humans. This conclusion was mirrored by the results of the naked DNA vaccine study presented in Chapter 3. Significant, but sub-optimal protection, particularly at higher challenge doses, was observed using DNA vaccines encoding DHBs and DHBpre-S/S antigens. This observation suggested that further investigations were necessary into methods that might increase the uptake, expression and hence immunogenicity of DNA vaccines.

To ensure efficient transfection and uptake of DNA vaccines, whole cell vaccines were developed as described in Chapter 4. In these studies, extensive optimisation of the isolation and transfection conditions for PDEF was performed prior to testing of the vaccine formulation in ducks. It was found that electroporation of PDEF with the DNA vaccines was by far the most economical and efficient method to produce the numbers of DHBcAg expressing cells required. For example, $1x10^9$ transfected PDEF of which 20-40% expressed DHBcAg (equivalent of 50 doses) were produced relatively simply 48 hr days prior to vaccination. Other methodologies to administer DHBcAg expressing cells such as vaccination with stably transfected chicken hepatoma cells (LMH) or infection of cells with rFPV that express DHBV antigens were not tested in these preliminary studies described in Chapter 4 but could be readily applied in the future.

In three separate vaccine experiments, ducks were vaccinated intramuscularly with PDEF expressing DHBcAg. Significant protection was afforded to ducks vaccinated with this regimen as 17/22 ducks resolved their DHBV infection whereas 1/11 non-transfected PDEF vaccinated ducks resolved its infection. The remaining ducks in this study developed chronic DHBV infection with readily detectable DHBsAg in the bloodstream and widespread DHBsAg-positive hepatocytes throughout the liver. Neutralisation of inoculum was not responsible for the observed protection as anti-DHBc antibodies are non-neutralising. Also, on day 4 p.c. the percentage of DHBV-positive hepatocytes in the ducks vaccinated with DHBc-expressing PDEF was not significantly different to the numbers of DHBsAg-positive hepatocytes present in the control ducks. Again it was impossible for us to establish the mechanism for clearance of DHBV-infected hepatocytes in vaccinated ducks, but we can hypothesise that clearance was probably the result of a CTL response. If we extrapolate from other models we can presume that if macrophages, dendritic or $CD8^+$ T cells, primed as the result of vaccination with PDEF expressing DHBcAg encounter DHBcAg peptide(s) presented with MHC I complexes on the surface of DHBV-infected hepatocytes, a CMI response will occur. Typically, this response is characterised by increased secretion of locally produced type I IFN such as IFN-$\alpha$ and IFN-$\gamma$ resulting in degradation of viral nucleic acid, up-regulation of MHC I molecules and apoptosis of neighbouring DHBV-infected cells. Increased secretion of IL-12 also should occur resulting in stimulation of additional IFN-$\gamma$ synthesis, further promoting the CMI bias. Dendritic cells, DHBcAg-specific $CD8^+$ CTL and NK cells (presuming that similar lineages of cells are functional within the duck) would also be stimulated by the IFN response resulting in perforin-mediated lysis of DHBV-infected hepatocytes by direct antigenic engagement to the infected hepatocyte. In the case of NK and dendritic cells lysis would most probably

occur following Fc-dependant recognition of anti-DHBc antibodies bound to DHBcAg on the surface of DHBV-infected hepatocytes (Figure 7).

Having hypothesised that the mechanism of clearance was probably mediated primarily by CMI, it is clear that the whole-cell regimen also stimulated humoral immunity, as anti-DHBc antibodies were readily detectable in serum of vaccinated ducks prior to DHBV challenge. This presumably is the result of uptake of exogenous PDEF-expressed DHBcAg via the endocytic pathway which is then degraded and presented by APC in context with MHC II (Figure 7). Although the ELISA to detect anti-DHBc and anti-DHBs antibodies are different, the data presented in Chapters 3 and 4 show that following vaccination with the naked DHBs encoding DNA vaccines, anti-DHBs antibodies were not detectable until after challenge with DHBV (10 days). In contrast, anti-DHBc antibodies were detected in the serum of ducks as early as 4 days after the initial vaccination with PDEF expressing DHBcAg. This observation therefore raises the question as to how effective a vaccination using a whole-cell vaccine expressing DHBsAg would be. Given that anti-DHBs antibodies, unlike anti-DHBc antibodies, bind and neutralise DHBV infectivity, it is probable that the efficacy of a whole-cell vaccine expressing DHBsAg may exceed that of a naked DNA vaccine encoding DHBs. Therefore, vaccination with PDEF transfected with DHBsAg alone or in combination with DHBc DNA vaccines warrants further investigation in future studies.

Whether the MHC haplotype of the fibroblasts expressing DHBcAg or cell survival affected presentation of DHBcAg derived peptides remains unresolved. The breeding flock that provided the experimental birds consists of approximately 2000 layers hens. It is a “closed-colony”, but not inbred. Therefore, several different MHC haplotypes are likely to be present in the population. Since the whole-cell vaccines were prepared from

pooled fibroblasts isolated from approximately twelve eggs per vaccine dose, it is possible that a mixture of MHC haplotypes were present in the vaccine. In the case of cells sharing MHC alleles with the recipient, direct presentation of processed, endogenously synthesised antigen in association with MHC I could occur. In the case vaccine cells that do not share MHC alleles with the recipient, viral antigen might be taken up and presented indirectly in association with MHC II by host APC. Nevertheless, host APC could also present vaccine antigens in association with MHC I via cross-presentation. As inbred strains of ducks are not available, further investigation into the roles of MHC haplotype matching, and resultant generation of immunity, would be more appropriately tested in established inbred rodent models.

## 6.3 ETV Treatment in Combination with Prime-Boost Vaccine Strategies

ETV is a guanosine nucleoside analogue which is highly effective against HBV, WHV and DHBV (Colonno, Genovesi *et al.* 2001; Levine, Hernandez *et al.* 2002; Marion, Salazar *et al.* 2002; Foster, Miller *et al.* 2003; Foster, Miller *et al.* 2005). ETV competes with cellular guanosine triphosphate for incorporation into nascent DNA chains ultimately resulting in premature DNA chain termination, and hence prevention of conversion of the input RC hepadnaviral genomes into cccDNA (Yamanaka, Wilson *et al.* 1999). In the case of chronic hepadnaviral infection, nucleoside analogues are able to lower the viral load but do not target the pre-formed and highly stable cccDNA, which is the replicative source of the virus.

As discussed in relation to Chapters 3 and 4 of this thesis, and in previous publications, DNA vaccines expressing DHBcAg *or* DHBsAg can provide protective immune responses against DHBV infection in ducks (Triyatni, Jilbert *et al.* 1998; Rollier,

Sunyach *et al.* 1999; Miller, Halpern *et al.* 2006; Miller, Kotlarski *et al.* 2006). Theoretically then, inclusion of both DHBc and DHBpre-S/S into a DNA vaccine schedule should have additive effects on efficacy. However in an earlier study, vaccination of chronically DHBV-infected ducks with DNA vaccines encoding DHBpre-S/S, DHBs, DHBc and DHBpre-C/C antigens in combination with ETV did not provide additional therapeutic effects over ETV alone (Foster, Miller *et al.* 2003). In this study chronically DHBV-infected ducks received daily doses of ETV in combination with five doses of the above DNA vaccines. Treatment with ETV resulted in a 4-$\log_{10}$ drop in serum DHBV DNA levels and a 2- to 3-$\log_{10}$ drop in serum DHBsAg. Disappointingly, withdrawal of ETV resulted in rebound of levels of serum DHBV DNA and DHBsAg to those in the water-treated animals within 40 days suggesting that additional immune pressure would be required if therapeutic effects were to be achieved.

It was also shown previously that ETV administered alone at the time of infection could protect against chronic infection in approximately 50% of ducks (Foster, Miller *et al.* 2005). This finding suggested that inclusion of novel vaccines designed to induce both enhanced humoral and cellular immune responses with ETV could increase the proportion of ducks that are able to resolve their DHBV infection. It had long been known that recombinant pox viruses were effective delivery vehicles for vaccine antigens (Roizman and Jenkins 1985; Boyle and Coupar 1986; Boyle and Coupar 1988; Moss 1991; Lancet 1992; Boyle and Heine 1993; Heine and Boyle 1993; Paoletti 1996; Heine, Foord *et al.* 1997) and more recently it was shown in mice that if a DNA vaccine priming immunisation was administered shortly prior to rVV immunisation the magnitude of $CD4^+$ and $CD8^+$ CTL responses increased above those animals that received either the DNA vaccine or rVV alone (Ramsay, Kent *et al.* 1999). Further to

this finding was the encouraging result where a chronically infected chimpanzee resolved chronic HBV infection shortly after being prime-boosted with DNA vaccines and recombinant canarypox vaccines expressing HBsAg (Pancholi, Lee *et al.* 2001).

Based on these results, the DHBpre-S/S and DHBc genes were cloned and introduced into the genome of the FPV vaccine strain FPV-M3 to create the strains FPV-pre-S/S and FPV-DHBc (Boyle and Coupar 1986; Boyle, Coupar *et al.* 1987; Boyle and Heine 1993; Boyle, Anderson *et al.* 2004; Coupar, Purcell *et al.* 2006). The ability of the resulting rFPV to express their respective DHBV antigen was initially assessed *in vitro*. In these experiments, PCEF and PDEF were infected with either rFPV strain. As expected, death of most cells of the monolayer was complete within 24 hr following rFPV infection of PCEF where as PDEF monolayers were able to be cultured for up to 72 hr following infection with minimal impact on monolayer integrity, suggesting that rFPV were not able to replicate efficiently in duck cells. Antigen expression was evaluated by immunofluorescence staining of infected monolayers. At a MOI of 5:1, >95% of the cells within these monolayers expressed either DHBpre-S/S or DHBcAg respectively. This is in contrast to experiments where PDEF were transfected with the DNA vaccine encoding DHBcAg as desrcribed in Chapter 4. In these transfection experiments, only 20-40% of the monolayer expressed DHBcAg. In light of these results, it is hypothesised that vaccination of ducks with PDEF infected with our rFPV would be a straightforward and effective means to further test the potential of whole-cell vaccines in the future.

The newly derived rFPV expressing DHBc and DHBpre-S/S antigens were then tested in 7-day-old ducks for safety. Ducks were inoculated into the wing-web with a bifurcated needle then monitored on a daily basis for two weeks. It was expected that a

"pox lesion" would be evident at the site of inoculation as is observed in chickens (Boyle and Coupar 1988; Boyle and Heine 1993; Boyle and Heine 1994; Boulanger, Smith *et al.* 2000). No lesions were observed over this time period further confirming that the rFPV did not actively replicate in ducks. As no adverse reactions were observed in this small-scale study it was decided that the rFPV expressing DHBV antigens should be tested in prime-boost studies in ducks infected with DHBV.

In these experiments as presented in Chapter 5, 14 day-old ducks were challenged with $5x10^8$ DHBV genomes then immediately given ETV and the DNA vaccine "priming" injection containing DNA vaccines expressing DHBpre-S/S, DHBs and DHBc (Triyatni, Jilbert *et al.* 1998; Miller, Halpern *et al.* 2006; Miller, Kotlarski *et al.* 2006). Seven days following, ducks received both the rFPV-DHBc and rFPV-DHBpre-S/S boosting injections. Using this regimen, 100% of the vaccinated ducks resolved their DHBV infection by day 14 p.i. In contrast, all of the control ducks that received water in replace of ETV and the vaccine vectors developed chronic infection. This was a highly significant result considering treatment was initiated shortly after the time of infection.

Certainly, the inclusion of ETV in our protocol provided additional benefits by inhibiting spread of input DHBV. In this current study, ~50% of the ducks that received the vaccine vectors and ETV were able to resolve DHBV infection. This result is in concordance with another study where ducks were treated with ETV alone (Foster, Miller *et al.* 2005). Therefore, this data strongly advocates that all subsequent prime-boost vaccine experiments should include ETV or other antiviral drugs for optimal efficacy.

Again, the mechanism of protection must be hypothesised upon. Several studies have been performed using similar prime-boost strategies albeit in the absence of antiviral drugs (Ramsay, Kent *et al.* 1999; Dale, Zhao *et al.* 2000; Dale, De Rose *et al.* 2004; Kent, Dale *et al.* 2005; Dale, Thomson *et al.* 2006; De Rose, Sullivan *et al.* 2006). In all of these studies, vaccination resulted in increases of IgG2a *in vivo* and following antigenic stimulation *in vitro*, proliferative responses in $CD8^+$ T cells and IFN-γ secretion from $CD4^+$ $T_H1$ cells were detected. It is only presumed that similar mechanisms are operational within the duck. As to why DNA vaccination followed by recombinant poxvirus boosting (DNA-poxvirus) provides enhanced responses over DNA-DNA, poxvirus-poxvirus or even poxvirus-DNA regimens is currently unknown and also warrants further investigation (Ramsay, Kent *et al.* 1999).

The importance of the DNA vaccine prime in achieving the therapeutic effect requires discussion. Infection with DHBV could itself have primed the immune response. Therefore, in this setting vaccination with the rFPV constructs alone might be sufficient to augment similar post-infection immunity. Therefore, additional experiments to assess the importance of DNA priming and the underlying mechanisms responsible (including which epitopes of DHBV elicit the best responses) are currently under investigation within the Hepatitis Research Laboratory. In these experiments, the efficacy of ETV treatment combined with DNA vaccines and rFPV vaccines alone and also “prime-boost” vaccination with DNA vaccines and rFPV strains expressing either DHBc or DHBpre-S/S as a single vaccine antigen are being tested. Although multiple vaccine antigens may induce broader humoral and CMI responses, it is possible that the DHBc or pre-S/S antigens alone may be sufficient to provide protective or therapeutic immune responses.

To achieve therapeutic vaccination against chronic DHBV infection is an extremely challenging goal. The efficacy of the combination ETV-DNA-rFPV therapy that is reported in this thesis is considerably more effective against DHBV than any other vaccine regimen reported in the literature (Triyatni, Jilbert *et al*. 1998; Rollier, Sunyach *et al*. 1999; Rollier, Charollois *et al*. 2000; Rollier, Charollois *et al*. 2000; Foster, Miller *et al*. 2003; Miller, Halpern *et al*. 2006; Miller, Kotlarski *et al*. 2006). However, while prevention of establishment of chronic DHBV infection is a significant step towards achieving this goal, treatment of chronically infected ducks presents an extra level of complexity. The large amount of viral antigen in the bloodstream and the liver of chronically DHBV-infected ducks neutralises circulating antibodies and appears to induce a state of functional tolerance or anergy. This is obviously a major hurdle to overcome if therapeutic vaccination is going to be successful. It is certainly clear from the studies presented in this thesis that a vaccine regimen with only a single modality will not cure chronic DHBV infection and that combination strategies will be required. Based on the results obtained using the ETV-DNA-rFPV regimen it would be desirable to first decrease the viral load to its lowest achievable point with the aid of antiviral agents. It would be prudent in future studies to include a “cocktail” of antiviral drugs to achieve the lowest possible number of DHBV-infected hepatocytes. Under a low-level DHBV load, prime-boost vaccination may then induce antigen-specific CTL responses that eliminate those remaining DHBV-infected hepatocytes and thus result in clearance of the pool of cccDNA from the liver. Absolute reduction in the numbers of DHBV-infected hepatocytes is also important if the generation of a robust CTL response is actually achieved as immune-mediated killing of large numbers DHBV-infected hepatocytes could worsen the disease state.

## 6.4 How these studies relate to HBV infection

A commercially available vaccine provides excellent protection against HBV infection. However, whether used alone or in combination with other treatments, such as pegylated IFN-α and/or antiviral drug it provides no additional long-term therapeutic effects against chronic HBV infection. Additionally, once drug is withdrawn, most recipients experience rapid rebound of virus to pre-treatment levels, and the existing drug resistant mutants are always going to be problematic (Tenney, Levine *et al.* 2004; Fung, Andreone *et al.* 2005; Fung and Fontana 2006; Ryu and Chung 2006). Therefore, different strategies are required to achieve a cure for chronically HBV-infected individuals.

Despite its promise in rodents, the efficacy of naked DNA vaccination has not translated successfully to humans. To address the issue of poor immunogenicity of naked DNA vaccination in larger animals, transfected embryonic fibroblasts were tested as an alternative as described in Chapter 4. Although this vehicle for administration of vaccine DNA was shown to elicit protective CMI in ducks, a number of huge regulatory hurdles would need to be addressed before this form of vaccination could be attempted in humans. Furthermore, it is not clear whether matching or mismatching of HLA haplotype of the donor cells is more advantageous in ensuring optimal response to the vaccine antigen. Autologous fibroblasts would be the safest option to avoid against transfer of infectious agents harboured within donor cells but there would still be a danger that transfection could lead to malignant changes in the cells. In the later sense, allogeneic fibroblasts might be a safer option, as the donor cells would be prevented from permanent engraftment. Finally, the issue as to whether living or dead transfected PDEF are required for induction of robust immune responses is also unanswered. As previously discussed, the electroporation protocol kills ~50% of PDEF, leaving roughly

equal numbers of both living and dead cells in the vaccine preparation. These cells, alive and dead, would certainly establish an adjuvant effect, resulting in sustained responses at the site of injection (Kwissa, Kasturi *et al.* 2007). Therefore, it has been hypothesised that simply coating dead or dying cells with DNA vaccines maybe equally efficacious at inducing protective immune responses as the adjuvant effect of injected cells alone may result in enhance uptake and expression of DNA vaccine constructs. If so, this would significantly simplify vaccine preparation and also alleviate the possibility of tumour development if it is the case that MHC class matched cells are required.

The combination therapy described in Chapter 5 of this thesis provides a promising protocol for testing as a therapeutic agent against chronic HBV infection. Some of the major hurdles associated with transferring this technology from basic research in animals to clinical trials in humans have already been addressed. DNA vaccination, rFPV and ETV have all been approved, albeit individually, for clinical use in humans (Coupar, Purcell *et al*. 2005; Coupar, Purcell *et al*. 2006; De Rose, Sullivan *et al*. 2006). It was observed throughout these studies that rFPV vaccines contained significant amounts of egg product. This suggests that vaccination of individuals with allergies to egg would be contraindicated. Unfortunately, removal of all traces of contaminating egg product significantly reduces the infective titres of rFPV due to inactivation of the virus during the purification process. However, as addressed previously with whole-cell vaccines, the presence of egg product could have elicited an adjuvant effect which may also partially explain the immuno-reactivity of antigens delivered by rFPV vaccines.

Recent publications suggest that optimised doses of DNA vaccines and rFPV are critical to induce robust immune responses in humans. That is, doses shown to be immunogenic

in non-human primate species have not been immunogenic in humans (Coupar, Purcell *et al.* 2006; De Rose, Sullivan *et al.* 2006). It was proposed in these studies that the immune responses generated following "prime-boost" vaccination correlated well with Estimated Body Surface Area (EBSA) (De Rose, Sullivan *et al.* 2006). To address this issue, the EBSA of a 14-to 21-day-old duck is approximately 0.04 to 0.05$m^2$. The quantity of 1mg of DNA vaccine and $1x10^8$ pfu rFPV per vaccine dose used in our duck studies falls well below the 4-8 mg DNA vaccine and $5x10^8$ pfu rFPV predicted to be required to induce similar immune responses in humans (De Rose, Sullivan *et al.* 2006). Therefore, research into methodologies to increase DNA vaccine and rFPV yields are certainly needed. Optimisation of the antiviral drug regime (duration and timing) is also likely to be important. This is suggested by the recent failure of a similar, but less controlled, combination therapy test in a single chimpanzee with high viral load (Shata, Pfahler *et al.* 2006). The results of this study suggest that antiviral drug compliance could be essential. It would also be logical to include a cocktail of antiviral compounds if possible in order to achieve maximum decreases in infected hepatocytes, at the same time minimise emergence of drug resistant mutants.

Other impediments do exist before the combination regimen could be tested in chronically HBV-infected humans. The practicality and costs involved in scaling up the combination treatment to the proposed doses for use in humans (De Rose, Sullivan *et al.* 2006) would initially limit the availability of the treatment to wealthier countries. However, considering the morbidity and large cost burdens to the health system associated with HBV-related cirrhosis and HCC, investment in a therapeutic vaccine would be worthwhile. Probably though, the greatest obstacle preventing transition of this protocol to human trials will be achieving interest from both antiviral drug

companies and vaccine manufacturers whose bottom-lines rely heavily on purchase of their currently available products.

## 6.5 Concluding remarks

In conclusion, the 4 manuscripts forming this thesis have described significant insights into the humoral immune responses generated following natural DHBV infection, and provided three novel vaccine strategies. Each study has built on the results and outcomes of the previous studies, culminating in the development of an extremely effective post-exposure DHBV vaccination regimen that, with optimisation, could be trialled in chronically HBV infected humans.

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