PERSISTENCE OF INFECTIOUS HEPADNAVIRUS IN
OFFSPRING BORN TO MOTHERS CONVALESCENT FROM
HEPATITUS IN THE WOODCHUCK MODEL
OF HEPATITIS B

CARLA S. COFFIN

PERSISTENCE OF INFECTIOUS HEPADNAVIRUS IN OFFSPRING

BORN TO MOTHERS CONVALESCENT FROM HEPATITIS IN THE

WOODCHUCK MODEL OF HEPATITIS B

by

Carla S. Coffin

A thesis submitted to the School of Graduate Studies

in partial fulfillment of the requirements for

the degree of Master of Science

Faculty of Medicine

Memorial University of Newfoundland

July 1997

St. John's   Newfoundland

ABSTRACT

Hepatitis B virus (HBV) is primarily an hepatotropic virus, although evidence of viral infection in lymphoid cells has also been observed. The virus induces life-threatening liver diseases, such as cirrhosis and hepatocellular carcinoma and is a major public health problem with more than 300 million chronically infected people worldwide. It is also evident that transmission of HBV from infected mothers to their babies is the most important mechanism by which the virus is maintained within the population. Recent findings have established the existence of a serologically undetectable persistent carrier state of HBV in apparently completely healthy individuals convalescent from an acute episode of hepatitis B. In these individuals, traces of HBV genomes were documented in serum and circulating lymphoid cells years after recovery. Related findings of the life-long hepadnaviral persistence after a transient exposure to woodchuck hepatitis virus (WHV) has been demonstrated in this laboratory in a woodchuck model of hepatitis B.

The current study was undertaken to learn about the risk of hepadnavirus transmission to newborn woodchucks from mothers with complete serological recovery from experimentally induced viral hepatitis and about natural course and molecular features of virus persistence in these offspring. The specific aims of this investigation were: (1) to determine whether hepadnaviral genomes can be transmitted from maternal woodchucks with a past episode of acute WHV hepatitis to their offspring; (2) if in fact this vertical transmission occurs, to identify reservoirs of hepadnavirus replication during long-term follow-up of these newborn animals; (3) to characterize physicochemical properties of molecules carrying WHV

DNA in sera of these offspring; (4) to test whether silent carriage of WHV genomes acquired after vertical transmission reflects the existence of biologically competent virus infectious to WHV-naive woodchucks, and (5) to determine whether the offspring carrying WHV traces are susceptible to WHV infection. In this work, 11 offspring born to 4 woodchuck mothers convalescent from an acute episode of viral hepatitis were investigated.

Our results have shown that serologically silent WHV carriage acquired after a self-limited episode of viral hepatitis is transmittable from mothers to newborns as an asymptomatic chronic infection. Importantly, all of the offspring tested carried WHV DNA through the entire follow-up, lasting for more then 3 years after birth, and remained nonreactive for immunovirological markers of WHV infection unless challenged with WHV. WHV DNA and RNA specific sequences were detectable both in the liver and lymphoid cells in the majority of the animals, although in some offspring WHV persisted exclusively at a extrahepatic location in the lymphatic system. Particles carrying WHV DNA in sera of offspring with WHV genomes in both the liver and lymphoid cells or the lymphatic system alone had physicochemical properties comparable to those of complete WHV virions. In addition, virus contained in offspring sera with or without WHV DNA expression in the liver as well as, culture supernatant from mitogen-stimulated peripheral blood mononuclear cells were infectious to WHV-naive woodchucks. Finally, despite silent carriage of WHV traces, the offspring were susceptible to WHV challenge. Since there are significant pathobiological similarities between HBV and WHV, it is possible that a comparable situation may exist in babies born from mothers with a past history of hepatitis B.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

xi

LIST OF TABLES

LIST OF FIGURES

Page

xiv

LIST OF ABBREVIATIONS

anti-HBc	antibody to HBcAg
anti-HBe	antibody to HBeAg
anti-HBs	antibody to HBsAg
anti-WHc	antibody to WHcAg
anti-WHs	antibody to WHsAg
AIDS	acquired immunodeficiency syndrome
bp	base pairs
cccDNA	covalently closed circular hepadnaviral DNA
cDNA	complementary DNA
CMV	cytomegalovirus
ConA	concanavalin A
CsCl	cesium chloride
CTLs	cytotoxic T lymphocytes
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
dpi	day post injection
DHBV	duck hepatitis B virus
EB	ethidium bromide
EBV	Epstein-Barr virus

EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
GGT	γ-glutamyltransferase
h	hour
HBV	hepatitis B virus
HBSS	Hanks' balanced salt solution
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HPV-16	human papillomavirus type 16
HSV	herpes simplex virus
IFN	interferon
i.v.	intravenous
kb	kilobases
LCMV	lymphocytic choriomeningitis virus
LPS	lipopolysaccharide
MHC	major histocompatibility complex
min	minutes

mo	month(s)
mRNA	messenger RNA
NK	natural killer cells
nm	nanometer
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RT	reverse transcriptase
RCDNA	relaxed circular hepadnaviral DNA
RNA	ribonucleic acid
SD	standard deviation
SLAH	self-limited acute hepatitis
TCR	T cell receptor
TE	1 mM EDTA in 10 mM Tris-HCl buffer, pH 8.0
TNF	tumour necrosis factor
VZV	varicella zoster virus
wks	weeks
WHcAg	woodchuck hepatitis virus core antigen
WHsAg	woodchuck hepatitis virus surface antigen
WHV	woodchuck hepatitis virus

CHAPTER ONE - INTRODUCTION

Hepatitis B virus (HBV) is considered to be one of the most important viral pathogens affecting mankind today. There are an estimated 350 million chronic carriers of HBV worldwide (Robinson, 1994), including approximately 250,000 Canadians or about 1% of the population in Canada (Sherman, 1996). Individuals chronically infected with HBV can transmit infection to susceptible individuals and they are at risk for significant morbidity and mortality. Exposure to HBV can lead to chronic hepatitis B, liver cirrhosis and hepatocellular carcinoma (HCC), which kill chronic HBV carriers at an annual rate of 1-2 million deaths worldwide. The source of infectious virus is usually blood, but HBV has also been detected in a variety of other body fluids, including semen, saliva, tears, and breast milk. Thus, it should be assumed that under certain conditions all biological fluids from HBV-infected patients may be infectious. In Canada and other western countries, the virus is mostly spread horizontally through a parenteral route, usually by intravenous drug use, occupational exposure or sexual contact. In the era before donor blood screening, transfusions of infected blood and blood derived products were the most common way of HBV spread. However, in endemic regions, most infections occur in children as a result of perinatal (vertical) transmission (Sherman, 1996). Almost all individuals infected since birth become chronic carriers of the virus. Therefore, mother to child transmission represents a very important mechanism for maintenance of HBV in the human population worldwide (Robson *et. al.*, 1994; Hollinger, 1996). Regions of the world which are most affected by HBV include sub-saharan Africa, south-east and far-east Asian countries, and the coastal belt of Greenland,

where it is estimated that 5% to 20% of individuals are chronically infected with HBV (Maupas and Melnick, 1981).

1.1 Viral Hepatitis

HBV is one of 6 presently known hepatotropic viruses that cause viral hepatitis in humans. These viruses, designated A, B, C, D, E and G all cause some form of liver inflammation. The mode of transmission, genome structure and course of infection significantly differ for each of these viruses, as summarized in Table 1.1.

Briefly, hepatitis A (HAV) and hepatitis E virus (HEV; Reyes *et al.*, 1990) are transmitted by the faecal-oral (enteric) route. Thus, they are usually spread under unsanitary conditions *via* contaminated food and water supplies and poor personal hygiene. Major outbreaks of enterically transmitted viral hepatitis typically occur in regions with inadequate public sanitation or malnourished populations. Infections with these viruses are usually self-limiting leading to complete recovery. For unknown reasons, 10-20% of pregnant women with acute HEV infection develop severe and fatal fulminant hepatitis (Koziel, 1996). A vaccine is available for HAV, but not for HEV.

Hepatitis B and hepatitis C viruses (HCV; Choo *et al.*, 1989) are spread mainly *via* blood, sexual contact or from mother to infant. These viruses are the two most important causes of viral hepatitis. Ten percent of patients acutely infected with HBV and up to 50% of those infected with HCV develop chronic hepatitis, placing these individuals at greater risk for development of life-threatening diseases, such as liver cirrhosis and HCC (Koziel, 1996).

Table 1.1 Comparison of the 6 known types of human viral hepatitis

Virus	Family and Nucleic Acid Type	Mode of Transmission	Clinical Effects
Hepatitis A (HAV)	*Picornaviridae* single-stranded RNA	Faecal-oral (enteric)	Incubation period 14-50 days; 99% recovery, acute or fulminant hepatitis; Vaccine available
Hepatitis B (HBV)	*Hepadnaviridae* double-stranded DNA	Horizontal transmission, blood (parenteral), sexual contact & body fluids; Vertical transmission	Incubation period 1-6 mo; 90% recovery, acute, fulminant & chronic hepatitis, cirrhosis, HCC, immune complex diseases; Vaccine available
Hepatitis C (HCV)	*Flaviviridae* single-stranded RNA	Same as HBV	Incubation period 14-18 days; Acute & chronic hepatitis, cirrhosis, HCC
Hepatitis D (HDV)	calcivirus (viriod-like) RNA	Mainly *via* blood and blood products (parenteral)	Incubation period 15-64 days; Only affects HBV infected patients; HDV super-infection increases risk of fulminant hepatitis, cirrhosis & liver failure
Hepatitis E (HEV)	*Flaviviridae* RNA	Faecal-oral (enteric)	Incubation period 15-60 days; Infection is mild and self-limiting in 99% of patients; Fulminant hepatitis in 10-20% of pregnant women
Hepatitis G (HGV)	*Flaviviridae* RNA	Mainly *via* blood and blood products (parenteral)	Generally a mild infection

Plasma-derived and recombinant vaccines are available for HBV (see Section 1.3.1), but none is yet available for HCV.

Hepatitis D virus (HDV) or delta virus has a similar mode of transmission as HBV. It is a defective ribonucleic acid (RNA) virus that lacks an envelope and requires HBV in order to complete its assembly. Thus, the virus only invades individuals already infected with HBV (Rizzetto *et al.*, 1980). Patients superinfected or co-infected with HDV are at increased risk for the development of fulminant hepatitis, cirrhosis and liver failure (Acorn *et al.*, 1995). Vaccination against HBV will also prevent HDV infection.

To date, little is known about the recently discovered hepatitis G virus, except that it can be transmitted *via* blood and blood products. It appears that the virus causes a mild self-limiting infection. The pathological significance of HGV infection remains unknown, but most likely is very limited (Linnen *et al.*, 1996).

In the past, a group of researchers claimed to have identified the enteric hepatitis F virus (Deka *et al.*, 1994), however, this finding could not be confirmed by others.

1.2 Natural History of HBV Infection

HBV primarily affects the liver of infected individuals, although extrahepatic viral replication has been well documented (*e.g.*, Baginski *et al.*, 1991; Mason *et al.*, 1993; Pardoe and Michalak, 1995; Yoffe *et al.*, 1990). It is generally accepted that the virus itself is not directly cytopathic, instead liver damage is caused by the host immune responses directed to the virus epitopes exposed on the surface of infected hepatocytes. Individual response to the

viral antigenic determinants varies a great deal, as symptoms of HBV infection can range from a complete absence to extremely severe, fatal liver injury. Following acute HBV infection, patients may appear to recover completely, progress to chronic hepatitis or develop fulminant hepatitis and die. In general, the clinical picture of HBV infection includes the following forms: (1) asymptomatic (subclinical) infection; (2) acute hepatitis; (3) fulminant hepatitis, and (4) chronic hepatitis, which histologically can be classified as persistent (mild) or active (aggressive) chronic inflammation.

Approximately 60-70% of individuals infected with HBV experience no symptoms, but produce specific antibodies to the virus antigens and develop an apparently lasting immunity to the virus. In these cases, clinical diagnosis can only be made after analysis of serological markers of HBV infection, as will be described in Section 1.3.3. Another 25% of people exposed to HBV develop acute hepatitis and experience symptoms which can range from a mild flu-like illness without jaundice to severe abdominal pain, extreme fatigue, and anorexia with jaundice. Fulminant hepatitis, which occurs in 1% of adults, is an extremely severe acute disease caused by rapid liver necrosis and is often fatal. However, most acute hepatitis infections of adults are self-limiting (self-limited acute hepatitis; SLAH). Therefore, approximately 90% of HBV infected individuals show complete serological recovery with apparent clearance of viral antigens from the circulation and liver, and the development of apparently permanent immunity (Hoofnagle *et al.*, 1987). It is estimated that a portion (5-10%) of acutely infected adult individuals develop serologically detectable chronic hepatitis B. In contrast, approximately 40% of young children infected at 5 to 10 years of age develop

chronic hepatitis (Sherman, 1996) and, in children infected since birth, 70-90% will progress to a serologically evident persistent hepatitis (Paré, 1996). Some chronic carriers infected since birth experience no clinical symptoms and report only occasional fatigue. However, symptoms in these individuals become more severe with age and increased liver damage. In more severe cases of chronic hepatitis, liver necroinflammation can progress to liver cirrhosis. Importantly, the risk of developing HCC is almost 100 times greater in chronic carriers of HBV (Paré, 1996) than in uninfected individuals. Seemingly, only tobacco is a more important human carcinogen than HBV (Hollinger, 1996). It is hypothesized that two independent mechanisms may contribute to the development of HCC in chronically infected patients. One pathway appears to be related to integration of HBV deoxyribonucleic acid (DNA) into host cell chromosomes which disrupts tumour suppressor gene functions or activates cellular oncogenes leading to uncontrolled cell proliferation. The second mechanism is most likely related to the continuous liver cell death and cell regeneration that increases the chance of mutations and subsequent tumour development (Robinson, 1994).

1.3 General Characteristics of HBV

1.3.1 Particle Types

HBV is classified as a member of the family *Hepadnaviridae* which reflects its liver tropism and DNA genome. The complete HBV particle or "Dane" particle is a double shelled, spherical structure that is only 42 nanometer (nm) in diameter (Dane *et al.*, 1970). The 7 nm outer shell or viral envelope is made up of viral surface proteins, as well as host

derived lipids. The viral inner shell consists of a 27 nm nucleocapsid (core) which encloses the viral genome. In addition to the infectious Dane particles, noninfectious subviral particles are also produced in HBV infected individuals. These are 20-nm diameter spheres or filaments that are composed of viral surface lipoproteins and carry virus surface antigen (HBsAg) specificity. They are lacking a nucleocapsid and viral genetic material and therefore, they are not infectious (Hollinger, 1996). HBsAg particles are produced and circulate in huge quantities in HBV carriers and are capable of inducing a strong antibody response which can confer immunity to infection. For this reason, subviral HBsAg particles purified from infected serum or generated by recombinant DNA technology, have been used as a successful vaccine against HBV infection (Hollinger *et al.*, 1986; Stevens *et al.*, 1987).

1.3.2 Genome Organization and Replication Strategy

HBV is one of the smallest DNA viruses known to man, as the complete DNA strand is only 3200 base pairs (bp). The unique, efficiently organized genome is circular, partially double stranded DNA that contains four overlapping open reading frames (ORF) encoding virus envelope or surface (S), nucleocapsid (C), polymerase (P) and X proteins (Figure 1.1). Unlike the genomes of the majority of other viruses, in which each ORF encodes one protein, one ORF of hepadnavirus can encode more than one protein product. For example, the S ORF encodes three surface proteins of variable length with different amino-ends, but a common carboxy-terminus. As well, elements which regulate hepadnaviral gene transcription are placed within the protein coding regions instead of a separate region of the genome

Figure 1.1 Schematic representation of HBV and WHV genomes. The inner circles represents the partially double stranded virion DNA with the first base pair marked by the *Eco*R1 cleavage site and subsequent marking at intervals of 400 base pairs. The positive DNA strand has a dashed line to indicate a variable 3' region and a wavy line at the 5' end to indicate the covalently attached RNA oligonucleotide primer. The minus strand is complete with a 5' attached protein primer (closed circle). The broad arrows surrounding the DNA strands represent viral open reading frames: core gene (preC and C regions), surface or envelope gene (preS1, preS2 and S regions), polymerase (P) and X genes. The arrowheads indicate the direction of gene transcription. The length of the translated protein products is shown as a number of amino acids (aa).

HBV

preS1 (108aa)
3174 preS2 (55aa) 157
S (228aa)
2850
2452
C (183 aa)
2309
833
P (832 aa)
3182/1
Eco RI
pre C
(29aa)
1623
X (154 aa)
1876
839

WHV

preS1 (148 aa)
719 preS2 (60aa) 206
S (222aa)
2699
2581
C (187 aa)
2427
961
P (684 aa)
3320/1
Eco RI
pre C
(30 aa)
1745
X (141 aa)
1605
966
2021
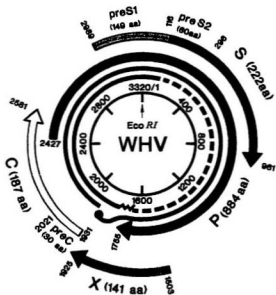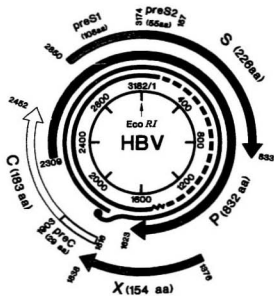
(Seeger *et al.*, 1991).

The circular structure of hepadnavirus genome is maintained by a short cohesive overlap between the 5' ends of the two DNA strands, hence this form of viral DNA is termed relaxed circular (RC) DNA. As shown in Figure 1.1, each HBV DNA strand is of different length. The minus strand, which encodes the viral proteins, is complete with defined 3'- and 5'-ends. In contrast, the incomplete plus strand has a variable 3'-end, creating a single-stranded gap region of approximately 200-300 bp. Another difference occurs at the 5'-end of both strands. The 5'-end of the minus strand contains a covalently linked protein, whereas the plus strand has an attached 5' RNA oligonucleotide primer (Ganem, 1996). Both the protein and RNA oligomer are important for viral replication, as described below.

The first step of viral replication occurs in the nucleus of an infected cell where the incomplete, RC DNA genome is converted, by addition of nucleotides to the 3'-end of the plus strand, into covalently closed, circular DNA (cccDNA). This cccDNA is then transcribed, using host RNA polymerase, into virus messenger RNA (mRNA) and RNA pregenomes. The mRNA transcripts are translated into viral proteins, whereas the RNA pregenomes are packaged in cores together with the polymerase and reverse transcribed, using the 5' protein primer, into minus strand DNA. After cleavage of RNA-DNA hybrids by RNaseH, the plus strand of DNA is synthesized from the minus strand using the 5' RNA oligomer as a primer. The mature cores, containing the incomplete double-stranded DNA genome, are packaged into envelope proteins along with host cell lipids to form the complete virions. The complete virus or Dane particle is then transported to the endoplasmic reticulum

(ER) and exported from the cell (Pugh and Bassendine, 1990).

The generation of cccDNA and the use of reverse transcription (a mechanism normally associated with RNA viruses), has enabled investigators to develop methods for studying hepadnaviral replication (Köck and Schlicht, 1993; Bréchot, 1993). The transformation of RC DNA into cccDNA is considered to be the first step in the hepadnavirus replication cycle. Therefore, detection of cccDNA can be used as an indicator of cells which support active virus replication (Tuttleman *et al.*, 1986). Accumulation of cccDNA occurs within infected cells due to the continuous recycling of cytoplasmic core particles, providing a template for production of new virions. Therefore, cccDNA is considered essential for persistent maintenance of virus replication and must be eliminated in order to eradicate the virus. After the cccDNA is transcribed into RNA, the mRNA transcripts, packaged in cores, are reverse transcribed into minus strand DNA. Investigators are able to detect this viral mRNA and hence, determine if virus replication is in fact occurring within particular cells or tissues. Hepadnaviruses are the only DNA viruses known that use reverse transcription in their replication cycle. Thus, like other RNA viruses, HBV lacks the 'proofreading capacity' of viruses that replicate using a DNA polymerase. Consequently, HBV has a high mutation rate in the transcription of RNA to DNA, estimated to be about 2×10^{-4} substitutions per site per year (Howard, 1995). Some mutations may enable HBV to persist and escape detection by the host immune system, as will be discussed below (Section 1.5.2e). However, the small size and overlapping genes of HBV limit the number of mutations which can occur without affecting virus viability.

1.3.3 Proteins of HBV

There are four principle translation products of the HBV genome. Thus, the C ORF encodes the virus nucleocapsid polypeptide carrying HBV core antigen (HBcAg) reactivity. This polypeptide self-aggregates into the inner 27 nm core of the Dane particle. The same ORF also encodes a protein with e antigen specificity (HBeAg). The HBe translation product is secreted as a result of the presence of an additional signal peptide, which directs the newly formed protein to the ER where it is cleaved and subsequently secreted from hepatocytes. Thus, although HBcAg circulates only as a part of Dane particles, HBeAg can freely occur in the blood. Detection of serum HBeAg indicates that the patient is highly infectious and HBV is replicating at its fastest rate.

The earliest antibodies to appear in the course of HBV infection are anti-HBc, which are directed to HBcAg. Anti-HBc are produced by almost all infected patients and persist indefinitely in serum after recovery. Thus, they are a very good indicator of exposure to HBV. The antibodies to HBeAg (anti-HBe) may appear in some patients after disappearance of HBeAg from the serum. Detection of anti-HBe is indicative of reduced infectivity and a decreased rate of viral replication. Anti-HBe can persist for approximately 2 or more years afterwards.

The envelope of HBV is formed by 3 related polypeptides: large (L or preS1), middle (M or preS2), and major or small (S) proteins. All are derived from the same S ORF by alternate use of three start codons. As mentioned before (Section 1.3.2), the three envelope proteins differ at their amino-ends, but they have a common carboxy-terminus. These

polypeptides make up the outer coat of the Dane particle (ratio of L:M:S is 1:1:4) and the 20-nm subviral envelope particles (mostly S with some M), which are assembled in the hepatocyte ER (Gerlich *et al.*, 1993). Overproduction of the large envelope polypeptides may lead to their retention within the ER, giving the cytoplasm the appearance of opaque ground glass on histological examination. These cells are called "ground-glass hepatocytes" (Hadziyannis *et al.*, 1973).

The earliest serological marker of HBV infection is the appearance of HBsAg. The antigen becomes detectable in serum within 2 to 6 months (mo) after exposure to the virus. The antibodies specific to this antigen (anti-HBs) appear during convalescence, when HBsAg has been cleared from the circulation. Detection of anti-HBs is considered as an indicator of complete recovery and life-long immunity to HBV.

The viral X protein is a small protein that has a transcriptional activating potential for triggering of many genes and thus may play a significant role in tumorigenesis. Transcription of the P ORF results in formation of a multidomain polypeptide with viral reverse transcriptase (RT), RNase, and DNA polymerase activities. This protein is also involved in genomic RNA encapsidation and DNA binding; thus, it plays an essential role in virus replication (Ganem, 1991).

1.4 The Hepadnavirus Family

1.4.1 General Features of Hepadnaviruses

HBV is the prototype member of a group of viruses which share similar morphology,

molecular structure, and pathobiological features. To date, the hepadnavirus family includes: HBV infecting humans, woodchuck hepatitis virus (WHV) in the eastern woodchucks (*Marmota monax;* Summers *et al.*, 1978), ground squirrel hepatitis virus (GSHV) in *Spermophilus beecheyi* (Marion *et al.*, 1980) and *Spermophilus richardsonii (*Tennant *et al.*, 1991*)* as well as, related viruses in tree squirrels (*Sciurus carolinesis*; Feitelson *et al.*, 1986) and Alaskan arctic ground squirrels (ASHV; *Spermophylus parryi kennicotti*; Testut *et al,* 1996). The avian hepadnaviruses include duck hepatitis B virus (DHBV) in *Anas domesticus* (Mason *et al.*, 1980) and heron hepatitis B virus (HHBV) in herons (*Ardea cinerea*; Sprengel et al., 1988).

In general, common features of hepadnaviruses are: (1) virion ultrastructure characterized by an envelope surrounding a spherical inner nucleocapsid, (2) small genome size (ranging from 3 to 3.3 kilobases, kb), (3) gene organization and nucleotide sequence homology, (4) replication strategy, (5) liver tropism, (6) narrow host range, and (7) pathogenic properties (Tiollais *et al,* 1985). The most divergent members of the family are avian hepadnaviruses. In these viruses, the X gene is absent, the C gene is larger than that of mammalian hepadnaviruses, the S gene does not encode preS1 domain, and surface antigen tubules do not form. Furthermore, unlike mammalian hepadnaviruses, there is no association between infection and HCC development (Cova *et al.*, 1993).

1.4.2 Woodchuck Hepatitis Virus (WHV)

It is generally accepted that WHV infection in woodchucks is the most suitable model

for the study of human HBV infection. WHV shows significant similarities with HBV with regard to morphology, genome structure, antigenic cross-reactivity of gene products, targeted organs, the course of infection, and the pathological features of the virus-induced liver disease (Roggendorf and Tolle, 1995).

The WHV genome is circular, but slightly larger than HBV. It has 3320 nucleotides in length compared to approximately 3200 bp of HBV and shares approximately 70% sequence homology with HBV (Figure 1.1). The intact WHV virion is also slightly larger (45 nm) than HBV (42 nm). Also, WHV constituent proteins demonstrate significant antigenic cross-reactivity with those of HBV. Consequently, this enables identification of serological markers of WHV infection by using the cross-reactivity of commercial assays for indicators of HBV infection (Werner *et al.*, 1979). For example, WHV surface antigen (WHsAg) and antibody to WHsAg (anti-WHs) can be detected using kits available from Abbott Laboratories (North Chicago, IL) developed for detection of human HBsAg and anti-HBs.

Both WHV and HBV induce acute hepatitis that in about 10-15% of cases progress to chronic liver disease associated with the development of HCC. However, among hepadnaviruses, WHV displays the highest oncogenic potential, believed to be caused by integration of viral sequences into host cell DNA resulting in activation of cellular oncogenes, in particular c-*myc* and n-*myc* (Hsu *et al.*, 1988 and Wei *et al.*, 1992).

WHV can be transmitted horizontally by blood or body fluids and vertically from infected mothers to offspring (Kulonen and Millman, 1988). In animals infected since birth, up to 90% develop a chronic serologically evident (*i.e.*, WHsAg and anti-WHc positive)

carrier state. Almost all of these offspring inevitably develop HCC, a situation which parallels that observed in man (Cova *et al.*, 1993). Although WHV is hepatotropic, the lymphoid system appears to be involved from the earliest stages of virus infection. A study by Korba *et al.*, 1989 reported that in woodchucks experimentally infected with WHV, viral DNA first appeared in lymphoid cells of the bone marrow, followed by the liver, spleen, peripheral blood lymphocytes, lymph nodes and finally the thymus. Furthermore, mitogen stimulation induced WHV replication in peripheral blood mononuclear cells (PBMC) from a chronically infected animal, as evidenced by presence of WHV DNA replication intermediates and WHV RNA specific transcripts in the stimulated lymphocytes (Korba *et al.*, 1988). Non-replicating viral DNA sequences have also been detected in other extrahepatic sites such as the pancreas, kidney, ovary and testis of chronically infected woodchucks (Korba *et al.*, 1990).

In summary, the WHV infected eastern woodchuck provides the most adequate model for studies on the natural course and the pathogenesis of HBV infection in humans. Infected woodchucks show similar liver disease profiles. Their relatively short life-span (approximately 5 years) enables the study of the development of each disease stage without having to wait for years. Furthermore, since WHV is not infectious for humans, investigators are not placed at risk by working with WHV. Thus, conclusions about the natural course of WHV infection, mode of WHV transmission, molecular and immunological mechanisms of WHV-induced liver injury and HCC, as well as cell tropism of WHV may be applicable to HBV infection in man (Paronetto and Tennant, 1990; Roggendorf and Tolle, 1995).

1.5 Viral Persistence After Adult Onset Infection

Viruses have evolved a wide variety of mechanisms in order to persist and avoid detection by the host's immune system. Many persistent viral infections do not cause any obvious disease symptoms, whereas others are symptomatic (Mahy, 1985). Viral infections can be divided into two general categories, cytopathic and noncytopathic. In a cytopathic (lytic) viral infection, virus proliferation disrupts cell membranes or inhibits protein synthesis causing tissue destruction. However, a cytopathic virus will eventually limit its own survival by eliminating cells supporting its replication. Therefore, many viruses have evolved to persist and reproduce in a host cell without killing it or causing excessive damage. These noncytopathic viral infections may not cause any overt injury or disrupt cell vital functions, but they may still interfere with cell differentiated functions (Oldstone, 1993a, 1993b). The long term effects of such persistent infections may lead to disturbances in homeostasis and eventually to diseases not traditionally associated with viral invasion. Furthermore, cell injury in these infections results predominantly from the host immune responses directed specifically to the virus antigens exposed on the surface of infected cells.

The following sections will discuss the antiviral immune responses and the different strategies which viruses have developed to evade both nonspecific and specific host immune surveillance, along with selected examples to illustrate each mechanism.

1.5.1 The Anti-Viral Immune Response

The primary function of the immune system is the recognition and elimination of

foreign antigens, including viral pathogens. Defence against a viral infection is mediated by both natural and specific immunity. In the first few days after invasion, the virus spread is controlled by the host's innate immune responses, called natural immunity. The main components of natural immunity involve physical barriers, such as skin and mucous membranes, and specialized lymphoid cells that include macrophages and natural killer (NK) cells. For example, macrophages phagocytose foreign particles and also produce cytokines, such as alpha interferon (IFN-α) and tumour necrosis factor alpha (TNF-α). These and other cytokines act in a complex network to eradicate viral infections by inhibiting viral replication, inhibiting proliferation of infected cells, mediating the inflammatory response or activating other immune effector cells. NK cells are lymphocytes which lyse virally infected cells, but the killing is not major histocompatibility complex (MHC) restricted or induced by a specific antigen. The mechanism of NK cell cytotoxicity appears to be perforin mediated, as described below (Abbas *et al.*, 1994).

There are two main effector arms of the specific immune responses involved in elimination of pathogens; namely, humoral and cellular immunity. Specific humoral immunity is mediated by B cells which upon encountering foreign antigens differentiate into antibody producing plasma cells. Antibodies are essential in the early defence against viral infections. Neutralizing antibodies may bind to viral envelope proteins preventing viral attachment and entry into host cells, enhancing phagocytosis of viral particles as well as, activating complement lysis of full virions. Thus, antibodies help to reduce the amount of freely circulating virus. Anti-virus specific antibodies can also mediate antibody-dependent cellular

cytotoxicity (ADCC). Therefore, they can bind to infected cells that express viral antigens on their surface. Then, the bound antibodies are recognized by Fc receptors of killer cells which in turn will preferenterially kill the coated target cells. Specific antibody responses are very important in preventing re-infection upon subsequent exposure to the same virus. Memory B cells that appear after antigen stimulation can persist long after the initial infection. Then, upon subsequent exposure to the same antigen, stimulation of memory B cell clones results in a rapid immune response and production of large amounts of specific antibodies with increased affinity for a particular foreign antigen (Abbas *et al.*, 1994). These antibodies can be passively transferred from mother to offspring *via* the placenta providing protection from infections until the newborn immune system matures (Zinkernagel, 1996).

Cellular immunity is mediated by two main groups of T cells, cytotoxic T lymphocytes (CTLs), which kill target cells, and helper T cells, which provide help to B cells and other immune effector cells. Almost all T cells mature in the thymus and express T cell receptors (TCR) that are specific for particular foreign antigens presented by MHC molecules. The MHC molecules are encoded by extremely polymorphic genes and they differ in their ability to bind and present antigenic epitopes. There are two types of MHC gene products, class I MHC, which is expressed on almost all nucleated cells (except neurons and only at low levels on hepatocytes), and class II MHC, which has a more restricted expression (i.e., B lymphocytes, macrophages and dendritic cells etc.). Infected cells which display viral epitopes in the context of class I MHC are killed by CTLs which express CD8 molecules. Helper T cells, which express CD4 molecules, interact with cells presenting phagocytosed viral antigens

in association with class II MHC (Abbas *et al.*, 1994).

The principle mechanism of specific immunity against established viral infections is virus-specific CD8$^+$ CTL response. However, full activation of CTLs requires cytokines produced by CD4$^+$ helper T cells and other cells of the immune system (*e.g.*, macrophages). The antiviral effects of CTLs include release of cytokines in the infected tissues and killing of infected target cells using either perforin or Fas-mediated pathways (Franco *et al.*, 1995). In the perforin-dependent pathway, CTLs secrete and deposit perforin granules onto the target cell membrane. Perforin undergoes assembly into trans-membrane pores and thus "punches holes" in the plasma membrane of the infected cell. In the second pathway, interaction of Fas ligand on the surface of the CTL with Fas receptor on the target cell membrane induces programmed cell death (apoptosis) of the target cell (Whitton and Oldstone, 1996). The T cell-mediated killing has an advantage over antibodies in that they can recognize low levels of viral peptides, including regulatory and nonstructural proteins which are not exposed on the virions (Kägi and Hengartner, 1996). Since the non-structural proteins are usually made early in infection, CTLs can act to eliminate cells before they start producing viral structural proteins and virions (Oldstone, 1994). Once an infection is established, it is much more difficult to clear virus and therefore, efficient and rapid, virus-specific immune responses are of primary importance in preventing virus persistence.

1.5.2 Strategies of Virus Escape from Host Immune Responses

Viruses have evolved multiple ways to avoid recognition by specific T cells and

antibodies and to establish a persistent infection. Each escape strategy can interfere with one or more mechanisms of the antiviral immune responses described above (Section 1.5.1). These viral escape strategies include: (1) silencing of viral gene expression (latency); (2) infection of immunologically privileged sites (*e.g.*, neurons); (3) infection of immune effector cells (*e.g.*, T cells); (4) interference with antigen presentation on infected cells (*e.g.*, modulation of MHC molecule assembly); (5) mutation of viral gene sequences to generate escape variants (*i.e.*, within T cell and antibody-recognition epitopes); and (6) induction of immunological tolerance (Franco *et al.*, 1995). It should be noted that most viruses which can persist have established a complex virus-host relationship and utilize more than one immune escape mechanism. However, in order to simplify descriptions of these mechanisms, a specific example is provided to illustrate each viral escape strategy. In many cases, the same virus example is used for different immune escape mechanisms.

1.5.2a Latent Infection

Latent infection is defined as a persistent carriage of virus in which the viral genome is present but only traces of infectious virus are produced (Banks and Rouse, 1992). In order for a virus to persist, it must be able to maintain its genome within the cell, even if the cell divides. For example, the retroviruses achieve this by integrating a DNA copy of their RNA genome into the host cell chromosomes. In this form, viral gene products are not expressed and the virus is undetected by the immune system since immunity is directed against foreign proteins and is not programmed to distinguish between "self" and "foreign" nucleic acid. In

general, the expression of viral proteins during any persistent viral infection is down-regulated relative to their levels during acute infection (Oldstone, 1989).

One of the best examples of viruses which develop a latent infection is herpes simplex virus (HSV) (Banks and Rouse, 1992). HSV establishes a latent infection in neurons where its gene expression is restricted, as only one region of the genome is transcribed. The latency associated transcripts (LAT) do not encode any protein products, but since neurons do not express MHC antigens, viral antigenic epitopes would not be recognized by T cells anyway. HSV also employs another strategy, *i.e.*, infection of immunologically privileged sites, as described below (Section 1.5.2b). Another member of the herpes virus family, cytomegalovirus (CMV), also establishes a latent infection but its transcripts are not detectable during the latency period (Bruggeman, 1993). Downregulation of the expression of viral proteins can also occur during chronic HBV infection. It is known that HBV DNA sequences can integrate randomly into host cell genome. This may result in extensive viral genomic rearrangements, which may reduce expression of virus gene products on the cell surface and consequently, their recognition by the host immune system (Chisari and Ferrari, 1995).

1.5.2b Infection of Immunologically Privileged Sites

Immunologically privileged sites are cells and tissues that are not easily accessible to the immune system. For example, many viruses such as HSV, lymphocytic choriomeningitis virus in mice (LCMV), varicella zoster virus (VZV) and measles establish persistence in the

central nervous system. This system appears to be a favourable site for virus persistence; most likely, because it is protected from lymphocyte recognition by the blood-brain barrier and contains neurons which do not express MHC molecules. Since neurons cannot be replaced once destroyed and are essential for brain function, they have evolved unique strategies to avoid injury, *e.g.*, failure to present viral peptides in the context of MHC molecules (Joly *et al.*, 1991). Similar strategy may be used by HBV during chronic infection of extrahepatitic tissues. HBV DNA sequences suggesting the presence of replicating virus have been detected in many organs, including lymph nodes, spleen, kidneys, pancreas, brain and some endocrine tissues, such as, testis, ovary, adrenal and thyroid gland (Mason *et al.*, 1993; Ogston *et al.*, 1989; Yoffe *et al.*, 1990). Thus, extrahepatic sites which could be inaccessible to CTLs due to microvascular barriers may serve as a reservoir of continuous virus replication and virus particles released from such immunologically privileged sites could reinfect the liver (Chisari and Ferrari, 1995).

1.5.2c Infection of Immune Effector Cells

It has been noted that all viruses known to be able to persist in their host are lymphotropic, as they infect cells of the immune system (Oldstone, 1990). These viruses can also abrogate functions of the immune system. The effector cells (lymphocytes and monocytes), which normally participate in clearing the virus, can be themselves infected, resulting in a selective immunosuppression against the virus itself or even a generalized suppression of cellular immune responses. For example, measles virus infects B and T

lymphocytes and their progenitors (and also monocytes), arresting all these cells in the G_1 phase and suppressing functions requiring cell terminal differentiation. This can result in generalized immunosuppression, but does not prevent acute infection (McChesney *et al.*, 1987). In rare cases, following acute measles infection, the virus can establish a slow persistent infection in the brain leading to a fatal neurological disease, subacute sclerosing panencephalitis. It has been postulated that this viral persistence is caused by a diminished CTL response (Dhib-Jalbut *et al.*, 1988), possibly *via* a similar mechanism seen during the acute stage of measles infection (Oldstone, 1990). Human immunodeficiency virus (HIV) is another well known example of a lymphotropic virus. The virus selectively infects $CD4^+$ helper T lymphocytes and establishes a latent infection with periodic reactivation (Embretson *et al.*, 1993). However, CD4$^+$ T cell levels eventually decline due to specific CD8 $^+$ CTL response against infected cells that express viral peptides in association with class I MHC. There are two possible consequences of CTL recognition; CTLs can either directly lyse infected cells or the CTLs can release cytokines (*e.g.*, IFN-γ and TNF-α) that interfere with viral replication. Eventually, the depletion of HIV- infected helper T lymphocytes can lead to generalized immunosuppression and severe disease (Oldstone *et al.*, 1996).

1.5.2d Interference with Presentation of Virus Antigenic Epitopes on Infected Cells

The above strategy can include: (1) blocking the assembly and/or transport of MHC molecules to the cell surface and (2) suppressing expression of accessory and adhesion molecules on the cell surface which ensure that the TCR effectively recognizes the MHC-

peptide complex and induces T-cell activation (Abbas *et al.*, 1994). Thus, impaired presentation of both MHC and cell recognition molecules on infected cells may decrease CTL recognition and consequently, virus can escape from immune clearance, as the following examples suggest (Rinaldo *et al.*, 1994).

Several different subgroups of adenoviruses are known to establish persistent infections in humans by interfering with class I MHC expression. For example, experiments performed with lymphoid and nonlymphoid cell lines infected with subgroup C adenovirus indicated that the virus early protein (E3/19K) forms complexes with class I MHC in the ER, preventing the MHC from reaching the cell surface (Korner and Burgert, 1994). In addition, evidence from studies of subgroup A adenovirus infection has shown that the virus directly interferes with class I MHC mRNA transcription (Shemesh *et al.*, 1991). Decreased expression of class I MHC also occurs in Burkitts lymphoma patients with persistent Epstein-Barr virus (EBV) infection by a mechanism known as "allele selective downregulation". These patients lack expression of one or more class I alleles and this defect is associated with resistance to killing by EBV-specific CTLs (Masucci *et al.*, 1989). Similarly, studies of human CMV infection *in vitro* showed that the virus disrupts expression of class I MHC molecules by causing their rapid degradation prior to export to the cell membrane. Consequently, the CMV infected cells are resistant to lysis by virus-specific CTLs (Warren *et al.*, 1994).

Downregulation of class II MHC is also known to occur during persistent CMV infection in humans. Sedmak *et al.*, 1994 presented evidence that CMV downmodulates IFN-

γ function, which is normally responsible for enhancing class II MHC mRNA and glycoprotein expression. The same mechanism has also been reported during infection with measles virus (Leopardi *et al.*, 1993) and HIV (Petit *et al.*, 1987).

Other studies have shown that decreased expression of adhesion molecules, such as lymphocyte function associated antigen (LFA-3) and intracellular adhesion molecule (ICAM-1), is involved in EBV escape from specific CTLs. However, the mechanism of suppression of these adhesion molecules is unknown.

Although there is no direct evidence that HBV can influence expression of the MHC or the cell surface accessory or adhesion molecules contributing to the TCR-MHC-peptide interactions, studies have shown that HBcAg can inhibit IFN-β gene transcription (Whitten *et al.*, 1991). In addition, the virus polymerase protein appears to be able to inhibit cellular immune responses induced by α and γ IFNs (Foster *et al.*, 1991). Thus, HBV may be able to indirectly downregulate expression of the class I MHC and accessory molecules (Chisari and Ferrari, 1995). Observations from our laboratory have shown that WHV interferes with expression of class I MHC heavy chain on infected hepatocytes in chronic WHV infection (Michalak *et al.*, unpublished).

1.5.2e Viral Variants

Many viruses can mutate rapidly, especially those which lack proof-reading enzymes during replication, as mentioned in Section 1.3.2. The variants which emerge may have increased ability to evade both T and B cell immunity. For example, the mutations can

interfere with antibody recognition or alter residues critical for MHC binding or TCR recognition (Ahmed *et al.*, 1996).

Antibody resistant viral variants are very common, as the alteration of viral proteins essential for antibody recognition allows effective escape from humoral immune responses. For example, amino acid substitutions in the envelope protein of HIV affects its recognition by neutralizing antibodies (Shioda *et al.*, 1994). In an analogous manner, viruses can also mutate epitopes which are presented to T cells. Thus, alterations in viral peptides can occur at residues which bind to MHC molecules or those that directly contact the TCR. These mutations can either fail to activate or may even antagonize the T cells responsiveness to wild antigenic peptide (Franco *et al.*, 1995). Such viral escape mutants have been demonstrated in lymphoid tissues during persistent LCMV infection of mice (Salvato *et al.*, 1991). It has been found that mice persistently infected since birth spontaneously give rise to viral variants with a single amino acid change in the TCR contact site, resulting in suppression of the CTL response and virus persistence. TCR antagonism is a process whereby amino acid substitutions of TCR contact sites creates variant peptides that can still interact with the TCR but are unable to deliver a full stimulatory signal, thus they may act as antagonists. These viral variants can also inhibit T cell activation by the normal (wild) stimulatory antigen. For example, HBV variants with one or two amino acid substitutions in the HBcAg T cell immunodominant epitope (amino acids 18-27) were shown to antagonize CTL recognition of the wild-type epitope in chronically infected patients (Bertoletti *et al.*, 1994). In this situation, cells which expressed both wild-type and mutant variants on their surface were

protected from elimination by CTLs specific for the wild-type epitope enabling persistence of HBV (Chisari and Ferrari, 1995).

1.5.2f Tolerance During Adult Onset Infection

In adult onset viral infection, immunological tolerance might be caused by the antiviral CTL response to a high virus load. An excess of viral antigens on numerous antigen presenting cells (APC) may induce a strong antigen-specific effector T cell response. Eventually, the mature effector cells will all die within a few days, resulting in the deletion of this specificity from the repertoire (Zinkernagel, 1996). In other words, virus-specific CD8$^+$ CTLs which are overstimulated by a high viral load can be driven to clonal exhaustion (deletion) in the periphery (Ahmed *et al.*, 1996). This situation has been reported during LCMV infection in mice, which fails to eradicate infection and leads to virus persistence (Moskophidis *et al.*, 1993). It is also possible that during adult onset of HBV infection some of the virus-specific T cells may be deleted in the periphery through overstimulation by high doses of viral antigen. Evidence for this theory comes from observations of chronic healthy HBV carriers who demonstrate large amounts of HBsAg in hepatocytes and in the circulation. It has also been documented that chronic WHV infection is associated with expression of large quantities of virus envelope in hepatocyte plasma membranes (Michalak and Lin, 1994). This may provide an immunologically resistant barrier at the hepatocyte surface, which may contribute to virus persistence by protecting virus from elimination within infected cells. Thus, in both examples of HBV and LCMV infections, the virus may successfully persist by

evading the immune responses through their overstimulation with an excess of viral antigens.

1.6 Virus Vertical Transmission

Mother to child transmission is a major cause of HBV spread in areas of the world with high incidence of the virus and is the main route for HBV to establish a chronic carrier state. As mentioned before (Section 1.2), 70-90% of children infected with HBV since birth become chronically infected, whereas less than 10% of adults exposed to HBV develop a persistent infection. Therefore, it is likely that the immune system maturity influences the host's response to HBV infection. The following section will discuss, among others, the development of persistent viral infection in the context of an immature immune system.

1.6.1 Factors Influencing Virus Vertical Transmission, Viral Persistence and Clinical Outcome

The probability of vertical transmission and the clinical outcome of viral infection in offspring is influenced by a number of factors. They include: (1) routes of transmission; (2) viral load (viraemia), (3) differences in viral strain, *i.e.*, its cell tropism and replication efficiency, and (4) host anti-viral immune response, *i.e.*, presence of maternal antibodies and anti-viral CTLs in the newborn and maturity of the immune system. Although all of these factors are known to influence the risk of mother to child infection and the clinical outcome of infection in the newborn, they may or may not have an effect on virus elimination or viral persistence. In some perinatal viral infections, the virus appears to be rapidly cleared after a

brief acute infection, whereas in others the virus can establish a chronic infection, without causing symptomatic disease. As it will be discussed, virus persistence in offspring is more likely to be determined by factors such as differences in viral strain and maturity of the fetal immune system.

1.6.1a Routes of Vertical Transmission

Vertical transmission from infected mothers to their children can occur by three possible routes: (1) *in utero* (congenital) *via* the placenta, (2) at birth (natal), *i.e.*, during passage through the birth canal and, (3) after birth but during the neonatal period (postnatal), *i.e.*, *via* maternal milk during breastfeeding. There are a number of viruses which can be transmitted from mothers to newborns. These perinatal viral infections in humans include: infections with: coxsackieviruses B, CMV, HCV, HSV, HIV, human T-lymphotropic virus type 1 (HTLV-1), measles, rubella, parvovirus B19, VZV, as well as LCMV in mice (Ueda *et al.*, 1992).

There are different routes of virus transmission. For example, HSV, HCV, HTLV-1 and coxsackieviruses B are transmitted during the natal and postnatal period, congenital infection occurs in rubella, CMV, VZV and parvovirus B19, whereas HIV is believed to be transmitted via all three routes (Ruff, 1994). In children born to mothers with acute or chronic HBV infection (*i.e.*, with serologically evident infection), transmission of the virus during the natal or neonatal period is known to occur, but congenital infection is not as common (Hollinger, 1996).

Generally, it appears that the earlier in development the infection occurs, the more serious it is (Donley, 1993). The clinical outcomes of congenital (*i.e. in utero*) viral infections tend be more severe, including embryo resorption, abortion, stillbirth, malformation, prematurity, and growth retardation. For example, during congenital rat parvovirus infection, the virus targets mitotically active cells. Thus, since embryos with rapid cell division are particularly susceptible to the virus, the early infection *in utero* can result in stillbirth, congenital malformations, and neonatal death (Gaertner *et al.*, 1996). However, the outcome can also be less serious and it could be more similar to viral infections occurring during the natal or postnatal period, which can result in acute disease, persistent symptomatic infection or in an asymptomatic infection in apparently healthy infants (Donley, 1993; Stamos and Rowley, 1994). HBV infected infants can display a number of symptoms ranging from severe acute hepatitis causing death, serologically evident chronic infection, self-limited disease or an apparent asymptomatic infection.

1.6.1b Viral Load

It was thought that threshold concentrations of a virus are required in order to breach the placenta and cause lethal infection of the fetus. For example, studies have shown that perinatal transmission of HBV is dependent on a high maternal viral load, using the presence of HBeAg as an indicator of viremia (Okada *et al.*, 1976). A more precise determinant for predicting risk of infection, according to Burk *et al.* (1994), is the level of HBV DNA in maternal serum. Infants born to mothers with circulating serum HBV DNA greater than 1.4

ng/ml had significantly higher rates of serologically evident infection. Similarly, babies born to HCV infected mothers with high levels of viraemia ($\geq 10^6$ HCV RNA /ml) had increased risk of infection (Ohto *et al.*, 1994; Aizaki *et al.*, 1996). In addition, pregnant women infected with human papillomavirus type 16 (HPV-16) and with a high viral load in cervical cells more frequently transmit HPV-16 DNA to their infants then those with a lower virus load (Kaye *et al.*, 1994; Cason *et al.*, 1995). Specifically, it has been determined that all women in which amplification by polymerase chain reaction (PCR) of cervical cells resulted in more than 325 HPV-16 genome copies per PCR sample transmitted infection to their infants, whereas all those with less than 22 HPV-16 genome copies failed to pass symptomatic infection to their children (Kaye *et al.*, 1994). However, the viral load does not influence development of a persistent infection. Infants found HPV DNA positive 24 hours after birth were still positive at 6 weeks irrespective of whether their mother had a high or low viral load. Comparable results were found in studies of rat parvovirus infection (Jacoby *et al.*, 1988). In this investigation, pregnant rats were inoculated either with 2×10^3 or 2×10^6 median tissue culture infective dose ($TCID_{50}$). Although virus was not detected in fetuses from dams given the lower dose of virus (*i.e.*, 2×10^3 TCID), fetuses from maternal rats challenged with higher doses (*i.e.*, 2×10^6 $TCID_{50}$) were severely malformed and carried infectious virus. Nevertheless, the researchers believed that small amounts of virus appeared to be adequate to sustain persistent infection, and that immunological status and genetic constitution may influence viral persistence.

1.6.1c Viral Variants

A characteristic feature of many viral infections is the presence of heterogeneous virus populations. These complex mixtures of related but non-identical genomes have been termed viral quasispecies. Such mutations are most typical of RNA viruses because their polymerases lack a proofreading function. Although HBV is a DNA virus, it uses reverse transcriptase in its replication cycle, thus it is also subject to a high mutation rate during replication (see Section 1.3.2). Heterogeneous viral populations have been detected in HBV, HCV and HIV-infected mothers, however, usually only a minor fraction of the many viral quasispecies from the mother is predominant in the infected infant (Wolinsky et al., 1992; Weiner et al., 1993; Von Weizsäcker et al., 1995). As the following examples suggest, selection of certain variant genomes could increase the rate of vertical transmission and hence influence the course of neonatal viral infection.

As mentioned above (Section 1.6.1c), previous studies have shown that infants born to HBeAg-positive carrier mothers usually become chronic carriers reactive for HBsAg and HBeAg. A HBV variant with mutation in the pre-core region of the C gene has been identified as unable to express e antigen. This variant is believed to be a HBV escape mutant from specific CTLs and is usually associated with chronic hepatitis and severe liver diseases (Carman et al., 1989). In babies born to anti-HBe-positive mothers, HBV DNA sequences showed a mixture of wild-type and this HBeAg non-expressing variant. In contrast, HBV DNA from babies born to HBeAg-positive mothers showed wild type sequences only. Newborns infected by mixed HBV populations seemingly recovered from hepatitis and

seroconverted to anti-HBs, while those with wild type alone became chronic carriers (Raimondo *et al.*, 1993). In general, only a small fraction of the mother's viral subpopulations appears to be transmitted to her child. It is possible that the strains transmitted have only recently appeared in the mother, thus an antibody response has not yet been mounted allowing their selective transmission to the child (Von Weizsäcker *et al.*, 1995).

Selective transmission of variant genomes have also been demonstrated for HCV infection. The hypervariable region (HVR) of the HCV genome encodes the viral envelope glycoproteins and is the most rapidly evolving region of the HCV genome. In addition, the HVR is known to encode protective epitopes that are subject to immune selection. In infants infected by vertical transmission, unique variants with mutation in the HVR differed significantly from the maternal viral population. It is suggested that these variants may have some selective advantage over other viral species in its ability to escape immune surveillance, cross the placenta or replicate efficiently in the host (Inoue *et al.*, 1992; Weiner *et al.*, 1993; Aizaki *et al.*, 1996).

Analysis of mother to child transmission of HIV has also revealed significant differences in the maternal and infant viral populations. Maternal viral sequences show greater sequence variation, whereas the prevalent virus genotype in the infant was derived from a single form present in its mother (Wolinsky *et al.*, 1992). Furthermore, viral variants which have been identified show sequence mutations which affect cell tropism and replication efficiency (De Rossi *et al.*, 1991; Scarlatti *et al.*, 1993). Studies by Scarlatti *et al.* (1993) have isolated two types of HIV viral isolates called "slow/low" and "rapid/high". The

"rapid/high" viral variant is able to productively infect cell lines and is efficiently transferred from mother to child. In addition, viruses with the "rapid/high" phenotype yield a higher viral titre in PBMC and can induce cell fusion. Both of these processes enhance virus spread and are correlated with vertical transmission and a poorer prognosis in infected children. A similar study by De Rossi *et al.* (1991) identified three types of HIV variants in children infected by vertical transmission. The rapid (R) type variant replicates rapidly, shows a high viral copy number in PBMC, and demonstrates a tropism for T lymphocytes. Infection with this variant correlated with severe clinical symptoms. Children infected with an intermediate (S/R) or the slow (S) variants have a reduced viral replication, a lower HIV copies per infected PBMC, and demonstrate monocytotropism resulting in a milder or even an asymptomatic viral infection.

1.6.1d Maternal and Fetal Immune Responses

The outcome of any viral infection is influenced by a struggle between the host immune response, which acts to recognize and destroy foreign materials, and strategies adapted by the virus to avoid elimination. In many cases, the clinical signs of viral infection are a result of the host's immune reactions toward the virus and destruction of infected cells (see Section 1.5.1). Thus, the maternal and fetal immune responses against a virus are critical determinants of vertical transmission, clinical effects of infection, and the likelihood of virus persistence.

For example, several studies have suggested that the presence of maternal antibodies

against the envelope glycoprotein of HIV is correlated with a lower vertical transmission rate (Rossi *et al.*, 1989; Goedert *et al.*, 1989; Devash *et al.*, 1990; Ugen *et al.*, 1997). The presence of these maternal antibodies may prevent mother to child transmission by providing immunological protection through virus neutralization, inhibition of virus binding to cell surface receptors or prevention of virus-cell fusion. In addition, passive transfer of HIV antibodies which can mediate ADCC appears to be correlated with a better clinical outcome in the newborn, such as providing protection against disease progression to acquired immunodeficiency syndrome (AIDS; Ljunggren *et al.*, 1990). A similar correlation was observed in the case of vertical transmission of HBV and the presence of maternal antibodies to the HBeAg (anti-HBe) (Okado *et al.*, 1976). Almost all HBeAg positive mothers transfer HBV to their infants, who usually develop a chronic carrier state. In children born to anti-HBe positive mothers, a symptomatic infection is rare but when it does occur, it is usually mild and self-limiting. It is possible that passive transfer of anti-HBe may play a role in neutralizing HBeAg and reduce its capacity to induce immunological tolerance, as it will be discussed below (Raimondo *et al.*, 1993). Virus-specific CTLs are an important arm of the cell-mediated immune responses against viruses. They act to destroy virally infected cells after infection and hence they may restrict virus spread (Whitton and Oldstone, 1996). For example, specific CTLs against HIV-1 have been detected in children of HIV-infected mothers. A correlation was observed between high activity of anti-HIV-1 CTLs detected less then 2 mo after birth and the lack of disease progression. However, with the onset of symptomatic AIDS in some children, the virus specific CTL response declined. In other

children, who became serologically negative for HIV, specific CTLs could still be detected (Cheynier *et al.*, 1992). Thus, it is believed that a strong CTL response helps to control virus infection in children born to HIV-infected mothers.

Further evidence for the importance of CTLs in the antiviral immune response is provided by experiments with athymic rats. It is apparent that both CTLs and helper T cells mature in the thymus and are essential to prevent persistent viral infection in some situations. For example, in neonatal rats, infected with rat parvovirus *via* vertical transmission, rats that lacked a thymus (athymic) had more difficulty with clearance of viral DNA than euthymic rats, in which case virus vertical transmission resulted in viral persistence (Gaertner *et al.*, 1996).

The effectiveness of the host immune response against a virus also depends upon the maturity of the immune system, thus viral persistence is affected by the age at which infection occurs. For example, rats infected at birth with rat parvovirus or during early infancy developed a persistent infection for up to 6 mo after infection, but in juvenile or younger rats, the symptomatic infection was usually quickly resolved (Jacoby *et al.*, 1991). Although the mechanisms of viral persistence in younger rats is not well understood, it has been attributed to immune system immaturity and the development of immunologic tolerance. It is believed that tolerance to an antigen is acquired during the development of the immune system. The antigens which are encountered during embryogenesis are regarded as self, whereas antigens seen after the immune system has matured are considered foreign and elicit an immune response. Thus, during congenital viral infections the developing immune system mistakenly

recognizes the virus as 'self' and any virus specific T cells are deleted or inactivated, resulting in the absence of both anti-viral CTL as well as, virus specific antibodies. This could possibly result in life-long viral persistence instead of virus recognition and its elimination by the immune system (Ahmed, 1989). Tolerance has also been suggested as a mechanism for viral persistence in other viral infections that persist after exposure of the fetus or neonate to such viruses as LCMV infection of mice, HBV, CMV, B19 parvovirus, HSV, HTLV-1, HIV, and rubella infection in humans (Ahmed and Stevens, 1990). Congenitally acquired LCMV infection in mice is one of the best studied models of tolerance to a viral infection. Mice infected since birth become life-long carriers and fail to develop a specific CTL response. However, experiments have demonstrated that it is possible to cure these mice of a chronic viral infection. Transfer of LCMV-specific CTLs into mice with congenitally acquired LCMV infection is able to clear viral antigens from the thymus and eliminate viral persistence. After the thymus is cleared of virus, LCMV-specific T cells which subsequently emerge are able to mount an efficient immune response and abrogate tolerance to the virus (Jamieson et al., 1991).

Induction of immunological tolerance is probably the reason why exposure to HBV during early development most often results in a life-long persistent viral infection (Ahmed, 1989). In this respect, a possible role for HBeAg has been suggested (Milich et al.,1990). Although the function of HBeAg in the HBV life cycle and in the pathogenesis of infection is unknown, some investigators believe that there is an association between transplacental passage of circulating maternal HBeAg and the induction of T cell tolerance to the HBV

nucleocapsid in infants born to chronically infected mothers. Both HBcAg and HBeAg share substantial amino acid sequence identity (see Section 1.3.3) and as a result they have common epitopes important for T cell recognition and hence they demonstrate significant antigenic cross-reactivity. Thus, *in utero* exposure to HBeAg could affect the immune response to both HBeAg and HBcAg at the T cell level, resulting in a failure to clear the virus and leading to the development of persistent infection in the newborn. In addition, these infants also fail to produce anti-HBe probably because of their exposure to circulating maternal HBeAg, which induced tolerance at the B cell level. Since the HBcAg does not freely occur in the circulation, most infected infants develop anti-HBc (Milich *et al.*, 1990 and 1995). However, it is postulated that the absence of anti-HBc in some infants is also caused by the induction of B cell tolerance, if there are sufficient levels of transplacental HBeAg and given the susceptibility of the neonatal immune system. This serological profile appears to be transient since in several cases reported, tolerance eventually wanes and seroconversion to specific viral antibodies occurs with immune system maturity (Ni *et al.*, 1993). Furthermore, newborns can produce specific antibodies after immunization against HBV with the HBsAg vaccine, indicating that tolerance induced by early exposure to HBV surface antigens may still be reversed (Milich *et al.*, 1990). However, in addition to HBeAg, exposure to other HBV antigens *in utero* could also be responsible for induction of immunological tolerance and the consequent development of a persistent viral infection (Chisari and Ferrari, 1995).

1.7 Long-Term Consequences of Persistent Viral Infections

In summary, many viruses which induce chronic infections are major human pathogens causing significant morbidity and mortality. Although the virus may not cause severe tissue destruction by interfering with a vital cell function, such as protein synthesis or cell membrane integrity, it may subtly alter the cell's differentiated or, so called, luxury function (*i.e.*, production of hormones or cytokines). Over time, this persistent disruption could lead to disturbances in homeostasis and eventually disease. In fact, many diseases of the lymphoid, endocrine and nervous systems, whose causes are currently unknown, may be caused by low-level persistent viral infections. For example, in mice persistently infected with LCMV since birth, the virus was able to replicate normally in the pituitary without causing any cell lysis or disrupting the cell morphology. However, the infected cells showed a deficiency in growth hormone synthesis which interfered with the animal's growth and development resulting in a significant decrease in the animal's body weight and length (Oldstone, 1993a). As mentioned before (Section 1.2), chronic persistence of HBV infection is clearly linked to the development of HCC, either by a direct mechanism of viral gene integration and activation of cellular oncogenes or indirectly, *via* the host immune system induced hepatocellular injury and hepatocyte turnover (Ganem, 1996). However, the viral persistence, especially at extrahepatic sites, could lead to the development of other diseases presently not being recognized as those associated with HBV infection.

1.8 Purpose of the study

Vertical transmission of HBV is an important mechanism by which the virus is maintained within the human population. The majority of infants infected since birth become chronic carriers of the virus and represent an important reservoir for infection of healthy people. Furthermore, they are at significant risk for the development of severe liver diseases, including cirrhosis and HCC. Recent studies have documented that apparently healthy individuals, years after complete clinical and serological recovery from acute HBV infection, still carry low levels of HBV in the circulation and in lymphoid cells. In addition, findings in a woodchuck model of hepatitis B have demonstrated that the hepadnavirus can persist at trace quantities for life in animals after complete spontaneous recovery from experimentally induced acute viral hepatitis. Therefore, it is conceivable that the virus persistently carried by convalescent mothers can be transmitted to their offspring. In the present study, the woodchuck model of HBV infection was used to determine whether such vertical transmission of hepadnavirus is possible and if so, what are the implications of this infection in the offspring.

The specific objectives of this investigation were as follows:

(1)   To determine whether vertical transmission of the hepadnaviral genome occurs from woodchuck mothers with a past history of recovery from acute viral hepatitis to their offspring.

(2)   To identify tissue reservoirs of persistent hepadnavirus replication in these offspring.

(3)   To characterize physicochemical properties of particles carrying WHV DNA in sera

and in circulating lymphoid cells of the offspring.

(4)   To test whether the offspring carry biologically competent WHV which is infectious to WHV-naive woodchucks.

(5)   To determine if these offspring are susceptible to challenge with infectious WHV and can develop serologically evident WHV infection and hepatitis.

CHAPTER TWO - MATERIALS AND METHODS

ANIMALS

Eleven offspring (3 males and 8 females) born to four woodchucks convalescent from a past episode of acute WHV hepatitis were the focus of this study. All the offspring were born in the woodchuck facility maintained by the Laboratory of Molecular Virology and Hepatology Research at Memorial University of Newfoundland, St. John's, Newfoundland. The animals were kept under dietary, environmental and biosafety conditions established specifically for this species in our colony. The newborns were nursed by their natural mothers until weaning at approximately 6 to 8 weeks (wks) of age. Then, they had unrestricted access to a standard herbivore woodchuck diet of fresh vegetables and water *ad libitum*.

2.1 Maternal woodchucks

Among four females whose offspring were investigated in this project, three animals (#A, #B and #C) were infected with WHV in this laboratory by intravenous (i.v.) injection with an infectious virus pool (Michalak *et al.*, 1989), whereas the fourth woodchuck (#D) was exposed under undetermined conditions to WHV prior to arrival to our colony. All animals inoculated with WHV developed acute hepatitis and subsequently completely recovered from the disease. Onset of acute infection was regarded as when WHV DNA and WHsAg or antibodies to WHV core antigen (anti-WHc) appeared in the circulation after administration of virus. In two mothers (#A and #B), acute hepatitis was confirmed by histological examination of liver biopsies which were taken approximately 6-8 wks after the

emergence of serum WHsAg or anti-WHc. Resolution of the acute episode of WHV infection was diagnosed when WHsAg was permanently cleared from the circulation. Importantly, all 3 WHV-inoculated woodchucks (#A, #B, and #C) developed anti-WHs following WHsAg disappearance and one of them (#B) remained antibody reactive until the birth of the investigated offspring. Overall, the episode of serologically evident (i.e., WHsAg positive) acute infection lasted between 3 and 15 wks (mean ± SD, 9 wks ± 6). The period between WHsAg clearance from the serum and parturition in these females ranged between 17 and 25 wks (mean ± SD, 21 wks ± 3.6). All these woodchucks were a part of the animal cohort participating in the study aimed at the determination of the longevity of WHV persistence and pathological consequences of WHV DNA carriage acquired after a self-limited episode of acute hepatitis (Michalak *et al.*, manuscript submitted) and they were the only animals which produced offspring. The fourth maternal woodchuck (#D) was anti-WHc reactive and WHV DNA positive and had no WHsAg or anti-WHs at the time of arrival to the laboratory. This animal cleared anti-WHc 7 wks prior to parturition. Two liver biopsies obtained at 12 and 17 wks after the animal's arrival showed normal liver morphology. Taken together, these data indicate that although #D woodchuck was evidently exposed to WHV in the past, it completely resolved the disease, as judged by both serological and histological criteria, but remained a persistent carrier of WHV DNA.

2.2 Offspring

The number of offspring, available for investigation, that were born to each of the

Table 2.1 Maternal woodchucks convalescent from acute WHV hepatitis and their offspring investigated in this study

Mother	Offspring		
	Animal	Total Observation Period (mo)[1]	WHV Challenge (mo)[1]
#A	1A/F[2]	1	n.a.[4]
#B	2B/F	4	n.a.
	3B/M[3]	15	n.a.
	4B/M	42	n.a.
#C	5C/F	22	n.a.
#D	6D/F	31	23
	7D/F	42	23
	8D/M	42	30
	9D/F	42	30
	10D/F	42	n.a.
	11D/F	42	n.a

[1] mo, months after birth
[2] F, female
[3] M, male
[4] n.a., not applicable

longer then a 6 mo period, liver biopsies were performed at least once yearly. In the case of autopsy (2B/F, 3B/M, 5C/F and 6D/F), serum, PBMC, and samples from liver and lymphoid organs (spleen, bone marrow, lymph nodes and occasionally, thymus) were collected. From animal 1A/F, only liver and fragments of spleen, thymus and lymph nodes were available for investigation.

2.3.1 Blood Sampling

Blood was collected under a general inhalant anaesthesia using isofluorane (CDMV Inc., St. Hyacinthe, Quebec, Canada). Approximately 10-15 ml of blood was collected from the digitalis vein in either leg using a butterfly catheter. The blood sample was divided into two parts, approximately 10 ml was aliquoted into vacutainers containing sodium ethylenediamine tetra-acetic acid (EDTA; lavender top; Becton Dickinson Vacutainer Systems, Rutherford, N.J.) from which plasma and PBMC were isolated, as described in Sections 2.3.3 and 2.3.4. The remaining approximately 5 ml sample was collected using a vacutainer which had no additives (red top; Becton Dickinson) and used for isolation of serum, as described in Section 2.3.2.

2.3.2 Serum Isolation

For isolation of serum, the blood sample collected was left for approximately 1 hour (h) at room temperature until a clot had formed. Then, each tube was spun at 720 x g for 10 minutes (min), the serum was removed and placed into sterile 1.5-ml eppendorf tubes. To

prevent contamination, each blood sample was handled separately and serum divided into small aliquots (approximately 0.5 ml each), under aseptic conditions, using disposable sterile plastic ware, and stored at -80°C.

2.3.3 Plasma Collection

Plasma was collected after density gradient centrifugation of whole blood used for PBMC isolation (see Section 2.3.4). The upper clear layer of supernatant (plasma) was carefully removed, poured into sterile plastic tubes (approximately 1-2 ml per tube) and stored at -80°C. To prevent contamination, the same precautions as those described above for serum collection were undertaken.

2.3.4 Isolation of PBMC and Splenocytes

Five ml of blood was layered over 4 ml of a Histopaque-1077 (Sigma Chemical Co., St. Louis, MO) density gradient and centrifuged at 400 x g for 30 min. Cells were harvested from the interface, washed 3 times by centrifugation at 400 x g for 10 min with a total of 45 ml of Hanks' balanced salt solution (HBSS; Gibco BRL, Gaithersburg, MD). The final wash was saved, spun at 720 x g for 30 min, decanted into a sterile 15 ml tube and stored at -20°C. Cells were counted in a haemocytometer after staining with trypan blue (Gibco BRL). Then, cells were aliquoted at a concentration of 1 x 10^7 per ml in 90% heat-inactivated fetal calf serum (FCS-HI; Immunocorp, Montreal, Quebec, Canada) and 10% dimethyl sulfoxide (DMSO; Sigma Chemical Co.) into sterile 1.8-ml polypropylene tubes (Nunc CryoTube™

vials; Gibco BRL). Finally, vials were frozen at -70°C overnight before storage in liquid nitrogen.

2.3.5 Laparotomies and Collection of Liver Biopsies

In animals observed for longer then 6 mo, serial liver biopsies were performed at yearly intervals to determine WHV genome expression and status of liver histology. In some offspring, up to 4 biopsies were taken during the course of this study. Each liver biopsy was performed by surgical laparotomy following standard aseptic methods. In preparation for surgery, the animal was sedated by an intramuscular injection of a mixture of ketamine (23 mg/kg, Ketaset; CDMV Inc.) and xylazine (10 mg/kg, AnaSed®; Lloyd Laboratories, Shenandoah, Ia), and then anaesthetised with 2-4% isofluorane (CDMV Inc.) provided by an anaesthetic machine (Pneumotech, Dartmouth, Nova Scotia, Canada) with a face mask. An incision was made along the midline of the abdominal wall (1.5-2.0 cm below the diaphragm) and a piece of liver tissue (approximately 0.5 cm^3) was taken using surgical forceps. After cessation of any bleeding with absorbable haemostat gauze (Johnson & Johnson Medical Inc., Arlington, Tx), the abdominal wall layers were closed by standard surgical procedure. Laparotomies were performed by Dr. T.I. Michalak with assistance from Ms. C.L. Trelegan and Mr. L. Grenning or the author in the Animal Surgery Unit, Faculty of Medicine, Memorial University of Newfoundland, St. John's, Newfoundland, Canada.

The liver tissue fragment was divided under sterile conditions into several pieces, which were preserved for DNA or RNA extraction and for histological and

immunohistochemical examinations. Preparation and storage of tissue samples for each of the above purposes is described in Section 2.3.8.

2.3.6 Collection of Specimens at Autopsy

For autopsy, animals were injected with a xylazine and ketamine mixture. Then, approximately 10 ml of blood was collected for serum isolation (as described in Section 2.3.1), followed by an injection into the same vein of 10 ml of an anticoagulant, citrate phosphate dextrose adenine solution (CPDA-1; Fenwal®; Baxter Healthcare Co., Deerfield, IL). Then, 50-100 ml of blood was collected by heart puncture and used for isolation of plasma and PBMC, as described in Sections 2.3.3 and 2.3.4. After opening the abdominal cavity, organs were removed under aseptic conditions. Typically, samples of the following tissues were collected; liver, spleen, lymph nodes, bone marrow, and skeletal muscle. In some cases, thymus was also obtained. In selected cases, most of the splenic tissue was used for isolation of lymphoid cells (splenocytes), as described below.

2.3.7 Isolation of Splenocytes

In some autopsy cases (3B/M, 6D/F, #260/M, #269/F, and #278/F) splenocytes, containing mainly lymphocytes, were prepared from spleen. For this purpose, spleen tissue obtained at autopsy under aseptic conditions was chopped into small pieces, washed with an excess of cold HBSS and then, gently pressed through a fine wire mesh as it had been previously described in detail (Michalak *et al.*, 1995). The suspension thus obtained was used

for isolation of splenocytes following the procedure described in Section 2.3.4.

2.3.8 Processing of Liver Biopsies and Autopsy Tissue Specimens

Tissue fragments obtained at autopsy, were preserved for DNA or RNA isolation, histological analysis and immunohistochemistry. DNA or RNA extraction was carried out by the author. For this purpose, several small (0.5 - 1 mm^3) tissue fragments, collected under aseptic conditions, were placed in Nunc CryoTube™ vials (Gibco BRL), snap frozen in liquid nitrogen, and stored at -80°C until use. Isolation of nucleic acids was done as described in Sections 2.5.2-2.5.3. Preparation of samples for immunohistochemistry or histology was done by other investigators in the laboratory, as described elsewhere (Michalak *et al.*, 1989 and 1990).

2.4 Serological Assays

2.4.1 Detection of Woodchuck Hepatitis Surface Antigen (WHsAg)

WHsAg was detected by a "sandwich" radioimmunassay (RIA) using cross-reactive AUSRIA-II kit for detection of HBsAg (Abbott Laboratories, N. Chicago, IL). Serum or plasma samples were tested directly for WHsAg or after fractionation through 15% sucrose layered onto a 60% sucrose cushion, as described in Section 2.6.3. Thus, 200 μl of each test and control sample was incubated for 16 h at room temperature with beads coated with anti-HBs, following a procedure recommended by the manufacturer. After washing, the beads were incubated for 1 h at 45°C with 200 μl ^{125}I-labelled anti-HBs. The bound radioactivity

was counted in a gamma counter. Specimens giving counts per minute (cpm) equal to or greater than the cutoff value, which was determined by multiplying the negative control mean (NC$\bar{x}$) count rate by a factor of 2.1, were considered WHsAg reactive. Based on detection of purified WHsAg, the assay sensitivity was estimated to be 3.25 ng protein/ml (Michalak *et al.*, 1989).

2.4.2 Detection of Antibodies to WHsAg (anti-WHs)

Anti-WHs was measured using a cross-reactive enzyme-linked immunoassay (ELISA; AUSAB®EIA, Abbott Laboratories), originally designed for the determination of anti-HBs in human serum or plasma. The assay applicability for detection of anti-WHs was established in previous studies (Michalak *et al.*, 1989 and 1990). Polystyrene beads coated with human HBsAg were incubated overnight at room temperature with 200 μl of either woodchuck serum or plasma or the appropriate positive (n = 2) and negative (n = 3) controls supplied by the manufacturer. Unbound material was removed by washing and the beads incubated with 200 μl of a mixture of HBsAg tagged with biotin and rabbit anti-biotin conjugated with horseradish peroxidase (HRPO) for 2 h at 40°C. Then, the beads were washed to remove any unbound conjugates and incubated with 300 μl of freshly prepared *o*-phenylenediamine solution containing hydrogen peroxide. After incubation at ambient temperature for 30 min, the beads were transferred to provided tubes and the enzyme reaction was stopped by addition of 1 ml of 1N H_2SO_4. The intensity of yellow colour which developed in the sample in proportion to the amount of bound HRPO-labelled antibody, was evaluated at 492 nm

wavelength using a Quantum II dual-wavelength analyser (Abbott Laboratories). The presence or absence of anti-WHs was calculated automatically using the spectroanalyser by comparing the absorbance values of the sample tested to the cutoff value, which was determined by adding a factor of 0.05 to the NCх̄. Samples with absorbance values greater than or equal to the cutoff value were considered anti-WHs reactive.

2.4.3 Detection of Antibodies to WHcAg (anti-WHc)

Anti-WHc was detected using a specific competition ELISA developed in this laboratory (Churchill and Michalak; unpublished). This assay is based on a principle that anti-WHc present in the test sample competes with HRPO-labelled anti-WHc for binding to immobilized woodchuck hepatitis virus core antigen (WHcAg). For this purpose, a 96-well, flat-bottom E.I.A. plate (Linbro/Titertek; ICN Biomedicals, Aurora, OH) was coated with woodchuck anti-WHc at 1 μg protein in 50 μl of phosphate buffered saline, pH 7.4 (PBS) per well, incubated at 4°C overnight and then washed three times with PBS. Nonspecific binding was blocked by filling the wells with 300 μl of 0.25% Tween-20 (Sigma Chemical Co.) in PBS (blocking buffer) and incubating at room temperature for 2 h. After washing, the plate was directly used or stored at -20°C. Before the assay, the plate that had been kept at -20°C was thawed and the wells washed with PBS. To each well, 0.5 μg of WHcAg in 50 μl of blocking buffer was added and the plate was incubated at ambient temperature for 2 h in a humid chamber. Then, the plate was washed 4 times with PBS, blotted dry and 20 μl of blocking buffer, 5 μl of the test serum sample or appropriate controls and 25 μl of anti-WHc

labelled with HRPO (diluted 1:2,500 in blocking buffer) was added to each well. After incubation for another 2 h, the wells were washed 3 times with PBS, and 50 μl of freshly prepared 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate (BioRad Laboratories, Richmond, CA) was added to each well. The reaction was stopped after 30 min by addition of 50 μl of 1N H_2SO_4. Absorbance was read at 450 nm using a microplate reader (BioRad Laboratories). As negative controls, sera from healthy animals were used. The positive controls included sera from WHV-infected woodchucks which had previously tested anti-WHc positive. The degree to which the test sample inhibited the binding of HRPO-labelled anti-WHc was calculated as follows: percent inhibition = 100 − (test sample OD ÷ negative control OD × 100). The assay results were accepted when the positive controls inhibited ≥95% of the HRPO-anti-WHc binding to WHcAg and the negative controls gave no inhibition. Samples that produced ≥50% inhibition were considered positive for anti-WHc.

2.4.4 γ-glutamyltransferase (GGT) Detection

Increases in the serum levels of GGT can occur during hepatic disease, such as viral hepatitis or HCC. Elevated serum GGT in woodchucks is considered a highly specific indicator of the developing HCC (Hornbuckle et al., 1985). GGT was tested using the Vettest assay system (Vettest S.A., Neuchatel, Switzerland). Sera obtained from the animals in this study showed normal GGT values, unless otherwise indicated (normal range 0-2 International Units, IU).

2.5 Molecular Biology Procedures

2.5.1 Slot-Blot Hybridization for Detection of WHV DNA

Fifty μl of serum was blotted onto a nylon membrane (Hybond-N; Amersham, Arlington Heights, IL) by vacuum suction using a Bio-Dot SF apparatus (BioRad Laboratories). After the sample aliquot had completely passed through, the membrane was placed, sample side up, onto 3MM Whatman filter paper (Whatman International Ltd., Maidstone, U.K.) soaked with 10% sodium dodecyl sulfate (SDS). Then, the membrane was sequentially transferred to three sheets of 3MM paper, each time leaving the membrane for 5 min. The first sheet was saturated with denaturing solution (1.5 M NaCl and 0.5 M NaOH), the second wetted with neutralizing solution (1.5 M NaCl in 1 M Tris-HCl, pH 8.0) and, the third saturated with 6X standard saline citrate (SSC; diluted from 20X SSC; 3 M NaCl in 0.3 M $Na_2Cit.2 H_2O$, pH 7.0). Then, the blot was air-dried, placed between two pieces of dry 3MM paper and baked for 2 h at 80°C in a vacuum oven. The nylon membrane was hybridized to a ^{32}P-labelled recombinant WHV probe, as described in Section 2.5.6c. Each assay was performed in parallel with serum from a chronic WHV carrier, as a positive control, and sterile PBS, as a negative control.

The assay limit for WHV DNA detection was determined using serial dilutions of recombinant WHV DNA (Section 2.5.6c). The assay sensitivity was 160 pg WHV DNA/ml that corresponded to a level of 10^7 WHV genome copies per ml.

2.5.2 DNA Extraction

2.5.2a Standard DNA Isolation Procedure

DNA was extracted from 100 μl of serum, 1 x 10^7 PBMC or splenocytes, or approximately 50 mg of tissue. Tissue fragments, PBMC and splenocytes were washed at least twice in 15 ml of HBSS by centrifugation for 5 min at 700 x g to remove any possible cell debris, and homogenized in a final volume of 100 μl HBSS. A mock sample of TE buffer (1 mM EDTA in 10 mM Tris-HCl, pH 8.0) was included as a standard control and extracted in parallel with DNA from the test sample.

For DNA isolation, a 100 μl sample of homogenized tissue, cells or serum was placed in a sterile 1.5-ml eppendorf tube and incubated at 42°C for 3 h with proteinase K (50 μg per reaction; Gibco BRL) in 200 μl of a lysis buffer containing 10 mM NaCl, 1 mM EDTA, 0.5% SDS in 10 mM Tris-HCl, pH 8.0. Then, each sample was mixed with 300 μl of phenol for 15 min at ambient temperature and centrifuged for 2 min at 10,000 x g. Approximately 300 μl of the clear aqueous upper layer was transferred to a new 1.5-ml tube, and mixed for 5 min at room temperature with 300 μl of a chloroform-isoamyl alcohol mixture, 24:1 (Sigma Chemical Co.). The upper aqueous layer was collected and DNA precipitated overnight at -20°C with 750 μl of 100% ethanol and 40.5 μl of 3 M sodium acetate. After precipitation, the sample was centrifuged at 16,000 x g for 30 min at 4°C. The supernatant was removed, the pellet of DNA washed with 750 μl of 70% ethanol and centrifuged again at 16,000 x g for 15 min at 4°C. After removal of the ethanol wash, the pellet was resuspended in TE buffer. DNA extracted from sera was suspended in 20 μl of TE. DNA from PBMC or

splenocytes was dissolved in TE to achieve a concentration of 4 μg of DNA per 10 μl, this usually corresponded to the amount of DNA obtained from extraction of 1 x 10^6 cells. Tissue derived DNA was first dissolved in 40 μl of TE and then, 2 μl of each sample was quantitated by spectrophotometric analysis at OD_{260} to determine DNA content.

2.5.2b DNA Isolation using TRIzol® Reagent

In some instances, DNA was extracted from PBMC using TRIzol reagent (Gibco BRL) following manufacturer's instruction. Thus, 1-2 x 10^7 cells were homogenized in 1 ml of TRIzol®, the supernatant was removed and used for isolation of RNA, as described in Section 2.5.3. The phenol phase was used for DNA isolation. For this purpose, 300 μl of 100% ethanol was added to the phenol phase and extensively mixed. The mixture was kept at room temperature for 3 min and then, centrifuged at 2000 x g for 5 min at 4°C to pellet DNA. After removal of supernatant, DNA pellet was washed twice with 0.1 M sodium citrate in 10% ethanol by centrifugation at 2000 x g for 5 min at 4°C. The final pellet was suspended in 1.5 ml of 75% ethanol, kept at room temperature for 20 min, and centrifuged again under the conditions described above. After vacuum drying, the DNA pellet was dissolved in 8 mM NaOH. Each sample was quantitated by spectrophotometric analysis as described previously (Section 2.5.2a). For amplification of DNA by polymerase chain reaction (PCR), the pH of the test sample was adjusted to 8.4 with 0.1 M HEPES (Sigma Chemical Co.) and checked using an alkacid litmus paper (Fisher Scientific Ltd., Nepean, Ontario, Canada).

2.5.3 RNA Extraction

Total RNA was extracted from tissue or PBMC using a commercial TRIzol® reagent (Gibco BRL). A similar reagent, TRIzol® LS, was used for RNA isolation from liquid samples, such as plasma or serum.

A small fragment of tissue or 1-2 x 10^7 cells was homogenized in 1 ml TRIzol® reagent or 750 μl of TRIzol® LS was added to 250 μl of a liquid sample tested. Then, the sample was incubated at room temperature for 10 minutes. If the sample was incompletely solubilized, it was cleared by centrifugation at 12,000 x g for 10 min at 4°C and the supernatant transferred to a fresh tube. Two hundred μl of chloroform (Sigma Chemical Co.) was added to the supernatant and after vigorous shaking for 15 sec, the sample was kept at ambient temperature for 15 min and centrifuged at 12,000 x g for 15 min at 4°C. Approximately 600 μl of the upper aqueous phase was transferred to a new tube and RNA precipitated by mixing with 500 μl of isopropanol (Sigma Chemical Co.) for 10 min at room temperature. Then, the mixture was spun at 12,000 x g for 10 min at 4°C. The supernatant was removed and the RNA pellet washed with 1.0 ml of 75% ethanol, vortexed and centrifuged at 7,500 x g for 5 min at 4°C. The final pellet of RNA was air dried, dissolved in RNase-free water, prepared by treatment with diethyl pyrocarbonate (DEPC; Sigma Chemical Co.), and stored at -70°C until use. The quality of isolated RNA was assessed by using 2 μl for gel electrophoresis in 0.9% agarose with 0.5 μg/ml ethidium bromide (EB-agarose), as described in Section 2.5.6a and enabling analysis of the pattern of RNA bands representing 28S and 18S ribosomal RNA molecules. The amount of RNA in each sample

was determined by spectrophotometric analysis at OD_{260}.

2.5.4 Polymerase Chain Reaction (PCR) for WHV DNA Detection

PCR employing WHV DNA specific primers was used to amplify WHV gene sequences in isolated DNA samples. Due to the low levels of virus present in most of the samples tested, both direct and nested PCR were employed.

2.5.4a WHV DNA Specific Oligonucleotide Primers

In general, primers for PCR amplification of WHV gene fragments were selected based on a consensus of four previously reported complete nucleotide sequences of the WHV genome (Galibert et al., 1982; Kodoma et al., 1985; Cohen et al., 1988; Girones et al., 1989). Primers specific for 3 separate, non-overlapping genomic regions of WHV DNA, i.e., core (C), surface (S) and X genes, were designed (Figure 2.2). For each subgenomic sequence, two sets of oligonucleotide primers (external and internal) were selected (Table 2.2). The external primer pair was used for the first (direct) round of PCR amplification. If the PCR product was not detectable after electrophoresis in EB-agarose, an aliquot of the first reaction was amplified with an internal primer pair by nested PCR. Each pair of primers was matched according to GC content (approximately 50%) and length (17-25 bases) in order to ensure the most efficient amplification of the target sequence. Oligonucleotides were synthesized by the University Core DNA Services, University of Calgary, Calgary, Alberta, Canada.

Figure 2.2 Schematic representation of the approximate location of direct and nested oligonucleotide primer pairs specific for the WHV C, S and X genes superimposed on the map of the complete WHV genome.
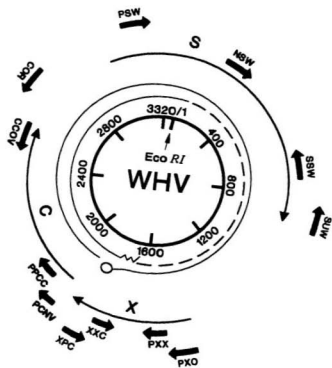
Table 2.2 Oligonucleotide primers used for amplification of WHV DNA sequences.

Primer pairs	Sequence 5'-3'	Position[1]	Target sequence (bp)
CORE GENE			
External primers			
PCNV (sense)	5'-TTCAAGCCTCCAAGCTGTGCCTTGG	1983-2007	623
COR (antisense)	5'-TTATGTACCCATTGAAG	2602-2586	
Internal Primers			
PPCC (sense)	5'-CCCTATAAAGAATTTGG	2033-2049	428
CCOV (antisense)	5'-GTATGTTCCGGAAGAGTCGAGA	2460-2439	
SURFACE GENE			
External primers			
PSW (sense)	5'-GGTAAACCATATTCTTGGGA	2947-2966	1290
SUW (antisense)	5'-ATGGCGGTAAGATGCTCAGAAGTG	917-894	
Internal primers			
NSW (sense)	5'-CATCAAGTCTCCTAGGACTC	303-322	501
SSW (antisense)	5'-TGAGCCAAGAGAAACGGGCTAAG	803-781	
X GENE			
External primers			
PXO (sense)	5'-GCCAACTGGATCCTGCGCGGGACGTC	1522-1547	386
XPC (antisense)	5'-ATGCCTACAGCCTCCTA	1907-1891	
Internal primers			
PXX (sense)	5'-CCTCAATCCAGCGGAC	1568-1584	193
XXC (antisense)	5'-GTTCACGGTGGAATCCAT	1760-1742	

[1]Numbers denote the position of the sequences in WHV (Kodoma *et al.*, 1985; GenBank accession number M11082).

2.5.4b Amplification of WHV Gene Sequences by PCR

For PCR amplification of WHV subgenomic fragments, 10 μl of DNA isolated from an equivalent of 50 μl of serum or 1 μg of DNA from PBMC and tissue samples was used, unless otherwise indicated. Samples were amplified in a 100 μl reaction volume containing 200 μmol/l of each deoxynucleotide triphosphate (i.e., deoxyadenosine triphosphate, dATP; deoxycytidine triphosphate, dCTP; deoxyguanosine triphosphate, dGTP and deoxythymidine triphosphate, dTTP), 100 μmol/l of each primer specific for WHV C, S or X gene sequences, 2 units of heat stable *Taq* polymerase (Promega Corporation, Madison, WI), 1.5 μmol/l MgCl$_2$, and 10 μl of reaction buffer (500 mM KCl, 1% Triton X-100 in 100 mM Tris, pH 9.0; Promega Corp.). The reaction was performed using a programmable thermal cycler (TwinBlock System, Ericomp Inc., San Diego, CA). For both direct and nested amplifications, samples were denatured at 92°C for 5 minutes, annealed at 52°C for 2 min, and elongated at 70°C for 3 min. Then, amplification was carried out for 30 cycles at 92°C, 52°C, and 70°C for 30 s at each step. The last cycle was followed by an elongation step at 70°C lasting 5 min. The second, nested round of PCR amplification was done using 10 μl of the reaction mixture obtained after the direct amplification and the same PCR conditions as described above. In general, the conditions for PCR amplification of WHV DNA were established in previous experiments done in this laboratory using recombinant entire WHV DNA genome (*e.g.*, Pardoe and Michalak, 1995) (see also Section 2.5.6c).

2.5.4c PCR Controls and Assay Sensitivity

All amplifications were carried out in parallel with contamination controls consisting of water added to the direct and nested PCR mixture instead of DNA, the "mock" extracted sample prepared in the absence of DNA (see Section 2.5.2a), and positive controls which, depending upon the type of material tested, consisted of DNA isolated from sera, PBMC or tissue specimens derived from WHsAg-positive chronic WHV carriers.

The PCR assay sensitivity was calculated based on amplification of serial dilutions of recombinant WHV DNA (Pardoe and Michalak, 1995). It was estimated that a direct PCR was able to detect 10^2-10^3 WHV genome copies per ml (10^{-3} pg WHV DNA/ml), whereas nested PCR between 10 and 10^2 genome copies per ml (10^{-5} pg WHV DNA/ml) were detected. To avoid contamination, rigorous laboratory precautions were undertaken, as described elsewhere (Michalak et al., 1994). For example, all assays were set up with sterile disposable plastic ware, gloves were worn at all times and changed frequently, and separate areas and instruments were used for PCR setup or handling of amplified PCR products.

2.5.5 Reverse Transcriptase PCR for WHV RNA Detection

For detection of WHV messenger RNA (mRNA), the total RNA was amplified by reverse transcriptase PCR (RT-PCR). In this method, random hexanucleotides were used to prime RNA species and reverse transcriptase from Moloney murine leukemia virus (MMLV-RT) was used for synthesis of the complementary DNA strand (cDNA). WHV cDNA was detected by a standard PCR with WHV specific primers, described in Section 2.5.4.

Approximately 1 μg of RNA isolated from PBMC or tissue samples that were previously found WHV DNA reactive by PCR, was mixed with 150 ng of random hexamers (100 ng/μl; New England Biolabs, Inc., Beverly, MA) and 4 μl of 5X RT buffer (375 mM KCl, 15 mM MgCl$_2$ in 250 mM Tris-HCl, pH 8.3; Gibco BRL) and incubated for 4 min at 75°C and then, quick chilled. Subsequently, each sample was mixed with 2 μl of dithiothreitol (DTT; 0.1 M; Gibco BRL), 2 μl of dNTP mixture (10mM of each dNTP; Promega Corp.), 10 U of RNase inhibitor (RNasin®; 40 units/μl; Promega Corp.) and 400 units of MMLV-RT (200 units/μl; Gibco BRL). After incubation at 37°C for 1 h followed by 5 min at 95°C, WHV cDNA specific sequences were amplified using PCR with C or S gene specific primers, as described in Section 2.5.4. Each reaction was set up in parallel with a negative control which had all the ingredients except reverse transcriptase, and a respective positive control, which consisted of total RNA isolated from the liver, spleen or PBMC of a chronic WHV carrier.

Detection of WHV mRNA in PBMC, liver and lymphoid organs was compared with that in serum. For this purpose, RNA was extracted from 1.5 ml of a serum sample collected from a woodchuck convalescent from self-limited acute WHV hepatitis and a WHsAg-positive, chronic WHV carrier. Two μg of serum-derived RNA and 1 μg of RNA isolated from spleen and liver of the same chronic WHV carrier were amplified in parallel. The RT reaction was performed, as described above, followed by standard PCR and Southern blotting of amplified WHV gene sequences.

2.5.6 Analysis of Amplified WHV Gene Sequences

2.5.6a Agarose Gel Electrophoresis

Twenty μl of the amplified WHV gene sequences during either direct or nested PCR was mixed with 1 μl of 6X blue/orange loading dye (Promega Corp.) and loaded into the wells of a 0.9% EB-agarose gel. A molecular DNA marker (PhiX 174 DNA/ Hae marker; 0.25 $\mu g/\mu l$; Promega Corp.) was also loaded in a parallel well to determine the size of amplified PCR products. Following electrophoresis in 1X TAE buffer (1 mM EDTA in 40 mM Tris-HCl, pH 8.0) at 100 V for 30 min, DNA bands were visualized by ultraviolet (UV) light and photographed using a UV transilluminator with camera (Fotodyne Inc., Bio/Can Scientific, Mississauga, Ontario, Canada) and Polaroid film (type 667; Polaroid Corporation, Cambridge, MA).

2.5.6b Southern Blot Analysis

To confirm authenticity of the amplified WHV genome sequences, Southern blot hybridization with a recombinant WHV DNA probe (see Section 2.5.6c) was performed. For this purpose, after completion of electrophoresis, the agarose gel was soaked in denaturing solution for 30 min then, in neutralizing solution for 1.5 h at room temperature with constant shaking (see Section 2.5.1). A capillary transfer of DNA from agarose to a nylon membrane (Hybond-N; Amersham) was done following the standard procedure described elsewhere (Maniatis *et al.*, 1989). Briefly, the gel was inverted onto a platform which has been placed in a reservoir filled with 10X SSC. The gel was covered with a nylon membrane, 2 layers of

3 MM filter paper (Whatman International) and then with a 5-8 cm high stack of paper towels. Glass plates were placed on the top of the stack to ensure a tight connection between the layers. After capillary transfer of DNA overnight, the nylon membrane was placed between 2 pieces of 3 MM paper and baked for 2 h at 80°C under vacuum. The baked membrane was immersed in approximately 10 ml of prehybridization buffer (6X SSC, 0.5% SDS and 50X Denhardts' solution containing 100μg/ ml denatured, sonicated salmon sperm DNA, sssDNA), heat sealed in a plastic bag and incubated for 2 h at 65°C while constantly shaking. After removal of all of the prehybridization fluid, the membrane was hybridized overnight at 65°C with 10 ml of 6X SSC, 0.5% SDS, 100μg/ ml sssDNA supplemented with 25-50 μl of ^{32}P-labelled, recombinant WHV DNA (see Section 2.5.6c). After hybridization, the membrane was incubated twice with 50 ml of 2X SSC, 0.1% SDS for 10 min at 65°C and with 0.1X SSC, 0.05% SDS for 15 min at room temperature. Then, the membrane was rinsed briefly with 0.1X SSC, air-dried, covered with plastic wrap, and exposed to X-ray film at -70°C overnight (XRP-1; Kodak, Eastman Kodak Company, Rochester, NY) with an intensifying screen.

2.5.6c Recombinant WHV DNA Probe

A plasmid containing the entire WHV genome cloned as an *Eco*RI fragment into a pSP65 plasmid vector (Galibert *et al.*, 1982), designated in our laboratory as pSP.WHV.1 or pSP.WHV.2, was used as a source of recombinant WHV DNA. This plasmid construct was kindly provided by Dr. J. Summers from the University of New Mexico, Albuquerque, NM.

WHV DNA labelling was done using a multiprime labelling kit (Amersham). In this labelling system, a relatively small amount of template DNA is primed by random sequence hexanucleotides and DNA is synthesised by the 'Klenow fragment' of DNA polymerase I. A radioisotope labelled-dNTP, added to the mixture, is incorporated into the newly synthesised DNA producing a labelled probe with, usually, very high specific activity. Thus, following manufacturers' instruction, 25 ng of DNA was boiled for 5 min and quickly chilled. Then, DNA was mixed with 10 μl of multiprime buffer solution (dATP, dGTP, dTTP, $MgCl_2$ and 2-mercaptoethanol in Tris-HCl buffer, pH 7.8), 5μl of primer solution (random hexacnuloetides), 18 μl of sterile water, 5 μl of ^{32}P- dCTP (50 μCi, specific activity 3000 Ci/mmol; Amersham) and 2 μl of Klenow enzyme (1 unit/μl in 50 mM potassium phosphate buffer, pH 6.5 with 10 mM 2-mercaptoethanol and 50% glycerol). After incubation for 5 h at room temperature, the mixture was diluted with 200 μl TE buffer and labelled DNA separated by centrifugation through a Sephadex G-50 column which was prepared according to standard procedure (Maniatis *et al.*, 1982). The recovered aliquot was stored at 4°C until use. The specific activity of the labelled probes was approximately 2.5×10^9 disintegrations per min (dpm)/μg DNA.

SPECIFIC EXPERIMENTS

2.6 Characterization of Physicochemical Properties of

Particles Carrying WHV DNA

2.6.1 Sedimentation Velocity and Buoyant Density Analyses

The sedimentation velocity of particles suspended in a solution can be analysed by a procedure called differential ultracentrifugation. In this method, the test sample is spun through a homogeneous supporting solution, *e.g.*, sucrose, in order to separate particles of different weight. In theory, heavier particles (*e.g.* WHV virions) should sediment to the bottom of the tube, but lighter molecules (*e.g.* protein-free DNA or DNA fragments), should remain at the top of the separative medium.

In another ultracentrifugation method, known as density gradient centrifugation, a test sample is layered over a supporting column of fluid whose density continuously or stepwise increases towards the bottom of the tube. Individual particles are separated during centrifugation by sedimenting to a position at which the gradient density is equal to their own density. Thus, denser particles *(e.g.* DNA) should migrate to the bottom, but buoyant, less dense molecules (*e.g.* WHV virions which contain a lipoprotein envelope) should sediment to the lower density closer to the top of the gradient.

In the present study, both sedimentation velocity and buoyant density of WHV DNA molecules present in test samples were determined and compared with those of intact WHV virions and recombinant protein-free WHV DNA. Each control was prepared as described below.

2.6.2 Preparation of WHV DNA-Reactive Controls for Ultracentrifugation Analyses

2.6.2a Purification of WHV Virions

Approximately 8 ml of pooled serum samples derived from a single WHsAg-positive chronic WHV carrier was spun for 10 min at 10,000 x g at 5°C in a J-20 rotor using a Beckman J-21B centrifuge (Beckman Instruments Inc., Palo Alto, CA) to remove any possible protein aggregates. Then, 7.5 ml of supernatant was collected and aliquoted to 2.5-ml samples which were layered over equal volumes of 30% sucrose (wt/vol) in TNE buffer (140 mM NaCl and 10 mM EDTA in 10 mM Tris-HCl buffer, pH 7.2,) and centrifuged at 200,000 x g for 30 h using a SW 50.1 rotor (Beckman Instruments Inc.). Subsequently, the supernatant was carefully removed, leaving approximately 300 μl aliquot in each tube, in which the pellets were resuspended. The suspensions were combined and enriched WHV particles washed with 5 ml of TNE buffer by centrifugation at 200,000 x g for 24 h under the above indicated conditions. The final pellet was resuspended in 1 ml TNE buffer and stored at 4°C until use. It has been previously established that this procedure resulted in isolation of the complete enveloped WHV particles (Michalak and Lin, 1994). This preparation was used as a reference to determine position of WHV virions after centrifugation in sucrose and cesium chloride (CsCl) gradients.

2.6.2b Preparation of Free WHV DNA Fragments

For preparation of protein-free, fragmented WHV DNA, DNA derived from a plasmid containing the complete WHV genome (described in Section 2.5.6c) was digested with

restriction enzyme Mnl-I (New England Biolabs). Computer-assisted analysis (PC/Gene; Intelligenetics, Mountainview, Ca.) of restriction enzyme sites within the WHV genome sequence, predicted that Mnl-I recognizes 32 sites. Therefore, digestion of WHV DNA with Mnl-I would lead to generation of multiple WHV DNA fragments with sizes that should range from 3 to 431 bp.

For this purpose, 5.4 μg of recombinant pSP.WHV was digested with 10 U (5 U/μl) of Mnl-I in 1X NE Buffer (50 mM NaCl, 10 mM MgCl$_2$, 1mM DTT in 10 mM Tris-HCl, pH 7.9; New England Biolabs) supplemented with 100 μg/ml of bovine serum albumin (BSA; Sigma Chemical Co.). The mixture was made up to 50 μl in sterile distilled water, incubated for 90 min at 37°C and then for 20 min at 65°C in a metal heating block to inactivate the enzyme. The digested plasmid was stored at 4°C until it was used as a marker of fragmented, protein-free viral DNA.

2.6.3 Analysis of Sedimentation Velocity of WHV DNA in Test and Control Samples

One hundred μl of serum from 4B/M offspring, obtained at 32 mo after birth, and from 7D/F offspring, collected at 22 mo after birth, was centrifuged through 4.5 ml of 15% (wt/vol) sucrose in TN buffer (140 mM NaCl in 10 mM Tris-HCl, pH 8.0) layered onto a 0.5 ml 60% sucrose cushion. Samples of purified WHV particles and Mnl-I digested WHV DNA were centrifuged in parallel. All tubes were spun at 200,000 x g for 4 h at 5°C in a SW50.1 rotor (Beckman Instruments Inc.). Fifteen fractions of approximately 340-μl each were collected using a 21-gauge needle inserted into the bottom of the tube. Sucrose concentration

was determined in each fraction by a refractometer (Fisher Scientific). Subsequently, DNA extracted from 100 μl of each fraction was tested for WHV DNA by PCR using WHV C or S gene specific primers. To further characterize the particles carrying WHV DNA in offspring sera, sucrose fractions which were found WHV DNA reactive were subjected to DNase digestion and analysed again by PCR (see Section 2.5.4). In addition, fractions 1-3 collected from the bottom of each gradient were assayed for WHsAg, as described in Section 2.4.1. Relevant sucrose fractions obtained after centrifugation of 100-μl sample of normal woodchuck serum (NWS) were used as negative controls.

2.6.4 Analysis of the Buoyant Density of WHV DNA in Test and Control Samples

Two hundred-μl samples of sera collected at 32 mo after birth from 4B/M offspring, a culture supernatant obtained after LPS-stimulation of PBMC collected from the same animal at 32-34 mo after birth (see Section 2.7.1), and serum from offspring 7D/F obtained at 22 mo after birth were layered separately onto 5-ml continuous gradients of 1.1-1.7 g/cm³ CsCl (Sigma Chemical Co.) in TE buffer. Two hundred μl of a purified WHV virion preparation (Section 2.6.2a) was centrifuged under the same conditions. After centrifugation at 200,000 x g for 18 h at 10°C in an SW50.1 rotor (Beckman Instruments Inc.), 13 fractions of approximately 400 μl each were collected beginning from the bottom of each gradient tube and evaluated for CsCl density (g/cm³) using a refractometer (Fisher Scientific). One hundred μl of fractions 1-13 recovered after centrifugation of the WHV virion preparation were used for DNA extraction and tested for WHV DNA by direct PCR using WHV C gene specific

primers. After identifying the WHV reactive fractions obtained after centrifugation of WHV virions, the corresponding fractions (*i.e.*, 9-13) obtained from the test offspring samples were examined for WHV DNA by nested PCR using WHV C gene specific primers.

2.6.5 Examination of the Effect of DNase Digestion on WHV DNA from Sucrose Fractions

There are a few possible forms of WHV particles which could occur in the circulation of a WHV infected animal. These forms may include intact (enveloped) WHV virions, fragments of unenveloped viral DNA, and probably defective virion particles or those with a damaged or a partial protein envelope. In order to determine whether WHV DNA reactivity detected in the sucrose gradient fractions could originate from intact virions, WHV DNA positive fractions were subjected to DNase digestion and then tested again for WHV DNA. In theory, WHV DNA contained within intact virions should be resistant to DNase digestion due to the protective lipoprotein envelope. In contrast, free DNA molecules and damaged WHV virions should be susceptible to enzyme treatment. Thus, a sample containing intact WHV virions should remain WHV DNA reactive after DNase treatment.

2.6.5a Preliminary Experiment

To establish conditions for differentiation of virions from freely circulating, unenveloped WHV DNA molecules , WHV DNA reactivity was examined both before and after DNase digestion in serum of a chronic WHV carrier containing circulating virions and

in a preparation of recombinant WHV DNA. Thus, 50 μl of serum from a WHsAg-positive chronic WHV carrier was supplemented with 10 μl of DNase digestion buffer (100 mM MgCl$_2$ in 500 mM Tris-HCl, pH 8.0), 5 μl of DNase (1 mg/ml, activity 20-50 x 10^3 U/ml; Boehringer Mannheim Canada, Laval, Québec, Canada) and 35 μl of deionized water and then, incubated for 1 hr at 37°C. Recombinant WHV DNA (i.e., unenveloped, free WHV DNA) at concentrations of 1.8 ng or 0.18 ng was suspended in 50 μl of NWS and digested under the same conditions as those for the serum of a chronic WHV carrier. After incubation, 100 μl of each digested sample was treated with proteinase K (see Section 2.5.2a) and DNA was extracted. In parallel, DNA was extracted from undigested samples that included 50 μl of serum from the same chronic WHsAg-positive woodchuck and 1.8 μg of recombinant WHV DNA suspended in NWS. After extraction, DNA was analysed by PCR using WHV DNA specific primers.

The results of this experiment revealed that WHV DNA reactivity detected in serum from a chronic WHV carrier was resistant to DNase treatment. The same treatment of recombinant WHV DNA, even at concentrations of 1.8 ng, completely eliminated WHV DNA reactivity as determined by nested PCR. Thus, we can conclude that at least part of the WHV DNA detectable in serum of a WHsAg-positive animal was protected from DNase action by an outer protein coat and therefore, reflects the existence of complete, intact virions.

2.6.5b DNase Treatment of Sucrose Fractions from Test and Control Samples

To differentiate the intact WHV virions from unenveloped WHV DNA in sucrose

fractions, fractions which had demonstrated the highest WHV DNA reactivity by PCR (i.e., bottom fractions 1-3 for WHV virion preparation, fractions 1-4 and 8-12 for 4B/M serum, and fractions 9-11 for *Mnl*-I digested recombinant WHV DNA; Section 2.6.3) were combined and examined for WHV DNA prior to and following digestion with DNase. For this purpose, 100 μl of the combined fractions was treated with 5 μl of DNase in 10 μl of DNase digestion buffer, as described in the preliminary experiment (Section 2.6.5a). After DNase digestion, the samples were treated with proteinase K and DNA was extracted. In parallel, DNA was extracted from 100-μl samples of the same sucrose fractions untreated with DNase. DNA from both DNase-digested and untreated fractions were tested for WHV DNA by PCR using primers specific for WHV S gene and the obtained products analyzed by Southern blot hybridization.

2.7 Determination of the Effect of Mitogen Stimulation
on WHV Transcription in PBMC

2.7.1 PBMC Stimulation with Lipopolysaccharide (LPS) and Concanavalin (ConA)

This experiment was designed to determine the effect of mitogen stimulation on WHV mRNA expression in PBMC of offspring born to mothers with a past history of acute WHV hepatitis. Cells were isolated, extensively washed and the final wash saved, as described in Section 2.3.4. Then, the cell number was adjusted to 2.0 x 10^6/ml. For stimulation, PBMC were cultured in RPMI 1640 medium (Gibco BRL) with 5% FCS-HI (Immunocorp), 2 mM glutamine (Sigma Chemical Co.), 50 μg/ml β-mercaptoethanol (Sigma Chemical Co.), and

a mixture of 500 U/ml penicillin and 500 μg/ml streptomycin (ICN Pharmaceuticals, Inc., Costa Mesa, CA) supplemented with 10 μg/ml LPS or 5μg/ml ConA for 3 days at 37°C in a 5% CO_2 atmosphere. Approximately 2×10^7 cells from the same isolation were pelleted in a 1.5-ml eppendorf tube and the pellet frozen at -70°C for further use as a control. PBMC isolated at 33.5 mo after birth from offspring 7D/F, 8D/M, 9D/F, 10D/F and 11D/F were stimulated with LPS whereas PBMC isolated at 34.5 mo after birth from the same woodchucks were stimulated with ConA, following the procedure described above. In addition, PBMC from 4B/M offspring harvested at 32, 33.5 and 34 mo after birth were cultured in the presence of LPS, whereas those obtained at 33 mo after birth were stimulated with ConA.

After 3-day culture, cells were pelleted at 400 x g for 10 min, supernatant decanted and saved. Subsequently, cells were washed with 15 ml of RPMI medium and counted. The final cell pellet was transferred to a 1.5-ml eppendorf tube and along with the pellet of unstimulated cells, extracted using TRIzol® reagent to isolate total RNA (described in Section 2.5.3). WHV RNA was transcribed to cDNA by RT-PCR and the amplified products were analysed by Southern blot hybridization, as described in Section 2.5.6b.

2.7.2 Evaluation of WHV DNA in Stimulated PBMC, Final Cell Wash and Culture Supernatant

To determine whether virus might be released by mitogen stimulated PBMC, WHV DNA reactivity was evaluated in the culture supernatant and compared to that in the final

wash obtained from the same cells prior to mitogen stimulation. Thus, for offspring 7D/F, 11D/F and 4B/M, both the final cell wash recovered before PBMC culture and PBMC culture supernatant collected after 72-h mitogen stimulation in the presence of LPS or ConA (approximately 9.0 ml each) were centrifuged at 140,000 x g for 18 h using a SW 40 rotor (Beckman Instruments Inc.). The resulting pellet was suspended in 1 ml of sterile 0.9% sodium chloride solution (Travenol Canada Inc., Mississauga, Ontario, Canada). DNA was extracted from 100 μl of each sample and then tested for WHV DNA by PCR using WHV S gene specific primers. The remaining portions of these samples were stored at -4°C until further use.

2.8 Evaluation of WHV DNA Expression in PBMC
after DNase and Trypsin Treatment

To determine whether WHV DNA reactivity detected in offspring PBMC was of an intracellular origin or the consequence of WHV virion or WHV DNA attachment to the cell surface, viable PBMC were subjected to DNase and limited trypsin digestion prior to DNA extraction. For this purpose, PBMC were isolated from approximately 12 ml of blood collected from 4B/M offspring at 40 mo after birth and from a WHsAg-positive carrier with histologically confirmed chronic hepatitis, as described in Section 2.3.4. The final PBMC wash after cell isolation was saved and stored at -20°C as well as, 1 x 10^7 untreated PBMC from each isolation were pelleted and stored at -70°C. The remaining cells (approximately 3 x 10^7 for 4B/M offspring and 1 x 10^7 for a chronic WHV carrier) were supplemented with

100 μl of 10X DNase buffer and 10 μl of DNase (1 mg/ml; Boehringer Mannheim) and digested for 1 h at 37°C. Then, 10 μl of 0.1 M CaCl$_2$ and 10 μl of trypsin (10 mg/ml; Sigma Chemical Co.) was added and the mixture treated for 30 min on ice. Finally, each sample was washed with three 15-ml changes of HBSS containing 1 mM EDTA and 1 mg/ml trypsin inhibitor (ovomucoid from chicken egg white containing ovoinhibitor; Sigma Chemical Co.) by pelleting cells for 10 min at 1130 x g. The supernatant after each wash was decanted, centrifuged for 30 min at 1130 x g and stored at -20°C. DNA was extracted from pelleted PBMC and from the final wash obtained after PBMC isolation but prior to digestion and from the final wash after PBMC treatment with DNase and trypsin. Then, each DNA sample was tested for WHV genome expression by PCR.

2.9 Determination of the Infectivity of WHV Persistently Carried by the Offspring

2.9.1 Preparation of Inocula from Offspring Plasma or Serum

Nine ml samples of sera or plasma collected from 3B/M offspring during autopsy at 15 mo after birth, 4B/M offspring at 32-34 mo after birth, and 7D/F offspring at 11-22 mo after birth were centrifuged at 200,000 x g for 18 h using a SW 50.1 rotor (Beckman Instruments Inc.). The supernatant was removed and the pellet from each sample suspended in 1 ml sterile distilled water. DNA extracted from 100 μl of each sample was tested for WHV DNA by PCR.

2.9.2 Challenge of WHV-Naive Woodchucks with Inocula Derived from Offspring Serum, Plasma or LPS-Stimulated PBMC

The suspended pellets from serum or plasma samples (Section 2.9.1) were injected i.v. into WHV-naive, completely healthy woodchucks. Thus, a sample derived from serum of 3B/M offspring was injected into #260/M woodchuck, whereas concentrated plasma from 7D/F and 4B/M were administered into #276/F and #269/F, respectively. Furthermore, WHV-naive woodchuck #278/M was inoculated with concentrated culture supernatant from LPS-stimulated PBMC of 4B/M offspring, as described in Section 2.7. Animals #260/M and #276/F were euthanised at 110 and 79 day post inoculation (dpi), respectively, whereas autopsy of woodchucks #269/F and #278/F was performed at dpi 215 and 218, respectively.

Serial serum samples were collected from woodchucks #269/F, #278/F and #276/F at 0, 2, 4, 7, 10, 14 and 21 dpi. Subsequent samples were obtained approximately every two weeks at 36, 49, 63, 78, 89, 104, and 119 dpi and then collected monthly until autopsy. PBMC were isolated at 0, 4, 10, 21 dpi and subsequently once a month until the end of follow-up. From #260/M woodchuck, sera were collected 0, 7, 13, 22, 35, 48 dpi and then monthly, whereas PBMC were isolated at days 0 and 7 and from then on, at monthly intervals up to the end of observation. Liver tissues were obtained from all woodchucks approximately 2 mo before and 2 mo after inoculation by laparotomy and at autopsy (Figure 3.3). Serum, PBMC and liver biopsy samples taken before and after administration of the inocula as well as, autopsy samples were analysed for WHV DNA by PCR (for details see Section 2.5.4). Sera and plasma samples were also assayed for WHsAg and anti-WHc as described above in

Section 2.4.

2.10 Inoculation of Offspring with Culture Supernatant from

PBMC of Woodchucks Convalescent from an Episode of SLAH

In another experiment, 10D/F and 11D/F offspring at 5 mo after birth were injected with a pellet of culture supernatant obtained after PBMC stimulation with LPS, as described in Section 2.7.1. The inoculum was derived from PBMC that were isolated from adult woodchucks months after complete recovery from an experimentally induced self-limited episode of acute WHV hepatitis (Michalak *et al.*, manuscript submitted). After inoculation, serum samples were collected 0, 2, 4, 7, 10 dpi, then biweekly for 4 months, and finally monthly, and analysed for immunovirological markers of WHV infection and WHV DNA, as presented in Sections 2.4 and 2.5.

2.11 Challenge of the Offspring Studied with Infectious WHV

The pool of infectious WHV used for inoculation of the offspring had been prepared from sera of a WHsAg-positive, chronically infected woodchuck. This pool was shown to be able to induce acute hepatitis in >95% of WHV-naive woodchucks (Michalak *et al.*, unpublished). The pool was sterilized through a 0.22 μm filter (Millex®-GS; Millipore Products Division, Bedford, MA), diluted 1:2 with sterile physiologic saline, and 0.75 ml administered i.v. under general anaesthesia to each woodchuck offspring. Thus, 6D/F and 7D/F offspring were inoculated at 23 mo after birth, whereas 7D/F and 8D/M offspring were

injected at 30 mo after birth. Sera from the inoculated animals were collected biweekly for 3 mo after inoculation and then monthly, and tested for WHsAg, anti-WHs, anti-WHc, and for WHV DNA, as described in Sections 2.4 and 2.5.

CHAPTER THREE - RESULTS

3.1 Transmission of Serologically Silent WHV Infection from Mothers to Offspring

All the offspring were examined for serological markers of WHV infection such as, WHsAg, anti-WHs, and anti-WHc, with the exception of 1A/F animal, as no serum samples were available from this woodchuck. In all cases tested, there were no serological indicators of WHV infection detected at any time point during the entire follow-up, except after experimental inoculation with WHV, as described in Section 3.10. In animals which were not subjected to WHV challenge, serum or plasma samples collected beginning at 2-3 mo up to 42 mo after birth were found negative for WHsAg, anti-WHs and anti-WHc (Figure 3.3). In addition, analysis of WHsAg reactivity in sucrose fractions obtained after centrifugation of selected offspring sera through 15% sucrose (Section 2.6.3) also gave negative results. This finding was in contrast to detection of WHsAg in late convalescent sera from adult woodchucks with complete recovery from an acute episode of WHV hepatitis, which were analysed under comparable centrifugation and assay conditions (Michalak *et al.*, manuscript submitted). Overall, the obtained data implies that serological markers of WHV infection tested were not present in any of the offspring investigated, or they occurred at levels below the detection limit of the assays used.

3.2 WHV DNA Remained Detectable in Sera Throughout
the Entire Follow-up of the Offspring

All sera from the studied offspring were negative for WHV DNA by slot-blot

Figure 3.3 Follow-up of maternal woodchucks convalescent from acute WHV hepatitis and their offspring. Sequential sera from four mothers (#A, B, C and D) and ten offspring (except 1A/F) were tested for WHsAg (red), anti-WHs (blue), anti-WHc (green) and WHV DNA by slot-blot hybridization (black) and by direct (dark grey) or nested PCR (light grey) with WHV genome specific primers, as described in Materials and Methods. The following charts illustrate the moment of birth (↑; time 0), the total observation period in months (mo), and the appearance and duration (shaded region) of serological markers of WHV infection and serum WHV DNA. The time-points of acquisition of liver biopsies (▼ ; Lbx) and autopsy tissues (↓ ; autopsy), challenge with an infectious WHV pool (∨; WHV) or with PBMC-derived inoculum (▼; PBMC/s) are indicated (pages 84-88).

A

WHsAg

anti-WHs

anti-WHc

WHV DNA

WHV Lbx1 Lbx2

1A/F

Autopsy (no serum available)

-12   -6   0   6   12 mo

Birth of offspring

B

C

WHV

| WHsAg |
| anti-WHs |
| anti-WHc |
| WHV DNA |

5C/F

Lbx1   Autopsy

| WHsAg |
| anti-WHs |
| anti-WHc |
| WHV DNA |

-6   0   6   12   18   24 mo

Birth of offspring
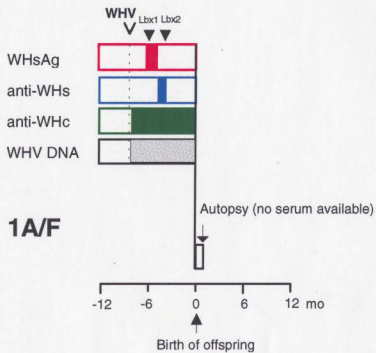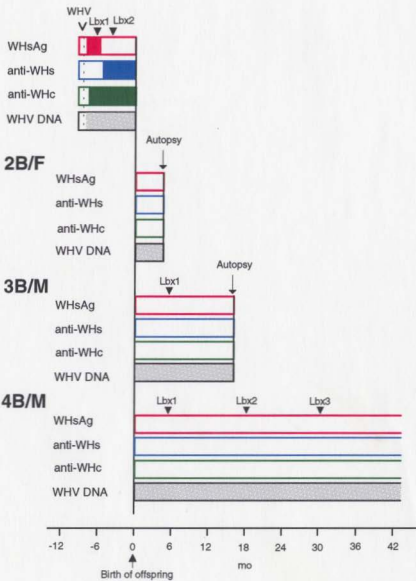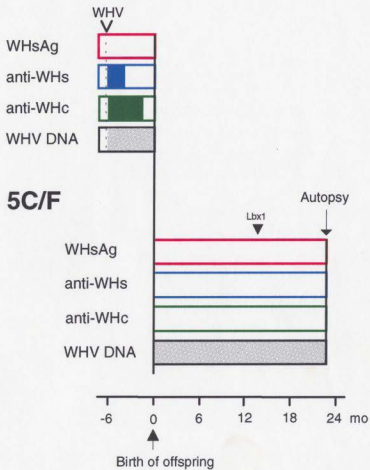
D

continued next page
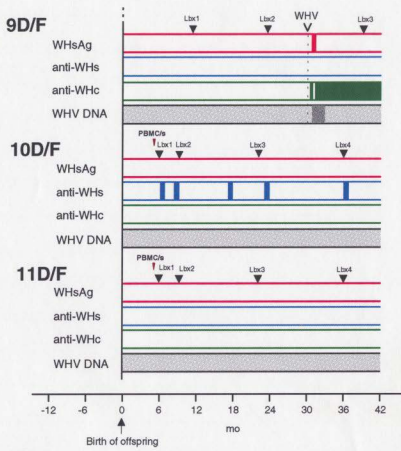
hybridization (sensitivity 10^7 genome equivalents/ml) except after experimental WHV reinfection, as described in Section 3.10. However, when the same sequential serum samples were tested by nested PCR with primers specific for WHV genes, all offspring were found WHV DNA-reactive. The sensitivity of a nested PCR assay was estimated to be $10-10^2$ genome equivalents per ml. Due to the generally very low quantities of WHV genomes in the circulation of the analysed animals, the direct PCR (sensitivity 10^3 genome equivalents/ml) was insufficient for detection of the amplified WHV DNA by EB-agarose. However, the direct PCR products could be occasionally identified by Southern blot hybridization with a WHV DNA specific probe (data not shown).

The results of nested PCR analysis revealed the persistent presence of virus DNA in the circulation of all offspring studied, beginning at the earliest sample up until the last serum sample collected (see Figure 3.3). In animals observed for longer then a 6 mo period, sera were tested at least once every 6 mo using nested PCR with C and S and/or X gene specific primers (mean monthly interval between samples tested ± standard deviation, SD; 2.0 mo ± 0.7). In all these animals, WHV genome was identified at least once during the 6 mo period (mean interval between WHV DNA positive samples ± SD; 3.2 mo ± 1.4). Table 3.3 summarizes results on WHV DNA assessment by PCR in sera from all cases investigated (except 1A/F and 2B/F). As shown, 48.1% of sera collected before WHV challenge were found WHV DNA-reactive when amplified with WHV C gene specific primers. However, when DNA from the same sera which were tested with WHV C gene primers, were also amplified with WHV S and/or X gene specific primers, 70.1% were found WHV DNA

Table 3.3 Detection of WHV gene sequences by nested PCR with WHV C gene primers or with C and X and/or S gene specific primers in offspring sera prior to and after WHV challenge.

Offspring	WHV C gene		WHV C and S and/or X gene	
	positive / total no. of sera tested		positive / total no. of sera tested	
	before WHV challenge	after WHV challenge	before WHV challenge	after WHV challenge
3B/M	3/7	n.a.[1]	4/7	n.a.
4B/M	9/14	n.a.	12/14	n.a.
5C/F	13/16	n.a.	13/16	n.a.
6D/F	6/12	5/5	8/12	5/5
7D/F	6/11	5/8	8/11	8/8
8D/M	3/10	7/7	6/10	7/7
9D/F	3/10	7/7	7/10	7/7
10D/F	14/26	n.a.	19/26	n.a.
11D/F	7/27	n.a.	17/27	n.a.
Total	64/133 (48.1%)	24/27 (88.9%)	94/133 (70.1%)	27/27 (100%)

[1] n.a., not applicable

positive (an increase of 22%). Importantly, all the serum samples that were tested with three pairs of WHV DNA specific primers (n = 25) were found to be WHV DNA positive with at least one primer set. In general, when the first and the final serum samples obtained from each animal were analysed by PCR with WHV C, S and X gene specific primers, all of the samples were found WHV DNA-reactive.

Four of the offspring (6D/F, 7D/F, 8D/M, and 9D/F) were challenged with an infectious WHV pool (see Section 3.10). DNA isolated from sequential sera of these offspring were evaluated for WHV genome expression at mean intervals ± SD of 1.6 mo ± 0.5. Only 88.9% of DNA samples amplified with C gene primers were found reactive but 100% of the samples tested positive when amplified with both C, S and/or X primer pairs (Table 3.3). This finding clearly demonstrated that WHV challenge increased WHV DNA detection in the circulation.

These results showed that amplifying the same DNA samples with primers specific for multiple, non-overlapping WHV genomic regions greatly improved detection of WHV DNA. They also revealed that identification of a given WHV gene sequence, either C, S or X genes tended to fluctuate during follow-up of a particular offspring. Also, in a given sample, WHV DNA could be detected with one set of primers, but not with another primer pair specific for a different part of the virus genome (see Figure 4.3). Serum samples collected from the same animal only a few months prior to or later than the tested sample could show the reverse situation. Nevertheless, all samples which were analysed using all three primer sets were found WHV DNA-reactive at least with one pair of primers. These fluctuations were most

Figure 4.3 Detection of WHV C, S and X gene sequences in serial serum samples collected from 4B/M offspring. Sera obtained between 2 and 36 mo after birth were tested for WHV DNA reactivity by nested PCR using WHV C, S and X gene specific primers and the amplified products analysed by Southern blot hybridization to a recombinant WHV DNA probe. Each assay was performed in parallel with DNA isolated from serum of a chronic WHV carrier (positive control) and water (negative control). The molecular sizes of the amplified WHV C, S and X gene fragments are indicated on the left of each panel.

	Negative Control	Mo After Birth							Positive Control
		2	8	14	19	25	30	36	

C

428 bp>

S

501 bp>

X

193 bp>
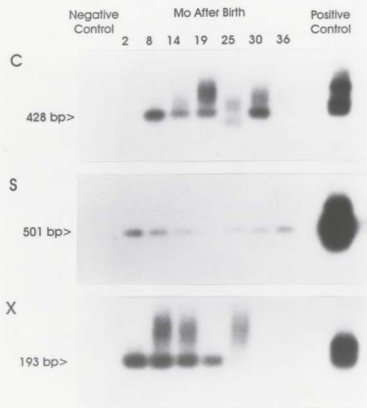
likely related to very low quantities of virus DNA in the sera tested that were at the detection limit of otherwise very sensitive assays used. Thus, due to technical limitations, it may not always be possible to detect each virus gene sequence in the analysed serum.

Figure 4.3 shows an example of WHV DNA detection in serial sera from offspring 4B/M which were collected from 2 up until 36 mo of age. In all samples examined, viral DNA could be identified by nested PCR with at least one set of oligonucleotide primers and by subsequent Southern blot hybridization with a WHV DNA specific probe. As illustrated, detection of a particular virus gene sequence in the test samples fluctuated from strongly positive, through moderately or weakly reactive to negative. This example demonstrates that the identification of WHV gene sequences circulating at trace quantities can drastically change and that the use of more than one primer pair greatly improves the chance of WHV DNA detection. In some cases, analysis of the amplified nested PCR products by Southern blotting with recombinant WHV DNA showed the presence of high molecular weight DNA species accompanying the WHV DNA band of predicted size. This unexplained phenomenon was also observed occasionally when DNA from offspring PBMC and tissue samples were tested (*e.g.*, Figures 5.3 and 12.3).

3.3 Expression of WHV Specific DNA and RNA Sequences
in PBMC from Offspring

PBMC were obtained from all offspring investigated except 1A/F and 2B/F. The first PBMC sample from offspring 3B/M, 4B/M, 5C/F, 10D/F, and 11D/F was collected between

3 and 4 mo after birth. In animal 6D/F, the first sample was taken at 7 mo of age, whereas in cases 7D/F, 8D/M and 9D/F the initial samples were not harvested until 10-11 mo after birth.

3.3.1 Persistent Carriage of WHV DNA in Offspring PBMC

DNA derived from sequential PBMC were tested for WHV DNA by nested PCR using primers specific for WHV C, S and X genes. In all offspring examined before WHV challenge, both the first sample and the last sample tested were found positive for WHV DNA using at least one of the primer pairs. On average, considering all animals tested, PBMC were assayed approximately every 6-8 mo (mean interval between tested samples ± SD; 3.4 mo ± 1.1) and they were found WHV DNA positive at least once during this period (mean interval between positive samples ± SD; 3.8 mo ± 1.1).

In PBMC evaluated for WHV DNA expression using only the C gene specific primers, 57.3% of the samples tested were found positive (Table 4.3). If the same samples, that were amplified with C primers were also tested with S and/or X primers, 80% of PBMC samples were reactive, as indicated in Table 4.3. In two offspring, 4B/M and 9D/F, DNA from sequential PBMC was amplified with all three WHV DNA primer pairs. Cells collected at 15, 17, 24, 26, 28 and 30 mo of age from offspring 9D/F, before re-infection with WHV, all tested positive with at least one out of three primer pairs (data not shown). Figure 5.3 illustrates an example of WHV DNA detection in serial PBMC from offspring 4B/M that were harvested from 3 to 34 mo after birth and analysed for WHV DNA with both C, S and

Table 4.3 Detection of WHV gene sequences by PCR amplification with WHV C gene primers or with C and X and/or S gene specific primers in offspring PBMC prior to and after WHV challenge.

Offspring	WHV C gene		WHV C and S and/or X gene	
	positive / total no. of PBMC tested		positive / total no. of PBMC tested	
	before WHV challenge	after WHV challenge	before WHV challenge	after WHV challenge
4B/M	7/11	n.a.[1]	10/11	n.a.
5C/F	3/7	n.a.	7/7	n.a.
6D/F	1/5	n.t.[2]	2/5	n.t.
7D/F	1/5	2/2	2/5	2/2
8D/M	4/4	2/2	4/4	2/2
9D/F	4/11	2/2	8/11	2/2
10D/F	5/6	n.a.	5/6	n.a.
11D/F	8/9	n.a.	8/9	n.a.
Total	35/61 (57.3%)	6/6 (100%)	49/61 (80%)	6/6 (100%)

[1] n.a., not applicable
[2] n.t., not tested

Figure 5.3 Comparison of WHV C, S and X gene sequences expression in serial PBMC samples of 4B/M offspring. Cells collected between 3 and 34 mo after birth were tested for WHV DNA reactivity by nested PCR using WHV C, S and X gene specific primers and the amplified products analysed by Southern blot hybridization to a recombinant WHV DNA probe. Each assay was performed in parallel with DNA isolated from serum of a chronic WHV carrier (positive control) and water (negative control). The molecular sizes of the amplified WHV nucleotide fragments are indicated on the left side of each panel.

	Negative Control	Mo After Birth							Positive Control

C

428 bp>

S

501 bp>

X

193 bp>
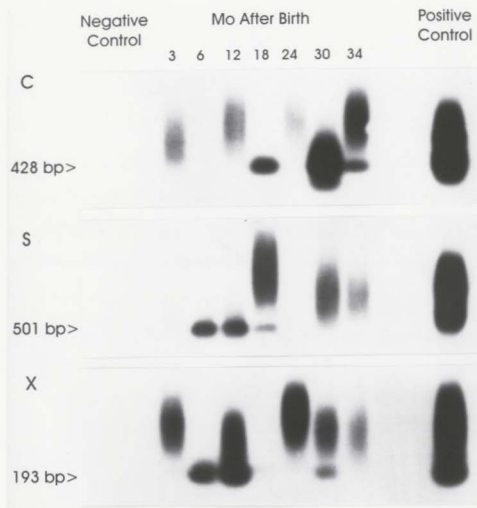
X gene primers. As shown, each cell sample was found WHV DNA positive with at least one primer pair. Thus, it is evident that the detection of WHV genome can be significantly improved by using two or more pairs of primers amplifying nucleotide fragments homologous to different regions of the WHV genome. Similarly, as it was observed in sequential serum samples, detection of each WHV gene sequence in serial PBMC samples tended to drastically fluctuate during follow-up (Section 3.2).

In offspring which were challenged with WHV (as described in Section 3.10), all PBMC samples examined after reinfection were found positive for WHV DNA (Table 4.3). As it was already demonstrated by the results from WHV DNA evaluation in sera (Section 3.2), WHV inoculation clearly increased expression of WHV DNA in circulating lymphoid cells.

3.3.2 Offspring PBMC Carried Transcriptionally Active WHV Genomes

The presence of WHV mRNA sequences was evaluated by reverse transcription reaction followed by nested PCR using WHV S or C gene specific primers in selected PBMC samples of 9 offspring. Transcriptionally active WHV genomes were identified in the cells derived from 5 of these offspring, *i.e.*, 4B/M, 7D/F, 8D/M, 9D/F, and 11D/F. In the remaining 4 cases, *i.e.*, 3B/M, 5C/F, 6D/F and 10D/F, WHV RNA sequences were not detected.

Figure 6.3 is an example of WHV RNA detection in sequential PBMC collected at 3 different time points during follow-up of offspring 4B/M and 7D/F. In offspring 4B/M,

Figure 6.3 Identification of WHV RNA sequences in consecutive PBMC samples from 4B/M and 7D/F offspring. PBMC were isolated at 18, 30 and 32 mo after birth from 4B/M. In the case of 7D/F, PBMC were collected at 18 mo after birth, and 9 and 10 mo after WHV challenge (32 and 33 mo after birth, respectively). Total RNA extracted from each cell sample was used for cDNA synthesis by reverse transcription (RT+) and the cDNA amplified by nested PCR with WHV S or C (*) gene specific primers. The PCR products obtained were analysed by Southern-blot hybridization to recombinant WHV DNA. As controls, RNA samples were subjected to the same reaction conditions in the absence of reverse transcriptase (RT−).

Mo After Birth

4B/M 18* 30 32 < 501 bp
                  < 428 bp

7D/F 18 32 33 < 501 bp

RT − + − + − +
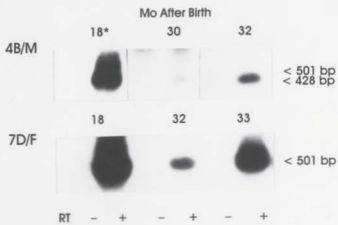
specific WHV RNA sequences were detected at 18, 30 and 32 mo after birth. Offspring 7D/F was challenged with WHV at 23 mo of age (see Section 3.10), however viral RNA was readily detectable before re-infection at 18 mo and after WHV administration 32 and 33 months of age. The negative control in each case consisted of PBMC RNA tested under identical conditions except RT was omitted during the cDNA synthesis step. As shown in this figure, no amplification products were observed in any of the RT negative controls.

3.3.3 Mitogen Stimulation Upregulated WHV Genome Expression in PBMC

In an attempt to determine whether mitogen stimulation might enhance expression of virus genome and its transcripts within PBMC in the offspring studied, freshly isolated cells were cultured in the presence of a nonspecific mitogen, such as ConA or LPS, for 72 h. After culture, total RNA was extracted and analysed for WHV mRNA. Results were compared with WHV RNA detection in unstimulated PBMC isolated at the same time from the same offspring. As shown in Figure 7.3, ConA stimulation of PBMC from animal 11D/F led to detection of viral RNA, although the same cells unstimulated were WHV RNA nonreactive. Similar results were seen in PBMC from offspring 8D/M and 9D/F after challenge with WHV (see Section 3.10). In Figure 8.3, WHV RNA was not detected in nonstimulated PBMC from offspring 8D/M, but PBMC stimulated with LPS for 72 h tested positive. Although WHV RNA signal was identifiable before stimulation of PBMC from offspring 9D/F, cells tested after LPS stimulation showed evident enhancement in the expression of WHV DNA transcripts. The negative control included cells isolated from a WHV-naive, healthy

Figure 7.3 Evaluation of WHV RNA expression in Concanavalin A stimulated and non-mitogen stimulated PBMC from 11D/F offspring at 34.5 mo after birth. Total RNA was extracted from unstimulated PBMC (−ConA) and from a parallel sample of the same cells that had been stimulated with ConA (+ConA) for 3 days before RNA isolation. ConA stimulated PBMC from a healthy, WHV-naive woodchuck were used as a negative control (control PBMC). RNA isolated from the spleen of a WHsAg-positive, chronic WHV carrier was used as a positive control for the RT reaction. 1 µg of the extracted RNA was used for cDNA synthesis with (+) or without (−) reverse transcriptase (RT) followed by nested PCR amplification of the resulting products using WHV C gene specific primers. The amplified 428-bp nucleotide sequences were identified by Southern-blot hybridization to recombinant WHV DNA.
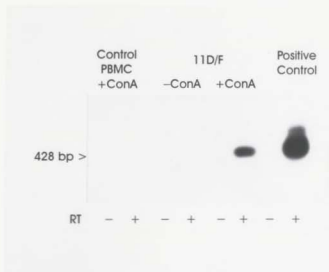
Figure 8.3 Evaluation of WHV RNA expression in lipopolysaccharide-stimulated and unstimulated PBMC from 8D/M and 9D/F offspring obtained at 3 mo after WHV challenge (33.5 mo after birth). In both offspring, total RNA was extracted from unstimulated PBMC (−LPS) and from parallel samples of the same cells that had been stimulated with LPS (+LPS) for 3 days before RNA isolation. LPS-stimulated PBMC from a healthy, WHV-naive woodchuck were used as a negative control (control PBMC). 2 μg of the extracted RNA was used for cDNA synthesis with (+) or without (−) reverse transcriptase (RT) followed by nested PCR amplification of the resulting products using WHV S gene specific primers. The amplified 501-bp nucleotide sequences were identified by Southern-blot hybridization to recombinant WHV DNA.

	Control PBMC	8D/M		9D/F	
	+LPS	− LPS	+LPS	− LPS	+LPS

501 bp>

RT   − +   − +   − +   − +   − +
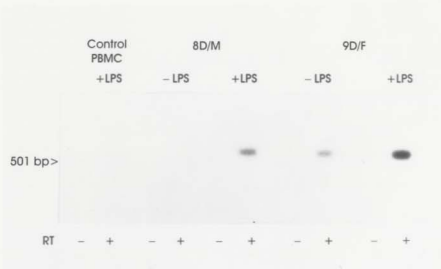
woodchuck which were stimulated with LPS or ConA under similar conditions as PBMC from the offspring. As illustrated in Figures 7.3 and 8.3, WHV specific RNA sequences were undetected in these PBMC. Total RNA purified from spleen of a WHsAg-positive, chronic WHV carrier was used as a positive control and produced amplicons of the predicted molecular size (Figures 7.3 and 8.3). Each RT reaction was performed in parallel with a negative control in which reverse transcriptase was omitted during cDNA synthesis. There were no products observed after amplification of any of these negative controls.

To determine whether virus might be secreted by PBMC due to mitogen stimulation, WHV DNA expression was evaluated in the concentrated supernatant of cells cultured in the presence of LPS and in the final wash from the PBMC that was obtained prior to their culture (see Section 2.7.2). Although WHV DNA was not detected in the wash, all PBMC culture supernatants tested positive (data not shown). In further experiments, described in Section 3.8, it was also tested whether the supernatant from mitogen-stimulated PBMC of offspring with long-term follow-up after birth from a WHV-convalescent mother contain infectious virus.

The above results indicate that lymphoid cells of the offspring support WHV replication. Thus, not only WHV DNA and transcriptionally active virus genomes were detected in PBMC for up to 32 mo after birth, but also mitogen stimulation of the cells yielded an increase in WHV RNA levels suggesting a higher rate of virus replication. Since, WHV DNA was undetectable in the final wash after PBMC isolation, this implies that virus genome sequences detected were of an intracellular origin. Further evidence has been

provided by the results presented below.

3.3.4 DNase and Trypsin Treatment had no Effect on WHV DNA Detection in Offspring PBMC

To further exclude the possibility that detection of WHV genome sequences in PBMC could be attributed to cell surface associated viral particles or free WHV DNA fragments, viable cells were subjected to limited DNase and trypsin digestion prior to DNA extraction. In both test PBMC from 4B/M and control PBMC from a WHsAg-positive, chronic WHV carrier, treatment of intact circulating lymphoid cells with DNase followed by trypsin before DNA isolation had no effect on the expression of WHV DNA signal. Furthermore, WHV DNA was undetected in the final wash from the PBMC isolation procedure as well as, in the final wash after DNase/trypsin treatment (Figure 9.3), indicating that there were no extracellular viral particles present which could be responsible for the observed positive signal after PCR amplification. Since, any extracellular viral DNA or virus DNA-protein complexes would have been removed or destroyed in this process, the WHV DNA detected is attributed to virus located inside the cell.

3.3.5 WHV RNA Sequences Carried in PBMC did not Originate from Serum

An additional control experiment was performed to compare WHV RNA detection in liver and lymphoid tissues with that in serum. The experiment was set up using 1 μg of RNA isolated from spleen and liver of a WHsAg-reactive, chronic WHV carrier, and 2 μg of

Figure 9.3 Effect of DNase and trypsin treatment on detection of WHV DNA in PBMC collected from 4B/M offspring at 40 mo after birth and from a WHsAg-positive, chronic WHV carrier. Both PBMC samples were extensively washed, digested with DNase and trypsin and washed again, as described in Materials and Methods. DNA extracted from cells (C) and final PBMC wash (W) obtained before and after PBMC enzymatic treatment was amplified by nested PCR using C gene specific primers and products hybridized to recombinant whole WHV DNA probe. Positive samples showed the expected 428-bp, nucleotide fragment.

4B/M Offspring   WHsAg carrier
Before   After   Before   After

428 bp >

W C W C   W C W C
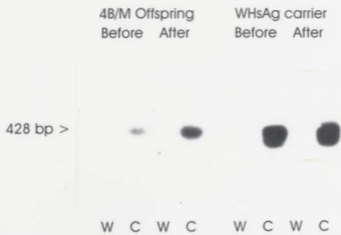
RNA from serum of the same chronic carrier and from an adult woodchuck convalescent from acute WHV hepatitis. Results of this experiment presented in Figure 10.3 showed that although liver and spleen from a chronically infected animal evidently carried transcriptionally active virus, WHV mRNA was not detected in a serum sample from the same animal or from another convalescent woodchuck, although they were tested at the quantities 2-fold greater than those from hepatic and splenic tissues. This provides further proof that WHV RNA sequences detected in circulating PBMC did not originate from extracellular virus which has adhered to the cell membrane, otherwise serum would also carry transcriptionally active virus genomes.

3.4 Offspring Livers with Different Status of WHV DNA Expression

Liver biopsies were obtained on at least yearly intervals from surviving offspring. In cases 1A/F and 2B/F, only autopsy specimens were available for investigation as both these offspring did not live long enough to perform liver biopsies. In the remaining offspring, up to 4 liver samples were collected during the reported follow-up. All liver tissue samples were tested for WHV DNA by nested PCR, with all three pairs of WHV genome specific primers (i.e. C, S and X). After evaluation of all liver samples, the 11 offspring investigated could be divided into 3 groups based on the status of WHV genome expression in the liver (Table 5.3).

The first group included 6 animals. In these offspring, WHV DNA could be detected in all sequential liver samples collected during the entire follow-up using at least one pair of virus genome specific primers for PCR amplification. This group constituted offspring 2B/F,

Figure 10.3 Analysis of WHV RNA in serum of a woodchuck convalescent from acute WHV hepatitis and in serum, spleen and liver from a WHsAg-reactive, chronic WHV carrier. Approximately 1μg of total RNA extracted from spleen and liver as well as 2 μg of total serum RNA was used for cDNA synthesis with (+) or without (−) reverse transcriptase (RT). The resulting products were amplified by nested PCR with WHV C gene specific primers and the 428-bp virus gene fragments detected by Southern-blot hybridization to recombinant WHV DNA probe.

Convalescent | Chronic WHV Carrier
Serum | Serum | Spleen | Liver

428 bp>

RT   −   +   −   +   −   +   −   +
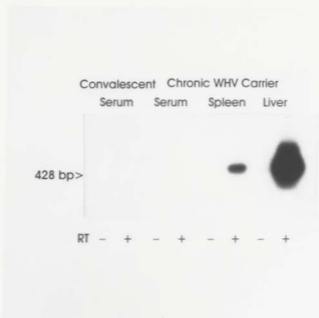
Table 5.3 Detection of WHV C, S and X gene sequences in serial liver samples collected during follow-up of the offspring studied[1]

Offspring	WHV gene primers	Liver sample number (offspring age in mo at sample collection)			
		1	2	3	4
1A/F	C	– (1)[2]			
	S	–			
	X	–			
2B/F	C	– (4)[2]			
	S	–			
	X	+			
3B/M	C	– (6)	– (15)[2]		
	S	–	–		
	X	–	–		
4B/M	C	– (6)	+ (19)	+ (31)	
	S	–	–	+	
	X	–	+	+	
5C/F	C	+ (14)	+ (22)[2]		
	S	+	+		
	X	+	–		
6D/F	C	+ (11)	+ (22)	+ (31)[2,3]	
	S	–	–	+	
	X	+	+	+	
7D/F	C	– (11)	– (22)	+ (37)[3]	
	S	–	–	+	
	X	–	–	–	
8D/M	C	+ (11)	+ (23)	+ (36)[3]	
	S	+	+	+	
	X	+	+	+	
9D/F	C	– (11)	– (23)	– (39)[3]	
	S	–	–	–	
	X	–	–	–	
10D/F	C	+ (6)	– (9)	– (22)	+ (36)
	S	+	–	–	+
	X	+	+	+	+
11D/F	C	+ (6)	+ (9)	– (22)	+ (36)
	S	+	–	–	+
	X	+	–	+	–

[1] WHV DNA sequences detected by nested PCR using WHV C, S and X gene specific primers
[2] Liver sample collected during autopsy
[3] Liver sample collected after WHV challenge

5C/F, 6D/F, 8D/M, 10D/F, and 11D/F.

Evaluation of WHV DNA in liver samples from 4 other animals gave very unexpected results. In this second group, which included offspring 1A/F, 3B/M, 7D/F and 9D/F, WHV DNA was not detected in the liver even up to 39 mo after birth, despite repeated testing by nested PCR using all three sets of WHV DNA specific primers and Southern blot analysis of the resulting reaction mixtures. The autopsy liver tissue obtained from offspring 1A/F at 1 mo of age was found negative for WHV DNA using C, S and X gene primer pairs. In case 3B/M, a liver biopsy collected at 6 mo of age and at autopsy performed at 15 mo after birth was also negative after repeated testing with all three pairs of primers. Before WHV challenge, WHV DNA was not identifiable in liver biopsies collected at 11 mo of age from offspring 7D/F and 9D/F, and in the liver biopsy collected at 22 mo of age from 7D/F and at 23 mo from 9D/F. After WHV challenge, liver biopsy from offspring 7D/F at 37 mo after birth was found WHV DNA-reactive. Even more surprising was finding that WHV challenge of 9D/F did not induce WHV DNA appearance in the liver and the third biopsy obtained at 39 mo after birth from this offspring remained negative.

The third category was represented by just one case, offspring 4B/M. In this animal, WHV DNA was undetectable in the first liver biopsy collected at 6 mo after birth. However, the remaining two biopsies obtained at 19 and 31 mo of age tested positive for WHV DNA using at least one of the virus genome specific primer pairs.

The data of WHV DNA testing of liver samples differed from the results on evaluation of the WHV DNA expression in PBMC or serum samples. Thus, WHV DNA could always

be detected in serum or PBMC by PCR amplification with at least one of the three WHV genome specific primers which were routinely used (Sections 3.2 and 3.3.1). Conversely, liver biopsies from some of the offspring (i.e. 1A/F, 3B/M, 7D/F, and 9D/F) were completely nonreactive for WHV DNA using all three primer pairs, even when the amount of total DNA analysed was increased to 5 μg per reaction (data not shown). These results are the first of this kind and they document that long-term virus persistence can be maintained exclusively at an extrahepatic location.

3.5 Comparison of WHV DNA Detection in Parallel
Serum, PBMC and Liver Samples.

In all offspring studied, serum, PBMC and liver samples collected at approximately the same time points of follow-up were evaluated for WHV DNA expression. Results showed a fluctuation not only in the detection of a particular WHV DNA sequence, either C, S or X genes, but also in the virus genome expression that varied at different cell or tissue sites in a given offspring when tested at the same time of follow-up. This observation is illustrated in Figure 11.3 whereby WHV C gene expression was compared in sequential serum, PBMC and liver biopsies collected at the same time from 11D/F offspring. Thus, at 6 mo after birth, C gene sequences were identified in serum and PBMC, but not in the liver. In contrast, the liver was WHV DNA positive at 9 mo of age, but serum and PBMC negative. By 22 mo, serum was reactive, whereas liver and PBMC were WHV DNA nonreactive. Finally, WHV C gene sequences were detected in both serum, PBMC and liver at 36 mo after

Mo After Birth
6   9   22   36

Serum

428 bp>

Liver
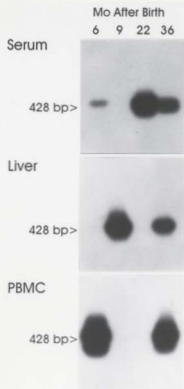
428 bp>

PBMC

428 bp>

Figure 11.3 Evaluation of WHV DNA expression in serum, liver and PBMC samples collected from 11D/F offspring at 6, 9, 22, and 36 mo after birth. Each sample was tested by nested PCR using WHV C gene specific primers and the amplified 428-bp nucleotide fragments were identified by Southern blot hybridization to a recombinant WHV DNA probe.

birth.

It became evident that a single negative result from testing of a particular serum or tissue sample does not mean that WHV DNA is in fact absent. For example, repeat testing of liver biopsy #1 from 11D/F offspring with C gene specific primers eventually yielded a positive result (compare Table 5.3 and Figure 11.3). WHV DNA was also readily detectable when nested PCR with S and X gene primers were used to test the same sample (Table 5.3). Consequently, a sample was considered to be truly WHV DNA negative only after extensive testing with all three WHV gene specific primers that was supported by negative results after Southern blot analysis of the nested PCR amplification mixtures. These results also indicate the importance of analysing different types of samples, such as serum, PBMC and liver, to completely exclude that the test animal is free of virus.

3.6 Expression of WHV DNA and RNA in Autopsy Liver and Lymphoid Tissues

Five offspring were autopsied during the course of this study (e.g., 1A/F, 2B/F, 3B/M, 5C/F and 6D/F). In 6D/F offspring, the autopsy was performed after WHV challenge (see Section 3.10). Liver and spleen tissues were collected from all the cases. Bone marrow was obtained from 3B/M, 5C/F and 6D/F, lymph nodes from 1A/F and 3B/M, and thymus from 1A/F and 6D/F woodchucks. As indicated in Table 6.3, WHV DNA expression was assessed by nested PCR with WHV C, S and X gene specific primers, whereas WHV specific RNA sequences were detected after reverse transcription reaction by nested PCR with WHV C or S gene primers.

Table 6.3 Detection of WHV specific DNA or RNA in liver and lymphoid tissues obtained during autopsy of offspring born to mothers convalescent from acute viral hepatitis

Tissue	WHV DNA[1] or WHV RNA[2]	WHV Gene Primers	Offspring				
			1A/F	2B/F	3B/M	5C/F	6D/F[3]
Liver	WHV DNA	C	−	−	−	+	+
		S	−	−	−	+	+
		X	−	−	+	−	+
	WHV RNA	C	x[4]	n.t.[5]	x	+	+
Spleen	WHV DNA	C	+	+	+	+	+
		S	+	+	+	+	+
		X	−	+	+	+	−
	WHV RNA	C or S	−	+	+	+	+
Bone Marrow	WHV DNA	C	n.a.[6]	n.a.	+	+	+
		S	n.a.	n.a.	−	−	+
		X	n.a.	n.a.	+	+	−
	WHV RNA	C	n.a.	n.a.	−	+	+
Lymph Node	WHV DNA	C	+	n.a.	+	n.a.	n.a.
		S	+	n.a.	−	n.a.	n.a.
		X	+	n.a.	+	n.a.	n.a.
	WHV RNA		n.t.	n.a.	n.t.	n.a.	n.a.
Thymus	WHV DNA	C	+	n.a.	n.a.	n.a.	−
		S	n.t.	n.a.	n.a.	n.a.	+
		X	−	n.a.	n.a.	n.a.	n.t.
	WHV RNA	C	−	n.a.	n.a.	n.a.	−

[1] Detected by nested PCR using WHV C, S and X gene specific primers.
[2] Detected by nested PCR with WHV C or S gene specific primers after reverse transcription.
[3] Autopsy performed after WHV re-infection
[4] x, denotes not applicable
[5] n.t., denotes not tested
[6] n.a., denotes not available

3.6.1 Lymphoid Restricted Expression of WHV Genome

Evaluation of WHV DNA sequences in autopsy liver and lymphoid tissue samples showed that all investigated offspring carried virus genome in the cells of the lymphatic system, but not all in the liver (Table 6.3). Therefore, in general the cases could be divided into two categories. In the first group (i.e., 2B/F, 5C/F and 6D/F), both liver and all lymphoid organs tested were found positive using at least one pair of WHV gene specific primers. In 2B/F offspring, the liver was only weakly reactive for WHV X sequences yet spleen was strongly WHV DNA positive when tested with all three WHV DNA primer pairs. The spleen from 5C/F offspring was found WHV DNA positive with all three pairs of WHV genome specific primers. However, virus gene sequences were only detectable in the liver and bone marrow after amplification with primer pairs specific either for C and S genes or C and X genes, respectively. 6D/F offspring was euthanised after WHV challenge (see Section 3.10). As expected, liver, spleen, bone marrow and thymus from this animal were evidently WHV DNA-reactive. Virus C, S and X gene sequences were detected in the liver, however only C and S gene sequences were identifiable in the splenic and bone marrow tissues of this offspring. The thymus was positive with WHV S gene specific primers, C gene sequences were undetectable, and X gene expression was not tested.

In the second group of autopsy cases (i.e., 1A/F and 3B/M), WHV genomes could not be identified in the liver despite repeated testing with both WHV C, S and X gene primers and Southern blotting. However, lymphoid tissues from both these offspring evidently carried WHV DNA, as detected by PCR using at least one of three WHV gene

specific primer pairs. Thus, in 1A/F animal C gene sequences were identified in the spleen, lymph node and thymus. The spleen and lymph node from this offspring were also WHV DNA-reactive when tested with S and/or X gene specific primers. Figure 12.3 illustrates an example of lymphoid tissue restricted WHV DNA expression in 3B/M offspring. In two liver samples from this animal, obtained at 6 and 15 mo after birth, WHV DNA was undetectable using both C and X gene primers as well as, with S gene primers (Table 6.3). In contrast, WHV DNA was readily detectable in the spleen, bone marrow and lymph node tissues with WHV C and X gene primers (Figure 12.3). Virus S gene sequences were also found in the spleen, but not in bone marrow and lymph node in this animal (Table 6.3).

In summary, WHV genome was detected in all lymphoid tissues collected from each of the autopsy cases analysed. However, virus DNA was evidently undetectable in the liver of 2 out of the 5 offspring examined.

3.6.2 WHV Genome Transcription in Autopsy Livers and Lymphoid Tissues.

Autopsy tissues were analysed for WHV RNA sequences by reverse transcription and the synthesized cDNA detected by nested PCR with WHV C or S gene specific primers (Table 6.3). WHV RNA was undetectable in all autopsy tissues obtained from 1A/F offspring. As shown in Figure 13.3, WHV RNA sequences were detected in the spleen from 2B/F and 3B/M offspring. In the case of 5C/F and 6D/F (6D/F after WHV challenge), WHV RNA could be identified in liver, spleen and bone marrow (Figures 14.3 and 15.3). In summary, transcriptionally active WHV gene sequences were found in the lymphatic system

Figure 12.3 Evaluation of WHV DNA expression in liver, PBMC and lymphoid tissues collected from 3B/M offspring. DNA was extracted from liver samples obtained by laparotomy at 6 mo of age and at autopsy performed at 15 mo after birth, from PBMC collected at 14.5 mo of age, and from spleen, lymph node, bone marrow and skeletal muscle acquired at autopsy. WHV gene sequences were identified by nested PCR using C or X gene specific primers followed by Southern-blot hybridization of the amplified products to recombinant WHV DNA. Positive samples showed the expected molecular size of the amplified virus C or X gene fragments, indicated on the left of the panel.

	Liver			PBMC	Spleen	Lymph Node	Bone Marrow	Skeletal Muscle
	6mo	15 mo	14.5 mo				

428 bp>
193 bp>

C X C X C X C X C X C X C X C X

Figure 13.3 Evaluation of WHV RNA expression in the spleen of three offspring born to mothers convalescent from a past episode of acute viral hepatitis. Total RNA extracted from the spleen of 2B/F, 3B/M and 5C/F obtained at 4, 15 and 22 mo after birth, respectively, was used for cDNA synthesis with (+) or without (−) reverse transcriptase (RT). After amplification of cDNA by nested PCR using WHV S gene specific primers, the resulting products were detected by Southern-blot hybridization to recombinant WHV DNA. The expected molecular size of the amplified WHV DNA sequences is shown on the right side of the panel.

	2B/F 4 mo		3B/M 15 mo		5C/F 22 mo	
RT	−	+	−	+	−	+

< 501 bp
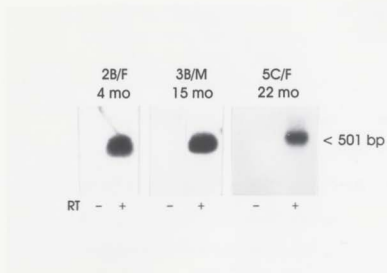
Figure 14.3 Detection of WHV RNA in the liver and lymphoid organs of an offspring born to a mother convalescent from acute WHV hepatitis. Total RNA extracted from liver, spleen and bone marrow obtained at 22 mo after birth from 5C/F offspring was used for reverse transcription reaction. The synthesized cDNA was amplified by nested PCR using C gene specific primers and the resulting products analysed by Southern blot hybridization to recombinant WHV DNA. The visualized bands showed the expected molecular size of 428-bp. As a control, each assay was performed with (+) and without (−) reverse transcriptase (RT).

428 bp>
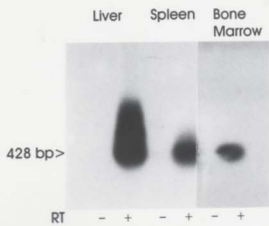
Figure 15.3 Evaluation of WHV RNA expression in the liver and lymphoid organs obtained from 6D/F offspring at 8 mo following challenge with WHV (i.e., 31 mo after birth). RNA extracted from liver, spleen, bone marrow, and thymus was used for cDNA synthesis with (+) or without (−) reverse transcriptase (RT). A control RNA sample obtained from spleen of a chronic WHV carrier (positive control) was subjected to the same treatment. The resulting products were amplified by nested PCR using WHV C gene specific primers followed by Southern-blot hybridization to recombinant WHV DNA. Positive samples showed the expected molecular size of 428-bp.

	Liver	Spleen	Bone Marrow	Thymus	Positive Control

428 bp>

RT − +   − +   − +   − +   − +
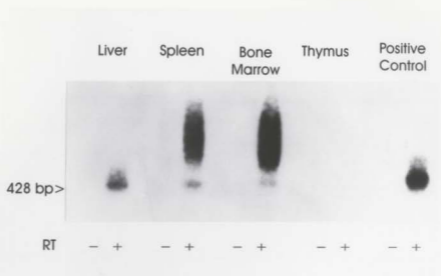
in 4 out of the 5 offspring analysed. However, in 2 out of 3 offspring reactive for WHV DNA in the liver WHV RNA specific sequences were also identified in hepatic tissues.

As mentioned previously (Section 2.5.5), each RT reaction was set up in parallel with a negative control that had all the ingredients except reverse transcriptase. The negative result of this control excludes the possibility that virus genome sequences detected in the tested samples were caused by WHV DNA contamination instead of virus specific mRNA. In addition, as shown in Section 3.3.5 WHV specific mRNA sequences were not found in serum either from a woodchuck convalescent from acute WHV hepatitis or a chronically infected animal, but were detected in total RNA prepared from the liver and spleen of the latter animal. Thus, the viral mRNA reactivity detected in the liver and lymphoid tissues could not originate from viral particles which may occur in the circulation.

3.7 Physicochemical Properties of WHV DNA-Reactive
Particles Carried by Offspring

3.7.1 Sedimentation Velocity of WHV DNA-Reactive Particles from Sera

To assess whether WHV DNA detected in the offspring sera could be contained within virion particles, the sedimentation velocity of WHV DNA in sera from 4B/M and 7D/F offspring was compared to that of purified WHV virions and fragments of recombinant WHV DNA. Sera from 4B/M and 7D/F animals were selected for this evaluation because they displayed opposite status of WHV genome expression in the liver. Thus, liver of 7D/F was repeatedly WHV DNA unreactive prior to collection of the analysed serum, whereas the liver

of 4B/M offspring displayed virus genome at the time of serum acquisition.

Figure 16.3 illustrates the results of WHV DNA determination in 15 fractions collected from the bottom of each gradient after ultracentrifugation of test sera and control preparations. The results of WHV DNA testing of each fraction, determined by PCR with S gene specific primers, is plotted in relation to the sucrose concentration of each fraction (as described in Section 2.6.3). In fractions collected after centrifugation of purified WHV virions, the peak of WHV DNA reactivity sedimented to the bottom of the sucrose gradient. Thus, WHV DNA could be detected in bottom sucrose fractions 1-3 and in a second peak located in the top fractions 13-15 when tested by direct PCR. In sucrose fractions collected after centrifugation of the recombinant WHV DNA, viral DNA could only be detected by nested PCR, with the strongest WHV DNA reactivity detected in the middle of the gradient in fractions 9-11 and in the top fraction 15. In sucrose fractions collected after sedimentation of sera from 4B/M and 7D/F offspring, two peaks of WHV DNA reactivity were detected by nested PCR. The first peak, found in bottom fractions 1-5, corresponded to WHV DNA-reactive fractions 1-3 from the WHV virion preparation. However, there was also the second peak observed in the middle of the gradient in fractions 8-12. The location of this peak was comparable to that of the WHV DNA positive fractions 9-11 obtained after centrifugation of recombinant WHV DNA.

Therefore, the above findings indicate that a part of WHV DNA reactivity detected in offspring sera was associated with particles migrating in sucrose with velocity similar to that of purified WHV virions, suggesting the presence of intact (complete) viral particles.

Figure 16.3 Analysis of sedimentation velocity of WHV DNA in sera collected from two offspring born to mothers convalescent from an acute episode of viral hepatitis. Test samples included: serum from 4B/M offspring with WHV DNA expression in both liver and PBMC at the time of serum acquisition at 32 mo after birth (blue), serum obtained at 22 mo after birth from 7D/F offspring with WHV DNA expression in PBMC but not in liver (yellow), purified WHV virions (red), and recombinant WHV DNA (green). All samples were centrifuged separately through 15% sucrose over a 60% sucrose cushion, as described in Materials and Methods. Fifteen fractions collected from the bottom of each gradient were tested for WHV DNA by direct or nested PCR using WHV S gene specific primers and evaluated by refractometry for sucrose concentration (%; grey). The degree of WHV DNA reactivity was assessed by the intensity of ethidium bromide staining of PCR product bands in agarose. WHV DNA detection was graded according to an arbitrary scale, which ranged from 0 (undetectable) to 3 (strongest ethidium bromide staining). Positive samples showed the expected WHV nucleotide fragment of 501-bp.

WHV DNA Positivity

Sucrose Concentration (%)

BOTTOM

Fraction Number

TOP
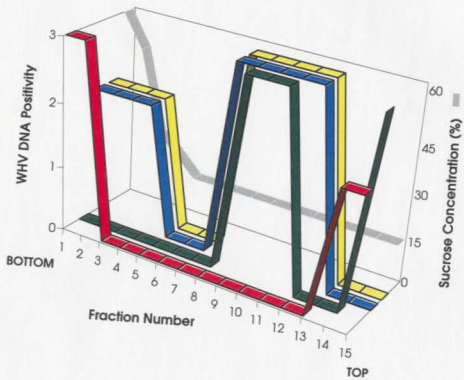
■ WHV virions ■ Recombinant WHV DNA ■ Offspring 4B/M ■ Offspring 7D/F

However, WHV sequences in the tested offspring sera sedimented also with velocity comparable to that of recombinant fragmented WHV DNA, indicating that viral nucleic acid fragments also occurred in the circulation of these animals. To further characterize the WHV DNA-reactive particles detected in the offspring sera, WHV DNA-reactive fractions were subjected to DNase treatment and analysed again by PCR, as described in Section 3.7.2.

3.7.2 Effect of DNase Digestion on WHV DNA Detected in Sucrose Fractions

Sedimentation analysis revealed that a WHV DNA-reactive serum sample could contain several distinct forms of viral particles, either intact WHV virions or free WHV DNA fragments. Undamaged WHV virions should be resistant to DNase treatment due to the presence of a protective, outer lipoprotein coat. In contrast, free WHV DNA, as well as damaged virions, should be susceptible to the DNase digestion and eliminated by the enzyme action.

In order to differentiate WHV virions from partially or unenveloped WHV DNA, sucrose fractions which had demonstrated the strongest WHV DNA reactivity by PCR were combined and examined for WHV DNA before and after treatment with DNase. As described in Section 3.7.1 and presented in Figure 16.3, the peaks of WHV DNA reactivity were detected in bottom fractions 1-3 for purified WHV virions, in fractions 1-4 and 8-12 for serum from 4B/M offspring, and in fractions 9-11 for *Mnl*-I digested recombinant WHV DNA. Figure 17.3 shows that WHV DNA was detectable before DNase treatment in all samples analysed. Following DNase digestion, the bottom fractions obtained after

Figure 17.3 Effect of DNase digestion on WHV DNA detection in pooled fractions collected after centrifugation through 15% over a 60% sucrose cushion of serum from 4B/M offspring, purified WHV virions, and recombinant WHV DNA. Sucrose fractions with strongest WHV DNA reactivity from the above samples were pooled and DNase treated (D), as described in Materials and Methods. Tested samples included: fractions 1-3 (bottom) for WHV virions, fractions 1-4 (bottom) and fractions 8-12 (top) for 4B/M serum, and fractions 9-11 (top) for recombinant WHV DNA (see Figure 16.3 for details). DNA extracted from each sample was tested for WHV S gene sequences by nested PCR and the amplified products were identified by Southern-blot hybridization to recombinant WHV DNA. For each digested sample (D), a sample of the same pooled sucrose fractions untreated with DNase (ND) was evaluated for WHV DNA reactivity. Positive samples showed the expected WHV nucleotide fragment of 501 bp.

	WHV Virions	4B/M Offspring		Recombinant WHV DNA
	Bottom	Bottom	Top	Top

501 bp >

ND D   ND D   ND D   ND D
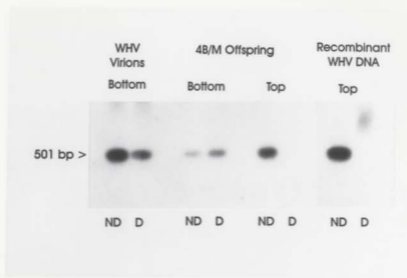
centrifugation of the WHV virions and the corresponding fractions collected after centrifugation of 4B/M remained WHV reactive. Conversely, the same DNase treatment of fractions harvested from the top of the gradients after fractionation of recombinant WHV DNA and 4B/M serum entirely eliminated WHV DNA reactivity. Since WHV DNA detected in the bottom fractions from offspring sera were resistant to DNase digestion, this experiment provides further evidence that intact WHV virions circulated in these offspring long after birth.

3.7.3 Buoyant Density Analysis of WHV DNA-Reactive Particles in Offspring Sera and PBMC-Derived Culture Supernatant.

The buoyant density of WHV DNA-reactive molecules was analysed in serum collected from 4B/M offspring at 32 mo after birth and in culture supernatant obtained after 72 h stimulation with LPS of PBMC from the same animal, and in serum from 7D/F offspring obtained at 22 mo after birth. In parallel, as described in Section 2.6.4, a preparation of purified WHV virions was also fractionated through CsCl gradient. Direct PCR testing of fractions obtained from WHV virion gradient revealed that WHV genome reactivity was detectable only in fractions 9-13, corresponding to CsCl density of 1.21-1.32 g/cm^3. All the tested samples from offspring 4B/M and 7D/F, centrifuged under the same conditions, demonstrated WHV DNA reactivity by nested PCR in fractions 9-11 showing a CsCl density of 1.21-1.32 g/cm^3. Thus, for 4B/M offspring serum, WHV DNA was detected in fractions 9-13, whereas in the culture supernatant from PBMC of the same animal fractions 10-13 were

WHV DNA positive. In the case of serum from 7D/F offspring, fractions 10-12 were found WHV DNA-reactive (Figure 18.3). Therefore, fractions collected from the offspring CsCl gradients which were found WHV DNA-reactive exhibited a buoyant density that is characteristic for WHV virions.

3.8 Infectivity of WHV Carried by Offspring

To determine whether silent carriage of WHV genomes by the offspring is infectious, either concentrated serum or plasma (Section 2.9.1) or culture supernatant from LPS-stimulated PBMC (Section 2.7) was administered intravenously into healthy, WHV-naive woodchucks. All serum samples collected from the healthy woodchucks prior to inoculation tested negative for anti-WHc by specific ELISA and were WHV DNA negative by nested PCR with virus gene specific primers. In addition, PBMC and liver biopsies collected from these normal animals were found WHV unreactive by nested PCR. All these results confirmed that the animals were WHV naive prior to the inoculation.

3.8.1 Inocula Derived from Plasma and PBMC of Liver WHV DNA Positive Offspring Induced Serologically Evident WHV Infection

WHV genome was expressed in both the liver, PBMC and serum of offspring 4B/M at the time of plasma and PBMC collection predestinated for preparation of inocula. To determine infectivity of WHV carried by this animal, plasma collected at 32-34 mo after birth was ultracentrifuged and the resulting pellet injected i.v. into woodchuck #269/F. PBMC

Figure 18.3 Analysis of buoyant density of WHV DNA in sera and a culture supernatant from LPS-stimulated PBMC of offspring born to mothers convalescent from acute WHV hepatitis. Test samples included: purified WHV virions, serum collected at 32 mo after birth and PBMC-derived supernatant at 32-34 mo after birth from 4B/M offspring which expressed WHV genome in both liver and PBMC at the time of sample collection, and serum obtained at 22 mo after birth from offspring 7D/F in which WHV DNA was present in PBMC but not in the liver. All samples were centrifuged separately through a 1.1-1.7 gm/cm³ CsCl gradients, as described in Materials and Methods. Thirteen fractions were collected from the bottom of each gradient and evaluated by refractometry for CsCl density (gm/cm³). All fractions recovered after centrifugation of the WHV virion preparation were tested for WHV DNA by direct PCR with WHV C gene specific primers. Only those fractions obtained after centrifugation of the offspring samples that corresponded to the WHV DNA positive fractions recovered after virion fractionation were tested by nested PCR with WHV C primers.

| CsCl density gm/cm³ | 1.70 | 1.67 | 1.63 | 1.61 | 1.53 | 1.48 | 1.44 | 1.39 | 1.32 | 1.28 | 1.24 | 1.21 | 1.21 |

Offspring 7D/F serum

Offspring 4B/M PBMC derived supernatant

Offspring 4B/M serum

WHV virions

Fraction Number

0 Bottom — 1 2 3 4 5 6 7 8 9 10 11 12 13 Top

■ nested PCR pos. ■ direct PCR pos. □ negative
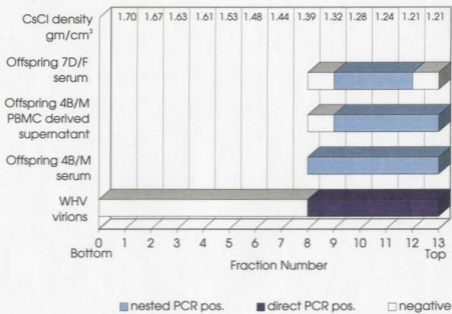
isolated at the same time were cultured for 72 h in the presence of LPS (Section 2.7) and the culture supernatant concentrated by ultracentrifugation and injected into WHV-naive woodchuck #278/F.

After inoculation, #269/F and #278/F sera were tested for WHsAg and anti-WHc (see Figure 19.3). In total, 9 sequential serum samples were evaluated from each animal beginning at 14 dpi. WHsAg was detected in sera collected at 49 and 63 dpi from #269/F and at 63 dpi from #278/F. Anti-WHc was found positive in #269/F beginning from dpi 49 until the end of follow-up, whereas animal #278/F became anti-WHc-reactive after 72 dpi and remained positive throughout the entire observation period.

WHV DNA was detected by nested PCR in sera obtained at dpi 10 from #278/F and by direct PCR beginning at dpi 49 in sera from #269/F and #278/F woodchucks. All subsequent serum samples from both woodchucks tested WHV DNA positive by direct or nested PCR amplification with virus genome specific primers.

PBMC from both #269/F and #278/F also carried WHV DNA. In #269/F, viral DNA was detected by nested PCR at dpi 49 and after direct PCR amplification at dpi 63, but could not be detected at dpi 215. In the case of #278/F, PBMC collected at dpi 10, 21, and 49 tested WHV DNA positive by nested PCR and even by direct PCR at dpi 72. However by dpi 218 a nested PCR was again necessary to detect WHV DNA in PBMC from #278/F (Figure 19.3).

Liver samples were obtained from #269/F and #278/F approximately 2 mo prior to inoculation and then at approximately 80 dpi and at autopsy. As indicated above, liver

Figure 19.3 Follow-up of WHV-naive, healthy woodchucks after inoculation with plasma, serum or a supernatant from LPS-stimulated PBMC (LPS-PBMC/s) derived from offspring born to mothers with a past episode of acute WHV hepatitis. Sequential sera obtained before and after inoculation were tested for WHsAg (red), anti-WHc (green) and WHV DNA by direct (dark grey) or nested (light grey) PCR, as described in Materials and Methods. The following charts illustrate the total observation period in days, the moment of inoculation (∨ ; time 0), and the appearance and duration (shaded region) of serological markers of WHV infection. The time of acquisition and WHV DNA expression (+ or −) in liver biopsies (▲), PBMC (▲) and autopsy tissues (↓) are indicated.

Figure 19.3 Follow-up of WHV-naive, healthy woodchucks after inoculation with plasma, serum or a supernatant from LPS-stimulated PBMC (LPS-PBMC/s) derived from offspring born to mothers with a past episode of acute WHV hepatitis. Sequential sera obtained before and after inoculation were tested for WHsAg (red), anti-WHc (green) and WHV DNA by direct (dark grey) or nested (light grey) PCR, as described in Materials and Methods. The following charts illustrate the total observation period in days, the moment of inoculation ($\vee$; time 0), and the appearance and duration (shaded region) of serological markers of WHV infection. The time of acquisition and WHV DNA expression ($+$ or $-$) in liver biopsies ($\blacktriangle$), PBMC ($\blacktriangle$) and autopsy tissues ($\downarrow$) are indicated.

269/F

Inoculum: 4B/M plasma

SERUM:		Autopsy
WHsAg		
anti-WHc		
WHV DNA		

PBMC: WHV DNA — − − − + + +

LIVER: WHV DNA − + +

SPLEEN: WHV DNA +
SPLENOCYTES: WHV DNA +
BONE MARROW: WHV DNA +
LYMPH NODE: WHV DNA +

-50   0   10   20   30   40   50   60   70   80   100   220

278/F

Inoculum: 4B/M LPS-PBMC/s

SERUM:		Autopsy
WHsAg		
anti-WHc		
WHV DNA		

PBMC: WHV DNA − − + + + + +

LIVER: WHV DNA − + +

SPLEEN: WHV DNA +
SPLENOCYTES: WHV DNA +
BONE MARROW: WHV DNA −
LYMPH NODE: WHV DNA

-50   0   10   20   30   40   50   60   70   80   100   220

biopsies collected before inoculation were WHV DNA negative by nested PCR. In both animals, liver tissue taken at about 2.5 mo after inoculation tested WHV DNA positive at the level of direct PCR whereas those obtained at autopsy were reactive by nested PCR. Furthermore, WHV DNA was detected by nested PCR in lymphoid tissues (*i.e.*, spleen, bone marrow and lymph node) from #269/F at autopsy (215 dpi). Interestingly, although the WHV genome could not be detected in the final PBMC sample, it was identifiable in lymphocytes from spleen. Evaluation of autopsy lymphoid tissues obtained at dpi 218 from #278/F yielded similar results. Thus, WHV DNA was detected by nested PCR in spleen and lymphoid cells derived from splenic tissue, however bone marrow and lymph node were WHV DNA unreactive (Figure 19.3).

In summary, the above experiment revealed that inocula derived from 4B/M offspring, either plasma or PBMC culture supernatant carried biologically competent WHV which was transmittable to healthy woodchucks. The induced infection was serologically evident since WHsAg was transiently detected and anti-WHc was persistently expressed in the circulation in both #269/F and #278/F. In some serum, PBMC and liver samples WHV DNA was detected even by direct PCR, indicating that the virus load was relatively high.

3.8.2 Inocula Derived from Plasma or Serum of Liver WHV DNA Negative Offspring were Infectious to Healthy Woodchucks

WHV genome sequences were undetectable in sequential liver samples obtained from both 3B/M and 7D/F offspring prior to WHV challenge, however both animals carried WHV

in circulating lymphoid cells and serum. To determine whether virus present in the offspring was infectious, inocula derived from serum of 3B/M and plasma of 7D/F was injected into WHV-naive woodchucks #260/M and #276/F, respectively. Following inoculation, both animals were assayed for serological markers of WHV infection, WHsAg and anti-WHc, as well as for WHV DNA in serum, liver and PBMC, as described in Section 3.8. Before inoculation, all WHV infection markers were not detectable in these woodchucks.

After inoculation, all serum samples collected from #260/M and #276/F tested negative for WHsAg and anti-WHc. However, WHV DNA was detectable in sera by nested PCR beginning at dpi 49 in #276/F and at dpi 22 of #260/M. PBMC became WHV DNA-reactive at 10 and 13 dpi from #276/F and #260/M, respectively. Thus, WHV DNA apparently appeared in PBMC of #260/M even prior to its appearance in serum.

As mentioned above, the first liver biopsy from #260/M, collected before inoculation, tested negative for WHV DNA. However, the liver biopsy obtained at dpi 76 from this animal as well as, that collected 110 dpi at autopsy were also found WHV DNA negative. Testing of these samples with pairs of primers specific for the WHV C, S and X genes gave repeatedly negative results confirming the complete lack of virus genome expression in the liver of this animal. Most interestingly, serum, PBMC, splenocytes, spleen and bone marrow obtained at autopsy from #260/M tested WHV DNA-reactive (Figures 19.3 and 20.3).

Animal #276/F was injected with inoculum derived from 7D/F offspring, in which WHV DNA could not be detected in the liver. As expected, WHV DNA was not detectable in the first liver biopsy obtained prior to inoculation, but in contrast to #260/M woodchuck,

Figure 20.3 Evaluation of WHV DNA expression in serum, liver, PBMC and lymphoid tissues of 260/M woodchuck obtained 3.5 mo after inoculation with serum from 3B/M offspring. Serum collected 15 mo after birth from 3B/M offspring with WHV DNA expression restricted to the lymphatic system was prepared as described in Materials and Methods and injected i.v. into a healthy, WHV-naive woodchuck 260/M. DNA extracted from liver biopsies collected at approximately 2 mo before (1) and after (2) inoculation, and from serum, liver (3), PBMC and lymphoid tissues obtained at autopsy of 260/M were tested for WHV DNA by nested PCR using WHV C gene specific primers. The resulting products were analysed by Southern blot hybridization to a recombinant WHV DNA probe. Each assay was performed in parallel with DNA isolated from serum of a chronic WHV carrier (positive control) and two negative controls which included water and a mock extracted sample (as described in Materials and Methods). Positive samples showed the expected molecular size of 428 bp, indicated on the left side of the panel.

| | Mock | Serum | Liver | | | Splenocytes | | Positive Control |
| | Water | | 1 | 2 | 3 | PBMC | Bone Marrow | |

428 bp>
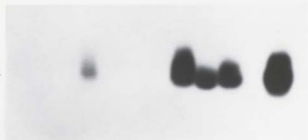
the liver obtained at autopsy at dpi 79 tested positive for WHV DNA. All lymphoid tissues from this animal, i.e., spleen, bone marrow and lymph node, as well as serum and PBMC were also WHV DNA-reactive (Figures 19.3 and 21.3).

In summary, serum or plasma derived from either offspring 3B/M or 7D/F, which did not carry WHV in the liver, was infectious to WHV-naive woodchucks. However, in contrast to infection induced by inocula prepared from 4B/M offspring with WHV DNA in the liver, the infection was serologically silent (i.e., WHsAg and anti-WHc negative) and WHV DNA was detectable only by nested PCR amplification. In #276/F inoculated with plasma from 7D/F offspring, both liver and lymphoid tissues tested positive for WHV DNA. In #260/M infected with 3B/M serum, the liver remained continuously WHV DNA negative. Overall, the obtained results indicate that inocula derived from animals with undetectable WHV DNA in the liver could carry infectious virus which is transmittable to healthy woodchucks. However, the triggered infection was serologically silent and virus DNA sequences occurred only at very low levels. In one of the inoculated animals, WHV DNA expression was restricted to the lymphatic system.

3.9 PBMC-Derived Inoculum from Adult Woodchucks with SLAH did not Infect Offspring

PBMC-derived inoculum from woodchucks after recovery from an episode of self-limited acute hepatitis was injected i.v. into 10D/F and 11D/F offspring (see Section 2.10). Sera collected from these animals were evaluated for WHsAg, anti-WHs, anti-WHc as well

Figure 21.3 Evaluation of WHV DNA expression in autopsy samples of 276/F obtained 3 mo after inoculation with plasma from 7D/F offspring. Plasma from 7D/F offspring that expressed WHV DNA in PBMC but not in liver was collected between 11 and 22 mo after birth, prepared as described in Materials and Methods, and injected i.v. into a healthy, WHV-naive woodchuck 276/F. DNA extracted from 276/F liver biopsy obtained at 2 mo before inoculation and serum, liver, PBMC, and lymphoid tissues acquired at autopsy were tested for WHV DNA by nested PCR using WHV C gene specific primers. The resulting products were analysed by Southern blot hybridization to a recombinant WHV DNA probe. Each assay was performed in parallel with DNA isolated from serum of a chronic WHV carrier (positive control). Positive samples showed the expected molecular size of 428 bp indicated on the left side of the panel.

| Serum | Liver Biopsy | Liver | PBMC | Spleen | Lymph Node | Bone Marrow | Positive Control |

428 bp>
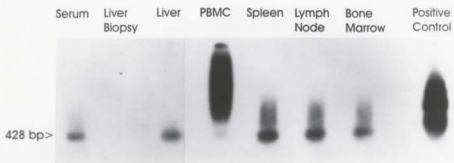
as, tested for WHV DNA. Anti-WHs was detected intermittently during follow-up of 10D/F, but no other serological marker of WHV infection appeared in this animal during the entire observation period after injection with the PBMC-derived supernatant. WHV DNA could be detected by nested PCR prior to inoculation and virus genome levels appeared to be unchanged following injection with inoculum (Figure 3.3). Thus, PBMC culture supernatant originating from woodchucks with SLAH was not infectious to these offspring.

3.10 Offspring Challenged with Infectious WHV Developed Acute Hepatitis

In this experiment, 6D/F and 8D/M offspring with WHV DNA positive liver, and 7D/F and 9D/F woodchucks with WHV DNA negative liver were challenged with a highly infectious WHV inoculum derived from serum of a WHsAg-positive WHV carrier. Following inoculation, all the offspring were tested for WHsAg, anti-WHs, anti-WHc, and serum WHV DNA (Figure 3.3).

After challenge of 6D/F offspring, anti-WHc was detected in the circulation at dpi 28, 42 and 56, anti-WHs transiently appeared at 82 dpi, and WHsAg remained undetectable. Levels of serum WHV DNA appeared to increase somewhat, although the virus genome was still only detectable by nested PCR and returned finally to pre-inoculation levels. GGT increased to 26 beginning at dpi 147, suggesting the possible development of HCC. However, liver obtained at autopsy (i.e., dpi 244) did not show evidence of liver tumour. WHV DNA sequences were identified by nested PCR in all autopsy tissues obtained from this animal, including liver, spleen, bone marrow and thymus, as described previously in Section

3.6. Autopsy liver, spleen and bone marrow also tested positive for WHV specific RNA, indicating presence of transcriptionally active WHV genomes, as illustrated in Figure 15.3.

In 7D/F offspring, WHsAg and anti-WHs remained undetectable after challenge with WHV. However, serum became anti-WHc-reactive at dpi 14 and remained positive until the end of follow-up (i.e., dpi 440). Serum WHV DNA levels transiently increased, as it could be detected by direct PCR amplification at dpi 14 and even by slot blot hybridization assay at dpi 28, which is approximately 10^5-fold less sensitive then direct PCR assay. Subsequently, as observed for 6D/F, serum WHV DNA levels ultimately decreased to the pre-inoculation level. As expected, liver biopsy collected from 7D/F at 14 mo after WHV challenge tested positive for WHV DNA, whereas two biopsies collected prior to inoculation were virus negative.

Similar results were obtained after WHV challenge of 8D/M offspring. Again, as observed for 7D/F, WHsAg and anti-WHs were not detected, but anti-WHc appeared in the circulation at dpi 14 and remained positive until the end of follow-up (i.e., dpi 216). WHV DNA could be detected in serum by direct PCR at dpi 35 but thereafter, a nested PCR was necessary to identify virus gene sequences. A liver biopsy was collected 6 mo after injection with WHV and, like the previous two biopsies from the same animal, was found WHV DNA-reactive.

The same pattern of WHV genome expression was also observed in 9D/F offspring following WHV challenge. However, unlike 6D/F, 7D/F and 8D/M, WHsAg could be detected during a short period between dpi 35 and 42. Anti-WHs was undetectable in this

animal, whereas its serum became anti-WHc-reactive beginning at dpi 14 and except for a brief interval of non-reactivity at dpi 35, remained positive until the last sample tested at dpi 216. WHV DNA was detectable by direct PCR at dpi 35, but sera collected following dpi 100 tested positive only after amplification by nested PCR. WHV DNA was not detected in any of three liver biopsies taken from this animal, including a sample collected 9 mo following WHV challenge.

In summary, 6D/F, 7D/F, 8D/M, and 9D/F offspring were susceptible to WHV infection after challenge with a highly infectious virus and there was no apparent difference in the pattern of the induced infection between offspring with and without WHV DNA expression in the liver, except 7D/F. In all cases, serum became anti-WHc-reactive. Also serum collected from 9D/F offspring became WHsAg positive and anti-WHs could be detected in 7D/F offspring serum. A transient increase in serum WHV DNA levels was observed in all the offspring injected with WHV, as it is illustrated for 9D/F animal in Figure 22.3. In this offspring, WHV S gene sequences were detectable by nested PCR in sera collected 60 and 30 days prior to WHV inoculation and at dpi 0 and 14 after WHV challenge. In contrast, a direct PCR was sufficient to amplify WHV DNA present in sera collected at dpi 35 and 42. Subsequently, serum WHV DNA levels returned to pre-inoculation titres, as shown for sample collected at dpi 251. As mentioned previously in Section 3.2 and shown in Table 3.3, a greater percentage of samples tested positive for viral DNA after WHV challenge. For example, 48.1% of all serum samples tested positive prior to injection with WHV by PCR with C gene specific primers, whereas 88.9% of serum samples examined were

Figure 22.3 Comparison of WHV DNA detection in sequential sera collected from 9D/F offspring before and after challenge with infectious WHV. DNA samples extracted from 9D/F sera at 60 and 30 days before and 0, 14, 35, 42, 251 days after WHV challenge were tested for WHV S gene sequence expression by direct and nested PCR. The PCR products obtained after direct (top panel) and nested (bottom panel) amplification, were detected by Southern-blot hybridization to a recombinant WHV DNA probe. Positive samples showed the expected molecular size of 1290-bp after direct PCR or 501-bp after nested PCR amplification. Water was added to the PCR mixture instead of DNA as a negative control (Neg Ctrl).

Days After WHV Challenge

	-60	-30	0	14	35	42	251	Neg Ctrl

Direct PCR

1290 bp>

Nested PCR

501 bp>
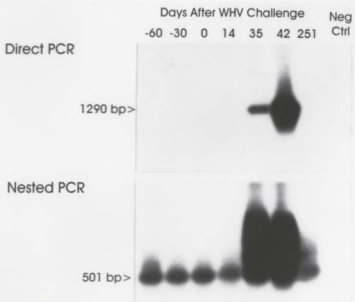
WHV DNA-reactive after WHV challenge. Similar results were obtained when WHV DNA expression was evaluated in PBMC. Prior to inoculation, PBMC were 80% positive when tested with C, S and/or X primers, but 100% of PBMC collected after inoculation were WHV DNA-reactive (Table 4.3). Liver samples were collected from each offspring analysed following WHV challenge. In two cases, 6D/F and 8D/M, liver had tested positive prior to and remained reactive after WHV inoculation, with no apparent change in the levels of WHV DNA. However, WHV DNA had not been detected in both liver biopsies taken before WHV challenge from either 7D/F or 9D/F offspring. The third liver biopsy, obtained after injection of WHV from 7D/F tested WHV genome positive, but in 9D/F the liver remained WHV DNA negative (Table 5.3). Thus, results indicate that WHV re-infection led to increased expression of WHV genome sequences in serum and PBMC from all the offspring. However, only offspring 7D/F showed a significant increase of virus load in the liver. The level of WHV DNA in hepatic tissues remained unchanged in all other cases. Since liver samples were collected from 6 up to 14 mo after WHV challenge, it is possible that WHV DNA in the liver transiently increased in 6D/F and 8D/M woodchucks or appeared in offspring 9D/F but has subsequently returned to pre-inoculation levels.

CHAPTER FOUR - DISCUSSION

4.1 Offspring Born to Mothers Convalescent from Viral Hepatitis are
Serologically Silent Carriers of WHV

For the first time, this study provides evidence that vertical transmission of hepadnavirus to offspring occurs despite the apparently complete recovery of mothers from viral hepatitis. We have demonstrated in the woodchuck model of hepatitis B that the virus can persist for a very long time, if not for life, in such offspring as an asymptomatic infection characterized by the absence of all WHV serological markers. In these newborn animals, the only indication of WHV infection was the continuous presence of trace amounts of viral genomes in the circulation, liver and lymphatic organs.

The persistence of low-levels of WHV DNA despite the absence of any serological marker of WHV infection was observed in all of the offspring analysed, except those animals which were challenged with infectious WHV in the late phase of our study. Although, we could not detect any WHV antigens or relevant anti-viral antibodies, it is possible that they were present but at levels below the detection limits of the assays used. However, even after concentration of offspring sera by fractionation in sucrose we failed to detect WHsAg. This is in contrast to the findings in sera from maternal woodchucks convalescent from acute WHV hepatitis (Michalak *et al.*, manuscript submitted) and from patients after recovery from acute HBV infection (Michalak *et al.*, 1994) which documented presence of the antigen traces after similar fractionation. Thus, it is evident that although the offspring lacked all conventional serological markers of infection they persistently carried WHV genomes. This

situation could be explained by a combination of a few coinciding events, *i.e.*, infection very early in life, or even *in utero*, with very low levels of the virus which caused a low rate of persistent virus replication and induced immune tolerance to viral antigens. This immune tolerance could be because antigens encountered during the development of the fetal immune system are regarded as "self" and fail to elicit specific cellular and humoral immune responses (Ahmed *et al.*, 1989). Virus-induced immunological tolerance is accepted as a reason for the development of long-term infection in children born to mothers with chronic hepatitis B (see Section 1.6.1d). However, unlike previous investigations showing that tolerance eventually wanes with immune system maturity, in our study, the animals did not develop any immune response during follow-up.

In sequential serum and PBMC samples obtained as early as 2 mo after birth and up until 42 mo from the offspring studied, WHV genome sequences could be detected by nested PCR followed by Southern blot analysis. In order to increase the sensitivity of our detection and not to miss mutated viral genome sequences, WHV DNA expression was routinely evaluated using oligonucleotide primers specific for three non-overlapping regions of the WHV genome, namely C, S and X genes. As indicated in Tables 3.3 and 3.4, by using this approach, we were able to improve the detection of low-levels of viral DNA in serum by at least 2-fold and in PBMC by a third in comparison to amplification with WHV C gene primers only. These findings parallel results reported by other investigators in HBV-infected patients (Chazouilleres *et al.*, 1994; Jilg *et al.*, 1995; Paterlini *et al.*, 1990). We also observed significant variability in the detection of different WHV gene sequences in the offspring

investigated. For example, WHV DNA could be identified with one primer pair but not detected using another pair specific for a different part of the WHV genome in the same serum or PBMC sample. Yet, the reverse situation could be found in the next sample from the same animal. This is not surprising given the very low levels of template viral DNA present in these samples. Therefore, even small fluctuations in virus levels or slight variations in recovery of total DNA from test sample and in PCR conditions could affect WHV DNA detection, especially when the two-step PCR procedure is employed. However, it is also possible that the observed variations are caused by the presence of virus mutants. As mentioned before (Section 1.3.2), WHV uses reverse transcription in its replication cycle thus, the virus is naturally subjected to a high mutation rate and can generate many genomic variants which may predominate at a given time during infection. If mutations occur within a primer binding site, this may consequently affect WHV DNA amplification using that particular primer pair. On the other hand, identification of WHV DNA in the same sample with all three primer sets specific for distinct genomic regions may suggest that viral DNA existed in a complete uninterrupted form. This could be considered as evidence for the presence of complete virions.

The existence of intact WHV virions in the offspring was supported by the results from analysis of physicochemical properties of circulating WHV DNA-reactive molecules. As mentioned above, concentration of serum samples by fractionation in sucrose enabled WHsAg detection in maternal woodchucks, yet the same treatment failed to identify similar particles in the offspring sera. Nevertheless, WHV DNA-reactive particles in the same

offspring sera did show physicochemical properties similar to those of intact WHV virions. For example, the WHV DNA particles migrated in sucrose with a velocity identical to that of WHV virions and they were resistant to DNase digestion, suggesting that they were protected by protein coat. However, there was also evidence that incomplete viral particles as well as freely circulating WHV DNA were present in the same samples. This was based on the finding that migration of some of the WHV DNA-reactive particles in sucrose was compatible to that of protein-free DNA fragments obtained by digestion with restriction enzyme of recombinant WHV DNA. Furthermore, centrifugation in CsCl gradients indicated that WHV DNA-reactive particles in the offspring sera also exhibited a buoyant density which corresponded to that of WHV virions with an intact lipoprotein envelope. Taken together, these results convincingly indicate that at least part of WHV DNA reactivity detected in the offspring sera was contained within intact virions. These findings are comparable to those obtained from analysis of sera from individuals who completely recovered from acute hepatitis B (Michalak *et al.*, 1994). In these patients, serum HBV DNA-reactive molecules also displayed physicochemical characteristics similar to those of purified Dane particles. In addition, the sedimentation velocities and buoyant densities of the different HBV DNA-reactive particles detected in these convalescent sera (*e.g.*, intact virions and free HBV DNA) corresponded to those observed in the present study. This is not surprising since both HBV and WHV demonstrate very similar molecular, structural and physical properties (Section 1.4.2). However, definitive evidence for the presence of structurally complete and biologically competent WHV virions was established when inoculum prepared from the

concentrated plasma of the offspring studied induced serologically evident acute hepatitis in WHV-naive woodchucks (discussed in Section 4.5).

In adult woodchucks convalescent from acute WHV hepatitis, carriage of WHV genomes in the absence of all serological markers of WHV infection, except anti-WHc, was associated with the ultimate development of HCC in 2 out of 9 animals studied (Michalak *et al.*, manuscript submitted). Similarly, in the human situation, persistence of HBV DNA in the context of serological immunity to virus (*i.e.*, serum reactivity for anti-HBc and/or anti-HBs) has been implicated in the development of HCC (Blum *et al.*, 1991; Fong *et al.*, 1993; Liang *et al.*, 1990; Paterlini *et al.*, 1990). For example, in one well studied patient, HCC was seen with integrated HBV DNA in the tumorous part of the liver 23 yr after complete serological and clinical recovery from acute hepatitis B and continuous presence of virus-specific antibodies (Blum *et al.*, 1991). Furthermore, serum taken from this individual and injected into a chimpanzee resulted in serologically evident acute HBV infection (as discussed below). Other studies described development of HCC in patients without symptoms of hepatocellular injury or inflammatory changes in the liver and in the absence of serological markers of HBV infection (Kamito *et al.*, 1996). The only indication of previous HBV exposure in these individuals was the presence of HBV DNA in serum and liver tissue. In the above studies, it was postulated that the integration of HBV DNA into the host genome could result in cellular genome rearrangements and mutations which may eventually lead to tumour formation. Although, the WHV genome has persisted in the offspring from our study for more than 3 yr after birth in the absence of serological indicators of infection, we have not

observed any evidence of liver inflammation or HCC development. Thus, the possible long-term consequences of this low-level virus carriage have yet to be determined.

In addition, it is also possible that serologically immune individuals who silently carry HBV could be responsible for transmission of viral hepatitis to healthy recipients of blood or organ transplants (Chazouilleres et al., 1994; Lowell et al., 1995; Wachs et al., 1995). It has been suggested that HBV can persist at trace quantities in extrahepatitic tissues, especially in the lymphatic system, which may serve as the potential virus reservoir for re-infection of the liver (Chazouilleres et al., 1994; Féray et al., 1990; Jiang et al, 1994). Therefore, these data together with findings from the present study show that infectious hepadnavirus undetectable by all conventional assays can persist in the absence of serological markers of infection. It is evident that potential transplant donors and recipients should be tested for the presence of HBV using sensitive molecular techniques, i.e., PCR followed by Southern blot analysis of the amplified products.

As mentioned above, the offspring investigated were negative for all serological markers of WHV infection, including anti-WHc antibodies. Antibodies to WHV or HBV nucleocapsid appear soon after virus invasion and usually persist for life. Hence, they have been applied as a reliable serological indicator of current or past hepadnavirus infection. The lack of anti-HBc response is very rare and has been attributed to aberrant immunological responses of the host or infection with HBV variants. For example, in one study of children born to HBsAg-positive carrier mothers (Lee et al., 1989), it was postulated that the absence of anti-HBc was due to the babies' immune incompetence. These infants also failed to

develop anti-HBe and anti-HBs, although they were positive for HBsAg and HBeAg during a follow-up period of 3 to 5 years. Therefore, it appeared that these infants were incapable of producing antibodies to HBV antigens. It was not determined, however, whether this was caused by a generalized immunosuppression or a defective immune response specific for HBV, since the authors did not challenge the infants with unrelated antigens. The authors of the above study believed that these children will eventually develop antibodies against HBV when they become immunocompetent. A comparable finding was reported in an anti-HBc nonreactive adult chronically infected with HBV (Lee *et al.*, 1992). In this study, the investigators suggested that the lack of anti-HBc response was a consequence of HBV-specific immune defect probably involving antigen presenting cells. Although, the described individual appeared to be immunocompetent, his monocytes did not ingest or process beads coated with HBcAg.

In other investigations, it has been reported that even some immunocompetent individuals infected with HBV by either vertical or horizontal transmission failed to produce anti-HBc. In these cases, the lack of anti-HBc response has been attributed to transmission of a HBV variant, since sequencing analysis revealed mutations in the C gene region containing important B and T cell recognition epitopes (Fiordalisi *et al.*, 1994; Valliammai *et al.*, 1995; Zoulim *et al.*, 1996). In another work already mentioned above, it was reported that a chimpanzee acquired acute HBV infection after inoculation with serum from a convalescent individual that had developed HCC years after complete resolution of acute hepatitis B (Blum *et al.*, 1991; Liang *et al.*, 1990). Although the chimpanzee became positive

for other serological markers of HBV infection (*i.e.*, HBsAg and HBeAg and subsequently, anti-HBs and anti-HBe), the animal did not produce an early (immunoglobulin class M) anti-HBc response. In addition, DNA sequence analysis of the inoculum predicted a single amino acid change in a highly conserved fragment of the preC region of the C gene. Thus, it was postulated that this substitution could be responsible for altering the early immune response to the C gene immune dominant epitope. Overall, the above mentioned studies suggest that the lack of an antibody response to the hepadnavirus nucleocapsid could be due to virus specific immune defect or viral variants. Whether these or other mechanisms underlie the observed antibody unresponsiveness to WHV antigens in the offspring studied needs to be determined in future investigations. One of the first approaches should be determination of the nucleotide sequence of WHV occurring in these offspring.

4.2 Lymphatic System is Intimately Involved in the
Persistence of Vertically Transmitted WHV

In all offspring from which serial serum samples were available for analysis (n=10), WHV genome was detected during the entire follow-up period. In most of the cases, WHV DNA were also found in the liver and lymphoid cells (n = 7). Most interestingly, in a significant number of offspring (n = 4), the life-long persistent carriage of hepadnavirus was restricted to the lymphatic system.

Hepadnaviral detection in lymphoid cells is well documented (Harrison, 1990; Lamelin *et al.*, 1995). It has been reported that during the natural course of WHV infection, viral

DNA appeared in lymphoid cells of the bone marrow at least 4 weeks before being detectable in the liver (Korba *et al.*, 1987a). Subsequently, WHV genome was detected in the spleen and lymph nodes, and then in PBMC. In our laboratory, woodchucks tested for WHV genome expression in PBMC by nested PCR with primers specific for the WHV C gene and followed by Southern blot hybridization showed that WHV DNA appears as early as 10 days after inoculation with virus, this was 1-2 wks prior to detection in the liver by the same method (Pardoe and Michalak, unpublished data). Another recent study from our laboratory identified an amino acid sequence in the preS1 domain of WHV envelope which recognizes in a strictly host specific manner woodchuck hepatocytes and lymphoid cells (Jin *et al.*, 1996). Interestingly, synthetic analogs of this epitope interacted with the host lymphoid cells to a much greater extent (approximately 1000-fold) than with hepatocytes. This study suggests that lymphoid cells may provide more favourable targets than hepatocytes for WHV invasion. Nevertheless, there is still some debate on whether hepadnaviruses can actually replicate in lymphoid cells and establish a productive infection.

4.2.1 Lymphoid Cells Support WHV Replication

The results from the current study indicate that WHV establishes a productive infection in lymphoid cells. We base this conclusion on several observations, namely: (1) Both WHV DNA and RNA specific sequences could be detected in lymphoid organs and in serial PBMC samples collected for more than 3 yr after birth, implying continuous presence of not only viral DNA but also transcriptionally active (replicating) virus genomes in the host

lymphoid cells; (2) No WHV RNA was detected in sera of a woodchuck convalescent from acute WHV infection and a WHsAg-positive chronic carrier with a high virus load. This finding indicates that virus-specific RNA does not circulate freely and therefore, WHV RNA detected in PBMC has to originate from a virus pool replicating intracellularly; (3) WHV DNA was detectable in PBMC that had been extensively treated to remove any adsorbed virus, demonstrating that the detected WHV DNA was unlikely of an extracellular origin; (4) Stimulation of the offspring PBMC with non-specific mitogen increased levels of WHV RNA expression, confirming that the cells maintained replicating virus; (5) The supernatant obtained from mitogen-stimulated PBMC carried WHV particles with physicochemical properties similar to those of intact virions and, most importantly, (6) Culture supernatant derived from offspring mitogen-stimulated PBMC was infectious to WHV-naive woodchucks. In the following paragraphs, the findings mentioned above will be discussed in more detail.

The detection of WHV DNA in serial PBMC, as well as in the spleen, bone marrow, thymus and lymph nodes, provides strong evidence for WHV infection of the lymphatic system. This correlates well with previous reports on WHV DNA replication in lymphoid cells (Korba *et al.*, 1987a, 1987b; Michalak *et al.*, manuscript submitted; Ogston *et al.*, 1989; Pardoe and Michalak 1995). However, the actual transcription of viral DNA was confirmed by the detection of WHV mRNA in lymphoid tissues and PBMC by using virus-specific PCR with a reverse transcription step (Sections 3.3.2 and 3.6.2). Some investigators argue, however, that the viral genomes detected in PBMC may originate from circulating viral particles adhered to the cell surface (Köck *et al.*, 1996). We do not believe that this is true

because WHV mRNA was not detected in serum from a control woodchuck with serologically evident chronic WHV infection, but it was detected in half the amount of total RNA from corresponding liver and spleen samples from the same animal. Similarly, total RNA isolated from serum of a woodchuck with complete recovery from acute hepatitis also tested negative for WHV mRNA (Section 3.3.5). These findings indicate that WHV RNA in PBMC unlikely originate from serum.

To further address the possibility of contamination of PBMC with circulating viral particles, viable PBMC were isolated from a chronic WHsAg carrier and from a representative offspring. These cells were thoroughly washed and then treated with DNase and trypsin to remove any free viral DNA or virus particles from the cell surface (Section 3.3.4). Even after this procedure, PBMC remained positive for WHV DNA and there was no detectable viral DNA signal in the cell washes obtained before and after enzymatic treatment, implying that the detected viral genome was of an intracellular origin. Additional proof for active virus replication in the offspring PBMC was obtained when mitogen-stimulated cells were analysed for WHV-specific mRNA (Section 3.3.3). This stimulation unregulated viral mRNA expression implying an increase in viral genome transcription in these cells. The induction of hepadnaviral replication in lymphoid cells upon stimulation with nonspecific mitogens has also been reported by other authors (Korba *et al.*, 1988; Baginski *et al.*, 1991). However, some critics argue that the activation of PBMC may induce only low-level viral replication, but not necessarily release of complete virions. Therefore, in the current study, the WHV DNA-reactive molecules detected in the PBMC supernatant after

mitogen stimulation were evaluated for their physical properties and infectivity. Our results demonstrated that the WHV particles in the PBMC culture supernatant exhibited sedimentation velocity in sucrose and buoyant density in CsCl gradients, similar to those of intact WHV virions. Even more convincing evidence documenting the release of infectious virions by stimulated PBMC was the transmission of WHV infection by inoculation of WHV-naive woodchucks with this culture supernatant (see also Section 4.5). In summary, it is evident that the offspring not only show persistent WHV replication within the lymphatic system but that the proliferating virus is complete and biologically competent.

Although the above experiments conclusively prove the existence of infectious virus in the offspring lymphoid cells, the detection of WHV DNA replicative intermediates and WHV cccDNA could provide an indication whether the virus employs the same replication strategy in lymphoid organs as in the liver. As mentioned in Section 1.3.2, virus DNA replicative intermediates include RCDNA (incomplete DNA genome before conversion into cccDNA), single stranded DNA fragments (present after reverse transcription of RNA pregenomes), and linear double stranded DNA (synthesized using the single stranded DNA as a template before circularization and packaging). The levels of single stranded and linear double stranded DNA species are very low in comparison to mature, circularized, double stranded WHV DNA and in fact, they are detectable only in chronic carriers with a high virus load. Since detection of WHV DNA in the samples analysed in this study required very sensitive nested PCR assays followed by Southern blotting, we could not expect that replicating DNA intermediates would be identifiable in the offspring samples. Thus, there was

no point in any attempt to find these DNA species in our samples.

We also did not observe any evidence for the presence of WHV cccDNA either in the liver or in extrahepatic tissues in the offspring analysed. As described previously in Section 1.3.2, formation of cccDNA species, generated by repair of RCDNA of invading virus, represents the earliest step in hepadnavirus replication. The cccDNA remains within infected cells and serves as a constant template for transcription into viral mRNA. Thus, detection of this replicating DNA form would provide further evidence of an active, continuous viral infection (Tuttleman *et al.*, 1986). Even though cccDNA has been detected within livers of chronic HBV carriers, its presence has not been documented in PBMC from the same patients (Lamelin *et al.*, 1995). The difficulty with cccDNA detection could be attributed to their low copy number, estimated to be 30-40 genome copies per infected hepatocyte in chronic hepatitis B which is at least 1000-fold less then that of HBV DNA. Thus, given the already very low-levels of WHV DNA in the animals investigated (10-10^2 genome copies per $1 \mu g$ of total DNA), it would be surprising if we could detect cccDNA in lymphoid cells and livers of the offspring. Hence, we decided not to test for WHV specific cccDNA in the offspring studied. On the other hand, it is possible that the virus employs a yet unknown replication strategy while propagating in the host lymphatic system. It has been noted that since HBV cccDNA cannot be detected in PBMC of chronic HBV carriers, there might be some other mechanism by which viral genome is replicated and maintained at extrahepatic sites (Lamelin *et al.*, 1995). The results from a HBV transgenic mice lineage containing 1.3 kb construct of the HBV genome apparently support this hypothesis (Guidotti *et al.*, 1995). In this

transgenic mice model, animals show high levels of viral replication in the liver and kidneys, comparable to that in the infected livers of patients with chronic hepatitis B. There is no difficulty with the detection of HBV DNA replicative intermediates and viral mRNA in hepatocytes and kidneys of these mice, yet the investigators cannot detect any viral cccDNA. In the same study, cccDNA was readily detectable in a human liver sample showing equivalent levels of HBV replication. The authors also noted that these HBV transgenic mice showed high levels of HBsAg and HBeAg in sera and urine as well as, HBcAg in liver and kidneys. Furthermore, potentially infectious complete viral particles, which were indistinguishable from human Dane particles, were found in the mice sera (Guidotti *et al.*, 1995). Thus, despite the lack of HBV cccDNA, high level viral replication can still occur, perhaps by a mechanism which does not require the normal viral transcriptional template.

4.2.2 Lymphoid Tissue-Restricted Expression of WHV

The presence of WHV genomes was tested in liver samples collected from all the offspring studied by using three different sets of oligonucleotide primers specific for the virus C, S and X gene sequences (see Table 5.3). In most of the cases, liver samples were obtained at yearly intervals. As expected, the majority of the animals with detectable WHV DNA in serum and/or PBMC (2B/F, 5C/F, 6D/F, 8D/M, 10D/F, and 11D/F) showed persistent carriage of virus genome in the liver during the entire observation period. However, we noticed occasionally a discrepancy in the detection of a particular WHV gene sequence between liver, PBMC and sera obtained at the same time points of follow-up. For example,

WHV C gene sequences could be detected in PBMC, but not in liver or serum (Figure 11.3). Again, as postulated above (Section 4.1), the most likely reasons could be the varied amounts of viral DNA within samples used for analysis and the lack of PCR reproducibility when testing for very low-levels of viral DNA. However, it is also possible that viral variants with tropism for either hepatocytes or lymphoid cells or with mutations in primer binding sequences can predominate at certain times.

Analysis of WHV DNA in autopsy tissues from 5 out of 11 woodchucks studied also revealed presence of the virus in lymphatic organs, including spleen, bone marrow, lymph node and thymus. In addition, replicating forms of the WHV genome could be detected in spleen and bone marrow. Most surprisingly, in 4 of the offspring (1A/F, 3B/M, 7D/F and 9D/F), WHV DNA was not identifiable in the liver, despite repeated testing by nested PCR and Southern blot analysis of the resulting reaction mixtures. It is noteworthy that offspring born to the same mother could have very different tissue patterns of WHV expression. For example, 2 offspring born to maternal woodchuck #D (7D/F and 9D/F) did not have any detectable WHV DNA in sequential liver biopsies taken before challenge with WHV, yet the viral genome could be detected in livers of 4 other offspring from the same litter (6D/F, 8D/M, 10D/F and 11D/F). Unfortunately, we could not obtain liver samples until at least 6 mo after birth from some of these animals. Thus, we cannot completely rule out the possibility that the liver had initially been positive but then cleared the virus. We do not believe, however, that this was the case, since even the earliest liver sample collected at 1 mo after birth from 1A/F was found negative for WHV DNA. In addition, we have never

observed WHV clearance from the liver that had been found previously reactive for WHV DNA. In contrast, there was a case (offspring 4B/M) when the initially lymphoid-restricted WHV infection spread to the liver at 19 mo after birth and the virus was readily detectable thereafter in hepatic tissue.

In the offspring with undetectable WHV sequences in the liver, WHV DNA and RNA were relatively easily identifiable in PBMC and lymphoid organs. The serum from these offspring also carried intact WHV virions, since WHV DNA-reactive particles exhibited properties similar to those of purified virions. Furthermore, injection of WHV-naive woodchucks with sera from the liver WHV-DNA negative offspring resulted in the lymphoid cell-restricted pattern of WHV infection in one of two inoculated woodchucks (#260/M; Figures 19.3 and 20.3). Thus, it is evident that this lymphotropic virus is transmittable to uninfected animals and induces infection apparently limited to lymphoid cells (see Section 4.5). Although hepadnavirus infection within the lymphatic system has been well documented (Korba *et al.*, 1987a, 1987b, 1989; Mason *et al.*, 1993; Michalak *et al.*, 1994; Ogston *et al.*, 1989; Pardoe and Michalak, 1995), this is the first report of hepadnavirus absence in the liver during the course of long-term infection. In fact, a dissociation in hepadnavirus expression between liver and lymphoid tissue has only been described in HBV-infected patients transplanted with livers from baboons (Lanford *et al.*, 1995). In these patients, HBV genomes were detected exclusively at multiple extrahepatic sites. Since baboons are not susceptible to HBV, xenotransplantation of the baboon liver is considered to have a better prognosis then liver allotransplants which are invariably re-infected with HBV. In the same

study, it was reported that HBV DNA can persist for a long time in extrahepatic tissues without infecting the baboon liver graft. In another study (Fèray et al., 1990), 30 HBV-infected patients were subjected to liver transplantation and then treated with high doses of anti-HBs immunoglobulin. Twenty-three of them cleared HBsAg and remained HBV DNA negative in liver grafts by direct PCR and Southern blotting during 13 mo of follow-up. However, 7 of those patients retained HBV DNA in PBMC, demonstrating persistent presence of the virus in lymphoid cells in the absence of HBV in the liver. In another report, 67 patients who underwent chronic haemodialysis were studied to determine the risk of infection with HBV. None of these patients was found positive for HBsAg or HBV DNA in serum. However, in 5 of these individuals, who were also anti-HBc negative, the only evidence of HBV infection was the presence of viral genomes in PBMC (Oesterreicher et al., 1995). The above studies provide evidence that lymphoid cells may constitute a reservoir for reinfection of the liver and that testing of PBMC for HBV DNA could be useful in the positive identification of potentially infectious patients. In this context, our current study clearly demonstrates that cells of the lymphatic system in woodchuck hepatitis in fact constititute a site of active hepadnavirus replication and that replicating virus is biologically competent and able to invade the liver.

We do not know the mechanism which caused the lymphoid tissue-restricted viral expression in some of the offspring. This could be a consequence of transmission of a viral variant which specifically targets lymphoid cells. It is possible that maternally WHV- infected PBMC or freely circulating virions crossed the placenta, transmitting a virus which selectively

established a productive infection of lymphoid cells. Other investigators have suggested that vertical transmission of HBV infected maternal PBMC could be associated with the onset of acute or fulminant hepatitis in infancy (Shimizu *et al.*, 1991). As mentioned above (Section 4.2.1), previous results from our laboratory have demonstrated that lymphoid cells could be favourable targets for WHV attack, given that they demonstrate higher affinity for virus binding than hepatocytes (Jin *et al.*, 1996). Perhaps, because of very low doses of the invading virus, WHV may preferentially recognize high affinity binding sites on lymphoid cells and establish infection in the lymphatic system. Concomitantly, higher levels of virus might be required to invade hepatocytes. However, it is possible that lymphoid cells of the liver carry the virus even if hepatocytes are not infected.

Vertical transmission of viral variants is considered to be a cause of many perinatal viral infections, including HBV, HCV and HIV (see Section 1.6.1c). It is postulated that transmission of a particular variant can influence virus organ tropism and outcome of infection in the newborn. LCMV provides one of the best examples of how viral variants can differ in their tissue tropism and influence the natural course of infection (Ahmed *et al.*, 1984 and 1988). In this regard, LCMV infection of mice resembles HBV infection because affected newborns develop a mild disease and a life-long viral persistence in the brain, liver, kidneys and the lymphatic system, whereas infection of adult mice leads usually to more severe disease and virus clearance. LCMV isolates from the brain, liver and kidneys have the wild-type phenotype and when inoculated into adult immunocompetent mice induce a vigorous anti-viral immune response, which normally results in virus clearance. In contrast, viral isolates

from lymphoid cells are characterized by single amino acid changes in viral polymerase and glycoprotein. In comparison to the wild-type strain, these two mutations confer enhanced ability of the virus for attachment, penetration and replication in macrophages (Matloubian *et al.*, 1993). Furthermore, inoculation of adult LCMV-naive mice with this particular variant induces chronic infection associated with suppressed T cell responses and susceptibility to opportunistic pathogens. Since macrophages are critical components of both natural and virus-specific cellular immune responses (see Section 1.5.1), this LCMV strain avoids immune surveillance by infecting macrophages. A comparable situation may exist in the woodchuck offspring in our study. It is possible that a specific viral variant/s has invaded the lymphatic system early in life leading to the life-long asymptomatic infection. However, it is also conceivable that organ-specific WHV genetic variants could be selected during the progression of infection. This could explain why in offspring 4B/M WHV infection restricted initially to the lymphatic system spread subsequently to the liver. At this point, we do not know whether different viral variants were present in the offspring studied, if there were differences in viral sequences occurring in the liver and lymphoid tissues of the same host, and whether viral populations in maternal woodchucks were different from those in offspring. Sequencing analysis, which is currently in progress, is necessary in order to answer these questions.

4.3 Challenge of Offspring with WHV Induces Serologically Evident Infection

Four of the offspring were selected for challenge with a serum pool containing infectious WHV. Two of these woodchucks carried WHV genomes in the liver and lymphatic system (6D/F and 8D/M), whereas in the other 2 (7D/F and 9D/F) WHV DNA was not detected in liver biopsies obtained prior to WHV challenge. The purpose of this experiment was to determine susceptibility of these animals to re-infection with WHV. Another purpose, which is beyond the aims of this thesis, is to assess long-term pathological consequences of re-exposure to virus in carriers with traces of WHV. Injection of the offspring with the WHV serum pool induced serologically evident infection in all 4 offspring. Thus, shortly after inoculation, we observed transient increases in serum WHV DNA levels and the appearance of anti-WHc. The antibodies remained positive in offspring 7D/F, 8D/M and 9D/F up until the end of the observation period, but they were cleared from offspring 6D/F at dpi 56. Other immunovirological markers of WHV infection, such as WHsAg and anti-WHs, were only transiently detected in offspring 9D/F and 7D/F, respectively. We also noticed that after challenge with WHV a greater percentage of serum and PBMC samples were found positive for WHV DNA (see Tables 3.3 and 4.3), demonstrating that the re-exposure increased virus replication. Furthermore, liver biopsy collected from offspring 7D/F at 14 mo after WHV challenge tested WHV DNA positive, although all biopsies obtained previous to the challenge had been virus negative. This finding demonstrates that the animal was evidently reinfected. Strangely, we did not observe the same situation in offspring 9D/F after WHV inoculation. This animal had also tested negative for WHV DNA in sequential liver biopsies taken prior

to WHV challenge, but liver tissue obtained 9 mo following virus administration remained negative. This result is surprising, since the animal became transiently serum WHsAg positive, indicating evident re-infection with WHV. Unfortunately, liver biopsy from the WHsAg-positive phase was not available for analysis. It is rather unlikely that in this case injection with WHV led to transient liver infection. As mentioned above (Section 4.2.2) and reported in our previous study (Michalak *et al.*, manuscript submitted), we have never observed WHV genome clearance from the liver once it was infected.

Overall, the results of this experiment clearly indicate that the animals with silent persistent carriage of WHV genomes acquired from mothers convalescent from hepatitis were not protected from WHV infection. This observation is in contrast to our previous findings in adult woodchucks carrying low levels of WHV after recovery from experimentally induced acute hepatitis (Michalak *et al.*, manuscript submitted). In these adult animals, challenge with the same WHV infectious pool as the offspring, did not induce any increase of virus expression, indicating their unresponsiveness to WHV re-infection. These woodchucks also carried virus genomes in sera, PBMC and in livers at levels comparable to those detected in the offspring. Considering all serological markers of WHV infection, the main difference between the offspring in the present study and in the adult woodchucks in the previous investigation was persistent presence of anti-WHc in the adult animals. It is possible that the carriage of the viral genome alone does not coincide with immunological protection, but the presence of anti-WHc is reflective of life-long immunity to WHV. It is generally accepted that antibodies to hepadnavirus nucleocapsid (*i.e.*, anti-HBc or anti-WHc) do not neutralize

viral infectivity and they are present at high titres during both acute and chronic infection. Instead, antibodies to viral envelope (*i.e.*, anti-HBs or anti-WHs) are believed to play a critical role in viral clearance and in providing immunity to virus. Resolution of acute infection is associated with the development of this protective antibody response and they are not detected in chronic hepatitis.

The potential protective importance of the host immune response to hepadnavirus nucleocapsid has been suggested in the recent report by Menne *et al.* (1997). The authors have shown that immunization with a WHcAg-derived peptide can induce protection against WHV despite the absence of anti-WHc. They suggest that the core antigen may stimulate helper T cells, which in turn activate virus-specific CTLs able to eliminate infected hepatocytes and therefore, prevent chronic viral infection. Furthermore, it is also postulated that vigorous helper T cell response to multiple epitopes of the HBV nucleocapsid is of critical importance in virus clearance in humans (Chisari and Ferrari, 1995).

It has been reported that the persistence of WHV DNA traces is evidence of low-level ongoing WHV replication and viraemia in the adult woodchucks convalescent from SLAH (Michalak *et al.*, manuscript submitted). This is consistent with similar findings in individuals analysed long after recovery from acute hepatitis B (Michalak *et al.*, 1994; Rehermann *et al.*, 1995, 1996a, 1996b; Sánchez-Quijano *et al.*, 1993; Scully *et al.*, 1994). These patients not only carried minute amounts of virus genomes in serum and PBMC years after recovery from acute HBV infection, but they also demonstrated a vigorous CTL response against viral antigens (Penna *et al.*, 1996; Rehermann *et al.*, 1995, 1996a, 1996b). This could be a

consequence of the continuous presence of long-lived memory CTLs specific for hepadnavirus in the absence of viral antigens. However, it is most likely a result of sustained CTL response due to protracted stimulation with trace quantities of antigens generated by low-level chronic virus replication. It is also conceivable that virus can escape to immunologically privileged sites (*i.e.*, dendritic cells, macrophages) and be periodically released into the circulation in sufficient amounts to maintain a CTL response. Although, the cellular immune response has not yet been examined in the woodchucks convalescent from WHV infection, given the similarities to hepatitis B, we would expect a similar sustained CTL response which may provide protection against WHV re-exposure.

4.4 WHV Expression in Offspring is not Affected by Challenge
with PBMC-Derived Inoculum

A previous study in our laboratory had shown that mitogen-stimulated PBMC isolated from adult woodchucks convalescent from SLAH transmitted WHV to normal animals and induced serologically detectable acute infection (Michalak *et al.*, manuscript submitted). We were interested to test whether the offspring in our study could also be infected with the same type of inoculum. For this purpose, 10D/F and 11D/F offspring were challenged with the same PBMC-derived inoculum as the normal woodchucks mentioned above. Following inoculation, WHV DNA levels in serum and PBMC remained unchanged and we did not detect WHsAg or anti-WHc in either 10D/F or 11D/F. The only immunological response observed was periodic appearance of anti-WHs in offspring 10D/F (Figure 3.3). These results

revealed that although virus carried in lymphoid cells was infectious to normal, WHV-naive woodchucks, it could not infect the offspring. We do not know a reason for this difference, however there could be a few possible explanations. Although the offspring were apparently healthy, they carried trace amounts of WHV and perhaps this was associated with some degree of protective anti-virus specific immune responses undetectable by the assays used. It is possible that this limited response was not sufficient to protect against higher doses of WHV (Section 4.3), but provided protection against lower amounts of virus derived from PBMC. Another possible explanation could be that viral variants carried within the lymphoid cells caused a pattern of WHV infection different from that induced by serum-derived inoculum. If this is the case, it can be further hypothesized that the particular WHV variants carried by the offspring provided protection against the lymphoid cell-derived virus, but not against the heterologous population of variants that may occur in sera. As mentioned above, it is necessary to perform sequencing analysis to determine if such variants indeed exist. Furthermore, the observed development of anti-WHs response in 10D/F offspring after injection with this PBMC-derived supernatant may suggest that the inoculum contained sufficient amounts of WHsAg to induce this antibody response, but not enough virus to reinfect the offspring.

4.5 WHV Carried by Offspring is Infectious to Virus-Naive Woodchucks

In the preceding sections, we have discussed a variety of data supporting our conclusion that the offspring born to mothers convalescent from viral hepatitis were

persistently infected with WHV. Thus, we have documented that transcriptionally active viral genomes were carried for a very long time, if not for life, in these animals. Furthermore, biophysical analysis of WHV DNA-reactive particles in sera and PBMC-derived supernatant showed that some of them exhibited properties identical to those of intact WHV. However, definitive proof for the presence of fully biologically competent virions in these offspring was obtained by demonstration of WHV transmission to healthy animals.

In order to determine whether the offspring were infectious, we used two types of inocula: (1) derived from an offspring with persistent carriage of WHV both in the liver and the lymphatic system (liver-positive infection; *i.e.*, 4B/M offspring) and (2) obtained from offspring with WHV infection restricted to lymphatic tissue (liver-negative infection; *i.e*, 3B/M and 7D/F). It was shown that inocula from both liver-positive and liver-negative animals could transmit WHV infection, however, there was one important difference (Section 3.8). Namely, normal woodchucks injected with concentrated plasma and PBMC supernatant from the liver-positive offspring developed serologically evident acute WHV infection, whereas those injected with inocula from the liver-negative offspring became serologically silent virus carriers. The reason for this difference can only be speculated. We do not think that there was a difference in the amounts of infectious virus present in these two types of inocula, since their PCR analysis revealed approximately similar levels of WHV DNA. It is more likely, however, that inocula from liver-positive and liver-negative offspring contained different virus variants capable of inducing different patterns of WHV infection. This could be due to the existence of a viral mutant/s with preferable or exclusive tropism for lymphoid

cells or the presence of virus with higher binding affinity for receptors expressed by lymphoid cells than for those on hepatocytes (see also Section 4.2.1).

The effect of virus mutations on the pattern of HBV-induced disease has been described in some patients. As summarized in Section 1.6.1c, HBV isolates unable to synthesize HBeAg because of a mutation in the stop codon of the virus pre-core region were found in some individuals with fulminant hepatitis or severe chronic liver disease leading to cirrhosis (Carman *et al.*, 1989). In other cases, HBV variants transmitted from mother to child have been shown to influence the course of perinatal infection (Raimondo *et al.*, 1993; Von Weizsacker *et al.*, 1995). Therefore, in cases of symptomatic, serologically evident HBV infection, the disease profile could be affected by the genetic heterogeneity of the virus. Viral variants may also determine the course of WHV infection in woodchucks.

On the other hand, the contribution of host factors to the outcome of hepadnaviral infection needs to be considered. In the human situation, it is commonly observed that patients who receive HBV-infected blood from the same donor and children born to the same infected mothers can develop distinct disease patterns (Foster and Thomas, 1993). Because of different genetic background, woodchucks may also be predisposed to react differently to virus and hence, develop variable patterns of infection. In the present study, this possibility is suggested by the fact that some of the offspring born to the same mother developed liver-negative infection, but others were WHV DNA liver-positive. As described in Section 1.5.1, a vigorous CTL response to specific viral epitopes presented by MHC class I molecules is one of the important factors determining resolution of acute hepadnaviral infection. Since the

viral epitope for CTL recognition varies in patients with different MHC backgrounds, the CTL response can be affected both by genetic make-up of the host and mutations in virus CTL epitopes. This mechanism could also be an important element in a complex network of virus-host interactions determining different patterns of WHV infection in the offspring studied.

4.6 Suggestions for Future Studies

The results of this study have raised several important questions which warrant future investigation of offspring born to mothers convalescent from hepadnaviral hepatitis. Some of these questions include:

(1) What are the possible long-term consequences, if any, of low-level virus persistence in these offspring? For example, further follow-up may prove that asymptomatic virus carriage can lead to the development of HCC or result in other extrahepatic diseases which are not traditionally associated with hepadnaviral infection.

(2) Are there any differences between the virus genome subpopulations of the mother and her offspring? Future work involving nucleotide sequence analysis of the maternal and offspring viral quasi-species may track transmission of particular variants as well as, determine the extent of genetic divergence in different offspring born to the same mother.

(3) Is there an association between different viral genomic variants and virus tissue tropism? For example, sequencing could determine if there are specific viral mutants present in the lymphatic system. It is possible that such genomic variants preferentially or exclusively

target lymphoid cells and induce lymphoid tissue-restricted infection, as was observed in some of the offspring.

(4) Why did inocula from different offspring induce either a serologically evident acute infection or cause a serologically silent WHV infection? Sequencing of the infectious pools from WHV DNA liver-positive and liver-negative offspring could identify WHV genetic variants which may induce different profiles and outcomes of infection.

(5) Finally and most importantly, does a similar situation exist in babies born to mothers with a past history of self-limited hepatitis B virus infection? Based on our findings, it is possible that vertical transmission of the virus from mothers convalescent from either clinically evident or serologically undetectable HBV infection could induce a low-level, asymptomatic infection of infants which cannot be detected by current (classical) serological or biochemical assays. Only very sensitive molecular tests (i.e., PCR amplification) would be able to detect the virus in these cases. Consequently, these babies may represent a reservoir of virus for transmission to healthy individuals and could be at the risk for distant development of liver diseases and possibly yet unidentified extrahepatic disorders.

4.7 Summary and Conclusions

This study documents for the first time that offspring born to mothers after complete recovery from an episode of hepadnavirus infection persistently carry traces of infectious virus. There have been no previous reports showing that such apparently healthy mothers could be infectious to their offspring. The results obtained in the course of our investigation

in the woodchuck model of hepatitis B can be summarized as follows:

(1) Complete serological and histological recovery from an experimentally induced episode of acute hepadnaviral hepatitis is followed by long-term persistence of virus which is transmittable and induces serologically undetectable chronic infection in the newborn woodchucks lasting for more than 3 years after birth.

(2) In the majority of the offspring studied, WHV genome sequences were detectable both in the liver and lymphoid tissues. However, in some of the offspring, the persistent hepadnavirus carriage was restricted only to cells of the lymphatic system. Liver tissue samples from these animals were WHV DNA negative despite extensive testing by nested PCR and Southern blotting. These results provide evidence that hepadnavirus can persist exclusively at an extrahepatic location throughout the entire course of infection.

(3) Analysis of physicochemical properties of WHV DNA-reactive particles occurring in sera from the offspring analysed revealed the presence of virus particles which resisted extensive DNase treatment and displayed sedimentation velocity in sucrose and buoyant density in cesium chloride gradients identical to those of purified intact virions, indicating that complete WHV was persistently carried by these offspring.

(4) Transmission of WHV infection and induction of serologically evident acute hepatitis in virus-naive woodchucks by inocula derived from the offspring sera or peripheral blood mononuclear cells prove that the offspring in fact carried biologically competent, infectious virus.

(5) The offspring born to mothers with a past episode of viral hepatitis were

susceptible to challenge with WHV present in serum of a symptomatic, chronic WHV carrier, but they did not respond to challenge with otherwise infectious inoculum prepared from mitogen-stimulated PBMC of woodchucks convalescent from acute viral hepatitis, suggesting that exposure to WHV as a newborn did not provide complete immunological protection against reinfection with this virus.

Since there are significant similarities between HBV and WHV and the natural patterns of infection induced by both viruses, this study raises the possibility that HBV can also be vertically transmitted from apparently healthy mothers convalescent from hepatitis B to their babies. Consequently, infants born to these mothers may represent a reservoir of virus for infection of healthy people and they may be at increased long-term risk for the development of liver and extrahepatic disorders.

REFERENCES

Abbas, A. K., A. H. Lichtman, & J. S. Pober. 1994. *Cellular and Molecular Immunology*, 2nd ed. W. B. Saunders Company, Philadelphia, Pennsylvania.

Acorn, C. J. M., C. C. Millar, S. K. Hoffman, & W. M. Lyle. 1995. Viral hepatitis: a review. *Optometry and Vision Science* 72:763-769.

Ahmed, R. 1989. Immunological tolerance in viral infections, pp. 302-310. In A. L. Notkins & M. B. A. Oldstone (eds.), *Concepts in Viral Pathogenesis III*, Springer-Verlag, New York.

Ahmed, R., L. A. Morrison, & D. M. Knipe. 1996. Persistence of viruses, pp. 219-249. In B.N. Fields & D. M. Knipe (eds.), *Fields Virology*, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Ahmed, R. & M. B. A. Oldstone. 1988. Organ-specific selection of viral variants during chronic infection. *Journal of Experimental Medicine* 167:1719-1724.

Ahmed, R., A. Salmi, L. D. Butler, J. M. Chiller, & M. B. A. Oldstone. 1984. Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. *Journal of Experimental Medicine* 60:521-540.

Ahmed, R. & J. G. Stevens. 1990. Viral persistence, pp.241-265. In B.N. Fields & D. M. Knipe (eds.), *Fields Virology*, 2nd ed. Raven Press, New York.

Aizaki, H., A. Saito, I. Kusakawa, Y. Ashiwara, S. Nagamori, G. Toda, T. Suzuki, K. Ishii, Y. Matsuura, & T. Mitamura. 1996. Mother-to-child transmission of a hepatitis C virus variant with an insertional mutation in its hypervariable region. *Journal of Hepatology* 25:608-613.

Baginski, I., A. Chemin, P. Bouffard, O. Hantz, & C. Trépo. 1991. Detection of polyadenylated RNA in hepatitis B virus-infected peripheral blood mononuclear cells by polymerase chain reaction. *Journal of Infectious Diseases* 163:996-1000.

Banks, T. A. & B. T. Rouse. 1992. Herpesviruses-immune escape artists? *Clinical Infectious Diseases* 14:933-941.

Bertoletti, A., A. Sette, & F. V. Chisari. 1994. Natural variants of cytotoxic epitopes are T cell receptor antagonists for antiviral cytotoxic T cells. *Nature* 369:407-410.

Blum, H. E., T. J. Liang, E. Galum, & J. R. Wands. 1991. Persistence of hepatitis B viral DNA after serological recovery from hepatitis B virus infection. *Hepatology* 14:56-63.

Bréchot, C. 1993. Polymerase chain reaction for the diagnosis of hepatitis B and C viral hepatitis. *Journal of Hepatology* 17 (suppl 3):35-41.

Bruggeman, C. A. 1993. Cytomegalovirus and latency: an overview. *Virchows Archiv B Cell Pathol* 64:325-333.

Burk, R. D., L. Hwang, G. Y. F. Ho, D. A. Shafritz, & R. P. Beasley. 1994. Outcome of perinatal hepatitis B virus exposure is dependent on maternal virus load. *Journal of Infectious Diseases* 170:1418-1423.

Carman, W. F., M. R. Jacyna, S. Hadziyannis, P. Karayiannis, M. J. McGarvey, A. Makris, & H. C. Thomas. 1989. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet* 2:588-591.

Cason, J., J. N. Kaye, R. J. Jewers, P. K. Kambo, J. M. Bible, B. Kell, B. Shergill, F. Pakarian, K. S. Raju, & J. M. Best. 1995. Perinatal infection and persistence of human papillomavirus types 16 and 18 in infants. *Journal of Medical Virology* 47:209-218.

Chazouillères, O., D. Mamish, M. Kim, K. Carey, L. Ferrell, J. P. Roberts, N. L. Ascher, & T. L. Wright. 1994. "Occult" hepatitis B virus as source of infection in liver transplant recipients. *Lancet* 343:142-146.

Cheynier, R., P. Langlade-Demoyen, M. Marescot, S. Blanche, G. Blondin, S. Wain-Hobson, C. Griscelli, E. Vilmer, & F. Plata. 1992. Cytotoxic T lymphocyte responses in the peripheral blood of children born to human immunodeficiency virus-1-infected mothers. *European Journal of Immunology* 22:2211-2217.

Chisari, F. V. & C. Ferrari. 1995. Hepatitis B virus immunopathology. *Springer Seminars in Immunopathology* 17:261-281.

Choo, Q., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, & M. Houghton. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 244:359-362.

Cohen, J. I, R. H. Miller, B. Rosenblum, K. Denniston, J. L. Gerin, & R. H. Purcell. 1988. Sequence comparison of woodchuck hepatitis virus replicative forms shows conservation of the genome. *Virology* 162:12-20.

Cova, L., I. Fourel, L. Vitvitski, V. Lambert, S. Chassot, O. Hantz, & C. Trépo. 1993. Animal models for the understanding and control of HBV and HDV infections. *Journal of Hepatology* 17 (suppl 3):143-148.

Dane, D. S., C. H. Cameron, & M. Briggs. 1970. Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. *Lancet* 1:695-698.

De Rossi, A. M., F. Pasti, L. Mammano, C. Ometto, C. Giaquinto, & L. Chieci-Bianchi. 1991. Perinatal infection by human immunodeficiency virus type-1 (HIV-1): relationship between proviral copy number in vivo, viral properties in vitro, and clinical outcome. *Journal of Medical Virology* 35:283-289.

Deka, N., M. D. Sharma, & R. Mukerjee. 1994. Isolation of the novel agent from human stool samples that is associated with sporadic non-A, non-B hepatitis. *Journal of Virology* 68:7810-7815.

Devash, Y., T. A. Calvelli, D. G. Wood, K. J. Reagan, & A. Rubinstein. 1990. Vertical transmission of human immunodeficiency virus is correlated with the absence of high-affinity/avidity maternal antibodies to the gp120 principle neutralizing domain. *Proceedings of National Academy of Science of United States of America* 87:3445-3449.

Dhib-Jalbut, S., H. F. McFarland, E. S. Mingiolo, J. L. Sever, & D. E. McFarlin. 1988. Humoral and cellular immune responses to matrix protein of measles virus in subacute sclerosing panencephalitis. *Journal of Virology* 62:2483-2489.

Donley, D. K. 1993. TORCH infections in the newborn. *Seminars in Neurology* 13:106-115.

Embretson, J., M. Zupancic, J. L. Ribas, A. Burke, P. Racz, K. Tenner-Racz, & A. T. Haase. 1993. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. *Nature* 362:359-362.

Feitelson, M. A., I. Millman, T. Halbherr, H. Simmons, & B. S. Blumberg. 1986. A newly identified hepatitis B type virus in tree squirrels. *Proceedings of National Academy of Science of United States of America* 83:2233-2237.

Féray, C., A. L. Zignego, D. Samuel, A. Bismuth, M. Reynes, P. Tiollais, H. Bismuth, & C. Bréchot. 1990. Persistent hepatitis B virus infection of mononuclear blood cells without concomitant liver infection. *Transplantation* 49:1155-1158.

Fiordalisi, G., D. Primi, E. Tanzi, E. Magni, C. Incarbone, A. R. Zanetti, & E. Cariani.

1994. Hepatitis B virus C gene heterogeneity in a familial cluster of anti-HBc negative chronic carriers. *Journal of Medical Virology* **42**:109-114.

Fong, T., A. M. D. Bisceglie, M. A. Gerber, J. G. Waggonner, & J. H. Hoofnagle. 1993. Persistence of hepatitis B virus DNA in the liver after loss of HBsAg in chronic hepatitis B. *Hepatology* **18**:1313-1317.

Foster, G. R., A. M. Ackrill, R. D. Goldin, I. M. Kerr, H. C, Thomas, & G. R. Stark. 1991. Expression of the terminal protein region of hepatitis B virus inhibits cellular responses to interferons alpha and gamma and double stranded RNA. *Proceedings of National Academy of Science of United States of America* **88**:2888-2892.

Foster, G. R. & H. C. Thomas. 1993. Recent advances in the molecular biology of hepatitis B virus: mutant virus and the host response. *Gut* **34**:1-3.

Franco, A., C. Ferrari, A. Sette, & F. V. Chisari. 1995. Viral mutations, TCR antagonism and escape from the immune response. *Current Opinion in Immunology* **7**:524-531.

Gaertner, D. J., A. L. Smith, & R. O. Jacoby. 1996. Efficient induction of persistent and prenatal parvovirus infection in rats. *Virus Research* **44**:67-78.

Galibert, F., T. N. Chen, & E. Mandart. 1982. Nucleotide sequence of a cloned woodchuck hepatitis virus genome: comparison with the hepatitis B virus sequence. *Journal of Virology* **41**:51-65.

Ganem, D. 1991. Assembly of hepadnaviral virions and subviral particles. *Current Topics in Microbiology and Immunology* **168**:61-83.

Ganem, D. 1996. *Hepadnaviridae* and their replication, pp. 2703-2737. In B. N. Fields & D. M. Knipe (eds.), *Fields Virology*, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Gerlich, W. H., X. Lu, & K. H. Heermann. 1993. Studies on the attachment and penetration of hepatitis B virus. *Journal of Hepatology* **17**(suppl.3):10-14.

Girones, R., P. J. Cote, W. E. Hornbuckle, B. C. Tennant, J. L. Gerin, R. H. Purcell, & R. H. Miller. 1989. Complete nucleotide sequence of a molecular clone of woodchuck hepatitis virus that is infectious in the natural host. *Proceedings of the National Academy of Science of the United States of America* **86**:1846-1849.

**Goedert, J. J., H. Mendez., J. E. Drummond, M. Robert-Guroff, H. L. Minkoff, S. Holman, R. Stevens, A. Rubinstein, W. A. Blattner, A. Willoughby, & S. H.

Landesman. 1989. Mother-to-infant transmission of human immunodeficiency virus type 1: association with prematurity or low anti-gp120. *Lancet* 2:1351-1354.

Guidotti, L. G., B. Matzke, H. Schaller, & F. V. Chisari. 1995. High-level hepatitis B virus replication in transgenic mice. *Journal of Virology* 69:6158-6169.

Hadziyannis, S., M. A. Gerber, C. Vissoulis, & H. Popper. 1973. Cytoplasmic hepatitis B antigen in "ground-glass" hepatocytes of carriers. *Archives of Pathology* 96: 327-330.

Harrisson, T. J., 1990. Hepatitis B virus DNA in peripheral blood leukocytes: a brief review. *Journal of Medical Virology* 31:33-35.

Hollinger, F.B., C. L. Troisi, & P. E. Pepe. 1986. Anti-HBs responses to vaccination with a human hepatitis B vaccine made by recombinant DNA technology in yeast. *Journal of Infectious Diseases* 153:156-159.

Hollinger, F. B. 1996. Hepatitis B virus, pp. 2739-2807. In B. N. Fields & D. M. Knipe (eds.), *Fields Virology*, 3rd ed. Lippincott-Raven Publishers, Philadelphia.

Hoofnagle, J. H., D. A. Shafritz, & H. Popper. 1987. Chronic type B hepatitis and the healthy HBsAg carrier state. *Hepatology* 7:758-763.

Hornbuckle, W. E., E. S. Graham., L. Roth., B. H. Baldwin, C. Wickenden, & B. C. Tennant. 1985. Laboratory assessment of hepatic injury in the woodchuck (*Marmota monax*). *Laboratory Animal Science* 35:376-381.

Howard, C. R. 1995. The structure of hepatitis B envelope and molecular variants of hepatitis B virus. *Journal of Viral Hepatitis* 2:165-170.

Hsu, T-Y., T. Möröy, J. Etiemble, A. Louise, C. Trépo, P. Tiollais, & M-A. Buendia. 1988. Activation of c-*myc* by woodchuck hepatitis virus insertion in hepatocellular carcinoma. *Cell* 55:627-635.

Inoue, Y., K. Takeuchi, W. Chou, T. Unayama, K. Takahashi, I. Saito, & T. Miyamura. 1992. Silent mother-to-child transmission of hepatitis C virus through two generations determined by comparative nucleotide sequence analysis of the viral cDNA. *Journal of Infectious Diseases* 166:1425-1428.

Jacoby, R. O., D. J. Gaertner, P. N. Bhatt, F. X. Paturzo, & A. L. Smith. 1988. Transmission of experimentally-induced rat virus infection. *Laboratory Animal Science*

38:11-14.

Jacoby, R. O., E. A. Johnson, F. X. Paturzo, D. J. Gaertner, J. L. Brandsma, & A. L. Smith. 1991. Persistent rat parvovirus infection in individually housed rats. *Archives of Virology* **117**:193-205.

Jamieson, B. D., T. Somasundaram, & R. Ahmed. 1991. Abrogation of tolerance to a chronic viral infection. *Journal of Immunology* **147**:3521-3529.

Jiang, J. J., A. Brind, M. Gigou, D. Samuel, C. Féray, C. Bréchot, &. D. Kremsdorf. 1994. Evidence for the role of PBMC in the infection of liver graft of hepatitis B variants after liver transplantation (Abstract). *Hepatology* **20**:138A

Jilg, W., E. Sieger, R. Zachoval, & H. Schätzl. 1995. Individuals with antibodies against hepatitis B core antigen as the only serological marker for hepatitis B infection: high percentage of carriers of hepatitis B and C virus. *Journal of Hepatology* **23**:14-20.

Jin, Y-M., N. D. Churchill, & T. L. Michalak. 1996. Protease-activated lymphoid cell and hepatocyte recognition site in the preS1 domain of the large woodchuck hepatitis virus envelope protein. *Journal of General Virology* **77**:1837-1846.

Joly, E., L. Mucke, & M. B. A. Oldstone. 1991. Viral persistence in neurons explained by lack of major histocompatibility class I expression. *Science* **253**:1283-1285.

Kägi, D. & H. Hengartner. 1996. Different roles for cytotoxic T cells in the control of infections with cytopathic versus noncytopathic viruses. *Current Opinion in Immunology* **8**:472-477.

Kamito, H., K. Nakata, K. Hamasaki, M. Daikoku, F. Mawatari, T. Ueki, Y. Hayashida, K. Nakao, Y. Kato, & S. Nagataki. 1996. Detection of hepatitis B virus genome in hepatocellular carcinoma from both hepatitis surface antigen- and antibody to hepatitis C virus-negative patients: a study using polymerase chain reaction. *Oncology Reports* **3**:619-623.

Kaye, J. N., J. Cason, F. B. Pakarian, R. J. Jewers, B. Kell, J. Bible, K. S. Raju, & J. M. Best. 1994. Viral load as a determinant for transmission of human papillomavirus type 16 from mother to child. *Journal of Medical Virology* **44**:415-421.

Köck, J. & H. J. Schlicht. 1993. Analysis of the earliest steps of hepadnavirus replication: genome repair after infectious entry into hepatocyte does not depend on viral polymerase activity. *Journal of Virology* **67**:4867-4874.

Köck, J., L. Theilmann, P. Galle, & H. Schlicht. 1996. Hepatitis B virus nucleic acids associated with human peripheral blood mononuclear cells do not originate from replicating virus. *Hepatology* 23:405-413.

Kodoma, K., N. Ogasawara, H. Yoshikawa, & S. Murakami. 1985. Nucleotide sequence of a cloned woodchuck hepatitis virus genome: evolutionary relationship between hepadnaviruses. *Journal of Virology* 137:390-399.

Korba, B. E., T. L. Brown, F. V. Wells, B. Baldwin, P. J. Cote, H. Steinberg, B. C. Tennant, & J. L. Gerin. 1990. Natural history of woodchuck hepatitis virus infections during the course of experimental viral infection: molecular virologic features of the pancreas, kidney, ovary and testis. *Journal of Virology* 64:4499-4506.

Korba, B. E., P. J. Cote, & J. L. Gerin. 1988. Mitogen-induced replication of woodchuck hepatitis virus in cultured peripheral blood lymphocytes. *Science* 241:1213-1216.

Korba, B. E., P. J. Cote, F. V. Wells, B. Baldwin, H. Popper, R. H. Purcell, B. C. Tennant, & J. L. Gerin. 1989. Natural history of woodchuck hepatitis virus infections during the course of experimental viral infection: molecular virologic features of the liver and lymphoid tissues. *Journal of Virology* 63:1360-1370.

Korba, B. E., B. C. Tennant, E. Gowans, P. J. Cote, F. Wells, B. Baldwin, & J. L. Gerin. 1987a. Infection and tissue tropism of WHV during the natural course of viral infection of woodchucks. In W. Robinson, K. Koike, & H. Will (eds.), *Hepadna Viruses*, pp. 419-428. Alan R. Liss, New York.

Korba, B. E., F. Wells, B. C. Tennant, P. J. Cote, & J. L. Gerin. 1987b. Lymphoid cells in the spleens of woodchuck hepatitis virus-infected woodchucks are a site of active viral replication. *Journal of Virology* 61:1318-1324.

Korner, H., & H. G. Burgert. 1994. Down-regulation of HLA antigens by the adenovirus type 2 E3/19K protein in a T-lymphoma cell line. *Journal of Virology* 66:1442-1448.

Koziel, M. J. 1996. Immunology of viral hepatitis. *American Journal of Medicine* 100:98-109.

Kulonen, K. & I. Millman. 1988. Vertical transmission of woodchuck hepatitis virus. *Journal of Medical Virology* 26:233-242.

Lamelin, J. P., F. Zoulim, & C. Trépo. 1995. Lymphotropism of hepatitis B and C viruses: an update and a newcomer. *International Journal Clinical Laboratory Research*

25:1-6.

Lanford, R. E., M. G. Michaels, D. Chavez, K. Brasky, J. Fung, & T. E. Starzl. 1995. Persistence of extrahepatic hepatitis B virus DNA in the absence of detectable hepatic replication in patients with baboon liver transplants. *Journal of Medical Virology*. 46:207-212.

Lee, J., T. G. Paglieroni, P. V. Holland, & J. B. Zeldis. 1992. Chronic hepatitis B virus infection in an anti-HBc nonreactive blood donor: variant virus or defective immune response? *Hepatology* 16:24-30.

Lee, S., K. Lo, Y. Tsai, J. Wu, & T. Wu. 1989. HBsAg carrier infants with serum anti-HBc negativity. *Hepatology* 9:102-104.

Leopardi, R., J. Illonen, L. Mattila, & A. A. Salmi. 1993. Effect of measles virus infection on MHC class II expression and antigen presentation in human monocytes. *Cell Immunology* 1993 147:388-396.

Liang, T. J., H. E. Blum, & J. R. Wands. 1990. Characterization and biological properties of a hepatitis B virus isolated from a patient without hepatitis B virus serologic markers. *Hepatology* 12:204-212.

Linnen, J., J. Wages, Z-K. Zhen-Yong, K. E. Fry, K. Z. Krawczynski, H. Alter, & E. Koonin. 1996. Molecular cloning and disease association of hepatitis G virus: a transfusion-transmissable agent. *Science* 271:505-508.

Ljunggren, K., V. Moschese, P. Broliden, C. Giaquinto, I. Quinti, E. Fenyö, B. Wahren, P. Rossi, & M. Jondal. 1990. Antibodies mediating cellular cytotoxicity and neutralization correlate with better clinical stage in children born to human immunodeficiency virus-infected mothers. *Journal of Infectious Diseases* 161:198-202.

Lowell, J. A., T. K. Howard, H. M. White, S. Shenoy, P. C. Huettner, D. C. Brennan, & M. G. Peters. 1995. Serological evidence of past hepatitis B infection in liver donors and hepatitis B infection in liver allografts. *Lancet* 345:1081-1085.

Mahy, B. W. J. 1985. Strategies of virus persistence. *British Medical Bulletin* 41:50-55.

Maniatis, T., E. F. Fritsh, & J. Sambrook. 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Marion, P. L., L. S. Oshiro, D. C. Regnery, G. H. Scullard, & W. S. Robinson. 1980.

A virus in Beechey ground squirrels that is related to hepatitis B virus in humans. *Proceedings of National Academy Science of United States of America* 77:2941-2945.

Mason, A., M. Wick, H. White, & R. Perrillo. 1993. Hepatitis B virus replication in diverse cell types during chronic hepatitis B virus infection. *Hepatology* 18:781-789.

Mason, W. S., G. Seal, & J. Summers. 1980. Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. *Journal of Virology* 36:829-836.

Masucci, M. G., N. J. Stam, S. Torsteinsdottir, J. J. Neefjes, G. Klein, & H. L. Ploegh. 1989. Allele-specific down-regulation of MHC class I antigens in Burkitt lymphoma lines. *Cell Immunology* 120:396-400.

Matloubian, M., S. R. Kolhekar, T. Somasundaram, & R. Ahmed. 1993. Molecular determinants of macrophage tropism and viral persistence: importance of single amino acid changes in the polymerase and glycoprotein of lymphocytic choriomeningitis virus. *Journal of Virology* 67:7340-7349.

Maupas, P. & J. L. Melnick. 1981. Hepatitis B infection and primary liver cancer. *Progress in Medical Virology* 27:1-5.

McChesney, M. B., J. H. Kehrl, A. Valsamakis, A. S. Fauci, & M. B. A. Oldstone. 1987. Measles virus infection of B lymphocytes permits cellular activation but blocks progression through the cell cycle. *Journal of Virology* 61:3441-3447.

Menne, S. J. Maschke, T. K. Tolle, M. Lu, & M. Roggendorf. 1997. Characterization of T-cell response to woodchuck hepatitis virus core protein and protection of woodchucks from infection by immunization with peptides containing a T-cell epitope. *Journal of Virology* 71:65-74.

Michalak, T. L, N. D. Churchill, D. Codner, & W. H. Marshall. 1995. Identification of woodchuck class I MHC antigens using monoclonal antibodies. *Tissue Antigens*, 45:333-342.

Michalak, T. I. & B. Lin. 1994. Molecular species of hepadnavirus core and envelope polypeptides in hepatocyte plasma membrane of woodchucks with acute and chronic viral hepatitis. *Hepatology* 20:275-286.

Michalak, T. I., B. Lin, N. D. Churchill, P. Dzwonkowski, & J. R. B. Desousa. 1990. Hepadnavirus nucleocapsid and surface antigens and the antigen-specific antibodies associated with hepatocyte plasma membranes in experimental woodchuck acute hepatitis. *Laboratory*

Investigation 62:680-689.

Michalak, T. I., C. Pasquinelli, S. Guilhot, & F. V. Chisari. 1994. Hepatitis B virus persistence after recovery from acute viral hepatitis. *Journal Clinical Investigation* 93:230-239.

Michalak, T. I., R. L. Snyder, & N. D. Churchill. 1989. Characterization of the incorporation of woodchuck hepatitis virus surface antigen into hepatocyte plasma membrane in woodchuck hepatitis and in the virus-induced hepatocellular carcinoma. *Hepatology* 10:44-55.

Milich, D. R., J. E. Jones, J. L. Hughes, J. Price, A. K. Raney, & A. McLachlan. 1990. Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance in utero? *Proceedings of National Academy of Science of United States of America* 87:6599-6603.

Milich, D. R., F. Schödel, D. L. Peterson., J. E. Jones, & J. L. Hughes. 1995. Characterization of self-reactive T cells that evade tolerance in hepatitis B e antigen transgenic mice. *European Journal of Immunology* 25:1663-1672.

Moskophidis, D., F. Lechner, H. Pircher, & R. M. Zinkernagel. 1993. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature* 362:758-761.

Ni, Y., H. Hsu, M. Chang, D. Chen, & C. Lee. 1993. Absence or delayed appearance of hepatitis B core antibody in chronic hepatitis B surface antigen carrier children. *Journal of Hepatology* 17:150-154.

Oesterreicher, C., J. Hammer, U. Koch, F. Pfeffel, G. Sunder-Plassmann, D. Petermann, & C. Müller. 1995. HBV and HCV genome in peripheral blood mononuclear cells of patients undergoing chronic hemodialysis. *Kidney International* 48:1967-1971.

Ogston, C. W., E. M. Schechter, C. A. Humes, & M. B. Pranikoff. 1989. Extrahepatic replication of woodchuck hepatitis virus in chronic infection. *Virology* 169:9-14.

Ohto, H., S. Terazawa, N. Sasaki, N. Sasaki, K. Hino, C. Ishiwata, M. Kako, N. Ujiie, C. Endo, A. Matsui, H. Okamoto, & S. Mishiro. 1994. Transmission of hepatitis C from mothers to infants. *New England Journal of Medicine* 330:744-750.

Okado, K., I. Kamiyama, M. Inomata, M. Imai, Y. Miyakawa, & M. Mayumi. 1976. e antigen and anti-e in the serum of asymptomatic carrier mothers as indicators of positive and negative transmission of hepatitis B virus to their infants. *New England Journal of Medicine*

294:746–749.

Oldstone, M. B. A., 1989. Viral Persistence. *Cell* **56:**517-520.

Oldstone, M. B. A. 1990. Viral persistence and immune dysfunction. *Hospital Practice* **25:**81-98.

Oldstone, M. B. A. 1993a. Viruses and diseases of the twenty-first century. *American Journal of Pathology* 143:1241-1249.

Oldstone, M. B. A. 1994. The role of cytotoxic T lymphocytes in infectious disease: history, criteria and state of the art. *Current Topics in Microbiology and Immunology* 189:1-8.

Oldstone, M. B. A. 1996. Virus-lymphoid cell interactions. *Proceedings of National Academy of Science of United States of America* **93:**12756-12758.

Oldstone, M. B. A. & G. F. Rall. 1993b. Mechanism and consequence of viral persistence in cells of the immune system and neurons. *Intervirology* **35:**116-121.

Pardoe, I. U. & T. I. Michalak. 1995. Detection of hepatitis B and woodchuck hepatitis viral DNA in plasma and mononuclear cells from heparinized blood by the polymerase chain reaction. *Journal of Virological Methods* **51:**277-288.

Paré, P. 1996. The clinical consequences of chronic hepatitis B. *Canadian Journal of Gastroenterology* 10: Hepatitis Update (issue 6).

Paronetto, F. & B. C. Tennant. 1990. Woodchuck hepatitis virus infection: a model of human hepatic diseases and hepatocellular carcinoma. *Progress in Liver Diseases* 9:463-483.

Paterlini, P., G. Gerken, E. Nakajima, S. Terre, A. D'Errico, W. Grigioni, B. Nalpas, D. Franco, J. Wands, M. Kew, E. Pisi, P. Tiollais, & C. Bréchot. 1990. Polymerase chain reaction to detect hepatitis B virus DNA and RNA sequences in primary liver cancers from patients negative for hepatitis B surface antigen. *New England Journal of Medicine* **323:**80-85.

Penna, A., M. Artini, A. Cavalli, M. Levero, A. Bertoletti, M. Pilli, F. V. Chisari, B. Rehermann, G. Del Prete, F. Fiaccadori, & C. Ferrari. 1996. Long-lasting memory T cell responses following self-limited acute hepatitis B. *Journal Clinical Investigation* **98:**1185-1194.

Petit, A. J. C., F. G. Terpstra, & F. Miedema. 1987. Human immunodeficiency virus infection down-regulates HLA class II expression and induces differentiation in promonocytic U937 cells. *Journal of Clinical Investigation* 79:1883-1889.

Pugh, J. C. & M. F. Bassendine. 1990. Molecular biology of hepadnavirus replication. *British Medical Bulletin* 46:329-353.

Raimondo, G., E. Tanzi, S. Brancatelli, S. Campo, M. A. Sardo, G. Rodinò, M. Pernice, & A. R. Zanetti. 1993. Is the course of perinatal hepatitis B virus infection influenced by genetic heterogeneity of the virus? *Journal of Medical Virology* 40:87-90.

Rehermann, B., C. Ferrari, C. Pasquinelli, &. F. V. Chisari. 1996a. The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response. *Nature Medicine* 2:1104-1108.

Rehermann, B., P. Fowler, J. Sidney, J. Person, A. Redeker, M. Brown, B. Moss, A. Sette, & F. V. Chisari. 1995. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. *Journal of Experimental Medicine* 181:1047-1058.

Rehermann, B., D. Lau, J. H. Hoofnagle, & F.V. Chisari. 1996b. Cytotoxic T lymphocyte responsiveness after resolution of chronic hepatitis B virus infection. *Journal of Clinical Investigation* 97:1655-1665.

Reyes, G. R., M. A. Purdy, J. P. Kim, K. Luk, L. M. Young, K. E. Fry, & D. W. Bradley. 1990. Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. *Science* 24:1335-1339.

Rinaldo, C. R. Jr. 1994. Modulation of major histocompatibility complex antigen expression by viral infection. *American Journal of Pathology* 144:637-650.

Rizzetto, M., M. G. Canese, J. L. Gerin, W. T. London, D. L. Sly, & R. H. Purcell. 1980. Transmission of the hepatitis B virus-associated delta antigen to chimpanzees. *Journal of Infectious Diseases* 141:590-602.

Robinson, W. S. 1994. Molecular events in the pathogenesis of hepadnavirus -associated hepatocellular carcinoma. *Annual Review Medicine* 45:297-323.

Robson, S. C., B. Schoub, & S. S. Abdool Karim. 1994. Viral hepatitis B - an overview. *South African Medical Journal* 84:530-535.

Roggendorf, M. & T. K. Tolle. 1995. The woodchuck: an animal model for hepatitis B virus infection in man. *Intervirology* **38**:100-112.

Rossi, P., V. Moschese, P. A. Broliden, C. Fundaró, I. Quinti, A. Plebani, C. Giaquinto, P. A. Tovo, K. Ljunggren, J. Rosen, H. Wigzell, M. Jondal, & B. Wahren. 1989. Presence of maternal antibodies to human immunodeficiency virus 1 envelope glycoprotein gp120 epitopes correlates with the uninfected status of children born to seropositive mothers. *Proceedings of National Academy of Science of United States of America* **86**:8055-8058.

Ruff, A. J. 1994. Breastmilk, breastfeeding, and transmission of viruses to the neonate. *Seminars in Perinatology* **18**:510-516.

Salvato, M., P. Borrow, E. Shimomaye, & M. B. A. Oldstone. 1991. Molecular basis of viral persistence: a single amino acid change in the glycoprotein of lymphocytic choriomeningitis virus is associated with suppression of the antiviral cytotoxic T-lymphocyte response and establishment of persistence. *Journal of Virology* **65**:1863-1869.

Sánchez-Quijano, A., J. I. Jauregui, M. Leal, J. A. Pineda, A. Castilla, M. A. Abad, M. P. Civeira, F. García de Pesquera, J. Prieto, & E. Lissen. 1993. Hepatitis B virus occult infection in subjects with persistent isolated anti-HBc reactivity. *Journal of Hepatology* **17**:288-293.

Scarlatti G., V. Hodara, P. Rossi, L. Muggiasca, A. Bucceri, J. Albert, & E. M. Fenyö. 1993. Transmission of human immunodeficiency virus type 1 (HIV-1) from mother to child correlates with viral phenotype. *Virology* **197**:624-629.

Scully, J. L., H. Sung, R. Pennie, & P. Gill. 1994. Detection of hepatitis B virus DNA in the serum of Canadian hepatitis B surface antigen negative, anti-HBc positive individuals, using the polymerase chain reaction. *Journal of Medical Virology* **44**:293-297.

Sedmak, D. D., A. M. Guglielmo, D. A. Knight, D. J. Birmingham, E. H. Huang, & W. J. Waldman. 1994. Cytomegalovirus inhibits major histocompatibility class II expression on infected endothelial cells. *American Journal of Pathology* **144**:683-682.

Seeger, C., J. Summers, & W. S. Mason. 1991. Viral DNA synthesis. *Current topics in Microbiology and Immunology* **168**:41-59.

Shemesh, J., R. Rotem-Yehudar, & R. Ehrlich. 1991. Transcriptional and posttranscriptional regulation of class I major histocompatibility complex genes following transformation with human adenoviruses. *Journal of Virology* **65**:5544-5548.

Sherman, M. 1996. The epidemiology of hepatitis B in Canada. *Canadian Journal of Gastroenterology* **10**: Hepatitis Update (issue 5).

Shimizu, H., T. Mitsuda, S. Fujita, & S. Yokota. 1991. Perinatal hepatitis B virus infection caused by antihepatitis Be positive maternal mononuclear cells. *Archives of Disease in Childhood* **66**:718-721.

Shioda, T. S. Oka., S. Ida, K. Nokihara, H. Toriyoshi, S. Mori, Y. Takebe, S. Kimura, K. Shimad, & Y. Nagai. 1994. A naturally occurring single basic amino acid substitution in the V3 region of the human immunodeficiency virus type I ENV protein alters the cellular host range and antigenic structure of the virus. *Journal of Virology* **68**:7689-7696.

Sprengel, R., E. F. Kaleta, & H. Will. 1988. Isolation and characterization of a hepatitis B virus endemic in herons. *Journal of Virology* **62**:3832-3839.

Stamos, J. K. & A. H. Rowley. 1994. Timely diagnosis of congenital infections. *Pediatrics Clinics of North America* **41**:1017-1033.

Stevens, C. E., P. E. Taylor, M. J. Tong, P. T. Toy, G. N. Vyas, P. V. Nair, J. Y. W Weissman, & S. Krugman. 1987. Yeast-recombinant Hepatitis B vaccine. *Journal of American Medical Association* **257**:2612-2616.

Summers, J., J. M. Smolec, & R. Snyder. 1978. A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. *Proceedings of National Academy of Science of United States of America* **75**:4533-453.

Tennant, B. C., N. Mrosovsky, K. McLean, P. J. Cote, B. E. Korba, R. E. Engle, J. L. Gerin, J. Wright, G. R. Michenner, E. Uhl, & J. M. King. 1991. Hepatocellular carcinoma in Richardson's ground squirrel (*Spermophilus richardsonii*): evidence for association with hepatitis B-like virus infection. *Hepatology* **13**:1215-1221.

Testut, P., C. Renard, O. Terradillos, L. Vitvitski-Trépo, F. Tekaia, C. Degott, J. Blake, B. Boyer, & M. A. Buendia. 1996. A new hepadnavirus endemic in arctic ground squirrels in Alaska. *Journal of Virology* **70**:4210-4219.

Tiollais, P., C. Pourcel, & A. Dejean. 1985. The hepatitis B virus. *Nature* **317**:489-495.

Tuttleman, J. S., C. Pourcel, & J. Summers. 1986. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. *Cell* **47**:451-460.

Ueda, K., K. Tokugawa, & K. Kusuhara. 1992. Perinatal viral infections. *Early Human*

Development 29:131-136.

Ugen, K. E., V. Srikantan, J. J. Goedert, R. P. Nelson Jr., W. V. Williams, & D. B. Weiner. 1997. Vertical transmission of human immunodeficiency virus type 1: seroreactivity by maternal antibodies to the carboxy region of the gp141 envelope glycoprotein. *Journal of Infectious Diseases* 175:63-69.

Valliammai, T., J. M. Echevarria, P. León, C. Tusets, & T. J. Harrison. 1995. Amplification and sequence analysis of the precore and core region of the HBV genome from sera of Spanish patients with HBV₃-like infections. *Journal of Medical Virology* 46:375-379.

Von Weizsäcker, F., I. Pult, K. Geiss, S. Wirth, & H. E. Blum. 1995. Selective transmission of variant genomes. *Hepatology* 21:8-13.

Wachs, M. E., W. J. Amend, N. L. Ascher, P. N. Bretan, J. Emond, J. R. Lake, J. S. Melzer, J. P. Roberts, S. J. Tomlanovich, F. Vincenti, & P. Stock. 1995. The risk of transmission of hepatitis B from HBsAg(-), HBcAb(+), HBIgM(-) organ donors. *Transplantation* 59:230-234.

Warren, A. P., D. H. Ducroq, P. J. Lehner, & L. K. Borysiewicz. 1994. Human cytomegalovirus-infected cells have unstable assembly of major histocompatibility complex class I complexes and are resistant to lysis by cytotoxic T lymphocytes. *Journal of Virology* 68:2822-2829.

Wei, Y., G. Fourel, A. Ponzetto, M. Silvestro, P. Tiollais, & M-A. Buendia. 1992. Hepadnavirus integration: mechanisms of activation of the N-*myc*2 retroposon in woodchuck liver tumours. *Journal of Virology* 66:5265-5276.

Weiner, A. J., M. M. Thaler, K. Crawford, K. Ching, J. Kansopon, D. Y. Chien, J. E. Hall, F. Hu, & M. Houghton. 1993. A unique, predominant hepatitis C virus variant found in an infant born to a mother with multiple variants. *Journal of Virology* 67:4365-4368.

Werner, B. G., J. M. Smolec, R. Snyder, & J. Summers. 1979. Serological relationship of woodchuck hepatitis virus to human hepatitis B virus. *Journal of Virology* 32:314-322.

Whitten, T. M., A. T. Quets, & R. H. Schloemer. 1991. Identification of the hepatitis B virus factor that inhibits expression of the beta interferon gene. *Journal Virology* 65:4699-4704.

Whitton, J. L. & M. B. A. Oldstone. 1996. Immune response to viruses, pp. 345-374. In B.N. Fields & D. M. Knipe (eds.), *Fields Virology*, 3ʳᵈ ed. Lippincott-Raven Publishers,

Philadelphia.

Wolinsky, S. M., C. M. Wike, B. T. M. Korber, C. Hotto, W. P. Parks, L. L. Rosenblum, K. J. Kunstman, M. R. Furtado, & J. Muñoz. 1992. Selective transmission of human immunodeficiency virus type-1 variants from mothers to infants. *Science* **255**:1134-1137.

Yoffe, B., D. K. Burns, H. S. Bhatt, & B. Combes. 1990. Extrahepatic hepatitis B virus DNA sequences in patients with acute hepatitis B infection. *Hepatology* **12**:187-192.

Zinkernagel, R. M. 1996. Immunology taught by viruses. *Science* **271**:173-178.

Zoulim, F., X. Zhang, C. Pichoud, & C. Trépo. 1996. Heterogeneity of hepatitis B virus (HBV) core gene in a patient with HBV-associated cirrhosis and serum negativity for anti-HBc. *Journal of Hepatology* **24**:155-160.