MECHANISMS OF THE PATHOGENESIS OF CELL INJURY AND VIRAL PERSISTENCE IN THE WOODCHUCK MODEL OF HEPATITIS B

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by

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ABSTRACT

Hepatitis B virus (HBV) persistently infects approximately 400 million people worldwide. It has several natural histories including silent infection, acute hepatitis and a serologically detectable chronic carrier state, which commonly leads to liver cirrhosis and hepatocellular carcinoma. Woodchucks infected with woodchuck hepatitis virus (WHV) have remarkably similar spectra of progression of infection and liver disease. This model is, therefore, invaluable for *in vivo* studies that are difficult or impossible to conduct in humans. The studies that comprise this thesis were aimed at determining some of the viral and host factors that may influence the outcome of experimental hepadnavirus infection of adult woodchucks. However, due to the relatively poor characterization of the woodchuck model, we first established a number of gene sequences relevant to the woodchuck immune system. This led to a gene discovery program that identified 14 novel woodchuck genes. Our first set of experiments studied Fas and perforin-mediated lymphocyte cytotoxicity to see if differences in the types of immune cell activation could predict the outcome of viral hepatitis. Our results demonstrate that lymphoid cells from woodchucks with acute WHV infection have an augmented capacity to elicit perforin-dependent cell killing when compared to woodchucks with chronic hepatitis (CH). This suggests that nonspecific cellular immunity, presumably NK cells, may play a role in early recovery from WHV infection. A second set of experiments investigated the hepatic and splenic expression of MHC class I in acute and chronic WHV infections. We have found that CH is accompanied by severely diminished hepatocyte and lymphoid cell

MHC class I surface expression despite upregulated transcription of affiliated genes. This provides evidence that the virus posttranscriptionally disrupts MHC class I display and, this may protect infected cells from T cell-mediated immune clearance. This would contribute to viral persistence and possibly deregulate the MHC class I-dependent functions of the host's immune system. Our final set of experiments studied intrahepatic cytokine expression and T cell influx in the course of, or after, experimental WHV infection. This work demonstrated a positive correlation between recovery from adult WHV hepatitis and upregulated interferon γ (IFNγ), tumor necrosis factor α (TNFα) and CD3 gene expression. In addition, the same markers of immune activation were found to endure for years after resolution of acute infection. This suggests that antiviral cytokines, such as IFNγ and TNFα, may play a central role in recovery from acute hepatitis, as well as in the long term control of occult hepadnavirus persistence. The processes identified in the present studies could be critical for perpetuation of liver damage and evasion of anti-viral immunological surveillance in chronic hepadnavirus infection. The data obtained and the investigative tools generated should enable a better understanding of the immunopathogenesis of HBV infection and aid in the development of more effective anti-viral agents.

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"Brains first and then hard work."
A. A. Milne (Winnie the Pooh)

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I dedicate this thesis to all the people who have had a positive influence on my life, particularly my family and my closest friends. Immediately standing out in my mind are my parents Wayne and Christine, my two sisters Jennifer and Julie, my grandparents who were able to encourage my education (Douglas and Vera Thorley and Ethel Hodgson) and my best friend Greg Boyde. You have all provided the unconditional support that I will always remember.

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All of the original research work presented in the following thesis has been published in peer reviewed papers. The details of these publications are outlined in each respective chapter. Briefly, the study presented in Chapter 3 entitled "Perforin and Fas/Fas Ligand-Mediated Cytotoxicity in Acute and Chronic Woodchuck viral Hepatitis" was published in Clinical and Experimental Immunology volume 118, issue 1 in October 1999. I wish to thank Dr. Michael Grant, in whose laboratory I performed the described cytotoxic assays and who provided his expertise in the evaluation of the results. The major components of Chapter 4, entitled "Posttranscriptional Inhibition of Major Histocompatibility Complex Class I Presentation on Hepatocytes and Lymphoid Cells in Chronic Woodchuck Hepatitis Virus Infection" were published in volume 74, issue 10 of the May Journal of Virology in 2000. I wish to acknowledge the contribution of cc-author Ms. Norma Churchill who generated immunoblot data. Finally, Chapter 5 entitled "Augmented Hepatic IFNγ Expression and T-cell Influx Characterize Acute Hepatitis Progressing to Recovery and Residual Lifelong Virus Persistence in Experimental Adult Woodchuck Hepatitis Virus Infection" was published in the November 2001 issue of Hepatology volume 34, number 5.

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ABBREVIATIONS AND SYMBOLS

ADCC	antibody-dependent cellular cytotoxicity
AH	acute hepatitis
AIDS	acquired immunodeficiency syndrome
ALT	alanine aminotransferase
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
ASGPR	asialoglycoprotein receptor
AST	aspartate aminotransferase
ATP	adenosine triphosphate
bp	base pairs
cccDNA	covalently closed circular DNA
cDNA	complementary DNA
CH	chronic hepatitis
CMV	cytomegalovirus
ConA	concanavalin A
cpm	counts per minute
CsCl	cesium chloride
CTL	cytotoxic T lymphocyte
CW	woodchuck
d.p.i.	days post inoculation
dCTP	deoxycytosine triphosphate
DEPC	diethylpyrocarbonate
DHBV	duck hepatitis B virus
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EB	ethidium bromide
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immunoassay
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
FASL	Fas ligand
FCS-HI	heat inactivated fetal calf serum
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GGT	γ-glutamyltransferase
GSHV	ground squirrel hepatitis virus

GTE	glucose/Tris/EDTA
h	hour
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HPM	hepatocyte plasma membranes
HPV	human papillomavirus
HSV	herpes simplex virus
i.v.	intravenous
IFNβ	interferon beta
IFNγ	interferon gamma
Ig	immunoglobulin
IL	interleukin
kb	kilobases
kDa	kiloDaltons
KPM	kidney plasma membranes
LB	Luria-Bertani medium
LCMV	lymphocytic choriomeningitis virus
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC	major histocompatibility complex
min	minutes
MMLV	Moloney murine leukemia virus
mo	month
MOPS	(N-morpholino)-propanesulfonic acid
mRNA	messenger RNA
NC	nitrocellulose
NK	natural killer cells
NKT	natural killer T cells
nm	nanometer
nt	nucleotide
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PHA	phytohemagglutinin
p.i.	post inoculation

rcDNA	relaxed circular DNA
RIA	radioimmunoassay
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcription
SD	standard deviation
SDS	sodium dodecyl sulfate
SE	standard error
sec	seconds
SLAH	self-limited acute hepatitis
SPM	splenocyte plasma membranes
SSC	standard sodium citrate
SSPE	standard sodium phosphate EDTA
TAE	Tris-acetate-EDTA
TAP	transporter associated with antigen processing
Taq	Thermus aquaticus
TCR	T cell receptor
TE	Tris-EDTA
Th	T helper cell
TNFα	tumor necrosis factor alpha
UV	ultraviolet
vge	viral genome equivalents
VZV	varicella zoster virus
WHcAg	woodchuck hepatitis virus core antigen
WHeAg	woodchuck hepatitis virus e antigen
WHsAg	woodchuck hepatitis virus surface antigen
WHV	woodchuck hepatitis virus
wks	weeks
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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CHAPTER ONE:

INTRODUCTION

1.1 EPIDEMIOLOGY OF HEPATITIS B INFECTION

The term "virus" (Latin: poison) is defined as an infective agent that depends on living host cells for its replication. Hepatitis is derived from the Greek "hepar" (liver) and "-itis" (inflammation). It is most commonly caused by one of 5 hepatotropic viruses, called hepatitis A, B, C, D, and E, although a number of other viruses can also induce hepatitis (e.g., adenovirus, human cytomegalovirus [HCMV], echoviruses, Epstein-Barr virus [EBV], rubella virus, and varicella zoster virus [VZV]). Viral hepatitis is characterized by hepatocyte injury, lymphomononuclear cell infiltrations, and liver cell regeneration. It is well recognized that chronic infection with the hepatitis B virus (HBV) is a major worldwide cause of morbidity and mortality, primarily due to the development of liver cirrhosis and hepatocellular carcinoma (HCC). This is despite the availability of a prophylactic vaccine that is highly effective in preventing HBV infection of healthy individuals. Currently, of the more than 2 billion living people who have been infected with the virus, the estimated global number of chronic HBV carriers (i.e., serum HBV surface antigen [HBsAg]-positive individuals) is approaching 400 million (World Health Organization, 2000). Of the people chronically infected, about 25% terminate in untreatable HCC (Beasley, 1988).

The geographic distribution of HBV infection varies greatly. Endemic areas include Southeast Asia and sub-Saharan Africa where up to 15% of the

total population are chronically infected with HBV (Maynard *et al.*, 1989). In these endemic areas, HBV is primarily transmitted vertically from mother to child. In the developed world, including North America and western Europe, where infection rates are usually below 1%, virus transmission occurs largely due to intravenous drug use and sexual contact, although occupational exposure is still a concern (Gerberding, 1996). It is estimated that at least 270,000 Canadians are chronically infected with HBV, but the prevalence of HBV infection varies considerably due to the heterogeneity of our population. There is an estimated chronic carrier rate of 4% among natives, 4.3% among immigrants, and 0.2-0.5% in non-immigrants (Canadian consensus on the management of viral hepatitis, 1999).

1.2 NATURAL HISTORY OF HBV INFECTION

HBV infection can be classified into five clinically distinct appearances: (1) asymptomatic 'subclinical' infection, (2) acute hepatitis (AH), (3) fulminant hepatitis, (4) chronic hepatitis (CH), and (5) a HBsAg-positive healthy chronic carrier state. It is estimated that up to 70% of adults infected with HBV develop clinically asymptomatic infection. This is usually identified by coincidental blood testing, although some of these individuals do demonstrate mild non-specific manifestations, such as fatigue and flu-like symptoms (reviewed by Hoofnagle *et al.*, 1987). The remaining 30% of the exposed individuals will develop clinically evident liver disease and experience flu-like symptoms, jaundice, abdominal

pain, fatigue and anorexia, diagnosed as AH . Approximately 1% of people with acute HBV infection will develop a very severe form of liver damage, termed fulminant hepatitis (Sarraco *et al.*, 1988). This form of liver disease is associated with severe and rapidly progressing hepatocyte necrosis accompanied by the development of encephalopathy and is almost always fatal (Kumar and Pound, 1992).

After the acute phase of disease, about 90% of adults spontaneously recover (self-limited acute hepatitis; SLAH) and enter the convalescent stage with apparent permanent immunity (Hoofnagle *et al.*, 1987). However, recent studies have demonstrated that recovery, which is characterized by the disappearance of clinical symptoms, the normalization of biochemical indicators of liver function (e.g., alanine aminotransaminase [ALT] and aspartate aminotransferase [AST]), the disappearance of serum HBsAg, and the rise of circulating antibodies to HBsAg (anti-HBs), does not reflect the complete elimination of the replicating virus (Michalak *et al.*, 1994, Rehermann *et al.*, 1996).

Patients with a continued presence of serological markers of active HBV infection (i.e., positive for HBsAg and antibodies to HBV core antigen [anti-HBc]) and with biochemical indicators of liver injury (e.g., ALT, AST) for longer than six months are deemed to have chronic hepatitis type B. Interestingly, the proportion of individuals who become chronic carriers is predominantly determined by the age of the patient when infected. For example, greater than

90% of infected neonates become symptomatic chronic carriers, as compared to 30-60% of children infected before the age of 4 years. This is in contrast to the 5-10% of individuals infected with HBV as adults (Moyer and Mast, 1994). In these chronically infected patients, the pattern of CH can be variable. According to classical serological and histological diagnostic criteria, it includes chronic active (aggressive) hepatitis, chronic persistent (mild) hepatitis, and a HBsAg-positive healthy chronic carrier state without apparent morphological features of liver injury (Hoofnagle *et al.*, 1987).

Chronically infected individuals usually have progressive liver inflammation which often leads to liver cirrhosis, the most common precursor of HCC (Beasley, 1988). Importantly, the risk of developing HCC is almost 100 times greater in chronic HBV carriers than in uninfected individuals (Beasley, 1988). It is hypothesized that at least three independent factors contribute to the development of HCC. One of them appears to be integration of HBV DNA into hepatocyte chromosomes, which potentially disrupts tumour suppressor gene functions or activates cellular genes (e.g., oncogenes and growth factors) leading to uncontrolled cell proliferation. The second factor is related to the continuous liver cell death and regeneration that increases the chance of mutations and subsequent tumour development (Robinson, 1994). Additionally, HBV X protein has been implicated, due to its transactivating capabilities, in the development of HCC (reviewed by Murakami, 1999).

HBV can also induce extrahepatic disorders through the deposition of

complexes of viral antigens and their specific antibodies. The pathogenic role of these immune complexes has been documented in glomerulonephritis and polyarteritis nodosa, vasculitis and arthritis, and is described in Section 1.8.1.

1.3 CHARACTERISTICS OF HBV

1.3.1 Molecular organization

The infectious virion of HBV is referred to as the Dane particle (Dane *et al.*, 1970). This complete virus is a spherical structure 42 nanometers (nm) in diameter. It consists of a lipoprotein outer envelope made of three virus surface proteins all of which carry HBsAg specificity. The envelope protein surrounds an electron dense nucleocapsid (core) which contains the genome (Kaplan *et al.*, 1973). In addition to Dane particles, subviral particles composed of envelope proteins, in the form of spheres and tubules, are also produced in large quantities and circulate in HBV-infected individuals. These particles do not contain genetic material and are, therefore, not infectious (Gavilanes *et al.*, 1982). Prior to the introduction of recombinant DNA technology these subviral HBsAg particles were purified from the plasma of infected individuals and used as HBV vaccines (Hollinger *et al.*, 1986).

The virus genome, is a circular 3.2 kilobase (kb)-long, partially double stranded DNA structure, commonly referred to as relaxed circular DNA (rcDNA). The circular structure is maintained by a short cohesive overlap between the 5' ends of plus and minus DNA strands. The 5'-end of the DNA minus strand

contains a covalently linked protein, whereas the plus strand has a 5' RNA oligonucleotide primer attached. Both the protein and the primer appear to be essential for viral replication (Ganem, 1991). The genome contains four overlapping open reading frames (ORF) encoding the four principal translation products, the virus envelope or surface (S), core (C), polymerase (P) and X proteins. The three envelope proteins are encoded by the same ORF containing three in-frame start codons, but they are derived from two different overlapping mRNA species (2.4 and 2.1 kb). These proteins have a common carboxy-terminus, but differ at their amino-ends, and are referred to as large (preS1), middle (preS2), and major or small.

The C ORF encodes the virus nucleocapsid protein (HBcAg) and a protein, which, due to post-translational modifications, displays e antigen specificity (HBeAg) (Uy *et al.*, 1986). Although the amino acid sequences of the core and e proteins are nearly identical, they stimulate distinct antibodies (Salfeld *et al.*, 1989). In contrast to the core, which only occurs in infected hepatocytes and virions (Schlicht and Schaller, 1989), HBeAg is also detectable in hepatocytes and in a soluble form in the circulation, but not on virions.

Transcription of the P ORF results in formation of a multidomain polypeptide with viral reverse transcriptase (RT), RNase, and DNA polymerase activities (Bavand and Laub, 1988; Mack *et al.*, 1988). Additionally, this protein contains a packaging signal (Bartenschlager *et al.*, 1990) and acts as a primer for reverse transcription of the viral pregenome (Wang and Seeger, 1992).

The smallest ORF and its derived mRNA (0.7 kb) encodes the X protein. This protein has transcriptional trans-activating properties that may play a role in virus tumor genesis (reviewed by Murakami, 1999). Additionally, the transactivating properties of the X-protein affect many other cellular proteins, including the expression of major histocompatibility complex (MHC) class I in cultured hepatocytes (Zhou *et al., 1990)*

1.3.2 Replication cycle

After virion recognition of a putative cell surface receptor and its penetration into the cytoplasm, it is postulated that only the virus nucleocapsid migrates to the nucleus. Here HBV rcDNA is repaired by host DNA polymerases and ligases to form covalently closed circular DNA (cccDNA). This is considered to be the first step of hepadnavirus replication (Tuttleman *et al.*, 1986). Four HBV mRNA transcripts (i.e., 3.5, 2.4, 2.1, and 0.7 kb) are transcribed from the cccDNA using host RNA polymerase. They are exported from the nucleus and translated into the aforementioned viral proteins (Section1.3.1). In addition to being a template for polymerase translation, the 3.5 kb RNA is also packed into core particles together with the viral polymerase. This process occurs within the nucleus of the infected cell. The pregenomic RNA is then reverse transcribed into minus strand DNA, which subsequently serves as a template for plus strand DNA synthesis. Once the plus strand is synthesized, HBV rcDNA is formed. The mature nucleocapsid particles containing rcDNA are either packaged into virions,

which are exported from the cell, or recycled to the nucleus. Presently, hepadnaviruses are the only known mammalian DNA viruses that use reverse transcription in their replication cycle.

Although the primary site of HBV replication is the liver, HBV can also replicate in the lymphatic system. HBV mRNA was detected by PCR with a reverse transcription step (RT-PCR) in PBMC from patients with CH type B (Baginski *et al.*, 1991). More recently, the presence of HBV cccDNA and all HBV mRNA transcripts were identified in circulating lymphoid cells from patients with active chronic HBV infection using PCR (Stoll-Becker *et al.*, 1997). Interestingly, the X mRNA (0.7 kb) was detected at the highest amount in these cells. The detection of HBV nucleic acid sequences in lymphoid cells was supported by identification of viral antigens in or on the cells. In one of the reports, most of the individuals with CH type B were reported to express HBsAg and HBcAg on their PBMC (Parvaz *et al.*, 1987). Additionally, trace amounts of HBV-specific DNA and RNA sequences were identified in PBMC from patients years after complete clinical and serological recovery from AH type B, indicating the persistence of replicating virus in lymphoid cells in convalescent, anti-HBs positive individuals (Michalak *et al.*, 1994; Rehermann *et al.*, 1996; Penna *et al.*, 1996; Yotsuyanagi *et al.*, 1998). It was also reported that HBV DNA sequences can persist in PBMC of patients with either spontaneous or therapy-induced recovery from CH type B (Trippler *et al.*, 1996; Oesterreicher *et al.*, 1995).

Taken together, these findings demonstrate that HBV also infects

lymphoid cells and that these cells can support long-term virus replication. Therefore, as in other persistent viral infections, the lymphoid system may play an important role in the establishment and maintenance of hepadnaviral persistence (reviewed by Michalak, 2000). However, the molecular basis of HBV lymphotropism and its pathogenic implications for the outcome of infection are not yet fully recognized.

1.4 HEPADNAVIRUS FAMILY

HBV is the prototypic virus of the hepadnavirus family. This family is divided into two genera: *Orthohepadnaviridae* (mammalian viruses) and *Avihepadnaviridae* (avian viruses). As molecular techniques improve and simplify, new hepadnaviruses are being discovered with increasing frequency. Key members of the mammalian genus are HBV, woodchuck hepatitis virus (WHV), and ground squirrel hepatitis virus (GSHV) (Marion *et al.*, 1980). The best characterized member of the avian hepadnaviruses is the duck hepatitis B virus (DHBV) (Mason *et al.*, 1980). Although the duck model is commonly used to study the hepadnavirus replication cycle and putative cellular receptors, it is not generally utilized as a model for analysis of the immunopathogenic mechanisms of liver injury and viral persistence (see below).

With some exceptions, the mammalian hepadnaviruses have similar molecular, structural, antigenic, and pathogenic properties. This includes virion ultrastructure and molecular organization, replication strategy, and an overall

similar course and pathological forms of virus-induced liver disease (Michalak, 1998). However, mammalian and avian hepadnaviruses also have distinct characteristics, including the fact that avian hepadnaviruses lack an X gene, only synthesize two envelope proteins (pre-S and S) and their envelope proteins do not form freely circulating filaments. Furthermore, liver disease induced by avian hepadnaviruses do not appear to be associated with the development of HCC (Cova *et al.*, 1994).

1.5 THE WOODCHUCK MODEL OF HEPATITIS B

1.5.1 Woodchuck hepatitis virus

A high frequency of HCC in North American woodchucks (*Marmota monax*) housed in the Philadelphia Zoological Gardens led to the discovery of the WHV as the first animal hepatitis B-like virus (Summers *et al.*, 1978). Among hepadnaviruses, WHV has the most similar genomic organization, and overall nucleotide sequence homology (~70%) to HBV (Roggendorf and Tolle, 1995; reviewed by Michalak, 1998). The high level of amino acid sequence similarity allows the use of commercial immunoassays designed for detection of HBV antigens and antibodies to identify corresponding markers of WHV infection (e.g., WHV surface antigen [WHsAg] and antibodies to WHsAg [anti-WHs] and WHV core antigen [WHcAg] and antibodies to WHcAg [anti-WHc]). The greatest differences in sequence homology between WHV and HBV lie within the X gene and preS1 region of the S gene. Interestingly, the X gene is apparently

indispensable in WHV replication and infection, but not in HBV replication (Chen *et al.*, 1993; Zoulim *et al.*, 1994). Additionally only two RNA transcripts, with molecular sizes of 3.7 and 2.1, have been detected in WHV-infected woodchucks (Moroy *et al.*, 1985).

1.5.2 Characteristics of WHV infection

The progression of WHV and HBV-induced liver disease parallel each other. Adult woodchucks infected with WHV have similar courses of infection and comparable histological characteristics of liver disease as humans infected with HBV. Both WHV and HBV induce AH, which in 10-15% of adult cases progresses to serologically evident chronic liver disease. Both viruses can be transmitted by blood or body fluids, as well as vertically from infected mothers to their offspring (Kulonen and Millman, 1988, Coffin and Michalak, 1999).

Although WHV is highly hepatotropic, WHV DNA and mRNA sequences have been detected in lymphoid cells from the earliest stages of virus infection (Korba *et al.*, 1989; reviewed by Michalak, 1998). It has been reported that even transient WHV hepatitis results in latent infection that involves both the liver and the lymphatic system (Michalak *et al.*, 1999; Coffin and Michalak, 1999). Recently, our laboratory demonstrated that woodchuck mothers convalescent from AH can still be infectious to their offspring, as well as to healthy animals (Coffin and Michalak, 1999; reviewed by Michalak, 2000).

On a cellular level WHV and HBV surface and core antigens have

essentially the same distribution patterns in infected hepatocytes (reviewed by Michalak, 1998). Hepatocyte plasma membranes (HPM) purified from WHV-infected woodchucks were shown to express both envelope and core specific polypeptides as peripheral and integral membrane proteins (Michalak and Churchill, 1988; Michalak *et al.*, 1990; Michalak and Lin, 1994). These proteins incorporated into the lipid bilayer may help the virus evade both cytotoxic T lymphocyte (CTL) and natural killer (NK) cell based killing (Section 1.6.2).

Among hepadnaviruses, WHV has the highest oncogenic potential, believed to be caused by the preferential integration of the viral genome near the proto-oncogenes *c-myc* and *N-myc* leading to their constitutive expression (Fourel *et al.*, 1990; Wei *et al.*, 1992). Inevitably, almost all serum WHsAg-reactive, chronically infected animals will progress to HCC; a situation that occurs much less frequently in humans (Cova *et al.*, 1994). Analogous to HBV-infected humans, woodchucks persistently infected with WHV can have chronic liver inflammation with different degrees of hepatocyte injury and lymphomononuclear cell infiltrations (reviewed by Michalak, 1998). However, in contrast to CH type B, woodchucks chronically infected with WHV rarely develop liver fibrosis or cirrhosis (Summers, 1981). Overall, due to the existing similarities, the woodchuck model is accepted as the most valuable natural system to study immunobiological events, pathogenesis of liver injury and HCC in hepadnavirus infection, molecular mechanisms of hepadnavirus persistence, and to test the efficacy of novel anti-HBV drugs. This model overcomes several of the

limitations posed by the use of chimpanzees for hepatitis B research.

1.6 GENERAL CONSIDERATIONS REGARDING THE ANTIVIRAL IMMUNE RESPONSES

The primary function of the immune system is the recognition and elimination of pathogens, including viruses. The immune system of higher vertebrates consists of natural (innate) and adaptive (specific) immunity. After a virus first enters a host, it encounters the innate immune system that includes monocytes and macrophages, NK cells, NKT cells and polymorphonuclear cells (Section 1.6). These cells recognize pathogen-associated molecules causing the activation of cellular processes including phagocytosis, induction and synthesis of antimicrobial agents (e.g., nitric oxide, lysomsomes), and cytokines (e.g., interferons [IFN] and tumor necrosis factor alpha [TNFα]). If the primary defence mechanisms do not eliminate the virus, the adaptive (specific) immune response becomes involved. This response exhibits the properties of memory and antigen specificity. Fundamentally, the adaptive immune responses can be divided into humoral, which mainly target extracellular pathogens, and cellular, which primarily combats those inside the cell. Pathogen-specific cellular immunity is mediated by two main groups of effector cells; CD4+ T helper cells, which provide help to B lymphocytes and to other immune effector cells, and CD8+ CTL, whose main function is elimination of infected host cells. In many cases of infections with noncytopathic viruses, the clinical symptoms are a result of the

host's immune response against the virus-infected cells and the subsequent destruction of these cells.

1.6.1 Humoral immune responses

Specific humoral immunity is mediated by antibodies produced by plasma cells derived from antigen-stimulated B cells. Antibodies are essential in the early defence against viral infections. Neutralizing antibodies can help reduce the amount of freely circulating virus by preventing viral attachment and entry into host cells. They may also act as opsonins and enhance phagocytosis. Furthermore, anti-virus specific antibodies can be involved in the elimination of infected cells that express viral antigens on their surface by complement dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) (reviewed by Lachmann and Davies 1997). Specific antibody responses are also very important in preventing re-infection with the same virus, although this has been reported to be ineffective in some viral infections (e.g., hepatitis C virus) (Farci *et al.*, 1992). Stimulation of specific memory B cells which persist after the initial infection results in a rapid secondary immune response and production of large amounts of specific antibodies with increased affinity for the particular viral antigen (Abbas *et al.*, 2000).

1.6.2 Cellular immune responses

1.6.2.1 CD4+ T cells

The activation of CD4+ cells requires recognition by the highly polymorphic T-cell receptor (TCR) of exogenous viral peptides presented by MHC class II molecules. These molecules are located on the surface of professional antigen presenting cells (APC), including dendritic cells, macrophages (designated in the liver as Kupffer cells), and activated B lymphocytes (Abbas *et al*, 2000). The 18-22 amino acid-long antigenic peptides presented by MHC class II are usually derived from extracellular antigens that are proteolytically processed in acidified endosomes or lysosomes after endocytosis by the APC (Cresswell, 1996).

There are two distinct subsets of CD4+ cells, each associated with a different arm of the immune system (Bottomly, 1988). The T-helper type 1 (Th1) subset, which produce cytokines such as interleukin (IL)-2 and IFNγ, are known to be involved in the classic cell-mediated functions, such as clonal expansion of CTL. In contrast, T-helper type 2 (Th2) cells, which secrete IL-4, IL-5 and IL-10, are primarily involved in the maturation and differentiation of B cells (Mosmann and Sad, 1996). Results from a murine model of leishmaniasis and from human leprosy demonstrate that Th1 responses are particularly effective against intracellular pathogens, whereas protection against extracellular microbes require a Th2 profile (reviewed by Paul and Seder, 1994).

1.6.2.2 CD8+ T cells

It is important to note that because viral encoded peptides are synthesized intracellularly they are processed by the class I MHC presentation pathway. Therefore, the principle mechanism of specific immunity against established viral infections is a virus-specific MHC class I-restricted, CD8+ CTL-mediated response (Whitton and Oldstone, 1989). The function of CD8+ CTL requires the interaction of the TCR with viral peptides associated with MHC class I molecules on the surface of infected cells.

MHC class I molecules consist of a transmembrane glycoprotein heavy chain, a soluble light chain, termed β_2-microglobulin (β_2m), and a processed peptide (Hill and Ploegh, 1995). Generation of viral peptides for loading of the MHC class I molecules is primarily through proteasome degradation. The resulting 8-12 amino acid-long peptides are translocated into the lumen of the endoplasmic reticulum (ER) to empty class I heavy-light chain heterodimers by the transmembrane transporter proteins, referred to as TAP1 and TAP2 (Spies *et al.*, 1990). The created trimeric complexes are transported through the Golgi apparatus to the cell surface where they are recognized by the TCR on CD8+ T-lymphocytes (Fig. 1-1).

Once triggered CTL can kill the targeted cell by two principal, contact dependent mechanisms. These cytotoxic pathways are mediated by Fas/Fas ligand (FasL) interaction and by perforin-granzyme release (Shresta *et al.*, 1998; Ando *et al.*, 1993). In the Fas/FasL pathway, ligation and trimerization of Fas

(CD95) receptors on target cells by effector cells (i.e., CTL) expressing FasL causes secondary messenger-induced programmed death (apoptosis) of the infected cells (Fig. 1-1). In the perforin-dependent pathway, CTL secrete the pore forming protein, perforin, that acts on the target cell membrane providing access for granzymes. After entering the cell, the granzymes are thought to pass into the cytoplasm where they may act on specific substrates involved in the ultimate death of the cell and/or they are transported to the nucleus where they may activate death substrates (Shresta *et al.*, 1998). In addition, activated CTL secrete antiviral cytokines (such as TNFα and IFNγ) which can directly kill the target cell (Kagi *et al.*, 1996) (Fig 1-1).

The CTL-mediated killing has an advantage over antibodies in that they can recognize low levels of viral peptides, including internal regulatory and nonstructural proteins which are not normally exposed on virions (Kagi and Hengartner, 1996). Since non-structural proteins are usually made the earliest after invasion, CTL can theoretically eliminate infected cells before they can assemble complete virions and are, therefore, of primary importance in preventing virus persistence (Oldstone, 1994).

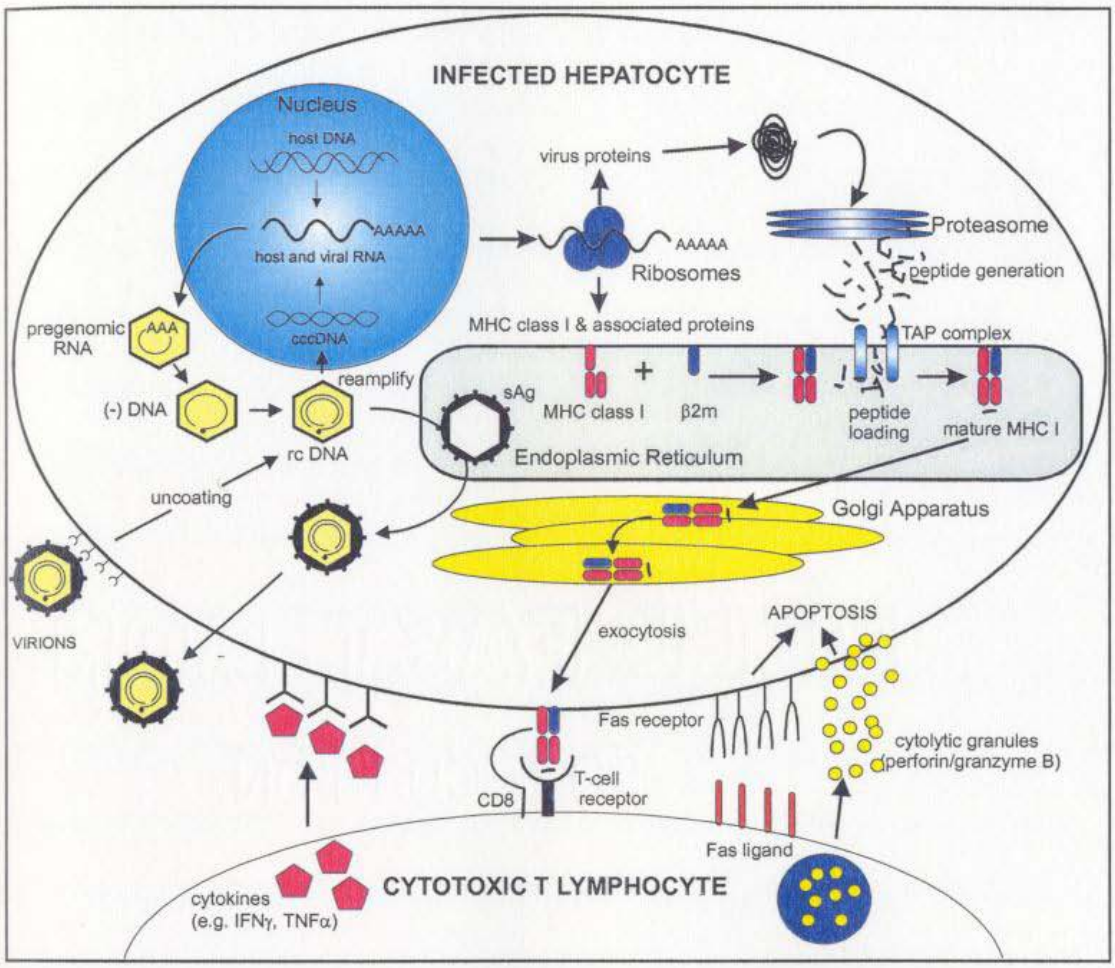


Figure 1-1. Major histocompatibility complex class I processing and presentation pathway. Following the synthesis of antigenic proteins, peptides are generated after proteasome degradation. Newly synthesized MHC class I heavy chain assembles with β2-microglobulin then with TAP. Viral peptides transported into the ER by TAP associate with MHC class I molecules. This stable 'trimolecular' complex is expressed on the hepatocyte surface where it is recognized by the CTL (via TCR). In response to activation by Th and NK cells, CTL release cytolytic granules and cytokines, and express Fas ligand.

1.6.2.3 Natural killer (NK) and NKT cells

The peak of NK cell cytolytic activity and proliferation usually occurs shortly after viral infection and is, therefore, an important element of natural resistence to many viruses (See *et al.*, 1997). NK cells lyse infected targets, but the killing is not MHC restricted nor directed toward a specific antigen. Rather, NK cells detect reduced or aberrant expression of MHC class I molecules (Karre *et al.*, 1986). NK cells are normally prevented from killing their targets by inhibitory signals provided through interaction of receptors on NK cells with self-MHC products (Karre and Welsh, 1997). Therefore, virus-infected cells with decreased surface expression of MHC class I renders them susceptible to NK-mediated elimination (Miller and Sedmak, 1999). Considerable evidence has accumulated to conclude that the principal mechanism by which NK cells eliminate targeted cells is through perforin-dependent cytotoxicity (Kagi *et al.*, 1994; Sayers *et al.*,1998). Several reports indicate that the ability of the host to mount a strong cytotoxic NK cell response very early in infection plays a decisive role in controlling virus spread and limiting progression of the disease (Biron, 1997; Welsh and Zinkernagel, 1979). Additionally, it is known that IFNγ, secreted by activated NK cells, may dramatically enhance the defence against certain virus infections (Orange *et al.*, 1995), including murine cytomegalovirus (CMV).

A second type of NK cell, which expresses both NK and T cell markers is the NKT cell. Although the exact characteristics of this cell subset remain

controversial the key features are suggested to include a biased Vα/Vβ TCR repertoire, the presence of one of the defined NK cell markers (e.g., CD16 and Ly49) and high levels of cytokine production, including IL-4 and IFNγ (Takahashi *et al.*, 2000). The IFNγ production suggests that these cells have the potential to control viral infections if they become activated. In addition to cytokine production, NKT cells also exhibit both Fas and perforin dependent lytic activity (Arase *et al.*, 1994; Smyth *et al.*, 2000). Both human and mouse NKT cells recognize non-classical MHC class I-like β2 microglobulin-associated molecules (CD1d) present on professional APC (Burdin and Kronenberg, 1999) via their TCR. Interestingly, NKT cells represent up to 50% of the mature T cells in the liver (Watanabe *et al.*, 1995), therefore, this cell type could be instrumental in the outcome of liver specific diseases.

1.7 STRATEGIES OF VIRUS ESCAPE FROM HOST IMMUNE RESPONSES

The outcome of any viral infection is influenced by a struggle between the host immune response, which acts to recognize and destroy the virus, and mechanisms adapted by the virus to avoid recognition and killing. Viruses that evade immune elimination and establish chronic infections (e.g., AIDS caused by human immunodeficiency virus [HIV] and CH caused by HBV and hepatitis C virus [HCV]) are considered to be among the most deadly infectious agents worldwide accounting for millions of fatalities per year (World Health Organization, 2000). In general, viruses that persist in a host need to possess

two fundamental characteristics. First, they must not be overly cytolytic and be able to maintain the genome in the host cells for a prolonged period of time. Second, the virus has to avoid detection and elimination by the hosts immune response (Oldstone, 1998). Viruses have evolved multiple mechanisms to avoid recognition by immune effector cells and antibody immune responses. Most viruses that persist have established a complex virus-host relationship and often use multiple mechanisms to avoid the antiviral immune response. The following sections will briefly outline some examples of the strategies used by viruses to evade host immune surveillance.

1.7.1 Infection of immunologically privileged sites

One immune escape strategy used by viruses is the infection of tissues and cell types that are not readily accessible to the immune system. A site of persistence favored by many viruses, including herpes simplex virus (HSV), lymphocytic choriomeningitis virus in mice (LCMV), varicella zoster virus (VZV) and measles, is the central nervous system (reviewed by Oldstone and Rall, 1993). Cells in the central nervous system are favored for at least two reasons: the presence of the blood-brain barrier that limits lymphocyte trafficking and the lack of MHC class I expression on neurons which, consequently, cannot be recognized by virus-specific CTL (Joly *et al.*, 1991).

Several viruses also infect lymphocytes, the effector cells which normally participate in viral clearance (Oldstone, 1989). This may disrupt the function of

these cells and result in virus-specific or even generalized immunosuppression. HIV is a relatively well characterized example of a lymphotropic virus which persistently infects CD4+ T helper cells and macrophages leading to their decline, generalized immunosuppression and ultimately death due to secondary infections (Embretson *et al.*, 1993). The measles virus is another example of a lymphotropic virus which causes generalized immunosuppression, although the molecular basis of this event is not yet characterized. It has recently been suggested that measles virus impairs both the primary and secondary T cell responses by inhibiting their proliferation, but not the effector functions (e.g., cytolysis or cytokine secretion) of the T cells (Niewiesk *et al.*, 2000).

1.7.2 Antigenic variation

The emergence of viral variants is another characteristic feature of many viruses that cause persistent infection, particularly those with high genomic mutation rates (Holland *et al.*, 1992). This usually occurs in viruses which replicate through reverse transcription due to the lack of proofreading ability of the reverse transcriptase. This mechanism can lead to significant genomic changes in the virus, causing protein modifications and providing a means for evading virus-specific B and T-cell mediated responses.

The mutation of viral proteins at epitopes critical for antibody recognition allows an effective escape of the virus from humoral immune responses. For example, mutations in hypervariable region 1 of the HCV E2 glycoprotein may

generate viral species able to escape recognition by specific antibodies (van Doorn *et al.*, 1995). In HIV infection, a single amino acid substitution in the conserved region of the envelope glycoprotein, gp 120, can lead to a loss of recognition by antibodies (Watkins *et al.*, 1993).

In an analogous manner, mutations in epitopes involved in the binding of viral peptides to the MHC molecules or in recognition of MHC/viral peptide complex by the TCR can create escape mutants. The presence of CTL escape mutants was first demonstrated in LCMV infection in transgenic mice carrying a TCR for a single LCMV epitope (Pircher *et al.*, 1990). This study revealed that a single amino acid substitution in the peptide that constitutes the TCR contact site resulted in suppression of the CTL response and virus persistence. Whether a similar advantage occurs when the CTL response is polyclonal and multispecific is not yet known. However, it has been shown that CTL escape variants occur during natural HIV infection (Borrow *et al.*, 1997) and HBV infection (Bertoletti *et al.*, 1994).

Another mechanism by which antigenic variation can aid in viral escape of immune recognition is TCR antagonism. This involves interaction of mutant viral peptides with the receptor and, in this case, the peptide renders the T-cell unresponsive by an unknown mechanism. This situation has been suggested to occur in influenza (Ostrov *et al.*, 1993) and HIV infections (Klenerman *et al.*, 1994). Although the exact mechanism of this event is not yet understood, it can provide a highly effective means of evading the virus specific CTL response. An

interesting aspect of this strategy is that the variants could block CTL-mediated lysis of cells that are co-infected with wild type virus. This could allow the survival of the wild type virus in the presence of an ongoing CTL response.

1.7.3 Induction of immune tolerance

The effectiveness of the host immune response against viruses also depends upon the maturity of the immune system at the time of exposure. The hypothesis that has endured time suggests that antigens encountered in early stages of development are more likely to be regarded as self, whereas the same exogenous antigens seen by a mature immune system will be immunogenic (Ahmed, 1989). In this regard, congenitally acquired LCMV infection in mice is one of the best studied models of tolerance. Mice infected since birth become life-long carriers and fail to develop a virus-specific CTL response (Jamieson *et al.*, 1991).

In adult viral infection, viral antigens may initially induce a strong antigen-specific effector T cell response. However, in later phases of the infection, the virus can overwhelm the CD8+ CTL virus-specific response causing their clonal exhaustion (deletion) from the repertoire (Ahmed *et al.*, 1996; Zinkernagel, 1996). This has been suggested to occur during LCMV infection in adult mice who fail to eradicate the virus leading to its persistence (Moskophidis *et al.*, 1993).

1.7.4 Interference with cytokine function

Cytokines are an important part of antiviral immunity. They act in a complex network to inhibit viral replication, inhibit proliferation of infected cells, mediate the inflammatory response and activate other immune effector mechanisms. Studies have demonstrated that proteins from several viruses can interfere with these cytokine functions. Three adenovirus early proteins (i.e., E3-14.7K, E3-10.4K/14.5K and E1B-19K) can protect mouse cells which are sensitive to TNFα- induced apoptosis. The mechanism by which these adenovirus proteins counteract TNFα appears to be through inhibition of cytosolic phospholipase A2 (Krajcsi *et al.*, 1996).

A different mechanism, used by Epstein-Barr virus (EBV), is to block antigen presentation. EBV expresses the protein BCRF1 which is similar in sequence to the human IL-10. It was shown that BCRF1 inhibits the expression of TAP1 to a similar extent as human IL-10 (Zeidler *et al.*, 1997). This would hamper the transport of peptide antigens into the ER causing a general reduction of MHC class I molecules on the surface of infected cells (Section 1.6.2.2).

Viral peptides that mimic host cytokine receptors or inhibit activation of cytokines have also been described. For example, vaccinia viruses encode proteins capable of binding IL-1 (Spriggs *et al.*, 1992), IFNα (Liptakova *et al.*, 1997), and TNFα or TNFβ (Hu *et al.*, 1994; Smith *et al.*, 1996). Additionally, vaccinia also encodes a protease inhibitor (crmA) that prevents the cleavage of IL-1β precursor to its active form by inhibiting the IL-1β converting enzyme (Ray

et al., 1992).

Recently, it was demonstrated that HCMV is capable of inhibiting IFNα-stimulated antiviral and immunoregulatory responses in infected fibroblasts and endothelial cells by blocking multiple levels in the IFNα signal transduction pathway (Miller *et al.*, 1999). For example, HCMV inhibits the expression of IFNα-stimulated MHC class I and 2',5'-oligoadenylate synthetase (OAS) genes by decreasing the expression of Jak 1 and p48, two essential components of the IFNα signal transduction. This mechanism may be a principal means by which HCMV is capable of escaping host immunity and establishing persistence.

1.7.5 Inhibition of MHC class II and accessory molecule function

Among the viruses infecting humans, HCMV, HIV and measles viruses have been shown to interfere with MHC class II expression. Most of the viruses inhibit the IFNγ-induced upregulation of MHC class II transcription rather than affecting the basal level of MHC class II gene expression. This mechanism has been reported for infection with measles virus (Leopardi *et al.*, 1993) and HCMV (Miller *et al.*, 1999). For example, inhibition of MHC class II expression in HCMV infection is due to disruption of the IFNγ-stimulated Jak/STAT signal transduction pathway (Miller *et al.*, 1999). Because the induced expression of MHC class II by IFNγ is likely to play a key role in antigen presentation, interference with this step could prevent the generation of an effective immune response against the virus.

Other studies have shown that accessory molecules, such as lymphocyte

function associated antigen-3 (LFA-3) and intercellular adhesion molecule-1 (ICAM-1), are involved in viral escape from CTL recognition. Specifically, a reduced level of LFA-3 and ICAM-1 on EBV-positive Burkitt's lymphoma cells allowed escape of the cells from virus-specific CTL lysis (Gregory *et al.*, 1988). However, the mechanism of suppression of these adhesion molecules is not currently known.

1.7.6 Interference with MHC class I antigen presentation

One of the major mechanisms of viral persistence is to prevent the presentation of viral peptides by MHC class I molecules on the surface of infected cells. Because the stable cell surface expression of MHC class I molecules requires association between the heavy chain and β_2m, as well as an endogenously synthesized viral peptide (Section 1.6.2.2; Fig. 1-1), any interference with antigen processing or presentation will interfere with CTL surveillance. Regarding this, numerous viral proteins have been reported to interact with TAP and other components of MHC class I antigen presentation (Section 1.7.6.2). However, relatively few viruses have been found to inhibit proteolysis of viral peptides by proteasomes or by decreasing MHC class I heavy chain or β_2m transcription. The following sections will briefly summarize key mechanisms used by viruses to modify MHC class I antigen presentation because this subject is directly related to one of the aims of the present study.

1.7.6.1 Decrease in MHC class I mRNA transcription

It has been documented that the oncogenic adenovirus type 12 can suppress the cell surface expression of class I molecules by affecting MHC class I mRNA. Cells transformed with this virus have decreased levels of MHC class I transcripts (Schrier *et al.*, 1983; Ackrill *et al.*, 1988; Shemesh *et al.*, 1991). This process appears to be dependent on the presence of the virus E1A oncogene and can be reversed by treatment with IFNγ (Eager *et al.*, 1989). Another example of a virus decreasing MHC class I mRNA has been demonstrated in human dermal fibroblasts transformed with the Rous sarcoma virus. The virus induced a reduction of cell-surface MHC class I and β_2m expression, and this reduction correlated with a markedly diminished amount of MHC class I transcripts in the infected cells (Gogusev, 1988). Further, it has been shown that the C-terminal domain of the HIV Tat protein, a transactivator of HIV transcription, represses transcription of MHC class I genes. The inhibition results from the interaction of Tat with a component of the general transcription factor referred to as TAFII250 (Weissman *et al.*, 1998) that acts on the MHC class I promoter. Thus, Tat-dependent repression of MHC class I transcription could be one of the mechanisms by which HIV avoids immune surveillance (Howcroft *et al.*, 1983; Weissman *et al.*, 1998).

1.7.6.2 Modulation of intracellular peptide transport

It was noticed that infection with the HSV reduces surface levels of MHC class I molecules (Jennings *et al.*, 1985). The decrease in the expression was due to peptide transport interference by a 9-kDa protein product of the immediate early virus gene, US12, referred to as infected cell protein 47 (ICP47) (York *et al.*, 1994). This cytosolic protein physically associates with the peptide binding domain of TAP, inhibiting peptide translocation to the ER. The lack of viral peptides in the ER results in empty and thus, unstable MHC class I molecules (Hill *et al.*, 1995). This effect is reported to be species specific, as ICP47 only minimally inhibits peptide translocation by murine TAP (Tomazin *et al.*, 1998). This is an excellent example of virus adaptation to its natural host. A second protein known to target the TAP complex is US6, a product of HCMV. US6 is a type I membrane glycoprotein that binds the TAP complex and inhibits its peptide-transporting function (Ahn *et al.*, 1997).

1.7.6.3 Rapid degradation of MHC class I heavy chains

Another known mechanism of viral downregulation of surface MHC class I expression is through the degradation of the heavy chain in the cytosol of infected cells. For example, primary infection with the HCMV is usually asymptomatic and followed by lifelong persistence, unless the host is immunocompromised or infection occurs in a fetus, in which case the infection is

quite severe, even fatal (Erlich, 1997). It is commonly accepted that MHC class I expression is decreased on HCMV infected cells and that the reduction correlates with resistence of the infected cells to virus-specific CTL-mediated lysis. Because the level of class I heavy chain mRNA is not decreased in HCMV-infected cells, this suggests that the downregulation is posttranscriptional. Recent studies have shown that two HCMV-encoded proteins are involved in the degradation of MHC class I in HCMV-infected cells. The viral early genes US2 and US11 encode glycoproteins that mediate translocation of MHC class I heavy chains from the ER to the cytosol, where the heavy chains are rapidly degraded by the proteasomes (Jones and Sun, 1997; Wiertz *et al.*, 1996).

1.7.6.4 Disruption of MHC class I assembly

In the case of adenoviruses belonging to groups B, C, D, E and F, a prime inhibitor of class I MHC expression is the early region 3 transmembrane glycoprotein termed E3/19K. This protein binds to the alpha 1 and alpha 2 helices of the MHC class I heavy chain (Flomenberg, 1994), preventing its transport and cell surface expression (Andersson *et al.*, 1985; Burgert and Kvist, 1985). Importantly, this protein has also been shown to cause a drastic suppression of MHC class I expression when transfected into human lymphoid cells, which are the site of adenovirus persistence (Korner and Burgert, 1994). Another example is the HCMV US3 gene product (Jones *et al.*, 1996). US3 is transcribed abundantly immediately after HCMV infection and forms a complex

with β_2m-associated class I heavy chains, which then accumulate in the ER. Interestingly, the product of the US3 gene has a short half-life, but is followed by the translation of US2 and US11 genes whose products cause degradation of the accumulated class I heavy chains (Section 1.7.6.3). HCMV also encodes a protein, UL18, that is homologous to the MHC class I heavy chain and is capable of binding to β_2m and forming an inactive complex (Fahnestock *et al.*, 1995). On the other hand, it was recently shown that HCMV infected cells can escape lysis by NK cells. This presumably is mediated by interaction between the UL18 protein and NK cell killer inhibitory receptors (Reyburn *et al.*, 1997).

1.7.6.5 Internalization of MHC class I surface molecules

Evidence suggests that virus-specific CTL are an important component of the protective immune response to HIV infection. However, despite a vigorous CTL response, the CTL are not successful in eliminating virus infection (McKinney *et al.*, 1999). One potential mechanism is that the HIV Nef protein reduces the expression of MHC class I antigens and this decrease is dependent on phosphoinositide kinase activity (Swann *et al.*, 2001). The transcription and translation of MHC class I molecules is not affected, rather Nef blocks transport of MHC-I molecules to the cell surface and their internalization in endosomal vesicles where they are subsequently degraded. This MHC class I downregulation has been confirmed in primary T cells, where Nef induced a 300-fold reduction of surface class I molecule expression and protected the cells from

HIV-specific CTL cytolysis (Collins *et al.*, 1998). Therefore, persistence of HIV in peripheral CD4+ T cells may be due to a reduced MHC class I display on these cells make them poor CTL targets.

1.8 IMMUNOPATHOGENESIS OF HEPADNAVIRAL INFECTION

As outlined above, a truly effective immune response utilizes a combination of humoral, cellular and innate immunity. Although the cellular immune response appears to be the main contributor in the pathogenesis of diseases caused by non-cytolytic viruses, other immune responses, such as cytokines, specific antibodies and possibly virus-induced autoimmune reactions, may also play a role (Chisari, 2000). The mechanisms responsible for tissue injury in acute and chronic hepadnaviral infection are not completely understood. Accumulating evidence implies that hepatocellular injury is caused by the host immune responses, presumably by T cells specifically directed against viral epitopes exposed on the surface of infected liver cells (Chisari and Ferrari, 1995; Curry and Koziel, 2000). Readily detectable HBV-specific polyclonal CTL in the peripheral blood has been identified as a distinctive feature of acute infection (Rehermann *et al.*, 1995). In contrast, CH is accompanied by a weak or undetectable HBV-specific CTL response in the blood (Ferrari *et al.*, 1990, Rehermann *et al.*, 1996). Recently, a new method of CD8+ T cell detection (via MHC class I tetramers complexed to viral peptides) has suggested that the number of HBV specific T cells in CH is far greater than initially suspected (Maini

et al., 1999, Boni *et al.*, 2001). The following sections will briefly summarize the major contributions of investigations performed using materials from HBV-infected patients, as well as the transgenic mouse and woodchuck models, to our current understanding of the molecular mechanisms of hepatitis B immunopathology.

1.8.1 Humoral immunity against HBV infection

The antibody response to HBV envelope glycoproteins is thought to be important in HBV neutralization and clearance. The loss of HBsAg and the rise in anti-HBs is the hallmark of disease resolution in patients acutely infected with HBV, yet it is not observed in individuals with CH type B unless specific assays that detect antibodies complexed to viral antigens are used (Maruyama *et al.*, 1993). The importance of these neutralizing antibodies in HBV infection has been demonstrated by the universal success of the HBsAg-based HBV vaccine (Beasley *et al.*, 1983). The anti-HBs may limit viral spread by removing virions from the circulation and/or by preventing virus attachment and uptake by susceptible cells. However, the antibody response to the envelope proteins could also be a contributor to liver injury. Immunocomplex deposits containing HBsAg, immunoglobulin (Ig) and complement were detected in infected hepatic tissue from various stages of hepatitis B (Nowoslawski *et al.*, 1972). Therefore, not only does the humoral response to HBsAg remain operational in chronic infection, it may also promote undesirable pathological effects caused by intra

and extra-hepatic deposition of immune complexes. In fact, it has been shown that about 10-20% of patients with HBV infection have extra-hepatic immunocomplex-associated disorders (Gocke, 1975), including glomerulonephritis, most frequently found in children (Combes *et al.*, 1971; Nowoslawski *et al.*, 1975; Slusarczyk *et al.*, 1980), polyarteritis nodosa (Gocke *et al.*, 1970; Michalak, 1978), and arthritis (Csepregi *et al.*, 2000). One of the unique features of HBV infection is accumulation of an excess of large envelope proteins in the ER causing a dramatic expansion of the hepatocyte. This change is a characteristic of 'ground-glass' hepatocytes and is found in the livers of chronically infected patients (Hadziyannis *et al.*, 1973).

The biological roles of the antibody responses to HBcAg and HBeAg are less clear. Anti-HBc and antibodies to HBeAg (anti-HBe) do not appear to be neutralizing antibodies, because they co-exist with the virus during both AH and CH type B. It is interesting, however, that passive administration of anti-HBe protects chimpanzees against HBV infection (Stephan *et al.*, 1984). Also, seroconversion to anti-HBe positive state has been associated with less infective and perhaps more benign state of hepatitis (Schmilovitz-Weiss *et al.*, 1993). Additionally, it has been suggested that the decline of virus titer, which is usually observed after seroconversion from HBeAg to anti-HBe, might be the result of the elimination of infected cells via an antibody recognition of membrane bound HBe protein (Schlicht *et al.*, 1991). The humoral response against HBeAg and HBcAg may also promote undesirable effects because the extrahepatic

deposition of these immunocomplexes have been observed in glomerulonephritis (Slusarczyk *et al.*, 1980; Ohba *et al.*,1997). In regard to the anti-HBc response, the occurrence of these antibodies in chronically infected patients might be because HBcAg can elicit a T cell-independent antibody response (Milich and McLachlan, 1986). This is in contrast to HBeAg which is exclusively a T cell-dependent antigen. It has also been postulated that complement dependent cytotoxicity of hepatocytes expressing HBcAg, HBsAg or asialoglycoprotein receptor (ASGPR) may also contribute to the injury of HBV-infected hepatocyte, particularly in patients with severe CH (Michalak *et al.*, 1995).

The antibody response to the HBV polymerase and X proteins are not routinely monitored in clinical situations, although they have been detected. It has been reported that antibody to the carboxy terminus of virus polymerase may be an early marker of infection and reflects active HBV replication (Weimer *et al.*, 1990). Antibodies to HBxAg have been reported to show qualitative and quantitative heterogeneity. They have been found at the highest levels and most frequently in patients with CH and usually at lower levels in acutely infected patients and asymptomatic carriers of HBV (Stemler *et al.*, 1990).

1.8.2 T cell-mediated immunity in HBV-infected patients

Due to the restricted host range of HBV infection and the lack of cell cultures that efficiently support viral replication, studies on T cell involvement in the immunopathogenesis of HBV-induced hepatocellular injury have focused on

the *in vitro* analysis of CTL and Th cells derived from patients with clinically evident hepatitis. In general, the T cell specific response to HBV epitopes is vigorous, polyclonal, and multispecific in patients with AH, but weak and epitope restricted in patients with chronic infection (reviewed by Chisari, 2000). A recent development in the study of viral pathogenesis is the establishment of techniques sensitive enough to detect virus-specific CTL without *in vitro* clonal expansion. One of these techniques involves the use of a fluorochrome-labeled peptide-MHC class I tetrameric complex that directly binds to the TCR of peptide-specific CTL (reviewed by Ogg and McMichael, 1998). Using this assay, the frequency of virus-specific CTL in HBV infection was found to be 30 to 45-fold greater than that estimated by conventional limiting dilution analysis (Maini *et al.*, 1999). It has been suggested that because this assay utilizes *in vitro* clonal expansion of CTL, it may under-estimate CTL that expand poorly or undergo apoptotic death during the intense antigenic stimulation in culture (Alexander-Miller *et al.*, 1996). However, it is also possible that subsets of peptide-specific CD8+ T cells do not kill infected cells, but rather secrete antiviral cytokines that directly inhibit viral replication without damaging the infected cell.

1.8.2.1 MHC Class II-restricted T cell response in acute hepatitis type B

The HBV antigen-specific proliferative responses of CD4+ T cells are usually measured by stimulating PBMC from patients with either AH or CH type B using purified virus antigens, recombinant proteins or synthetic peptides, and

subsequently assessing their proliferation via ^{3}H-thymidine incorporation. These assays have demonstrated that a vigorous class II-restricted T cell response is produced against several HBcAg epitopes in virtually all patients with AH (Ferrari *et al.*, 1990; Jung *et al.*, 1991, Ferrari *et al.*, 1991). Although some HBV core peptides are recognized by CD4+ T cells in the context of a particular MHC background, the immunodominant peptides appear to be recognized by CD4+ T-cells from patients with multiple MHC backgrounds (Ferrari *et al.*,1990). Among the epitopes identified, the HBc/eAg amino acids at positions 50-69 are the most commonly recognized by CD4+ T cells from patients with acute infection, irrespective of their MHC haplotype (Ferrari *et al.*, 1990, Ferrari *et al.*, 1991).

An increased HBcAg-specific CD4+ T cell response in acutely infected patients was found to be associated with clearance of HBsAg from the serum and decreases in viral DNA to levels undetectable by slot-blot hybridization assays, suggesting that these cells could play an important role in virus elimination (Ferrari *et al.*,1986, Jung *et al.*, 1991). The antiviral effect of CD4+ T cells appears to be primarily through their ability to help initiate and sustain the CTL response to HBV. This is mediated by the secretion of proliferative cytokines (e.g., IL-2) and antiviral cytokines (e.g., IFNγ), as described in Section 1.6. A functional study of HBcAg-specific T cells derived from peripheral blood of patients with AH type B suggested that a prevalent Th1 cytokine pattern and high levels of IFNγ may contribute to the successful control of HBV infection (Penna *et al.*, 1997).

The CD4+ T cells may also contribute to long term control of trace levels of HBV replication persisting after recovery from hepatitis, as demonstrated by the detection of HBcAg-specific proliferative T-cell responses years after resolution of AH (Penna *et al.*, 1996, Rehermann *et al.*, 1995). Along this line, identification of HBV DNA by highly sensitive nested PCR in the sera and in PBMC many years after self-limiting AH suggests that the HBV-specific CD4+ T-cell response is maintained indefinitely by minute amounts of persistently replicating virus (Michalak *et al.*, 1994; Rehermann *et al.*, 1996; Penna *et al.*, 1996; Yotsuyanagi *et al.*, 1998).

The CD4+ T-cell proliferative response to HBV envelope antigens is relatively weak compared to HBc/eAg in patients with self-limited AH or CH (Ferrari *et al., 1990)*. The molecular basis for this observation is not understood, in particular due to the observation that HBsAg is immunogenic in healthy volunteers (Ferrari *et al.*, 1989; Celis *et al.*, 1988). It is possible that high levels of circulating HBsAg present early in the course of infection could exhaust or anergize the specific CD4+ T cells responses to virus envelope proteins.

1.8.2.2 MHC class II-restricted T-cell responses in chronic hepatitis type B

In general, the HBV-specific peripheral blood CD4+ T-cell response in patients with CH type B are much weaker than in those with AH (Ferrari *et al.*, 1990; Jung *et al.*, 1991; Marinos *et al.*, 1995). However, the strength of the core-specific CD4+ T-cell responses may be accentuated during reactivation phases

of CH (Marinos *et al.*, 1995, Tsai *et al.*, 1992). It is possible that the increased MHC class II restricted core-specific CD4+ T-cell response and the associated disease flare are dependent on the viral replication rate. In contrast to HBcAg, the proliferative T cell response to HBsAg during these transient episodes remain unaltered. It is postulated that HBsAg-specific T-cells might be suppressed by the persistently elevated levels of circulating HBsAg that typically occur in CH type B whereas CD4+ T cells specific for HBcAg continue to play a key immunoregulatory role. In keeping with this theory, 12 chronically infected patients taking lamivudine (3TC) to suppress HBV replication (Dienstag *et al.*, 1995), had a rapid restoration of their circulating HBV nucleocapsid-specific T cells after 1-2 weeks of treatment leading to a marked reduction of viremia, but not in the serum levels of HBsAg and HBeAg (Boni *et al.*, 1998). After cessation of lamivudine treatment, HBV replication returned to pretreatment levels.

Although data have indicated that in chronic HBV infection a Th1-like response is strongly represented in the intrahepatic infiltrate (Barnaba *et al.*, 1994; Lohr *et al.*, 1995; Penna *et al.*, 1997), it has been reported that these cells should be classified as Th0 phenotype because they secrete IL-4 and IL-5, in addition to IFNγ (Bertoletti *et al.*, 1997). Additionally, neither cells from HBeAg-positive patients with CH type B or patients with high levels (>300 pg/ml) of HBV DNA were capable of producing IFNγ after *in vitro* stimulation with recombinant IL-12 and HBV peptides (Schlaak *et al.*, 1999). This could be one of the explanations for the poor response to immunostimulatory therapy in patients with

a high HBV load.

1.8.2.3 MHC class I-restricted T-cell response in acute hepatitis type B

CTL are thought to be primary mediators in virus elimination due to their ability to recognize and kill the infected cells that express viral epitopes in the context of class I MHC molecules (Section 1.6.2.2; Fig 1-1). However, because serial liver biopsies from patients with AH are not obtainable due to ethical considerations, HBV-specific CTL are rare in CH type B and biopsies from individuals with AH that progress to CH are not routinely available, it is difficult to reconstruct the complete profile of intrahepatic CTL responses during the course of HBV infection. To circumvent these difficulties, CTL from the peripheral blood are clonally expanded *in vitro* in the presence of HBV-derived synthetic peptides (Nayersina *et al.*, 1993). This technique involves stimulation of PBMC with synthetic immunogenic viral epitopes and recombinant IL-2, resulting in CTL clonal expansion and increased killing of target cells, as determined by ^{51}Cr release assays. This strategy has been validated by identification of numerous CTL epitopes in sequences of different viral pathogens (e.g., Bertoni *et al.*, 1997; Sidney *et al.*, 1996; Threlkeld *et al.*, 1997).

Several novel observations have been made in patients acutely infected with HBV by using the strategy mentioned above. It is now known that patients with AH type B display a vigorous, polyclonal, MHC class I-restricted CTL response against multiple HBV envelope, nucleocapsid and polymerase

epitopes. Many of the infected patients recognize the same spectrum of epitopes (e.g., HBV core amino acids 18-27, envelope amino acids 183-191, 250-258 and 335-343, and polymerase amino acids 455-463), whereas other epitopes were seen in a minority of patients (Bertoni *et al.*, 1997; Nayersina *et al.*, 1993; Rehermann *et al.*, 1995). This suggests the presence of a hierarchy in HBV-specific CTL response which may be influenced by the MHC class I/virus epitope binding affinity and the heterogeneity of viral sequences present (Sette *et al.*, 1994). Some HBV-specific CTL epitopes lie in protein sequences that are critical for virus specific functions. For example, the nucleocapsid nuclear localization and encapsulation signals contain a CTL epitope (core 141-151) that is recognized by at least two MHC haplotypes, A31 and Aw68 (Missale *et al.*, 1993). Similarly, the MHC-A2-restricted HBsAg epitope, that spans residues 250-269, overlaps an important topogenic sequence in the HBsAg transmembrane domain (Nayersina *et al.*, 1993).

The polyclonality and multispecificity of the CTL response, together with potential sequence constraints, mitigate against the emergence of CTL virus escape mutants, despite the high mutation rate of HBV. It is evident that a strong polyclonal HBV-specific CTL response is maintained for decades after the loss of conventional virus infection markers (e.g., HBsAg, HBeAg) (Rehermann *et al.*, 1996). This response could be a consequence of an ongoing stimulation with proteins derived from trace amounts of transcriptionally active HBV DNA that remain years after an apparent complete clinical, serological and biochemical

recovery from hepatitis (Michalak *et al.*, 1994; Rehermann *et al.*, 1995; Penna *et al.*, 1996; Rehermann *et al.*, 1996)

The vigorous and polyclonal CTL response appears to be a good predictor of recovery from AH type B. Experiments in the transgenic mouse of HBV (Section 1.8.5) and, more recently, in a chimpanzee model of hepatitis B suggest that the non-cytopathic clearance of the virus from infected hepatocytes may be principally mediated by the antiviral cytokines released from CTL (Guidotti *et al.*, 1996; Guidotti *et al.*, 1999). Indeed, the number of HBV-infected hepatocytes can vastly exceed the number of HBV-specific CTL in the body, so that antigen-specific destruction of all the infected liver cells by the CTL would not only to be physically impossible, but also very detrimental to the host (Guo *et al.*, 2000).

1.8.2.4 Studies on the antiviral MHC class I-restricted CD8 T-cell response in chronic hepatitis B

In contrast to the CTL response detected in patients with self-limited HBV infection, CH type B is usually associated with weak or undetectable CTL responses against the virus. In contrast to neonatal infection, where immunological tolerance can explain the weak T-cell responses and viral persistence, the mechanisms leading to weak CD8+ T-cell reactivity in adult onset of CH type B are not well understood. This weak, but continual, anti-viral cell cytotoxicity probably induces the injuries that are characteristic of chronic

liver inflammation (Michalak, 2000). Because HBV-specific CTL can still be detected in chronically infected patients, it is unlikely that their decreased activity is due to clonal deletion (Bertoletti *et al.*, 1994, Barnaba *et al.*, 1989). Additionally, the CTL occasionally become reactivated causing an exacerbation in the severity of liver disease. It has also been shown that patients with chronic HBV infection, who experienced a spontaneous or IFNα-induced HBeAg clearance display a vigorous and multispecific HBV-specific CTL response similar to that observed in patients with self limiting AH (Rehermann *et al.*, 1996). The dramatic difference in the strength of the antiviral CTL reactivity seen between patients with AH and CH type B suggests that this response is of primary importance in the determination of viral clearance.

1.8.2.5 Hepadnavirus infection and NK/NKT cells

Currently, the role of NK and NKT cells in hepadnaviral elimination is not well recognized. It has been reported that AH type B is associated with enhanced NK cell cytotoxicity (Chemello *et al.*, 1986) and that HBsAg can inhibit the NK cell-mediated cytotoxicity *in vitro* (de Martino *et al.*, 1985), but further studies are required to fully understand the importance of the NK cell mediated response. Suggestions have been made that it is the innate immune response that is responsible for the initial control of HBV infection in chimpanzees (Guidotti et al., 1999). Recently, there has been compelling evidence that NKT and NK cells control hepadnavirus replication in HBV transgenic mice via IFNα/β and

IFNγ release (Kakimi *et al.*, 2000). However, whether these responses are important in natural hepadnaviral infection must still be determined.

1.8.3 HBV immune evasion

Induction of immunological tolerance may be one of the key reasons why exposure to HBV during early development (i.e., neonatal) most often results in a life-long persistent viral infection (reviewed by Chisari and Ferrari, 1995). It is also possible that during adult onset of HBV infection some of the virus-specific T cells may be deleted or anergized through overstimulation by high doses of viral antigen. Evidence for this hypothesis comes from observations of chronic healthy HBV carriers, who demonstrate large amounts of HBsAg in hepatocytes and in the circulation, without apparent evidence of liver injury (de Franchis *et al.*, 1993). Thus, the virus may successfully persist by evading the immune responses through their overstimulation with an excess of viral antigens.

Although there is no direct evidence that HBV can influence the expression of important molecules, including MHC or other cell surface accessory molecules contributing to the TCR-MHC-peptide interaction, studies have shown that HBcAg can inhibit IFNβ gene transcription (Whitten *et al.*, 1991). In addition, it has been reported that HBV can inhibit cellular immune responses induced by IFNα and IFNγ, although the mechanism responsible for this has not been identified (Foster *et al.*, 1991). Thus, HBV may hypothetically be able to indirectly block a cytokine-induced upregulation of MHC class I and its

accessory molecules.

Because HBV uses a reverse transcription step in its replication cycle numerous viral mutants emerge during replication. This may allow the virus to have an increased ability to evade both T and B cell based immunity. For example, HBV variants with one or two amino acid substitutions were shown to antagonize CTL recognition of the wild-type virus epitope in chronically infected patients (Bertoletti *et al.*,1994). Additionally, variant T cell epitopes in the HBcAg immunodominant peptide (amino acids 18-27) which inhibit CTL response against wild type epitope have been derived from patients with CH type B (Bertoletti *et al.*,1994). The concomitant expression of virus wild type and antagonistic peptides on the surface of infected hepatocytes could contribute to viral persistence.

Another mechanism HBV may use to avoid immune recognition is the invasion of immunologically privileged sites. HBV DNA sequences have been detected in many extrahepatic sites that are potentially inaccessible to CTL due to microvascular barriers (Mason *et al.*,1993; Ogston *et al.*, 1989; Yoffe *et al.*,1990), as well as in lymphoid cells (Sections 1.3.2 and 1.5.2). These may serve as a reservoir of continuous virus replication and release infectious virions.

1.8.4 Cytokine-induced hepadnaviral clearance

As mentioned in Section 1.6, it was previously thought that the elimination of virus-infected cells relied exclusively on their destruction by the effector cells of

the immune system. However, it is now recognized that cytokine-dependent, non-cytolytic curative mechanisms may also contribute to controlling infections. This was initially demonstrated in a transgenic mouse model of HBV infection (Section 1.8.5). At this stage, the intracellular events causing the downregulation of viral expression following cytokine/receptor interaction are not well characterized, although suggested mechanisms include induction of a host endoribonuclease that cleaves HBV RNA, as well as the 2'5'-OAS system which induces RNase L (Guidotti and Chisari, 1999). Recently, data obtained from a chimpanzee experimentally infected with inoculum derived from transgenic mice that replicate infectious HBV demonstrated that decreases in markers of HBV infection precede the onset of T-cell infiltration and hepatitis. This may suggest that a non-inflammatory mechanism of hepadnaviral clearance is possible (Guidotti *et al.*, 1999).

1.8.5 The transgenic mouse model of HBV infection

Transgenic technology has allowed the creation of mice, which are not natural hosts for hepadnaviruses, to replicate the HBV genome and produce complete virions (reviewed by Chisari, 1996). Studies in the various types of HBV transgenic mice have significantly contributed to the current understanding of HBV pathogenesis and mechanisms of hepatocellular injury. Interestingly, HBV cccDNA has not been detected in any mouse lineage generated, including strains which produce infectious HBV virions. However, when inocula from these

mice was used to infect chimpanzees. HBV cccDNA became detectable. This suggested that species-specific differences at the level of generation of cccDNA may be important in determining the host range of the virus (Guidotti *et al.*, 1999). It is now apparent that the process of hepadnavirus replication and synthesis of virus proteins are not cytopathic. The recognized exception is transgenic mice which overexpress and retain the large envelope (preS1) protein of HBV. These mice can develop severe CH and HCC (Chisari *et al.*, 1987, Chisari *et al.*, 1989). In other HBV transgenic mouse lineages, which do not spontaneously generate liver cell injury, the retention of the large protein in the ER sensitizes the hepatocytes to cytokines, such as IFNγ and TNFα, leading to enhanced hepatocellular injury (Gilles *et al.*, 1992; Ando *et al.*, 1993).

Since transgenic mice can support HBV replication without any evidence of a specific immune response (Guidotti *et al.*, 1995), the components of the adaptive immune system that mediate liver damage cannot be studied directly. However, investigators were able to induce transient AH, as well as fulminant hepatitis when HBV-specific CD8+ T cell clones were adoptively transferred into these mice (Moriyama *et al.*, 1990; Ando *et al.*, 1993). Analyzing mice which developed fulminant hepatitis demonstrated that liver disease is an orderly, multistep process that involves both virus-specific CTL and antigen non-specific inflammatory responses which can amplify the local cytopathic effects of the CTL. It was concluded that when virus-specific CTL encounter HBV antigen they secrete IFNγ, which activates intrahepatic macrophages and induces a delayed

type hypersensitivity response. This reaction is able to destroy the liver and kills the animal. Interestingly, the pathogenic effect of the HBV-specific CTL and the mortality rate can be dramatically reduced (~97%) by prior administration of anti-IFNγ antibodies (Ando *et al.*, 1993).

In addition to causing hepatocyte cytolysis in transgenic mice which replicate HBV, specific CTL adoptively transferred into these mice can transiently abolish HBV gene expression and replication with minimal liver cell damage. This coincides with the induction of intrahepatic antiviral cytokines (e.g., IFNγ, TNFα, IL-2, IL-12) (Guidotti *et al.*, 1994; Guidotti *et al.*, 1996; Cavanaugh *et al.*, 1997). The existence of this non-cytolytic clearance mechanism suggests that hepatocyte injury may be an unfortunate consequence of a primarily non-cytopathic virus clearance mechanism. This may represent an important host strategy to control HBV infection in the liver where almost 100% of hepatocytes are frequently infected (Kajino *et al.*, 1994).

Although the transgenic mouse model of HBV has meaningfully contributed to our understanding of the immunopathogenesis of HBV infection, this model is artificial and, therefore, the data obtained need to be interpreted with caution. The processes identified in the HBV transgenic mouse system will have to be re-evaluated in a natural model of HBV infection to confirm the results.

1.8.6 The woodchuck model of HBV infection

Similar to HBV infection of humans, woodchucks persistently infected with WHV develop chronic liver inflammation with different degrees of hepatocellular injury and lymphomononuclear cell infiltrations (reviewed by Michalak, 1998). Additionally, as in HBV infection, most of the infected neonates develop serologically evident chronic infection and almost invariably HCC (Tennant and Gerin, 2001). The fact that animal age, virus strain and its dose may influence the rate of chronicity in experimental WHV infection has recently been shown (Cote *et al.*, 2000). In adult animals, WHV infection usually leads to acute self-limited hepatitis and clearance of virus serological markers (Korba *et al.*, 1989), but molecular indicators of residual WHV infection remain (Michalak *et al.*, 1999). However, suppressing the initial immune response against WHV in experimentally infected adult woodchucks with cyclosporin A dramatically increases the rate of chronic outcome depending on the time of drug administration (Cote *et al.*, 1991; Cote *et al.*, 1992). Additionally, these animals had reduced severity of liver disease, increased viremia, and only a transient hepatitis once the drug was discontinued. These data strengthen the concept that the immunological status of the host is critical in the pathogenesis of hepadnaviral chronicity and clearance.

At the current stage, the main constrains associated with the wide utilization of the woodchuck model for research on the pathogenesis of hepadnaviral infection are the outbred nature of the animals and the lack of

reagents recognizing woodchuck immune cell markers. However, the woodchuck model of hepatitis B is more accessible and substantially less expensive than studying HBV infected chimpanzees. Hepadnaviral clearance without massive immune mediated destruction of infected hepatocytes has been suggested to occur in WHV infection (Kajino *et al.*, 1994). Hepatocytes labeled during the peak of acute infection, when nearly all liver cells are infected, were still present after the elimination of WHV from the liver (Kajino *et al.*, 1994). This suggests that non-cytopathic viral clearance, perhaps via local cytokine release, also occurs in woodchucks infected with WHV. The correlation of hepatic IFNγ and TNFα expression and viral clearance has been recently established in neonatally acquired WHV infection (Guo *et al.*, 2000; Nakamura *et al.*, 2001)

The establishment of assays measuring the proliferative T-cell response in woodchucks has further demonstrated that CD4+ cells may play a role in preventing development of chronic infection. Similar to HBcAg, WHcAg and some of its synthetic peptides effectively stimulate proliferation of T cells derived from acutely infected woodchucks, but not those obtained from animals with CH (Menne *et al.*, 1997). Additionally, immunization with either recombinant WHcAg or the immunodominant core peptide (amino acid 97-110) can protect naive woodchucks against WHV infection (Menne *et al.*, 1998).

It has been documented that chronic WHV infection, but not acute WHV hepatitis, is associated with the incorporation of larger (saturable) quantities of virus envelope proteins in heaptocyte plasma membranes (HPM) (Michalak and

Churchill, 1988; Michalak and Lin, 1994). It was postulated that this, together with the abundant amounts of these proteins in the circulation, could provide a type of immunological barrier. In addition to WHsAg, both WHcAg and WHV e antigen (WHeAg) were also found to be associated with the outer membranes of infected hepatocytes (Michalak *et al.*, 1990; Michalak and Churchill, 1988; Michalak and Lin, 1994). Interestingly, while anti-WHc reactivity was readily detectable on hepatocyte surface membranes, anti-WHe antibodies could only be detected on the membranes from animals that had recovered from AH (Michalak *et al.*, 1990). This may suggest that, similar to HBV infection in humans, a humoral response against eAg may contribute to the resolution of acute infection presumably by the elimination of infected hepatocytes through antibody-mediated cytolysis, as in chimpanzees vaccinated with HBeAg (Section 1.8.1; Schlicht *et al.*, 1991).

Furthermore, it has been documented that WHV infection commonly triggers the production of both organ non-specific and liver specific (i.e., ASGPR) autoantibodies (Dzwonkowski and Michalak, 1990; Diao and Michalak, 1997). Anti-ASGPR antibodies, in particular, could be capable of inducing hepatocyte cytolysis in the presence of complement, as demonstrated by an *in vitro* assay (Diao *et al.*, 1998). If these autoantibody mediated cytopathic effects exist *in vivo*, they may contribute to the pathogenesis, aggravate severity and prolong recovery from liver injury in viral hepatitis (Diao *et al.*, 1998).

1.9 THERAPY OF VIRAL HEPATITIS

Although safe and effective prophylactic vaccines for hepatitis B are available, vaccination does not treat established HBV infections and there are no effective therapies for patients with CH type B (Lemon and Thomas, 1997). Attempts at treating chronic HBV infections have met with limited success. Until recently, IFNα remained the only agent with any beneficial effect for some groups of patients with CH type B (Alter and Mast, 1994). Retrospective multivariable analysis has shown that several features of HBV infection are associated with a higher likelihood of a response to IFNα treatment. They include: adult acquired infection, short duration of disease (longer than 6 months, but shorter than 2 years), elevated transaminases (e.g., 2-5 fold normal ALT level), significant inflammation on histological examination of liver biopsy, HBeAg positivity, low serum DNA levels, immunocompetence, female, and the absence of hepatitis delta virus infection (Lau *et al.*, 1998). Unfortunately, IFNα therapy has many other short comings, including the fact that it is administered parenterally, is poorly tolerated, is costly, and the fact that it results in sustained viral clearance in only one-third of patients, with primarily unsuccessful nonresponders retreatment (Hoofnagle, 1998). Therefore, new antiviral agents and immunomodulatory agents are being actively pursued, including nucleoside analogs and therapeutic vaccines. One of the most successful is lamivudine (3TC), an orally administered second generation nucleoside analogue which inhibits virus reverse transcriptase activity. Initially developed as a treatment for

HIV infection, this drug also inhibits hepadnavirus replication, including WHV (Mason *et al.*, 1998; Doong *et al.*, 1991; Dienstag *et al.*, 1999; Michalak *et al.*, unpublished data). For example, a 12-week course of lamivudine decreased serum HBV DNA levels to levels not detectable by hybridization assay in patients that received 100-300 mg daily (Dienstag *et al.*, 1995). However, only 6 of 32 treated patients remained serum HBV DNA-negative and only 4 of them became HBeAg-negative during post-treatment followup. It was also reported that lamivudine is able to restore the initially depressed proliferative T-cell responses to HBcAg, HBeAg and interestingly to tetanus toxoid and nonspecific mitogens (e.g., PHA, anti-CD3), within 1 to 2 weeks of therapy (Boni *et al.*, 1998). However, lamivudine becomes less effective due to a mutation in the highly conserved YMDD locus of the HBV polymerase gene in 20-25% of people after 1 year of treatment and in up to 60% of people after 3 years of treatment (Tipples *et al.*, 1996). The percentage of the lamivudine nonresponders increases.during long term therapy.

Theradigm® is an experimental lipopeptide vaccine designed to stimulate induction of HBV-specific CTL responses in individuals with the MHC class I-A2 subtype by inclusion of the highly antigenic HBcAg 18-27 epitope, a universal Th motif derived from the tetanus toxoid (peptide 830-843) and two palmitic acid molecules as the lipids (Vitiello *et al.*, 1995). Clinical trials have demonstrated that although Theradigm is safe and able to induce a primary HBV-specific CTL response in healthy volunteers, comparatively weak CTL responses were seen in

chronically infected HBV patients (Livingston *et al.*, 1999). Additionally, although the T-cell proliferative responses to the universal Th motif in the construct appeared normal, the cytokine profile observed suggested the induction of a Th0/Th2 response rather than a Th1 (Livingston *et al.*, 1999). This observation may support the importance a Th1 response in elimination of HBV infection.

The importance of *in vivo* testing of the new antiviral drugs in a suitable model, such as the woodchuck-WHV system, was demonstrated by the tragic failure of the anti-viral agent FIAU (Fialuridine). This second generation nucleoside analog showed promise against HBV DNA polymerase activity *in vitro* and in the duck hepatitis B model, however clinical trials resulted in the deaths of 6 of 24 patients chronically infected with HBV due to hepatic failure and lactic acidosis (McKenzie *et al.*, 1995). Drug testing conducted in woodchucks after the fact showed that these animals develop similar toxicity and death rates to those observed in humans (Tennant *et al.*, 1998). The above example demonstrates the importance of using appropriate animal models in anti-HBV drug testing. Interestingly, a derivative of FIAU, L-FMAU dramatically decreased WHV with no apparent toxicity in chronically infected woodchucks. In addition, there was no significant virus rebound after cessation of the drug treatment (Chu *et al.*, 1998).

1.10 PURPOSE OF THE STUDY

Chronic infection with HBV is an important cause of mortality within the human population due to the development of severe liver diseases, including chronic active hepatitis, cirrhosis and HCC. The pathogenesis of hepadnavirus-induced liver disease and the establishment of viral persistence are complex processes that involve both virus and host dependent factors. These processes remain only partially elucidated. WHV induced hepatitis in eastern North American woodchucks (*Marmota monax*) reflects, with a high degree of accuracy, virological, immunological and pathological events occurring in HBV infected patients. The woodchuck model of HBV infection was used in the present series of studies to investigate selected aspects of host immunity against WHV infection, molecular mechanisms by which the virus may persist, and factors of the immune response whose evaluation may allow prediction of the outcome after adult onset of WHV infection (i.e., recovery or progression to chronic infection and hepatitis). For this purpose, novel assays and molecular markers applicable for investigations in the woodchuck-WHV model were developed and used over the course of the presented studies.

The specific objectives of the current studies were as follows:

1. To develop species specific molecular biology reagents and assays to aid in determining how the status of the immunological response, including intrahepatic cytokine expression, influences the outcome of hepadnavirus infection and virus-induced liver disease in the woodchuck model of

hepatitis B.

2. To investigate the relative contributions of Fas/Fas ligand and perforin-based mechanisms of cytotoxicity mediated by woodchuck lymphoid effector cells during acute, chronic and convalescent phases of experimental hepadnaviral infection.

3. To elucidate the relationship between acute and chronic stages of experimental hepadnaviral hepatitis and the cell surface expression of MHC class I molecules and related genes in hepatocytes and lymphoid cells which are known to be natural sites of WHV replication.

4. To determine whether different stages of experimentally induced hepadnaviral infection are characterized by specific inflammatory and antiviral cytokine profiles in organs naturally targeted by the virus (i.e., liver and spleen) and whether the evaluation of these profiles can predict the long-term outcome of hepadnavirus infection in the woodchuck-WHV experimental system.

CHAPTER TWO :

GENERAL MATERIALS AND METHODS

2.1 ANIMALS

2.1.1 Woodchucks

All woodchucks used in this study were maintained by the Molecular Virology and Hepatology Research Laboratory at the Health Sciences Centre, Memorial University of Newfoundland, St. John's, Newfoundland under environmental and biosafety conditions specifically established for this species. Animals were fed a herbivore diet supplemented with fresh vegetables and given water *ad libitum* (Michalak and Churchill, 1988; Michalak and Bolger, 1989; Michalak *et al.*, 1990; Michalak and Lin, 1994; Michalak, 1998).

Animals infected with WHV were housed separately from healthy woodchucks. Healthy woodchucks had no serological markers of current or past WHV infection, i.e., they were negative for WHsAg, anti-WHc and anti-WHs antibodies (Sections 2.3.2 and 2.3.3). In addition, DNA extracted from sera, peripheral blood mononuclear cells (PBMC), and liver biopsies from these animals were WHV DNA non-reactive by nested PCR (Section 2.8.2) and subsequent Southern blot analysis of the resulting PCR products (Section 2.9.3).

The beginning of WHV infection was considered to be after the first appearance of detectable WHV DNA in the circulation (i.e., serum and/or PBMC) when assayed by PCR/Southern blot hybridization. The initiation of the acute

phase of WHV infection was diagnosed when WHsAg was detected for the first time in serum. The presence of AH was verified by histological examination of liver biopsies obtained by laparotomy after WHsAg appearance (Section 2.5). Recovery from AH was diagnosed when serum WHsAg permanently cleared and anti-WHs appeared.

Chronically infected animals were defined as having the continuous presence of circulating WHsAg, anti-WHc, and WHV DNA for at least 6 months before their utilization in experiments. Diagnosis of CH was confirmed by histological examination of liver biopsies that demonstrated typical features of protracted necroinflammatory liver injury (Section 2.5).

2.1.2 Other animal species

Tissues and serum from several other animal species were used as controls in this study. These species included rat, rabbit, mouse and frog (*Xenopus africanus*). All animals were housed in the Animal Care facility at Memorial University of Newfoundland.

2.2 SAMPLE COLLECTION

2.2.1 Blood sampling

Blood was obtained from the digitalis vein of woodchucks under isofluorane induced anaesthesia (CDMV Inc., St. Hyacinthe, Quebec) into tubes

with no additives (red top Vacutainer®; Becton Dickinson, Rutherford, New Jersey) for serum isolation (Section 2.2.2) or into tubes containing sodium ethylenediamine tetra-acetic acid (EDTA; lavender top Vacutainer®; Becton Dickinson) for plasma and PBMC isolation (Section 2.2.3).

2.2.2 Serum isolation

Untreated blood was allowed to clot at room temperature (RT) for approximately 1 hour (h). The tube was then centrifuged at 720 x g for 10 minutes (min). The isolated serum was aseptically aliquoted to small volumes and stored at -70°C for future use.

2.2.3 Isolation of peripheral blood mononuclear cells

Approximately 5 ml of EDTA-treated blood was overlaid on 3 ml of Ficoll-Paque® (Pharmacia Biotech, Baie d'Urfé, Quebec) and centrifuged at 330 x g for 30 min. The plasma layer was collected and stored at -70°C. The interface containing PBMC was removed and diluted with 10 ml of sterile phosphate buffered saline with EDTA (PBS-EDTA; Appendix A), and centrifuged at 330 x g for 10 min. Red blood cells remaining in the resultant pellet were lysed with 3 ml of buffered ammonium chloride solution (ACK; Appendix A) for 10 min, then washed with PBS-EDTA under the same conditions as indicated above.

Viable cells were counted with a hematocytometer using a trypan blue exclusion assay. Approximately 10^7 cells were pelleted by centrifugation using

the conditions described above and frozen in liquid nitrogen. Frozen cells served as a source for nucleic acid isolation (Section 2.6).

2.2.4 Liver biopsies

Liver biopsies were obtained by surgical laparotomy under sterile conditions. Each animal was sedated by an intramuscular injection of ketamine (23 mg/kg; Ketaset; CDMV Inc.) and xylazine (10 mg/kg; Lloyd Laboratories, Shenandoah, Iowa) and then anaesthetized with 2-4% isofluorane (CDMV Inc.). Each biopsy was divided aseptically into several fragments. Some of these pieces (1-2 mm^3 each) were immediately frozen in liquid nitrogen for future nucleic acid analyses. Other portions (about 5 mm^3 each) were fixed in 10% buffered formalin (Fisher) for histological examination or embedded in HistoPrep ® (Fisher Scientific, Canada) and then frozen in isopentane alcohol cooled in liquid nitrogen for immunofluorescent (IFL) examination.

2.2.5 Collection of specimens at autopsy.

For autopsy, animals were injected with an overdose of ketamine:xylazine. Blood was collected by cardiac puncture and used for isolation of serum (Section 2.2.2), plasma, and PBMC (Section 2.2.3). The liver, spleen, kidneys, pancreas, lymph nodes, bone marrow, and portions of the small intestine and skeletal muscle were removed aseptically. Tissue samples were preserved for further

investigations as described in Section 2.2.5.

2.3 ASSAYS FOR SEROLOGICAL MARKERS OF WHV INFECTION

2.3.1 Detection of woodchuck hepatitis surface antigen (WHsAg)

WHsAg was detected by a "sandwich" radioimmunoassay (RIA) using a cross-reactive AUSRIA-II kit for detection of HBsAg (Abbott Laboratories, N. Chicago, IL). Serum or plasma samples were tested directly for WHsAg following a procedure recommended by the manufacturer. Briefly, 200 µl of each test and control sample were incubated for 16 h at RT with beads coated with anti-HBs. After washing the beads to remove excess anti-HBs, they were incubated for 1 h at 45°C with 200 µl ^{125}I-labeled anti-HBs. The radioactivity was counted in a gamma counter. Specimens giving counts per minute (cpm) equal to or greater than the cutoff value were considered WHsAg reactive (Michalak *et al.*, 1989. Based on detection of purified WHsAg, the assay sensitivity was estimated to be 3.25 ng protein/ml (Michalak *et al.*, 1989).

2.3.2 Detection of antibodies to WHsAg (anti-WHs)

Anti-WHs antibodies were measured using a cross-reactive enzyme-linked immunoassay (ELISA: AUSAB® EIA, Abbott Laboratories) designed for the detection of anti-HBs. The cross reactivity with anti-WHs was established previously (Michalak *et al.*, 1989 and 1990). Briefly, polystyrene beads coated

with human HBsAg were incubated overnight at RT with 200 µl of either test or control 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 with water and the beads were incubated with 200 µl of a mixture of HBsAg tagged with biotin and then with rabbit anti-biotin conjugated with horseradish peroxidase (HRPO) for 2 h at 40°C. Next, the beads were washed to remove 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 colour intensity in proportion to the amount of bound HRPO-labeled antibody was evaluated at 492 nm using a Quantum II dual-wavelength analyser (Abbott Laboratories). The presence or absence of anti-WHs reactivity was calculated automatically by comparing the absorbance values of the sample tested to the cutoff value (NC $\bar{x}$ + 0.05). Samples with absorbance values greater than or equal to the cutoff value were considered positive.

2.3.3 Detection of antibodies to WHcAg (anti-WHc)

Anti-WHc was detected using a specific competition ELISA developed in this laboratory (Michalak *et al*, 1999). This assay is based on a principle that anti-WHc present in the test sample competes with HRPO-labeled anti-WHc for

binding to immobilized WHcAg. For this purpose, a 96-well, flat-bottom plate (Linbro/Titertek; ICN Biomedicals, Aurora, OH) was coated with woodchuck anti-WHc antibodies at 1 µg protein in 50 µl of PBS (pH 7.4) per well, incubated at 4 °C overnight, and washed three times. Nonspecific binding was blocked by adding 300 µl of 0.25% Tween-20 (Sigma Chemical Comp., St., Louis Mo) in PBS (blocking buffer) and incubating at RT for 2 h. After washing, the plate was directly used or stored at -20 °C. Before the assay, the plate was thawed and the wells washed briefly 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 the appropriate controls and 25 µl of anti-WHc labeled with HRPO (diluted 1:2,500 in blocking buffer) was added to each well. After a 2 h incubation, 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). Sera from healthy animals were used as negative controls. Positive controls included sera from WHsAg-positive woodchucks chronically infected with WHV. The degree to which the test sample inhibited the binding of HRPO-labeled 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.

2.3.4 Assay for serum γ-glutamyltransferase (GGT)

Elevated serum levels of γ-glutamyltransferase (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 available in this laboratory (Vettest S.A., Neuchatel, Switzerland). Sera from animals examined in this study showed normal GGT values (normal range 0-2 IU), unless otherwise indicated (reviewed by Michalak, 1998).

2.4 DETECTION OF SERUM WHV DNA BY DOT-BLOT HYBRIDIZATION

A 10 µl aliquot of serum was vacuum filtered blotted onto a nylon membrane (Hybond-N; Amersham, Arlington Heights, IL) using the Bio-Dot apparatus (Bio-Rad Laboratories). The membrane was denatured with 1.5 M NaCl/0.5 M NaOH for 10 min, then neutralized with 1.5 M NaCl in 1 M Tris-HCl buffer, pH 8.0 for 5 min. The blot was air-dried, baked for 2 h at 80°C under vacuum, and hybridized to a radiolabeled recombinant WHV DNA (rWHV DNA) probe as described in Section 2.9.3. The rWHV DNA was excised from a pSP65

vector kindly provided in the past by Dr. J. Summers from the University of New Mexico, Albuquerque, NM (Pardoe and Michalak, 1995).

Each hybridization assay used serum from a chronic WHV carrier and rWHV DNA as positive controls and serum from a healthy animal as a negative control. The assay sensitivity for WHV DNA detection was assessed using two-fold serial dilutions of rWHV DNA followed by densitometric analysis using the Cyclone phosphoimage system (Canberra Packard, Meriden, CT). The lowest detection limit was 30 pg of WHV DNA which corresponded to approximately 2 x 10^6 virus genome equivalents (vge)/ml (Lew and Michalak, 2001).

2.5 HISTOLOGICAL EXAMINATION OF LIVER TISSUE

Paraffin embedded liver fragments were sectioned to 5 µm and routinely stained with hematoxylin and eosin, Mason-trichrome, periodic acid shift or impregnated with silver. Liver examination included assessment and numeric scoring of three categories of histological lesions: hepatocellular, intralobular/extrahepatocellular and portal, as previously described by Michalak *et al.*, 1990. Briefly, liver tissue examination included assessment and numeral scoring of three categories of histologic lesions: hepatocellular, intralobular extrahepatocellular, and portal. In hepatocellular lesions, particular emphasis was placed on the determination of the localization of necrosis (i.e., necrosis affecting interlobular hepatocytes-lobular necrosis, assigned score 3;

centrilobular hepatocytes, score 4; periportal hepatocytes-piecemeal necrosis, score 8, and bridging necrosis, score 10) and its extent in each location through the examined section (score ranging from 0 to 3). In addition, evaluation of the severity of hepatocyte ballooning pleomorphism, swelling, eosinophilic and acidophilic degeneration, hyalinization, and the presence of acidophilic bodies were taken under consideration in the assessment of this category of liver tissue lesion (score of each alteration ranging from 0 to 3). In this scoring system, the maximal possible score assessing hepatocellular damage was 55. When mitoses and/or multinuclear cells were present, the score was diminished in range from 1 to 5. Overall, a hepatocellular lesion was graded according to the total score points using the following scale: 0 to 3 points, grade 0; 4 to 20 points, grade 1; 21 to 40 points, grade 2; and above 41 points, grade 3. The degree of the intralobular extrahepatocellular lesions was determined on the basis of the distribution of inflammatory infiltrations within lobules (i.e., infiltrations located mainly in one zone: interlobular, assigned score 3; centrilobular, score 4; perilobular, score 6; or spilled over into all zones, score 10) and their extent through the examined section (score ranging from 0 to 3). Assessment of Kupffer and endothelial cell proliferation, bile canalicular proliferation, and disorganization of reticular network was also included (score ranging from 0 to 3). On the basis of this scoring, the grade of an extrahepatocellular lobular lesion was determined according to the following scale: 0 to 3 points, grade 0; 4 to 15 points, grade 1; 16 to 30 points, grade 2; and above 31 points, grade 3.

Assessment of the portal lesions included evaluation of the portal tract enlargement owing to edema and inflammatory cell infiltrations (score ranging from 0 to 5), proliferation of bile ducts, and fibrosis (score of each alteration ranging from 0 to 3, dependent on its presence, frequency and/or extent). The grade of portal lesions was judged using the scale: 0 to 1, grade 0; 2-5, grade 1; 6-9, grade 2; and above 10 points, grade 3. An overall grade of disease severity, taking into consideration a global impression of the pathological picture of liver injury as a whole, rated in a scale from 0 to III and defined as "Histological Degree of Hepatitis", was assigned for each liver specimen examined. All histological evaluations were performed on coded samples on two different occasions, each time testing at least 10 lobules and 10 portal areas and was performed without the knowledge of serologic data.

2.6 ISOLATION OF NUCLEIC ACIDS

2.6.1 Isolation of cellular DNA

For DNA isolation, 100 mg tissue fragments were homogenized in 1.2 ml of lysis buffer containing proteinase K (Appendix A). The samples were incubated overnight in a 50°C shaking incubator, then extracted with an equal volume of Tris-HCl-buffered phenol/chloroform/isoamyl alcohol (25:24:1) following a standard procedure (Strauss, 1997). DNA was precipitated from the aqueous phase with one-half volume of 7.5 M ammonium acetate and two volumes of absolute ethanol (Sigma). After centrifugation, the resultant pellet

was washed with 70% ethanol. The DNA was then air dried and resuspended in TE buffer (Appendix A) to an approximate final concentration of 0.5 mg/ml.

If the tissue was in limited supply, DNA was recovered from the non-aqueous phase of TRIzol® following RNA extraction using the manufacturer's protocol (Gibco BRL, Grand Island, NY). Briefly, the DNA was precipitated from the interphase and phenol phase with 0.3 ml of absolute ethanol per ml of original TRIzol. Samples were kept at 30 °C for 5 min and then, DNA was pelleted by centrifugation at 2,000 x g for 5 min at 4 °C. The DNA pellet was washed three times for 30 min each, at 30°C in 1 ml of 0.1 M sodium citrate in 10% ethanol. After each wash, the DNA was pelleted at 2,000 x g for 5 min at 4°C. The final DNA pellet was suspended and washed by rotation in 2 ml of 75% ethanol for 20 min at 30°C and then pelleted at 2,000 x g for 5 min at 4°C. Subsequently, the DNA was air dried and resuspended in 8 mM NaOH to bring the final DNA concentration to approximately 0.5 mg/ml. To remove any remaining RNA, the DNA was treated with 2 µg of DNase-free-RNase (Boehringer Mannheim, Laval, Quebec) for 30 min at 37°C and then extracted with phenol/chloroform, as described above. DNA was quantitated as described in Section 2.6.3. Any DNA not used for slot-blot hybridization or PCR analysis was stored at -20°C.

2.6.2 RNA isolation

Total RNA was isolated from tissue samples, as well as naive and mitogen-stimulated PBMC using TRIzol® reagent (Gibco BRL), according to the manufacturer's instruction. Briefly, tissues or cells were pulverized in liquid nitrogen, homogenized in 1 ml of TRIzol® reagent and mixed for 30 min. After the addition of 200 µl chloroform, the tubes were shaken vigorously for 15 sec and the samples centrifuged at 12,000 x g for 15 min at 4°C. RNA was precipitated from the aqueous upper phase with 500 µl of isopropanol (Sigma) for 10 min at ambient temperature and collected by centrifugation at 12,000 x g for 10 min at 4°C. The RNA pellet was washed in 1 ml of RNase-free 75% ethanol. The final RNA pellet was briefly air-dried and resuspended in RNase-free water. RNA was quantitated as described in Section 2.6.3 and used immediately for Northern blot hybridization analysis or reverse transcription reaction or stored at -70°C.

2.6.3 Quantitation of nucleic acids

DNA, RNA and synthetic oligonucleotide primers or probes used in this study were quantitated based on the ultraviolet (UV) absorbance at 260 nm using a DU 530 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). DNA and oligonucleotides were read after suspension in TE buffer, whereas RNA was diluted in alkaline water to enhance accurate concentration readings (Wilfinger *et*

al., 1997). The concentration of RNA was determined assuming an optical density of 1 corresponded to 40 mg RNA/ml (Maniatis *et al.*, 1989). The actual calculation used was as follows: RNA (mg/ml) = (OD @ 260 nm - OD @ 320 nm) x dilution factor x 40 mg/ml. DNA concentrations were determined as above except that an optical density of 1 corresponded to 50 mg DNA/ml (Maniatis *et al.*, 1989). The concentration of oligonucleotides was determined using the specific weight per OD obtained in the certificate of analysis from the manufacturer (Gibco BRL). The evaluation of the purity of the nucleic acid preparations were based on the 260:280 nm absorbance ratio. Only RNA with a 260:280 nm ratio of greater than 1.8 and DNA greater than 1.6 were used.

2.7 REVERSE TRANSCRIPTION REACTION.

The reverse transcription (RT) reaction was used to convert total RNA to single stranded cDNA. To help eliminate RNA secondary structures, 1 µg of total RNA in 5.5 µl of RNase-free water was denatured at 70°C for 10 min and then ice chilled. Final assay conditions consisted of 1 µg of denatured RNA, 20 U RNasin® (Promega Biosciences Inc., Madison, WI), 1X reaction buffer (50 mM Tris-HCl buffer [pH 8.3], with 75 mM KCl and 3 mM $MgCl_2$), 10 mM dithiothreitol, 1 mM of each deoxynucleotide triphosphate (dNTP, Gibco BRL), and 200 U of Moloney murine leukemia virus reverse transcriptase (MMLV-RT; Gibco BRL) in 20 µl. The reaction proceeded at ambient temperature for 10 min and then at

42°C for 1 hr. After completion of the reaction, the sample was heated to 100°C for 5 min to inactivate remaining enzymes and then ice chilled. The cDNA samples were used for PCR amplification as outlined in Section 2.8.

2.8 POLYMERASE CHAIN REACTION (PCR)

In this study, nucleic acid amplifications by PCR were used for three different purposes. First, to test for the expression of WHV gene sequences in serum, PBMC, liver and spleen of infected animals, secondly, to amplify and clone woodchuck genes which were not identified prior to this work and, lastly, to quantitate the expression of woodchuck cytokine gene transcripts present at levels undetectable by Northern blot hybridization.

2.8.1 General criteria for primer design

Since many of the woodchuck gene sequences required in this study were undetermined prior to its initiation, the PCR primers initially used were based on the consensus of human, rat, mouse and rabbit sequences. At this initial stage, most of the oligonucleotide primers contained at least one degenerate base (referred to as degenerate primers). As the study progressed, the woodchuck specific sequences were established in our laboratory, or by other groups (Lohrengel *et al.*, 1998; Nakamura *et al.*, 1997) and the primer sequences were modified accordingly.

In order to ensure efficient amplification of the target sequences, each primer pair was matched according to GC content (approximately 50%), length (18-25 bases) and melting temperature (+/- 5°C). Matching was done with PC/Gene software (Intelligenetics, Geneva, Switzerland) and the BLAST search engine software (http://www.ncbi.nlm.nih.gov/BLAST; National Centre for Biotechnology Information, National Library of Medicine, National Institute of Health, Bethesda, MD). All oligonucleotides were synthesized and desalted by Gibco BRL. Primers used for the initial amplification and cloning of particular woodchuck genes are outlined in the respective chapters.

2.8.2 Standard PCR conditions for WHV DNA detection

Three sets of primers specific for non-overlapping genomic regions of WHV DNA, i.e., core (C), surface (S) and X genes were used for the detection of virus genome by direct PCR. In addition, 3 other primer pairs, internal to those mentioned above, were used for nested PCR when the results of the direct PCR were negative. Sequences of these primers and their location in the WHV genome have been outlined in previous studies from our laboratory (Coffin and Michalak, 1999; Michalak *et al.*, 1999).

In general, direct PCR detection of WHV utilized 2 µg of total DNA as template. The final reaction conditions consisted of 200 µM of each dNTP, 10 pmol of each oligonucleotide primer, 1X reaction buffer comprising 1.5 mM $MgCl_2$

with 50 mM KCl in 20 mM Tris-HCl buffer (pH 8.4) and 2.5 units of *Taq* DNA polymerase (all Gibco BRL) in a 100 µl total volume. The reaction mixture was layered with 100 µl of mineral oil (Sigma) to inhibit evaporation. DNA amplification proceeded in a programmable thermal cycler (TwinBlock System; Ericomp Inc., San Diego, CA) using the following program: 94°C for 5 min as the first step, then 94°C for 30 sec, 52°C for 30 sec, and 72°C for 30 sec for 30 cycles. A final extension step was performed at 72°C for 10 min. For each thermocycling reaction, rWHV DNA and liver DNA from a WHsAg-positive chronically infected animal were used as positive controls. In addition, both a water and a mock sample, containing all reagents used during the DNA extraction and PCR, were included as negative controls. For nested PCR, 10 µl of the direct PCR mixture was reamplified under the same conditions as the direct reaction. PCR amplifications were performed under conditions limiting the possibility of contamination, as outlined in detail in previous studies (Coffin and Michalak, 1999; Michalak *et al.*, 1999)

2.8.3 Amplification of woodchuck cDNA for cloning using degenerate primers

All PCR using degenerate primers utilized lenient PCR conditions and 5 µl of cDNA (Section 2.7) as the amplification template. The 100 µl final reaction mixture was the same as for WHV DNA amplification (Section 2.8.2) except 20

pmol of each synthetic oligonucleotide primer and high fidelity *Taq* polymerase (Boehringer Mannheim) with exonuclease activity were used. The following program was employed for all PCR using degenerate primer pairs: 94°C for 5 min for the first cycle and then 94°C for 1 min, 52°C for 2 min, and 72°C for 3 min for 35 cycles. The products were analyzed by gel electrophoresis as per Section 2.9.2, and product identity confirmed by Southern blot hybridization using specifically designed oligonucleotide probes or purchased cross-reactive heterologous DNA probes.

If the PCR product produced a single band after agarose gel electrophoresis and Southern blot hybridization, it was cloned without further manipulation (Section 2.11.2). However, if multiple bands were observed the PCR products were electrophoresed on a 0.8% low melting temperature agarose gel, the band corresponding to the expected molecular size was excised and then purified using the Wizard PCR purification kit (Section 2.11.1). The purified PCR product was cloned as described in Section 2.11.2.

2.9 DETECTION OF DNA

2.9.1 Slot-blot hybridization assay

To determine the levels of WHV DNA in tissue samples (e.g., liver, spleen) or cells (e.g., PBMC) from the animals investigated, a slot-blot hybridization assay was used. For this purpose, 5 µg of total liver DNA or 10 µg

of spleen DNA was heat denatured in 200 µl of 6X SSC (Appendix A) for 10 min, ice chilled and then vacuum blotted onto nylon (Hybond-N; Amersham) using a microfiltration apparatus (BioRad Laboratories). To aid in the quantification of WHV DNA test samples were done in parallel with serial two-fold dilutions of rWHV. After loading the DNA, the wells were washed twice with 400 µl of 6X SSC. The nylon membrane was removed, air-dried and baked for 2 h at 80°C in a vacuum oven. The nylon membranes were hybridized to a ^{32}P- random prime-labeled recombinant DNA probe, as described in Section 2.9.3. Hybridized blots were washed and exposed for autoradiography according to Section 2.9.3.3.

2.9.2 Agarose gel electrophoresis

To detect PCR products an 18 µl reaction aliquot (Section 2.8) or 1 µg of the appropriate *Eco*R I restriction enzyme-digested plasmid DNA as positive control (Section 2.10.1) was mixed with 2 µl of DNA loading dye (Appendix A) and poured into the wells of a 1.5% agarose gel (Gibco BRL). The gel was made with 1X TAE buffer (Appendix A) containing 0.5 ng/ml ethidium bromide. After electrophoresis at 80 V for 60 min the DNA bands were documented using a low-light imaging system (ChemiImager 4000, Alpha Innotech Corporation; San Leandro, CA)

2.9.3 Southern blot hybridization.

2.9.3.1 DNA blotting onto nylon membrane

To confirm the authenticity of DNA products and plasmid preparations or to estimate the quantity of the PCR products Southern blot hybridization using ^{32}P- labeled cloned DNA or synthetic oligonucleotide probes was performed. For this purpose, the agarose gel containing the PCR or restriction enzyme digestion products were denatured with 1.5 M NaCl with 0.5 M NaOH for 45 min. The DNA was then neutralized with 1.5 M NaCl in 1 M Tris-HCl buffer (pH 8.0) for 45 min. Blotting of DNA from the gel to the nylon membrane was performed using downward capillary transfer. After transfer, the membrane was baked at 80°C in a vacuum oven for 2 h.

2.9.3.2 Southern hybridization with DNA probes

Membranes to be hybridized with radio-labeled recombinant DNA probes were sealed in a plastic bag with 10 ml of hybridization buffer (Appendix A) containing 100 µg/ml sonicated salmon sperm (sss) DNA (Sigma). The membrane was prehybridized at 65°C in a shaking oven for 30 min. After prehybridization, 15 x 10^6 cpm of a heat-denatured radiolabeled probe was added and the blot was hybridized overnight at 65°C in a shaking oven.

For hybridization with oligonucleotide probes the nylon membranes were prehybridized at 42°C in a shaking oven for 1 h with 10 ml of hybridization buffer

(Appendix A). The blot was then incubated in a rotary shaker at 42°C for 18 h with 15 x 10^6 cpm of a ^{32}P end-labeled oligonucleotide probe (Section 2.10.3). After hybridization, all blots were washed according to Section 2.9.3.3.

2.9.3.3 Blot washing

All blots were washed twice in 2X SSC with 0.1% SDS for 5 min followed by two washes with 0.2X SSC with 0.1% SDS for 5 min each at RT. This was proceeded with two 15-min moderate stringency washes at 42°C using prewarmed 0.2X SSC with 0.1% SDS. To alleviate problems of high and low signal intensity when blots are analyzed on film the nylon membranes were exposed to a multipurpose Phosphor screen for 15 min to 2 h and analyzed on the Cyclone system (Canberra Packard). For final documentation, membranes were exposed to X-ray film (XRP-1; Eastman Kodak Co., Rochester, NY) at -70°C in cassettes equipped with intensifying screens. Multiple film exposure times were used to compensate for the fact that there is a sensitivity lag in films at low signals and saturation at high signal intensities.

2.10 GENERATION OF DNA PROBES

2.10.1 Excising recombinant DNA from plasmid vectors

All recombinant DNA fragments used in this study were excised from the plasmid vectors with *Eco*R I restriction enzyme. For this purpose, 10 µg of the

recombinant plasmid DNA was incubated with 100 U of *Eco*R I, 1X REACT 3 buffer (both from Gibco BRL; Appendix A), and 50 U of DNase-free-RNase (Boehringer Mannheim, Quebec, Canada) for 4 h at 37°C. After digestion, the DNA insert was separated from the plasmid by electrophoresis at 50 V in a 1% low-melting point agarose (Gibco BRL) made with 1X TAE containing EtBr (Appendix A). The band containing the DNA of interest was excised from the gel and purified using the Wizard™ PCR Prep DNA Purification System (Section 2.11.1).

2.10.2 Random prime labeling of recombinant DNA

To prepare a WHV DNA probe, a random primed DNA labeling system (Rediprime: Pharmacia Biotech) using ^{32}P-dCTP was employed. Briefly, 25 ng of the recombinant DNA of interest in 45 µl of TE was boiled for 5 min and then chilled on ice. The denatured DNA and 5 µl of ^{32}P-dCTP (3000 Ci/mmol) (Amersham) was added to a reaction tube containing freeze dried dATP, dGTP, dTTP, Klenow enzyme, and 9-mer random primers. The labeling reaction proceeded for 1 h at 37°C and was stopped with the addition of 2 µl of 0.5 M EDTA.

In later experiments, the strip-EZ ®, random primed probe synthesis procedure (Ambion Inc., Austin, TX) was used to prepare probes for Northern blot hybridization. Briefly, 25 ng DNA template was suspended in 9 µl of water,

boiled for 5 min, and then snap frozen in liquid nitrogen. After thawing, 2.5 µl of a 10X random decamer primer solution, 5.0 µl of dATP/dCTP-free 5X buffer, 2.5 µl modified 10X dCTP, and 5 µl ^{32}P-dATP (3000 Ci/mmol) (Amersham) were added. The labeling reaction proceeded at 37°C for 30 min after the addition of 2 U of exonuclease-free Klenow fragment.

2.10.3 End labeling of oligonucleotide probes

Synthetic oligonucleotides were labeled with ^{32}P-ATP (3000 Ci/mmol) using T4-kinase (Gibco BRL) following manufacturer's instructions. The final reaction mixture consisted of 5 pmol of template, 2.5 µl of ^{32}P-ATP (3000 Ci/mmol) (Amersham), 1X forward reaction buffer (Appendix A), and 10 U of T4 kinase in a total volume of 25 µl. The tube contents were mixed gently and incubated for 1 h at 37°C. The reaction was stopped with the addition of 2 µl of 0.5 M EDTA.

2.10.4 Purification of radiolabeled DNA probes

The ^{32}P-labeled recombinant DNA and oligonucleotide probes were separated from unincorporated ^{32}P by fractionation on Sephadex G-50 NICK™ columns (Pharmacia Biotech), as per the manufacturer's instructions. Briefly, the column was equilibrated with TE buffer, the probe mixture was applied onto the column and then washed through with 400 µl of TE buffer. The first wash was

discarded and 450 µl of fresh TE buffer was applied to the column. The recovered elutant, containing the labeled probe, was saved. The radioactivity of the probe (cpm/µl) was determined using a scintillation counter. Probes not immediately used were stored at -20°C.

2.11 DNA CLONING

2.11.1 Purification of PCR products

PCR products destined for cloning were purified using the Wizard ® PCR preps DNA purification system (Promega). After electrophoresis on a 1% low melting point agarose gel, the band containing the PCR product of interest was excised, placed in a 1.5 ml microtube and melted at 65°C. One ml of purification resin was added to the tube, mixed briefly, and filtered under vacuum through a Wizard ® minicolumn. The column was washed twice with 70% isopropanol, dried under vacuum for 30 sec, and then centrifuged at 12,000 x g for 2 min. The collected isopropanol was discarded. TE buffer (50 µl) prewarmed to 65°C was added to the column and left for 5 min. The minicolumn was centrifuged at 12,000 x g for 20 seconds and the elutant containing the DNA saved. The DNA concentration was determined by spectrophotometric analysis as described in Section 2.6.3.

2.11.2 TA cloning reaction

DNA fragments amplified by PCR using degenerative primers were cloned using a dual promoter TA Cloning© Kit (Invitrogen, Carlsbad, CA). The final cloning mixture consisted of 25 ng of PCR product, 1X ligation buffer (Appendix A), 50 ng of linearized pCRII ® plasmid, and 4 U of T4 DNA ligase in a total volume of 10 µl. The reaction proceeded at 14°C for 18 h. After incubation, 2 µl of the reaction mixture was added to 50 µl of INVαF' bacterial cells (Invitrogen) in the presence of 20 mM β-mercaptoethanol. The mixture was chilled for 30 min on ice, heat shocked for 30 sec at 42°C, and placed on ice for 2 min. Subsequently, 250 µl of SOC medium (Appendix A) was added and the cells were incubated at 37°C for 1 h in a rotary shaker (250 rpm).

2.11.3 Growth of plasmid-transfected bacteria

A 100 µl aliquot of each transformation reaction mixture (see above; Section 2.11.2) was spread onto 1.5 % Bacto-Agar (Difco Laboratories, Detroit MI) coated with 40 µl of X-Gal (Sigma; Appendix A) in 10 cm petri dishes. Petri dishes were incubated at 37°C for 16 h. Single bacterial colonies were collected and inoculated into 5 ml of sterile LB medium supplemented with 50 µg/ml kanamycin (Sigma). Bacteria were grown overnight at 250 rpm in a rotary shaker at 37°C .

2.11.4 Small scale (Mini) preparations of plasmid DNA

For preparation of small amounts of plasmid DNA, a 1.5-ml aliquot of transformed bacterial cells were pelleted at 20,000 x g for 20 sec. The cells were resuspended in 100 µl of Glucose Tris-EDTA (GTE; Appendix A) and left for 5 min at ambient temperature. The bacteria were lysed with 200 µl of freshly prepared 0.2 N NaOH/1% SDS solution and ice chilled for 5 min. To neutralize the solution and aid in the removal of chromosomal DNA and proteins, 250 µl of 3 M potassium, 5 M acetate (pH 5.5) was added to the tube, vortexed for 10 sec, and the mixture ice chilled for 5 min. The mixture was centrifuged at 20,000 x g for 3 min to pellet cell debris and chromosomal DNA. The supernatant was transferred to an eppendorf tube and the nucleic acids were precipitated with 1 ml of 95% ethanol for 2 min at RT. Plasmid DNA was pelleted by centrifugation at 15,000 x g for 1 min. The pellet was washed with 1 ml of 70% ethanol, air dried and resuspended in 30-50 µl of TE buffer. Plasmid mini-preparations were analyzed for the presence of the proper DNA inserts as described in Section 2.12.1.

2.11.5 Large-scale (Maxi) plasmid DNA preparation

A 50 µl bacterial aliquot containing the proper plasmid insert, as judged by miniprep analysis (Section 2.11.4), was grown to saturation in 500 ml of LB medium supplemented with 50 µg/ml of the appropriate antibiotic (kanamycin or

ampicillin). The bacteria were collected by centrifugation at 6,000 x g for 10 min at 4°C. The pellet was resuspended in 4 ml of GTE solution.

Bacteria were lysed with 25 mg of hen egg white lysozyme (Sigma) and 0.2 N NaOH with 1% SDS alkaline solution, following a standard procedure (Heilig *et al.*, 1998). The viscous mixture was supplemented with 7.5 M potassium acetate, stirred until a white precipitate formed and then centrifuged at 20,000 x g for 10 min at 4°C. The resulting supernatant was filtered through sterile surgical gauze into 50 ml tubes. Nucleic acid was precipitated by the addition of isopropanol and recovered by centrifugation at 15,000 x g for 10 min. The pellet was washed in 70% ethanol and resuspended in TE buffer. Remaining RNA was removed by incubation with 20 µg of DNase-free-RNase (Boehringer Mannheim) for 30 min at 37°C. Then, freshly prepared 0.2 M NaOH/1% SDS solution was added to the eppendorf tube and mixed for 10 min at RT. In the next step, 3 M potassium acetate was added to the DNA solution and mixed by inversion for 10 min at RT. After centrifugation for 10 min at 20,000 x g, the plasmid DNA was extracted using a standard phenol extraction procedure (Section 2.6.1). Plasmid DNA was recovered by centrifugation at 10,000 x g for 10 min at 4°C, and the resulting pellet washed with 70% ethanol and then dried briefly under vacuum. The DNA pellet was resuspended in 1 ml TE buffer, then 1.5 ml of 30% sterile filtered polyethylene glycol was added and the mixture left overnight at 4°C. The plasmid DNA was recovered by centrifugation at 10,000 x g for 20 min at 4°C and resuspended in 1 ml TE buffer.

The DNA was precipitated using absolute ethanol containing 3 M sodium acetate (pH 5.5), as outlined in Section 2.6.1. The fragment of interest was removed from the plasmid by *Eco*R I restriction enzyme digestion, as per Section 2.10.1.

2.12 DNA SEQUENCING

2.12.1 PCR amplification

Final confirmation of the identity of the cloned woodchuck cDNA fragments was done by nucleotide sequence analysis using the *fmol*® DNA cycle sequencing system (Promega Corp.). Briefly, a cocktail containing approximately 40 fmol of recombinant plasmid DNA, 1.5 pmol of a universal sequencing primer (T7 or M13 reverse) end-labeled with ^{32}P-ATP (3000 Ci/mmol) (Amersham) (Section 2.10.3), 2 mM of $MgCl_2$ in 50 mM Tris-HCl buffer, pH 9.0, (supplied as a 5X buffer), and 5 units of sequencing grade *Taq* DNA polymerase (Promega Corp.) were prepared. The cocktail was divided equally into each of 4 tubes containing 2 µl of either the G (guanine), A (adenine), T (thymine) or C (cytosine) terminating nucleotide mixture. Cycle sequencing was conducted using the following program: 95°C for 2 min, then 30 cycles consisting of 95°C for 30 sec, 42°C for 30 sec, and 70°C for 1 min. Upon completion, 3 µl of formamide stop solution (Appendix A) was added to each tube and the tube was stored on ice until electrophoresis in a sequencing polyacrylamide gel (PAGE) (Section 2.12.2).

2.12.2 Sequencing PAGE

Prior to loading on the sequencing gel each sample was heat-denatured for 2 min at 70°C, then chilled on ice. The DNA samples were separated at 50 V on an 8% denaturing polyacrylamide gel containing 7 M urea (Gibco BRL). After electrophoresis at 50°C (monitored by a thermal probe), the gel was fixed with a 10% methanol, 10% acetic acid solution and dried at 80°C in a slab gel dryer. Subsequently, the gel was exposed briefly to a multipurpose storage Phosphor® screen and the image analyzed using the Cyclone system (Canberra Packard). Final documentation was done by overnight exposure of the gel to X-ray film (Kodak).

The identity of the nucleotide sequences was confirmed by comparison with published sequences in GeneBank library (National Centre for Biotechnology Information, National Library of Medicine, National Institute of Health, Bethesda, MD) using the BLAST search software (http://www.ncbi.nlm.nih.gov/BLAST). Once confirmed, the complete sequences of the cloned woodchuck DNA were obtained by employing a commercial fluorescence-based automated DNA sequence analyzer (LI-COR; LiCor Inc., Lincoln, NB)(Department of Genetics, Hospital for Sick Children, Toronto, Ontario). All woodchuck sequences obtained through the course of this study were submitted to Genbank at the National Institutes of Health. They can be viewed in the order submitted in Appendix B or at www.ncbi.nlm.nih.gov.

2.13 DETECTION OF RNA

2.13.1 Denaturing gel electrophoresis

Prior to Northern blot hybridization, RNA was electrophoretically fractionated on a denaturing agarose gel following a standard protocol (Brown and Mackey, 1997). Briefly, 10-20 µg of total RNA (Section 2.6.2) was supplemented with 3 volumes of RNA denaturing solution (Appendix A) and incubated for 15 min at 65°C. Samples were then chilled on ice and 1/10 volume of formaldehyde gel loading buffer was added, and RNA electrophoresed at 5 V/cm on a 1% denaturing formaldehyde agarose gel. After electrophoresis, the formaldehyde was removed by washing the gel in RNase-free water (5 min) and twice in RNase-free 10X SSC solution (25 min each). The quality of the isolated RNA was assessed by visualization of ribosomal RNA on a UV transilluminator (Fotodyne Inc., Bio/Can Scientific, Mississauga, Ontario).

2.13.2 Northern blot analysis

After electrophoresis, the RNA was blotted onto positively charged nylon membrane (Hybond-XL ®; Amersham) by downward capillary transfer using RNase-free 10X SSC, as per Section 2.9.3.1. After a 4 hr transfer, the membrane was air-dried and then baked for 2 h at 80°C prior to hybridization with radiolabeled probe.

Nylon membranes containing RNA were pre-hybridized in 10 ml of

formaldehyde hybridization solution (Appendix A) at 42°C. After 30 min, 15 x 10^6 cpm of a heat-denatured ^{32}P-radiolabeled probe was added. The blots were hybridized overnight at 42°C and then the membranes were washed and signals were quantified the following day, as described in Section 2.9.3.3.

2.13.3 Removal of hybridized probes from Northern blots

To hybridize the same Northern blots with different probes, radioactive signals were removed from the blots using a degradation solution provided in the EZ-strip DNA labeling kit (Ambion Inc.), following the manufacturer's procedure. This technique provides mild stripping conditions which greatly enhance the lifespan of Northern blots compared to standard probe removal protocols.

CHAPTER 3:

PERFORIN AND FAS/FAS LIGAND-MEDIATED CYTOTOXICITY IN ACUTE AND CHRONIC WOODCHUCK VIRAL HEPATITIS*

* This study was published in Clinical and Experimental Immunology in October 1999 (vol. 118; pp.63-70). The cloning and sequencing of the woodchuck perforin gene was done after the publication of the manuscript. All the research reported in this study, with the exception of immunohistological analysis of liver sections, was performed by the candidate.

3.0 SUMMARY

The Fas/FasL and the perforin-granzyme cytotoxic pathways presumably play a central role in the development of hepatocellular injury in viral hepatitis. To recognize the potential contribution of FasL and perforin-based cell killing in hepadnaviral infection, we adopted a cytotoxic assay using murine Fas-positive P815 and human Fas-negative K562 cells as targets. Freshly isolated PBMC from woodchucks with newly acquired WHV infection (n=6), chronic WHV hepatitis (n=9) and from healthy animals (n=11) were used as effector cells. We have found that woodchuck lymphoid cells kill cell targets via both the FasL/Fas and the perforin death pathways. The contribution of the Fas-dependent cytolysis was ascertained in blocking experiments with anti-Fas antibody and by incubation of PBMC with cycloheximide to prevent *de novo* synthesis of FasL. The involvement of the perforin pathway was confirmed by treatment of effector cells with colchicine to inhibit the microtubule-dependent perforin release. Comparative analysis showed that peripheral lymphoid cells from acute WHV hepatitis, but not those from chronic WHV infection, are more cytotoxic and that

this increase seems to be entirely due to activation of perforin-mediated killing. The data indicate that acute infection in woodchucks is associated with the augmented capacity of lymphoid cells to elicit perforin-dependent killing, but in chronic infection, independent of the severity of liver disease and duration of chronicity, these cells have the same or lower cytotoxic potential as PBMC from healthy controls. These findings suggest a role for nonspecific cellular immunity, presumably NK cells, in the control of early WHV infection and in the progression of CH.

3.1 INTRODUCTION

Although the mechanisms of the action are not completely understood injury in hepatitis B is presumably caused by CTL which are readily detectable in the peripheral blood of patients with acute infection (Rehermann et al., 1995), but are apparently lacking or at low levels in CH (Ferrari et al., 1990, Rehermann et al., 1996). Activated immune effector cells use at least two independent mechanisms to kill targeted cells. These cytotoxic pathways are mediated by a Fas/FasL interaction and by perforin-granzyme release. In addition, it is assumed that cytokines, such as IFNγ and TNFα, secreted by activated lymphoid cells cause cell damage and death (Ando et al., 1993).

Hepatocytes are Fas bearing cells and are highly sensitive to FasL-induced injury. Cross linking of Fas molecules with an anti-Fas antibody has been shown to cause fatal hepatic failure in mice due to hepatocyte apoptosis

(Ogasawara et al., 1993). In viral hepatitis, the involvement of the FasL-Fas system was postulated based on the presence of activated T cells, which display FasL, in the intrahepatic inflammatory infiltrates and on the unregulated expression of Fas on hepatocytes during ongoing necroinflammation (Galle et al., 1995). Demonstration that soluble Fas can prevent CTL-induced hepatitis in transgenic mice retaining HBsAg in hepatocytes supported a notion about a principal role of the FasL-Fas interaction in the development of viral hepatitis (Kondo et al., 1997). However, other experiments in HBV transgenic mice in the absence of hepatic HBsAg retention showed that both FasL and perforin-based cytotoxicity contribute to hepatocyte injury and that both these pathways must be operative to kill targeted cells (Nakamoto et al., 1997). The importance of the perforin pathway in T cell-mediated liver injury was also substantiated in perforin knockout mice which fail to eliminate virus and are resistant to LCMV-induced hepatitis (Kagi et al., 1994). Until now, the contribution of this FasL and perforin-dependent killing had not been comparatively evaluated in acute and chronic phases of natural hepadnavirus infection.

In this study, we measured the cytolytic capacity of PBMC from woodchucks with acute and chronic WHV hepatitis and from healthy animals to investigate differences in the effector cell killing during hepadnaviral infection. To discriminate between FasL and perforin-based cytotoxicity and to assess the relative contribution of each of the mechanisms in acute and chronic WHV infections, we adopted an assay with heterologous FasL-sensitive and FasL-

insensitive cells as targets and manipulated the testing conditions using agents that selectively block individual reactant molecules of each pathway. Our results show that the circulating woodchuck lymphomononuclear cells kill cells by both FasL and perforin-dependent mechanisms. Furthermore, comparative analysis showed that AH, but not chronic WHV infection, is uniformly associated with an enhanced ability of the circulating lymphoid cells to induce cell death and that this increase is a consequence of activation of perforin but not the FasL-mediated pathway. These data indicate that an increased activity in the perforin effector system could be important in defence against early hepadnavirus infection and that its decreased efficiency may contribute to the establishment and/or perpetuation of chronic infection.

3.2 MATERIALS AND METHODS

3.2.1 Animals and categories of WHV infection

The study group comprised in total 20 adult woodchucks (6 males and 14 females) randomly selected from animals housed in our colony. Eleven of the animals were healthy and were investigated as controls (Table 3-1). Six of the initially healthy animals were subsequently inoculated with a WHV infectious pool (Michalak and Lin, 1994; Michalak et al., 1999) and analyzed for serological indicators of WHV infection (Section 2.3) at weekly intervals during the pre-acute and acute phases of hepatitis. The remaining 9 woodchucks were chronically infected with WHV and their sera typically tested monthly for WHV infection

Table 3-1. Details on WHV infection in adult woodchucks at the time of acquisition of circulating lymphoid cells analyzed in the cytotoxicity assays.

Characteristics	Healthy	Newly Acquired Infection	Chronic Infection
Animals (n)	11[a]	6	9
Male:Female	3:8	2:4	3:6
No. of PBMC samples tested	33	15	30
Serum WHV DNA positivity (n)			
Slot-blot hybridization [b]	0	2	9
PCR/Southern blot hybridization [c]	0	6	nt [d]
Serum WHsAg positivity (n)	0	5	9
Duration of WHs antigenemia (weeks)			
Range	0	1-4	34-192
Mean	0	2	83
Serum anti-WHs positivity (n)	0	1[e]	0
Serum anti-WHc positivity (n)	0	6	9
Serum GGT positivity (n)	0	0	0
Liver histology (n)			
Normal	11	1[f]	0
Acute hepatitis	0	5	0
Chronic hepatitis	0	0	9

[a] Includes 6 animals that were subsequently inoculated with WHV and developed preacute or acute WHV infection.
[b] Approximate sensitivity 10^6-10^7 vge/ml.
[c] Amplified by nested PCR with WHV core gene specific primers and detected by Southern blot hybridization; approximate sensitivity 10-10^2 vge/ml.
[d] nt, not tested.
[e] One animal became anti-WHs reactive 5 weeks after inoculation with WHV following a 3-week period of WHsAg positivity (see Fig. 3-4A; 838/AH animal).
[f] After WHV inoculation, one animal remained WHsAg non-reactive and had normal liver histology at the time of cytotoxic assay despite the presence of WHV DNA and anti-WHc in the serum (see Fig. 3-4A; 821/preAH animal).

markers. Of these, 5 had CH which had developed after experimental injection with WHV and 4 others were brought to the colony as juveniles with serologically and histologically evident chronic infection acquired in the wild. The stage of WHV infection and status of liver disease in the animals investigated was classified according to serological and histological criteria outlined in Sections 2.3 and 2.5. All animals in this study had normal levels of GGT excluding the likelihood of HCC.

Onset of preacute WHV infection in experimentally inoculated animals was recognized when WHV DNA and anti-WHc first appeared in the circulation but serum remained WHsAg negative.

3.2.2 Preparation of target cells

Murine mastocytoma P815 cells (ATCC #TIB-64), which constitutively express Fas (De Leon et al., 1998) and are susceptible to cross-species FasL and perforin-mediated killing (Takahashi et al., 1994), and human chronic myelogenous leukemia K562 cells (ATCC #CCL-243), which are Fas-negative (Montel et al., 1995) and are model cells for determination of perforin-induced cytotoxicity, were purchased from the American Type Culture Collection (ATCC; Rockville, MD). The cells were maintained in growth medium consisting of RPMI 1640 supplemented with 10% (v/v) fetal calf serum (FCS), 10 mM HEPES, 2 mM L-glutamine, 2% (v/v) penicillin/streptomycin (all Gibco BRL) and 2 μM β-

mercaptoethanol (Sigma). The cells were subcultured 24 h prior to cytotoxicity assay to allow for log-phase cell growth. To radiolabel the cells, approximately 5 x 10^6 cells were pelleted by centrifugation at 328 x *g* for 10 min and incubated in minimal volume with 200 µCi $Na_2{}^{51}CrO_4$ (Amersham) at 37 °C for 90 min. Labeled cells were washed four times in phosphate buffered saline, pH 7.4 (PBS) supplemented with 1% (v/v) FCS and resuspended in growth medium at a final concentration of 2 x 10^5 cells/ml. Labeled cells were used immediately for cytotoxicity assays.

3.2.3 Preparation of effector cells

Woodchuck PBMC were isolated by density gradient separation as described in Section 2.2.3. The viability of isolated PBMC was consistently greater than 90%, as evaluated by trypan blue exclusion. After isolation cells were washed with sterile PBS containing 1% FCS and resuspended at a final concentration of 10^7 viable cells/ml in reaction medium which consisted of growth medium (Section 3.2.2) supplemented with 5 µg/ml phytohemagglutinin (PHA; Murex Biotech Ltd. Dartford, U.K.). The cells were used immediately in cytotoxicity assays.

3.2.4 Cytotoxicity assay

The standard cytotoxicity assay was performed in 96-well round bottom

plates (ICN Pharmaceuticals, Montreal, Quebec, Canada) with 200 µl of reaction medium (described above) per well. Effector (E) cells were added to duplicate wells to achieve three different E:T ratios, equivalent to 50:1, 25:1 and 12.5:1. Subsequently, 1 x 10^4 ^{51}Cr-labeled target (T) P815 or K562 cells in 50 µl and an appropriate volume of growth medium were added to the wells to reach a final volume of 300 µl. The plates were incubated at 37 °C in a humidified 5% CO_2 incubator for 5 h. Following the incubation, 125 µl aliquots of cell free supernatant were transferred into 1-ml glass culture tubes and the radioactivity measured in a gamma counter. As a control, cells incubated in the absence of PHA were included in each assay. The maximum and spontaneous release were determined by incubation of 10^4 labeled target cells in 50 µl with 250 µl of 1 N HCl or 250 µl of reaction medium, respectively. The percent specific lysis was calculated from means of duplicate evaluations as follows: 100 x (experimental release - spontaneous release)/(maximum release - spontaneous release). In all assays, the spontaneous ^{51}Cr release was less than 20% of the maximum release.

3.2.5 Determination of the effects of anti-Fas antibody and regulatory agents on cytotoxicity

To discriminate between FasL and perforin-mediated killing of P815 cells caused by woodchuck PBMC, the hamster anti-mouse Fas Jo2 monoclonal antibody

(Jo2 mAb; purified IgG; PharMingen, Mississauga, Canada) was used. It has been previously established that some cells, including P815, resist direct lysis by Jo2 mAb despite the fact that the antibody specifically recognizes Fas on these cells and blocks FasL interaction with Fas (data not shown; Kuwano and Arai, 1996). Thus, prior to the cytotoxic assay, approximately 3 x 10^6 of ^{51}Cr-labeled P815 cells in a minimal volume were incubated with 5 µg of Jo2 mAb or an unrelated hamster antibody (control) at 37°C in a humidified atmosphere of 5% CO_2 for 30 min. The cells were resuspended in growth medium at a final concentration of 2 x 10^5 cells/ml, and then the standard cytotoxic assay was performed as described above.

To determine the effect of inhibition of *de novo* FasL synthesis on the PBMC cytotoxic activity, 0.177 mM cycloheximide (CHX; CalBiochem-NovaBiochem, La Jolla, CA) was added to test wells with E:T ratio of 50:1 at the start of the standard 5-h cytotoxic assay. In parallel experiments, the effect of a microtubule polymerization inhibitor, colchicine (Sigma), on the perforin-mediated killing of Fas-deficient K562 cells was tested. In these assays, reaction mixtures containing effector and target cells at 50:1 ratio were supplemented with colchicine at a final concentration of 1 mM and incubated for 5-h at 37 °C. In all assays, the results were compared to the data from assays done under identical conditions in the absence of the test agents.

3.3 RESULTS

3.3.1 Woodchuck lymphoid cells kill both Fas-positive and Fas-negative target cells

Initial experiments were aimed at determining whether heterologous cells which either express Fas or were Fas-negative could serve as woodchuck lymphoid cell targets and whether they could provide a means to differentiate between FasL and perforin-based cell killing caused by the woodchuck cells. For this purpose, murine P815 cells susceptible to cross-species FasL and perforin-mediated killing were used as the principal target cells. In addition, to ascertain the accuracy in discriminating perforin from FasL-mediated cytolysis, Fas-deficient human K562 cells, model cells for determining perforin-dependent toxicity, were used. Employing this two-target cell system, PBMC from healthy woodchucks lysed both P815 and K562 cells in the presence of PHA in a manner strictly dependent on the effector cell concentration. Thus, a linear decrease in the level of killing of both target cells was observed as the effector cell number decreased in the assay (Fig. 3-1). The above cytolysis did not occur in the absence of PHA (data not shown). This may suggest that in an *in vivo* situation direct contact between effector and target cells is required to facilitate both Fas and perforin-mediated cell killing.

At all three E:T ratios examined, Fas-deficient K562 cells were killed with an efficiency of about 25% of that observed with an equivalent number of P815

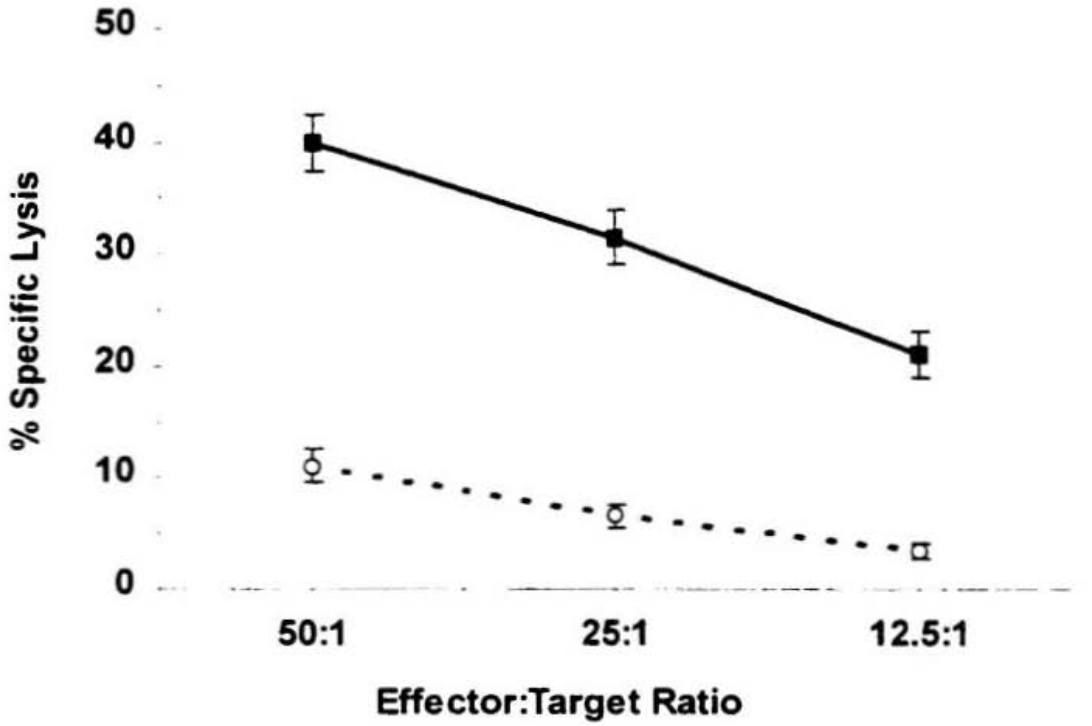


Figure 3-1. Cytotoxicity of normal woodchuck PBMC towards Fas-competent and Fas-deficient target cells. PBMC from 11 healthy, WHV-negative woodchucks were incubated with ^{51}Cr-labeled Fas expressing P815 cells (solid line) or Fas deficient K562 cells (dashed line) at indicated E:T ratios. Data from evaluations with 30 and 19 individual PBMC samples were used to determine P815 and K562 cell killing, respectively. The results are expressed as % specific lysis ± SEM.

cells (Fig. 3-1), suggesting that the woodchuck PBMC lyse targeted cells more efficiently via the FasL-Fas pathway. Preincubation of P815 cells with Jo2 mAb reduced P815 cell lysis by an average of 61% (see Fig. 3-2A), supporting the conclusion that the woodchuck cells eliminated P815 cells to a larger extent via the FasL-mediated mechanism. A similar level of inhibition of the P815 cell lysis (approximately by 72%) was seen in the presence of CHX, an inhibitor of *de novo* FasL synthesis (see Fig. 3-2A). This last finding provided additional evidence that the observed cytolysis resulted mainly from activation of the FasL-Fas pathway.

3.3.2 Acute but not chronic WHV hepatitis is associated with increased peripheral lymphoid cell killing activity

To establish whether the cytolytic potential of woodchuck effector cells varies in different stages of WHV infection, circulating lymphoid cells collected from animals in the pre or acute phases of WHV infection and during advanced chronic infection were analyzed in a 5-h ^{51}Cr release assay with P815 cells. Figure 3-3 shows that the PBMC from animals with newly acquired infection displayed approximately 50% greater capability to kill P815 cells than PBMC from healthy animals at all three E:T ratios. In contrast, peripheral lymphoid cells from woodchucks with CH lysed P815 targets at substantially lower levels than those found for animals with the recently acquired infection. In CH, the rates of PBMC-mediated killing were the same or, at 50:1 and 25:1 E:T ratios, even lower

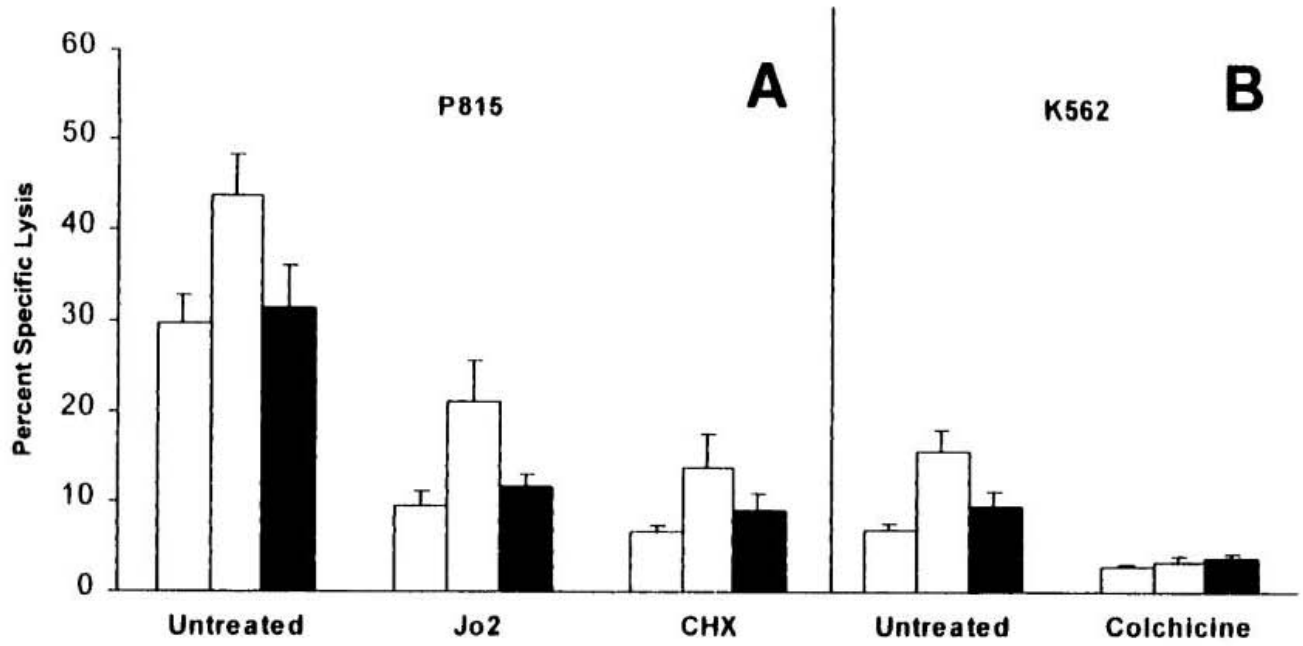


Figure 3-2. Non-Fas mediated killing of P815 and K562 target cells by PBMC from woodchucks with newly acquired and chronic WHV infections and from healthy controls. PBMC from WHV-negative, healthy animals (10 individual PBMC samples; open bars) and from woodchucks with recently induced WHV infection (14 PBMC samples; grey/dotted bars) or chronic WHV hepatitis (11 PBMC samples; solid bars) were incubated with ^{51}Cr-labeled P815 or K562 cells. (A) Fas-positive P815 cells were used untreated, pre-incubated with anti-Fas Jo2 mAb or tested in the presence of the protein synthesis inhibitor cycloheximide (CHX). (B) The Fas-negative K562 cells were used untreated or tested in the presence of the perforin release inhibitor colchicine. Results are presented at an E:T ratio of 50:1 from 5 experiments and expressed as a mean percentage of the specific lysis of P815 or K562 cells ± SEM.

than those detected for PBMC from healthy uninfected animals (Fig. 3-3). Of note is the fact that all PBMC samples from chronically infected woodchucks, independent of the severity of hepatitis and the duration of chronicity, uniformly displayed a low cytolytic activity comparable to that of PBMC from WHV-naive animals. Although the majority of PBMC samples from woodchucks with recently acquired pre-acute or acute WHV infection displayed markedly elevated killing of P815 targets, there were also some which induced cell death at levels similar to those typically found for PBMC from healthy woodchucks (see Fig. 3-4).

3.3.3 The increased PBMC cytotoxicity in acute WHV infection is perforin but not FasL-mediated

To uncover the mechanism of the augmented cell killing by lymphoid cells from acutely infected animals, P815 cells preincubated with anti-Fas Jo2 mAb were used as targets. In a parallel experiment, *the de novo* expression of FasL on woodchuck PBMC was inhibited by CHX before and during the cytotoxicity assay using P815 cells. As illustrated in Fig. 3-2A, anti-Fas antibody decreased killing of P815 targets by 52%, whereas CHX treatment of the same effector PBMC reduced killing of unaltered P815 cells by 68%. Thus, neither of these antagonistic treatments blocked the enhanced cytolysis caused by PBMC from acutely infected animals, indicating that the FasL-Fas interaction unlikely contributed to the increased killing. This was supported by the data obtained after subtracting values of the killing of P815 cells preincubated with Jo2 mAb

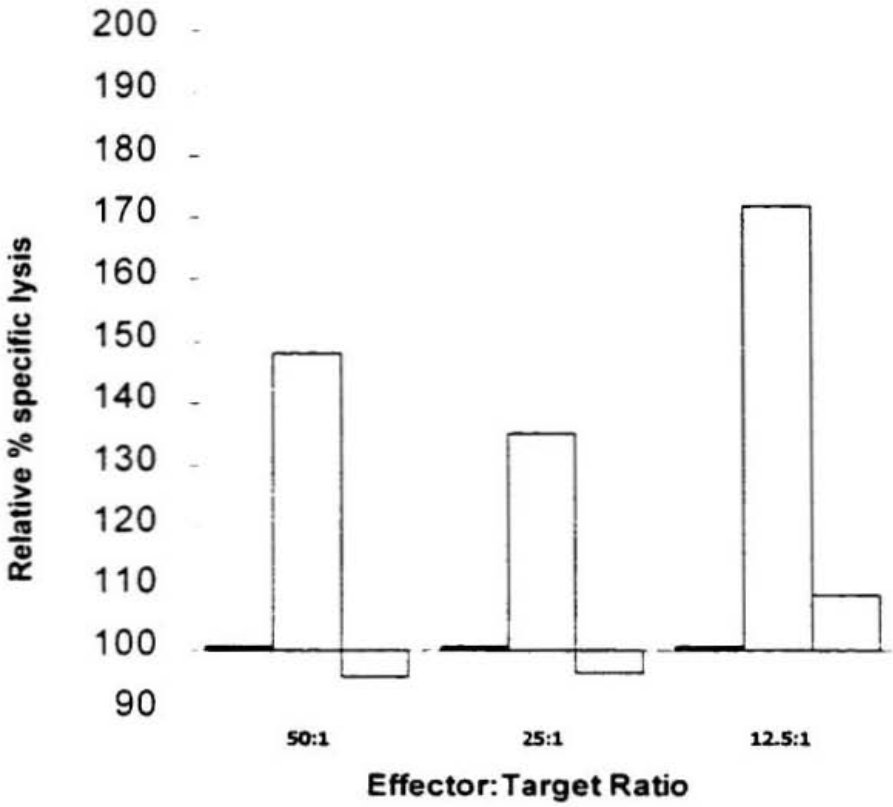


Figure 3-3. P815 cell killing by PBMC from woodchucks with newly acquired and chronic WHV infections. Circulating lymphomononuclear cells isolated from animals with newly induced preacute or acute WHV infection (14 PBMC samples; grey/dotted bars) or with chronic hepatitis (11 PBMC samples; open bars) and from healthy woodchucks (10 individual PBMC samples; solid bars) were incubated with ^{51}Cr-labeled P815 cells at indicated E:T ratios. Results for each E:T ratio are expressed as a percentage of the specific lysis induced by PBMC derived from healthy animals and tested at the same E:T ratio (taken as 100%).

(Fig. 3-2A; columns depicted as Jo2) from the values of the killing of untreated P815 cells (Fig. 3-2A; columns marked as untreated) by PBMC from different groups of the animals studied.

The final values were almost identical both for healthy animals (20.3%) and for woodchucks with recently acquired (22.6%) and chronic (19.8%) WHV infections. This suggested that Fas-mediated cytotoxicity participated to the same extent in the cytolysis caused by PBMC from either WHV-naive or infected animals and, therefore, that the enhanced cell killing in newly acquired infection was a consequence of the non-Fas-mediated pathway.

Circulating lymphoid cells from woodchucks with newly acquired WHV infection killed Fas-negative K562 cells with approximately twice the efficiency of PBMC from healthy or chronically infected animals (Fig. 3-2B). Only the perforin-release inhibitor colchicine blocked the increased cytotoxicity seen from PBMC from acutely infected animals. This finding provided strong evidence that the increased killing by PBMC from animals with recently acquired infection was non-Fas-mediated but most likely perforin related.

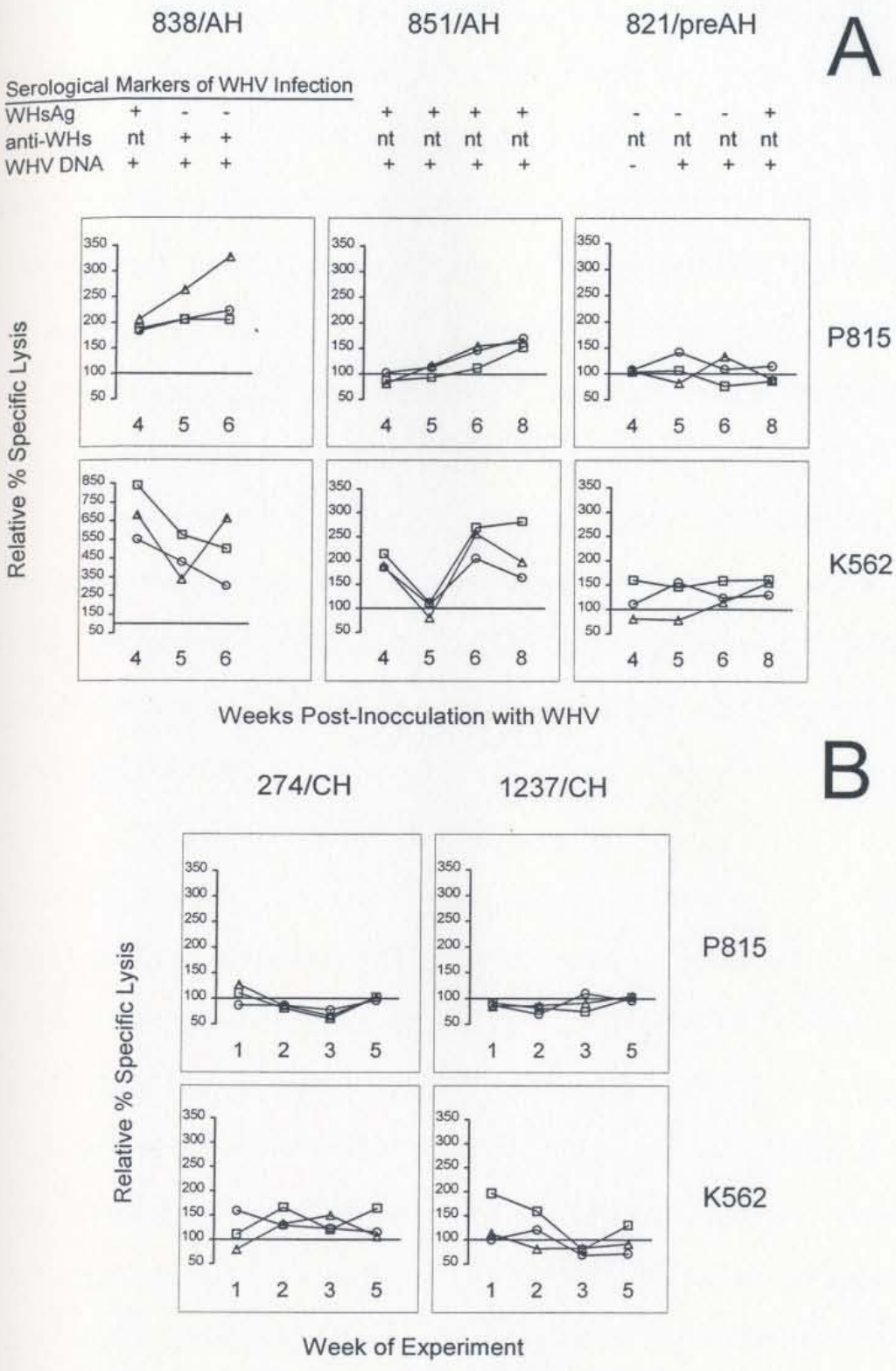
3.3.4 Profiles of Fas and perforin-mediated killing in newly acquired and chronic WHV infections

To investigate dynamics of the PBMC-mediated cell killing and the contribution of Fas and perforin-based cytotoxicity during newly induced and advanced chronic WHV infections, serial PBMC samples collected from 3

recently infected animals and 2 chronic WHV carriers were assayed using P815 and K562 cells as targets. Two of the acutely infected animals (838/AH and 851/AH; Fig. 3-4A), which were WHsAg reactive at 4 weeks post WHV inoculation (wpi), showed high and continuous increase in the cytotoxicity toward P815 cells at all three E:T ratios when sequential PBMC samples were investigated. One of these woodchucks (838/AH) cleared WHsAg and seroconverted to anti-WHs at 5 wpi, whereas the second (851/AH) remained WHsAg reactive during follow-up. Both animals showed histological features of AH in liver tissue samples obtained immediately after completion of the cytotoxic assays. In contrast, the killing exhibited by PBMC from 821/preAH animal, which was WHsAg negative until 8 wpi, fluctuated at levels comparable to those exhibited by PBMC from heathy controls tested in parallel (Fig. 3-4A). This animal showed normal liver morphology at the time of the cytotoxic assays despite the presence of WHV DNA and anti-WHc in the serum and was classified as being in the preacute phase of WHV hepatitis (Table 3-1). PBMC from woodchucks with CH (274/CH and 1237/CH) killed P815 cells at levels greatly below those found for woodchucks with acute infection and these levels were close to or slightly below those for healthy animals (Fig. 3-4B).

The level of non-Fas-mediated killing was determined in the same serial PBMC samples using K562 cell targets. In 838/AH animal, the lysis of these target cells decreased as the time from the WHV inoculation progressed and the anti-WHs developed. Nevertheless, despite the decrease in non-Fas-dependent

Figure 3-4. Profiles of PBMC-induced cytotoxicity toward P815 or K562 target cells determined in individual woodchucks during newly acquired and advanced chronic WHV infections. Sequential PBMC samples collected from 3 woodchucks between week 4 and 8 after WHV inoculation (A) and serial PBMC samples from 2 WHsAg-positive animals with chronic WHV hepatitis (B) were tested in parallel at given time points for cytotoxicity against Fas-positive P815 or Fas-negative K562 cells at E:T ratios of 50:1 (circles), 25:1 (squares) and 12.5:1 (triangles). Points represent the means of duplicate evaluations and are shown as a percentage of the cytotoxicity exhibited by PBMC from healthy animals against a given cell target and tested in the same assay. The results on serum WHsAg and anti-WHs reactivities and WHV DNA detection are presented for animals with newly acquired WHV infection in panel A. All animals chronically infected with WHV (shown in panel B) were WHsAg and WHV DNA positive at all time points tested.


838/AH
851/AH
821/preAH
A
Serological Markers of WHV Infection
WHsAg + - - + + + + - - - +
anti-WHs nt + + nt nt nt nt nt nt nt nt
WHV DNA + + + + + + + - + + +
Relative % Specific Lysis
P815
K562
Weeks Post-Inocculation with WHV
B
274/CH
1237/CH
Relative % Specific Lysis
P815
K562
Week of Experiment

killing over time, the level of cytolysis was evidently elevated (3 to 8.5-fold) over the cytotoxicity displayed by PBMC from healthy and chronically infected animals at all time points and E:T ratios tested (Fig. 3-4B). Killing exhibited by PBMC from 851/AH woodchuck was approximately two-fold greater at all E:T ratios than that for healthy controls and animals with CH, except 5 wpi (Fig. 3-4A). In contrast to 838/AH and 851/AH, 821/preAH animal demonstrated a slightly elevated (up to 1.5-fold) K562 cell killing and this level remained relatively stable over the 5-week test period.

The Fas-independent cytolysis exhibited by serial PBMC samples collected during the 5-week investigation period from chronically infected animals was at the same level or moderately augmented in comparison to PBMC from healthy woodchucks (Fig. 3-4B). In contrast to acutely infected animals, the level of the K562 cell killing in CH showed no or minimal fluctuations.

3.4 DISCUSSION

The present study demonstrates that circulating woodchuck lymphoid cells are capable of direct lysis of heterologous target cells through activation of both FasL/Fas and perforin death pathways, that this cytopathic effect requires PHA and, therefore, effector and target cell contact, and that AH is selectively associated with the enhanced capacity of lymphoid cells to induce cell death via the perforin-dependent mechanism. The employed two-cell target cytotoxicity assay system, which is based on the cross-species productive interaction

between woodchuck lymphoid cell FasL and Fas on murine P815 target cells and on the cross species perforin-mediated cell killing, showed that Fas-dependent cytotoxicity could be the main pathway by which woodchuck PBMC induce cell death. The accuracy of this determination was ascertained using Jo2 mAb which blocks cell surface Fas antigen and by treatments with CHX and colchicine to inhibit FasL expression and microtubule-dependent perforin release, respectively. In addition, the obtained results demonstrate that close cross-species compatibility exists in the FasL/Fas and perforin-granzyme systems between woodchuck, mouse and human, as has been previously shown between human and mouse (Takahashi et al., 1994; Tanaka et al., 1995; Smyth et al., 1996). In the case of FasL, this was further supported by sequence analysis of a 508 bp-fragment that included complete sequences of exons 2 and 3 and flanking fragments of exons 1 and 4 of the woodchuck FasL (Hodgson and Michalak; GeneBank accession number AF152368). This analysis showed 89% and 86% nucleotide homology with the human and mouse FasL sequences, respectively (data not shown). Additionally, a 700 bp fragment of woodchuck perforin (Hodgson and Michalak; GeneBank accession number AF298158) demonstrated 85% similarity to human perforin and 74% homology to mouse pore forming protein. These sequences are available in Appendix B.

Although woodchuck peripheral lymphoid cells killed virus uninfected cells mainly through the FasL/Fas pathway, the increased cytotoxicity found for animals with recently acquired WHV infection was entirely mediated by perforin

release. This indicates that the perforin-dependent killing is selectively activated in the early phase of WHV infection. In this respect, considerable evidence has been accumulated to conclude that the perforin-dependent cytotoxicity is the principle mechanism by which NK cells eliminate targeted cells (Kagi et al., 1994; Sayers et al., 1998), that the peak of NK cell cytolytic activity and proliferation usually occurs shortly after viral invasion (reviewed by See et al., 1997), and that NK cells are an important element of the natural resistance to many viruses (reviewed by See et al., 1997 and Smyth and Trapani, 1998). Considering our finding in the context of the data previously reported by others, it is likely that the increased perforin-mediated killing in the early stage of WHV infection was a consequence of NK cell activation, although, at this stage, we do not have reliable tools to separate woodchuck NK cells from other circulating effector cells. Nevertheless, the augmented killing of K562 cells by PBMC from acutely infected animals corroborates the previous observations on the increased cytotoxicity toward K562 cells by PBMC from patients with AH type B, which has been interpreted as indicative of enhanced NK cell cytotoxicity (Chemello et al., 1986; Echevarria et al., 1991). The ability of the host to mount a strong cytotoxic NK cell response very early in the course of hepadnavirus infection could play a decisive role in controlling virus spread and limiting progression of the disease. The observed co-occurrence of the initially very strong PBMC cytotoxic activity followed by a swift seroconversion to anti-WHs and recovery in one of the woodchucks with AH in our study (838/AH; Fig. 3-4a) could be interpreted in

support of this possibility.

The main advantage of our experimental approach is the ability to dissect the contribution of the FasL/Fas system from the perforin-dependent killing induced by the same effector cells and to assess simultaneously the cytopathic effect caused by PBMC derived from different stages of viral hepatitis and from healthy animals. By parallel examination of the effector cells from acute and chronic infections using the two-cell cytotoxicity assay system supplemented with selective inhibition of reactant molecules of both the FasL/Fas and perforin pathways, we have circumvented concerns raised by other authors that the target cell itself may have the last word in selecting its mode of execution (Nakamoto *et al.*, 1997). This allowed for an unbiased demonstration that chronic WHV hepatitis is accompanied by cytotoxic activity in the peripheral blood equal to or lower than in healthy controls and that there is no relation between the level of the PBMC-induced cell killing and the severity of hepatitis or the duration of chronic WHV infection. This finding appears to be comparable to the reported reduced cytotoxicity of circulating NK cells in patients with chronic active and persistent hepatitis B (Actis *et al.*, 1991; Ono *et al.*, 1996), although elevated levels of this cell activity has also been observed in an aggressive form of CH type B (Ono *et al.*, 1996). Our study also implies that, in contrast to acute infection, the levels of nonspecific cell killing remain relatively stable in chronic infection, as was shown by analysis of serial PBMC samples collected during the 5-week examination period of 2 of the chronically infected animals.

Of note, in the above context, is the earlier observation that HBsAg decreases, in a dose-dependent manner, NK cell cytotoxicity *in vitro* by interfering with their binding to target cells (De Martino *et al.*, 1985; Azzari *et al.*, 1992). If the continuous persistence of large quantities of circulating HBsAg and WHsAg (which is typical for chronically infected humans and woodchucks, respectively) in fact suppresses NK cells, it would be reasonable to expect relatively normal or decreased function of these cells reflected in the suppressed PBMC cytotoxic activity. The same could also be true for intrahepatic NK cells. It has been well documented that chronic WHV hepatitis is accompanied by an extensive, irreversible accumulation of WHsAg in the outer membranes of infected hepatocytes, as opposed to AH (reviewed by Michalak, 1998). It is possible that the incorporation of the saturable quantities of virus envelope material into hepatocyte surface, coexisting with the large amounts of virus envelope antigen in serum, acts as a negative modulator for *in situ* NK cell cytotoxicity in chronic WHV infection. This might be an important element of virus strategy devised to protect infected cells against immunocytolysis and could be in agreement with our recent observation of the impaired expression of the woodchuck MHC class I molecules on hepatocytes in chronic WHV infection (Michalak *et al.*, 2000), whose depletion is known to upregulate local NK cell activity (reviewed by Brutkiewicz and Welsh, 1995). Our present data suggest that virus nonspecific immunity may contribute to the induction and perpetuation of chronic liver disease in hepadnaviral infection. The detected difference in the

efficiency of the perforin-mediated cell killing between acute and chronic WHV infections provides basis for further investigations on the pathogenic role of this form of cytotoxicity in HBV-infected humans and in animal models of hepatitis B.

CHAPTER 4:

POSTTRANSCRIPTIONAL INHIBITION OF MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I PRESENTATION ON HEPATOCYTES AND LYMPHOID CELLS IN CHRONIC WOODCHUCK HEPATITIS VIRUS INFECTION*

*The major components of this chapter were published in the Journal of Virology, 2000 (vol.74: pp. 4483-4494). The exception to this is Figure 4-1 (Determination of conditions for quantitation of woodchuck IFNγ). My specific contributions to this study comprises all of the molecular biology data, including cloning of woodchuck genes, Northern blot hybridization, quantitative PCR analyses, and evaluation of WHV DNA by dot blot hybridization.

4.0 SUMMARY

WHV, similar to human HBV, causes acute liver inflammation that can progress to CH and HCC. WHV also invades cells of the host lymphatic system where it persists for life. We report here that acute and chronic hepadnaviral hepatitis is characterized by a profound difference in the expression of MHC class I molecules on the surface of infected hepatocytes and, notably, lymphoid cells. While acute WHV infection is accompanied by the enhanced hepatocyte surface presentation of MHC class I and upregulated transcription of the relevant hepatic genes, inhibition of class I antigen display on liver cells is a uniform hallmark of chronic WHV infection. This inhibition in CH occurs despite augmented (as in acute infection) expression of hepatic genes for MHC class I heavy chain, β2-microglobulin and transporters associated with antigen processing (TAP1 and TAP2). Further, the class I antigen inhibition is not related to histological severity of hepatocellular injury, the extent of lymphocytic infiltrations, the level of intrahepatic IFNγ induction or hepatic WHV load.

Importantly, the antigen expression is also inhibited on organ lymphoid cells of chronically infected hosts. The results obtained in this study demonstrate that the defective presentation of MHC class I molecules on cells supporting persistent WHV replication is due to viral posttranscriptional interference, mechanisms of which are described in Section 1.7.6. This event may diminish the susceptibility of infected hepatocytes to virus-specific T cell-mediated elimination, hinder virus clearance, and deregulate the MHC class I-dependent functions of the host's immune system. This multifarious effect could be critical for perpetuation of liver damage and evasion of the anti-viral immunological surveillance in chronic infection and, therefore, supportive of hepadnavirus persistence.

4.1 INTRODUCTION

The host's cellular immune responses directed against HBV peptides displayed on infected hepatocytes are considered to be crucial in the induction of hepatic damage and likely contribute to both cytopathic and noncytopathic elimination of virus from infected livers (Section 1.8; Chisari and Ferrari, 1995; Guidotti *et al.*, 1999). Although a diminished anti-virus CTL response is thought to be the main contributor to the pathogenesis of CH type B, the basis of this hindrance remains uncertain. Since triggering and strength of anti-viral CTL responsiveness depends to a significant degree on the efficient cell surface presentation of viral peptides by MHC class I, delineation of the changes and the

mechanisms modulating their expression in the course of viral hepatitis might be decisive for understanding the pathogenesis of protracted liver disease and virus persistence in hepadnaviral infection. The above reasoning has proven to be correct for other virus infections. These investigations have shown that virus-induced alterations in the MHC class I surface display play an important role in viral pathogenesis and persistence (Früh *et al.*, 1999; Miller and Sedmak, 1999; Oldstone, 1997; Rinaldo, 1994).

Lymphotropism is a common feature of many viruses, including hepadnaviruses, capable of induction of long-term infection in the host. However, the direct link between HBV lymphotropism and chronic infection is not yet established. It is conceivable that, like in other viral infections, invasion of the lymphatic system may have a detrimental effect on a variety of the host's immune responses. Among others, the virus may alter the display of MHC class I molecules on lymphoid cells and, in consequence, impair a variety of cell immune functions.

In this study, we investigated the WHV-woodchuck model to elucidate the relationship between acute and chronic phases of hepadnavirus infection and the MHC class I presentation on cells naturally supporting WHV replication. We have also searched for a molecular basis of the discovered disparities in the MHC class I expression. In contrast to the past evaluations, which brought conflicting conclusions based on immunohistochemical staining of liver tissue from HBV-infected patients (Chu *et al*, 1988; Lau *et al.*, 1993; Pignatelli *et al.*,

1986), purified cell plasma membranes, quantitative and highly sensitive immunoblotting techniques and molecular methods measuring MHC class I affiliated gene activity were used in this study. We report that acute and chronic WHV infections are characterized by a profound difference in presentation of class I antigen on hepatocytes and organ lymphoid cells. Hence, while AH is accompanied by an augmented expression of class I molecules on liver cells, but an unaltered display on lymphoid cells, inhibition of the antigen presentation on both cell types is a uniform characteristic of chronic WHV infection. Interestingly, this inhibition in CH occurs despite upregulated transcription of the hepatic genes encoding MHC class I heavy (α) and light (β2-microglobulin) chains, and transporters associated with antigen presentation (TAP1 and TAP2), implying that the defect in class I molecule presentation occurs posttranscriptionally.

4.2 MATERIALS AND METHODS

4.2.1 Animals and categories of WHV infection.

Fourteen woodchucks constituted the main study group (Study Group 1). Eight animals (WM 2070, WF 2112, WF 2114, WF 2131, WM 2121, WF 2160, WM 2167 and WM 2171) were infected intravenously with WHV (Michalak and Lin, 1994, Michalak *et al.*, 1999). Four others (WF 2020, WF 2030, WM 2040 and WM 2150) had naturally acquired, WHsAg-positive CH which was monitored for up to 21 months prior to the start of the experiment. In this study group, two healthy animals (WM 2075 and WF 2078) were examined as controls (Table 4-

1). All the woodchucks, except WF 2112 and WM 2121, were a part of our previous study aimed at identification of molecular species of WHV structural proteins and the nature of their interactions with hepatocyte plasma membranes (HPM) (Michalak and Lin, 1994).

The serological status of WHV infection was determined by testing sequential sera for WHsAg, anti-WHs and anti-WHc by immunoassays described in Section 2.3. Serum WHV DNA was determined by slot-blot hybridization (Section 2.4) and, when negative, by polymerase chain reaction (PCR) using WHV core gene specific primers, as described in Section 2.8.2.

Histological examination of liver samples, obtained by laparotomy 3 to 4 weeks prior to the experiment or at the time of liver perfusion, was done after conventional processing to paraffin. Paraffin sections (4 µm) were stained with hematoxylin and eosin, Masson-trichrome, periodic acid-Schiff or impregnated with silver (Michalak and Lin, 1994). Morphologic assessment of liver damage, referred to as histologic degree of hepatitis, was based on criteria described in our previous works (Michalak and Lin, 1994, Michalak *et al.*, 1990).

Based on serological and histological assessments, woodchucks in Study Group 1 were classified to three categories: (1) healthy or recovered from AH (n = 4); (2) with AH (n = 4), and (3) persistently infected with serologically and histologically evident CH (n = 6) (Table 4-1). Histological examination showed a highly variable degree of inflammatory liver injury in the animals examined (Table 4-1). The changes ranged from minor lesions (grade I) seen in 2 woodchucks

Table 4-1. Immunovirological and histological characteristics of WHV infection in animals studied at the time of analysis of MHC class I expression.

Category of disease and animal [a]	Duration of WHs antigenemia (wk)	WHV Serology and DNA				Histological degree of hepatitis [c]
		WHsAg	Anti-WHs	Anti-WHc	WHV DNA [b]	
GROUP I						
Healthy						
WM 2075	0	-	-	-	-	0
WF 2078	0	-	-	-	-	0
Resolution of AH						
WF 2131	3	-	-	+	(+) [d]	0/I
WF 2160	2	-	+	+	(+) [d]	0/I
AH						
WM 2070	8	+	-	+	+	I
WM 2167	8	+	-	+	+	II
WM 2121	6	+	-	+	+	III
WM 2171	10	+	-	+	+	III
CH						
WF 2114	46	+	-	+	+	I
WF 2030	>60	+	-	+	+	II
WM 2040	>26	+	-	+	+	II
WF 2020	>84	+	-	+	+	III
WF 2112	26	+	-	+	+	III
WM 2150	>49	+	-	+	+	III
GROUP 2						
Healthy						
WM 3069	0	-	-	-	-	0
WF 3299	0	-	-	-	-	0
AH						
WF 3392	1	+	-	+	+	I
WF 3838	3	+	-	+	+	II
WM 3158	6	+	-	+	+	III
CH						
WF 4832	>72	+	-	+	+	I
WF 3349	>83	+	-	+	+	II
WF 4980	>65	+	-	+	+	II
WF 4751	>55	+	-	+	+	III

a. WM, woodchuck male; WF, woodchuck female

b. Evaluated by slot-blot hybridization using WHV DNA as a probe (sensitivity 10^6 to 10^7 vge/ml).

c. Severity of hepatitis from 0 to III reflects the degree of liver injury determined on the basis of grades separately assigned for hepatocellular, extracellular intralobular and portal alterations (Michalak *et al.*, 1990).

d. Detected by nested PCR with WHV core gene-specific primers and Southern blot hybridization of the amplified virus sequences (sensitivity 10 to 10^2 vge/ml).

(WM 2070 and WF 2114), through mild acute or CH (grade II; WF 2030, WM 2040 and WM 2167) to severe liver injury with heavy lymphocytic infiltrations and prominent necrosis of parenchyma (grade III; WF 2020, WF 2112, WM 2121, WM 2150 and WM 2171). Liver biopsies from healthy animals (WM 2075 and WF 2078) did not show morphological alterations, whereas hepatic changes in woodchucks which resolved AH (WF 2131 and WF 2160) were minimal and consisted mainly of minor lymphomononuclear infiltrations in some portal areas and scanty intralobular infiltrations surrounding singular degenerating hepatocytes, as reported previously (Michalak *et al.*, 1999).

Liver and spleen specimens from 7 other woodchucks with detailed characterization of serological and histological profiles of AH (n =3) or CH (n =4) and from 2 healthy animals (Study Group 2) were also investigated (Table 4-1). Hepatic histological lesions in this group varied from none in healthy animals (WM 3069 and WF 3299), through minor (grade I; WF 3392 and WF 4832) and moderate (grade II; WF 3349, WF 3838, and WF 4980) to severe (grade III ; WM 3158 and WF 4751) in animals with either AH or CH. In addition, several other woodchucks with well defined status of WHV infection were used as a source of PBMC to test the MHC class I display on the surface of circulating lymphoid cells. For this purpose, freshly isolated PBMC from 2 animals with AH, 2 animals convalescent from AH, 4 with CH, and 4 healthy animals were examined by fluorescence activated cell sorting (FACS; see below). In a parallel experiment, PBMC collected from 2 woodchucks prior to WHV infection and then during AH,

and from 2 other animals before WHV administration, during AH and then in advanced CH were used for isolation of PBMC plasma membranes and evaluation of MHC class I heavy chain presentation by immunoblotting.

4.2.2 Cell and plasma membrane isolation.

Hepatocytes were isolated by two-step collagenase perfusion of livers from animals in Study Group 1 using methods reported previously (Michalak and Churchill, 1988, Michalak *et al.*, 1989, Michalak and Lin, 1994). HPM were purified from the isolated hepatocytes by differential fractionation in sucrose gradients (Michalak and Churchill, 1988). Purity of HPM was determined by measuring activities of marker enzymes for plasma membranes (5'-nucleotidase), microsomes (glucose-6-phosphatase), and mitochondria (cytochrome C oxidase)(Michalak and Churchill, 1988; Michalak and Lin, 1994). These evaluations showed that the HPM were essentially free from subcellular contamination and of comparable purity. Kidney plasma membranes (KPM) were isolated from the woodchuck kidney homogenates following the HPM isolation procedure.

Splenic lymphomononuclear cells (splenocytes), containing mainly lymphocytes, were prepared by two sequential density gradient centrifugations in Histopaque 1119 (Sigma) as described previously (Michalak *et al.*, 1995). After depletion of residual erythrocytes, spleen plasma membranes (SPM) were purified by hypotonic treatment and sucrose gradient centrifugation (Michalak *et*

al., 1995).

PBMC were isolated from freshly drawn EDTA treated blood by gradient centrifugation in Histopaque (Jin *et al.*, 1996; Michalak *et al.*, 1995). Plasma membranes were prepared by hypotonic shock and brief sonication, and subsequent removal of nuclei and cellular debris by centrifugation (Jin *et al.*, 1994). Protein content was determined by a bicinchoninic acid assay (Sigma).

4.2.3 Monoclonal antibody to woodchuck MHC class I heavy chain.

Mouse monoclonal antibody (B1b.B9 mAb) against a nonpolymorphic epitope of the woodchuck MHC class I heavy chain was generated and characterized in our previous study (Michalak *et al.*, 1995). This antibody recognizes two polypeptide species of woodchuck class I heavy chains with molecular masses of 43- and 39-kDa.

4.2.4 Western and dot imunoblotting.

Plasma membranes, subcellular fractions or tissue homogenates were immobilized at the desired protein concentration onto nitrocellulose (NC; 0.45-µm pore size; BioRad Laboratories), exposed to a blocking solution containing 3% bovine serum albumin, 1% normal goat serum, 0.05% Tween-20 and 0.001% sodium azide in PBS, and incubated with B1b.B9 mAb under conditions described before (Michalak *et al.*, 1995). After incubation and washing, the blots

were exposed to goat anti-mouse conjugated with alkaline phosphatase (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), washed, and reactions developed (Diao and Michalak, 1997).

For Western immunoblotting, purified membrane preparations were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 20 µg protein/lane and separated proteins electrotransferred onto NC, and immunoblotted as described before (Diao and Michalak, 1997, Michalak and Lin, 1994). Efficiency of protein transfer and molecular masses of the detected polypeptide species were determined using prestained molecular weight markers (BioRad Laboratories). The relative expression of MHC class I heavy and light chains was determined by densitometry using a computerized Chemi-Imager 4000 System (Canberra-Packard Canada Ltd.).

4.2.5 Immunohistochemical staining.

Cryostat sections 4 µm thick were cut from frozen liver and spleen tissue blocks, air-dried, and fixed in cold acetone-chloroform (1:1) mixture for 5 min at ambient temperature (Michalak *et al.*, 1995). Sections were hydrated in phosphate-buffered saline, pH 7.4 (PBS), incubated for 45 min with B1b.B9 mAb or PBS (control), and washed for 30 min in 3 changes of PBS. Subsequently, sections were incubated with FITC-conjugated anti-mouse IgG (H+L) antibody (Jackson ImmunoResearch Laboratories Inc.) for 30 min, washed 3 times for 10

min in PBS, mounted in 20% glycerol buffered in PBS, and examined using an epifluorescent Leitz-Diaplan microscope.

4.2.6 Fluorescence activated cell sorting

Freshly isolated PBMC, approximately 5 x 10^5 cells/sample with viability greater than 95% by trypan blue exclusion, were incubated with B1b.B9 mAb or PBS (control) and then with anti-mouse antibody labeled with FITC (Jackson ImmunoResearch Laboratories Inc.) by a procedure described previously (Michalak *et al.*, 1995). Cell analysis was done using a FACS Star-Plus flow cytometer (Becton-Dickinson, Mississauga, Ontario, Canada).

4.2.7 Nucleic acid extractions.

DNA and RNA were isolated using standard procedures described in Section 2.6. Nucleic acids were quantitated by standard spectroscopic analysis and stored in small aliquots at -80°C prior to use.

4.2.8 Dot-blot detection of tissue WHV DNA.

For WHV DNA hybridization, 5 µg of liver or 10 µg of spleen DNA was denatured by boiling for 10 min in 200 µl of 6X SSC, chilled on ice, and immobilized on a nylon membrane (Hybond-N; Amersham) using a BioDot SF apparatus (BioRad Laboratories). The membrane was hybridized for 16 h at

65°C to a full-length, linearized, cloned WHV DNA (Pardoe and Michalak, 1995) labeled with [^{32}P]-dCTP (3000 Ci/mmol) by a random primer method (Rediprime; Amersham). The blot was washed to final stringency of 0.2X SSC, 0.1% SDS for 30 min at 65 °C, and exposed to X-ray film (XRP-1 or XAR-5; Eastman Kodak Co.) with an intensifying screen or to a phosphor screen (Canberra-Packard Canada Ltd.). For estimation of the levels of WHV DNA expression, autoradiographic or phosphor images of hybridization signals were quantitated for equivalence with 10-fold serial dilutions of recombinant, complete WHV DNA using a chemi-image analyzer or a Cyclone Phosphor Imaging System (Canberra-Packard Canada Ltd.), respectively.

4.2.9 Cloning of woodchuck genes.

Total RNA isolated from spleen of a healthy woodchuck was reverse transcribed to cDNA then amplified by PCR using degenerate oligonucleotide primers. For amplification of woodchuck MHC class I heavy chain sequence, the sense primer MHC-W (5'-AGTCTTTCCGAGTGAACCTGCGGAC) and the antisense primer W-CHM (5'-TCCTTTCCCATCTGAGCTGTGCTTC) were used. The woodchuck β2-microglobulin was amplified with the sense and antisense degenerative primers β2M-plus (5'-ATGKCTCGCTCSGTGRCC) and β2M-minus (5'-TTACATGTCTCGRTCCCAS), respectively. The woodchuck TAP1 sequence was amplified with the sense primer APT-1 (5'-TTCTTYACRGGCCGCMTCACTGAC) and the antisense primer 1-PAT (5'-

AGGGCACTGGTGGCATCRTC), whereas TAP2 sequence with the sense primer APT-2 (5'-TTCGGGTCGTGTRATTGACATCC) and antisense primer 2-PAT (5'-CTTSACAGAACCSGAGAACAGCAC). For identification of the woodchuck CD3 gene, whose transcripts are specific for T lymphocytes, primers CD3P (5'-CTGGGACTCTGCCTCTTATC) and CD3M (5'-GCTGGCCTTTCCGGATGGGCTC) with sequences essentially identical as those reported by others (Nakamura *et al.*, 1997), were used. Woodchuck IFNγ was amplified with primers designed in this laboratory W-IFNG (5'-GGCCTAACTCTCTCTGAAACG) and W-GNFI (5'-GAGGACTGTTATTTGGATGC). In addition, an approximately 315-bp fragment of woodchuck β-actin and a 570-bp fragment of glyceraldehyde-3-phosphate dehydrogenase (GADPH) were generated by PCR using woodchuck liver cDNA and oligonucleotide primers published previously for human β-actin (Fuqua *et al.*, 1990) and mouse GADPH (Ju *et al.*, 1995). For PCR amplification of cDNA to be cloned, samples were denatured at 94°C for 5 min, then 35 cycles of 94°C for 1 min, 52°C for 2 min and 72°C for 3 min at each step were carried out. The last cycle was followed by an elongation step at 72°C lasting 10 min. PCR amplifications were carried out in a TwinBlock Thermal cycler (Ericomp Inc.) using 5 µl of the reverse transcription reaction product and a standard reagent mixture described previously (Michalak *et al.*, 1999). The specificity of the amplified woodchuck DNA fragments was verified by Southern blot analysis using internal oligonucleotide probes except for the MHC class I heavy chain

which was probed with a [^{32}P]-labeled fragment of rabbit MHC class I (exon 4) excised from plasmid pUC12-RLA-A (ATCC 77230; Marche *et al.*, 1985). After confirmation of specificity, the DNA amplicons were purified from low-melting point agarose using the Wizard PCR Preps DNA Purification System (Promega Corp.) and cloned into vector pCRII using a TA Cloning Kit (Invitrogen). After plasmid amplification, the specificity and orientation of the fragments cloned were validated by sequencing either by using the *f*mol DNA Sequencing System (Promega Corp.) or a fluorescence-based automated sequence analyzer (LI-COR).

4.2.10 Gene expression analysis.

Liver and spleen RNA was analyzed for MHC class I heavy chain, β2-microglobulin, TAP1 and TAP2, as well as, for CD3, WHV, β-actin and GAPDH expression by Northern blot hybridization (Section 2.13). The blots were hybridized for 18 h at 42°C to probes labeled with [^{32}P] using the Strip-EZ DNA kit (Ambion Inc., Austin, TX). After hybridization, membranes were washed to a final stringency of 0.2X SSC, 0.1% SDS at 42°C and exposed for autoradiography or phosphoimage analysis. Prior to rehybridization, probes were stripped from the membranes following Section 2.13.3. The signal intensity was quantified and equalized to β-actin expression by densitometry.

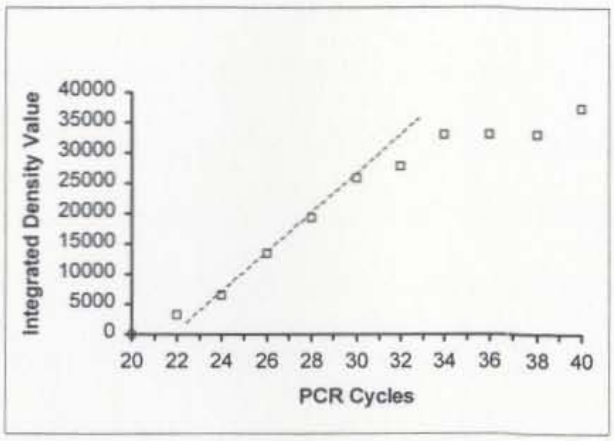
Intrahepatic IFNγ RNA expression was estimated by relative PCR using woodchuck liver cDNAs and oligonucleotide primers presented above. PCR was

performed in the linear amplification range under the following conditions: 94°C for 5 min, 51°C for 2 min and 72°C for 1 min in the first cycle, then 94°C for 1 min, 52°C for 1.5 min and 72°C for 1.5 min for 32 cycles, followed by the final extension at 72°C for 10 min (Fig. 4-1). As loading controls, the same cDNA samples were amplified with β-actin primers. The resulting PCR products were analyzed by Southern blot hybridization with appropriate cloned probes and compared to β-actin expression with a phosphoimage analyzer.

4.2.11 Nucleotide sequence accession numbers.

The accession numbers for the woodchuck nucleotide sequences derived in this study submitted to GenBank were as follows: MHC class I heavy chain, AF232723; TAP1, AF232724; TAP2, AF232725; β2-microglobulin, AF232726; CD3, AF232727; IFNγ, AF232728; GADPH, AF232729, and β-actin, AF232730.

A



B

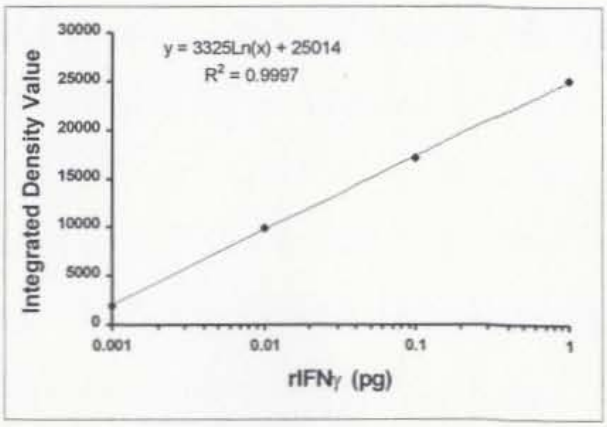


Figure 4-1. Determination of conditions for quantitation of woodchuck IFNγ.
A. PCR amplification of a constant amount of woodchuck recombinant IFNγ (rIFNγ) over increasing cycle number. Woodchuck rIFNγ at 1 pg was amplified for 20 to 40 cycles under the following conditions: denaturation, 1 min at 94°C; annealing, 1.5 min at 52°C; and extension, 1.5 min at 72°C. The integrated density values of the amplified products were determined by a chemi-imager, plotted and the linear range marked with a dashed line.

B. Amplification of 10-fold serial dilutions of rIFNγ at 32 PCR cycles. rIFNγ at concentrations between 1 pg and 1 fg were amplified for 32 cycles under conditions described in A. The densities of the amplified signals are presented as integrated density values. The coefficient factor (R^2) of the plotted slope is above 0.99 indicating a high degree of correlation.

4.3 RESULTS

4.3.1 Upregulated expression of MHC class I on hepatocyte surface is a hallmark of acute but not chronic WHV infection.

Liver sections from healthy and convalescent animals showed MHC class I heavy chain immunofluorescent staining of sinusoidal lining cells and the bile duct epithelia, but little or no expression on hepatocyte outer plasma membranes (Fig. 4-2A). In the livers of animals with AH, a strong staining of periportal and intralobular inflammatory infiltrates and membranes of the hepatocytes adjacent to these infiltrates were seen. The lobular hepatocytes, not associated with inflammatory cells, showed a membranous staining of part or the entire surface, while their cytoplasm essentially remained negative (Fig. 4-1B). Woodchucks chronically infected with WHV had an enhanced display of the class I antigen on hepatocytes only in the areas of inflammatory infiltrations. Hepatocytes distant from the infiltrates were nonreactive (Fig. 4-2C), however, some cells, usually occurring in clusters, had weak staining at their outer membranes. Overall, the MHC class I pattern was noticeably different in AH and CH (Fig. 4-2B, 4-2C), but there was no relation between this display and overall histological severity of liver injury (assessed by criteria outlined in Section 2.5). There also was no differences in the MHC antigen staining on sinusoidal lining endothelium or on the bile duct epithelium in livers from infected, healthy or recovered animals. Sections incubated using the second layer antibody alone as a control showed the same minimal background staining in all livers examined.

All HPM preparations isolated from Study Group 1 and probed with B1b.B9 mAb by Western blotting, demonstrated the MHC class I heavy chain 43-kDa polypeptide, although the protein was displayed at markedly higher density in HPM from animals with AH (Table 4-2 and Fig. 4-3). The HPM from healthy and convalescent woodchucks, as well as those from animals with CH, showed noticeably lower expression of the polypeptide. In addition to the 43-kDa species, the 39-kDa polypeptide was detected on hepatocyte outer membranes from AH (Fig. 4-3 and Table 4-2). This band was not identifiable on blots of the membranes from healthy, recovered or chronically infected woodchucks. The detection of the 39-kDa polypeptide was consistent with our previous finding that only woodchuck cells displaying surface MHC class I molecules at the highest densities (e.g., normal splenic lymphoid cells) show both 43 and 39-kDa heavy chain species (Michalak *et al.*, 1995).

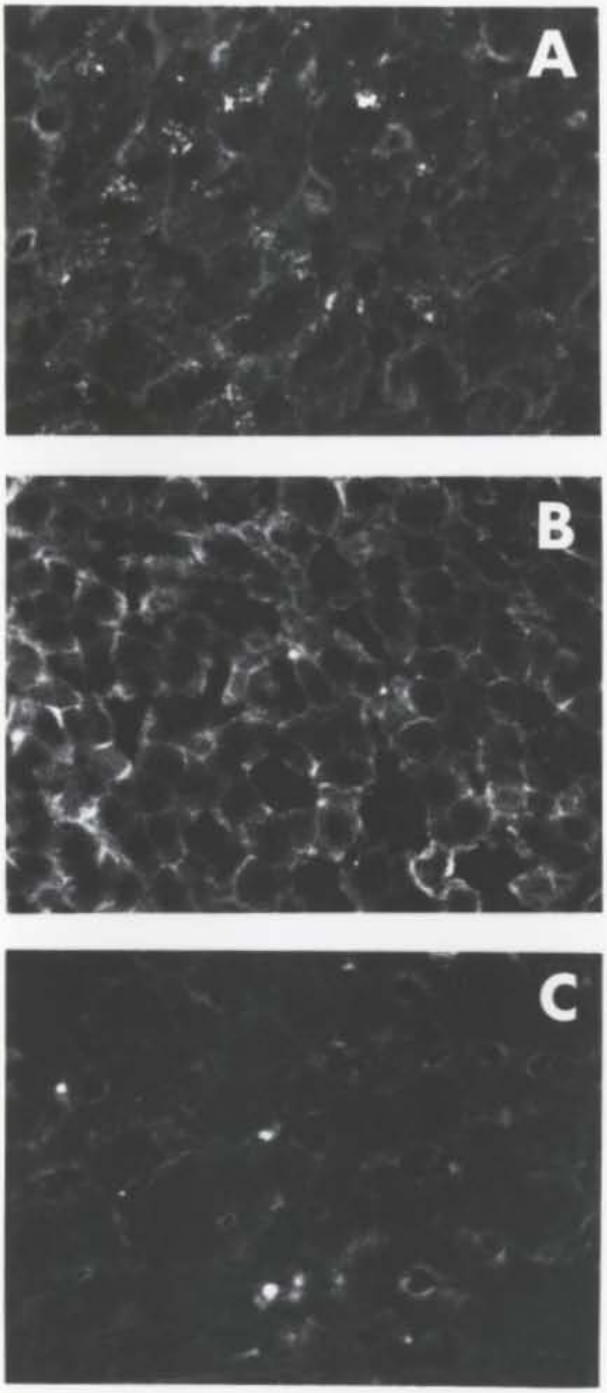
Densitometric quantitation of the class I heavy chain signals detected by Western (Fig. 4-3 and Table 4-2) or immunodot (Table 4-2) blotting confirmed a significant difference in the hepatocyte surface presentation of MHC class I between acutely infected and the healthy, recovered or chronically infected animals. Thus, HPM from either healthy or convalescent woodchucks, which had normal (WM 2075 and WF 2078) or nearly normal (WF 2131 and WF 2160) liver histology, displayed approximately the same amounts of class I heavy chain. In contrast, the quantity of MHC on HPM from AH was on average 3.5-fold greater

Table 4-2. Hepatocyte outer plasma membrane expression of MHC class I heavy chain in woodchucks with WHV hepatitis and in control animals.

Category of disease and animal[a]	Histological degree of hepatitis	HPM[b] MHC class I heavy-chain expression[c]	
		43 kDa protein	39 kDa protein
Healthy			
WM 2075	0	1.1	0
WF 2078	0	0.9	0
Resolution of AH			
WF 2131	0/I	1.4	0
WF 2160	0/I	1.1	0
AH			
WM 2070	I	3.1	0.3
WM 2167	II	3.0	0
WM 2121	III	3.5	0.6
WM 2171	III	4.0	3.3
CH			
WF 2114	I	0.6	0
WF 2030	II	0.8	0
WM 2040	II	1.0	0
WF 2020	III	0.7	0
WF 2112	III	1.4	0
WM 2150	III	0.9	0

[a] WM, woodchuck male; WF, woodchuck female
[b] HPM, hepatocyte plasma membranes
[c] Assessed by Western blotting with B1b.B9 mAb against woodchuck MHC class I heavy chain and expressed using a scale from 0 to 4, comparatively presenting the densities of the 39- and 42-kD polypeptide bands based on integrated chemi-image density values.

Figure 4-2. Immunofluorescent identification of the MHC class I expression in livers of healthy and WHV-infected woodchucks. Cryostat sections from hepatic tissue of a normal woodchuck (WM 2075) (A) and livers of animals with AH (WM 2121) (B) and CH (WF 2114) (C) were incubated with B1b.B9 mAb directed against woodchuck MHC class I heavy chain followed by FITC-labeled anti-mouse IgG. A plasma membrane-associated pattern of the MHC class I staining of intralobular hepatocytes is evident in the liver of the animal with AH but not in the livers from healthy woodchuck and that with CH. Magnification, x 400.


A
B
C

than that on the membranes from healthy and convalescent woodchucks (Table 4-3). When HPM from animals with AH and CH were compared, a 3.2-fold lower content of the heavy chain was found on the membranes derived from chronically infected animals (Table 4-3). Taken together, the hepatocyte surface expression of MHC class I was evidently elevated in AH, but was essentially the same in CH and in the healthy or convalescent woodchucks.

It is of note that probing of the whole liver homogenates with B1b.B9 mAb by immunodot and Western blotting did not show detectable variation in the hepatic MHC class I content between infected and healthy animals. This observation supported the conclusion that the identified difference was predominantly restricted to the hepatocyte surface. There was no variation in the class I heavy chain display on KPM purified from the animals examined (Table 4-3).

4.3.2 Inhibition of hepatocyte surface MHC class I expression is associated with chronic WHV infection but not with hepatic virus load, severity of hepatitis or intrahepatic IFNγ induction.

The level of the class I heavy chain expression on HPM was not related to the amount of WHV present in the liver. The average hepatic content of WHV DNA was $6.1 \times 10^7 \pm$ SEM 1.2×10^7 vge/μg liver DNA in acutely infected animals and $8.2 \times 10^7 \pm$ SEM 4.5×10^7 vge/μg in animals with CH. Also, the hepatic

expression of WHV specific mRNA, determined by Northern hybridization and densitometric analysis, were not meaningfully different between woodchucks with AH and CH, as illustrated in Fig. 4-7. In animals which recovered from AH (WF 2131 and WF 2160), traces of WHV genome were identifiable in livers by nested PCR followed by Southern hybridization of the amplified products (sensitivity < 10^2 WHV vge/ml). This result collaborates our previous findings which demonstrated that traces of replicating WHV persists in the liver for life after resolution of AH (Michalak *et al.*, 1999). In these serologically silently infected animals, hepatocyte membrane expression of class I antigen was not appreciably different from that in healthy woodchucks. Collectively, these data showed that comparable hepatic loads of WHV were accompanied by strikingly distinct hepatocyte surface display of MHC class I molecules that depended on whether HPM originated from acutely or chronically infected animals.

As illustrated in Fig. 4-3 and Table 4-2, there was also no correlation between the hepatocyte surface presentation of class I molecules and histological severity of liver disease. Albeit, HPM from animals with the most severe AH (WM 2121 and WM 2171) tended to display greater amounts of class I heavy chain than HPM from woodchucks with mild or moderate AH (WM 2070 and WM 2167). HPM from animals with chronic infection, which had histologically very mild (WF 2114), moderate (WF 2030 and WM 2040) or severe (WF 2020, WF 2112 and WM 2150) hepatitis, showed comparably low

Table 4-3. Relative expression of cell surface MHC class I heavy chain and its RNA in woodchucks with acute and chronic hepatitis.

Category	MHC heavy chain expression [a] in:			MHC heavy-chain mRNA level [b] in:	
	HPM	SPM	KPM	Liver	Spleen
Healthy and convalescent animals (n = 4)	100	100	100	100	100
Animals with AH (n = 4)	350	100	95	330	120
Animals with CH (n = 6)	110	<5	100	350	95

a: The dot blots of the indicated plasma membrane preparations were probed with B1b.B9 MAb for class I heavy-chain expression and the resulting signals were quantified by chemi-image densitometry. The average integrated density values were calculated for each animal group and membrane type and are presented as percentages of the average amounted detected in HPM, SPM or KPM derived from healthy and convalescent woodchucks, which were taken as 100%.

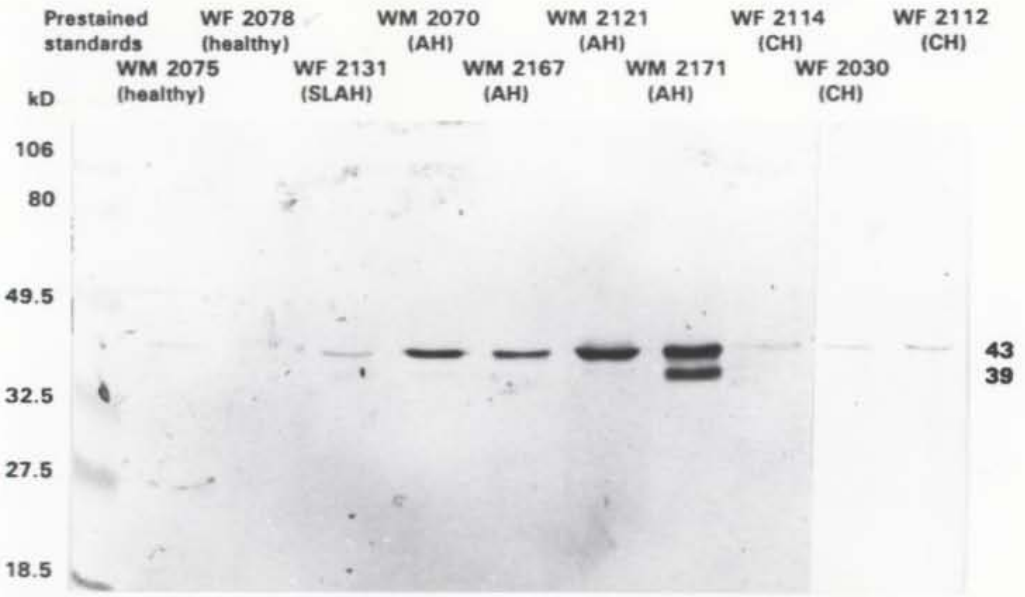
b: The MHC class I heavy chain mRNA levels were normalized to β-actin RNA signals in each tissue and are presented as percentages of the average amounts detected in livers or spleens of healthy and convalescent animals, which were taken as 100%.

levels of surface class I heavy chain. The intrahepatic CD3 RNA, an indicator of T lymphocyte infiltration, was barely detectable in healthy animals but elevated to the same level in woodchucks with AH and CH (see Fig. 4-7). Similarly, the levels of liver IFNγ RNA were comparable in both acutely and chronically infected woodchucks, but were on average approx 4.5-fold greater than that in healthy animals (Fig. 4-4). Overall, these data suggest that the status of the MHC class I display on hepatocyte surface in actively progressing hepatitis was not related to histological severity of hepatocellular injury, degree of lymphocytic infiltrations or intrahepatic IFNγ activity, but was clearly connected with chronicity of WHV infection.

4.3.3 Chronic but not acute WHV infection is associated with decreased MHC class I expression on lymphoid cells

Because WHV replicates in both hepatocytes and lymphoid cells, it was of interest to establish whether the class I antigen display differs in the lymphatic tissue in AH and CH. Immunohistochemical staining of spleen sections from healthy and WHV-infected animals showed the same intensity of the MHC class I expression on the cells lining splenic sinuses and blood vessels. The staining of the periarteriolar lymphoid sheaths, which are enriched in lymphoid cells, also was similar in healthy (Fig. 4-5A), recovered (data not shown) and acutely infected woodchucks (Fig. 4-5B). However, lymphoid cells in the same

Figure 4-3. Expression of the MHC class I heavy chain on HPM in animals with acute and chronic hepatitis, healthy woodchucks, and an animal convalescent from self-limiting acute infection. Purified hepatocyte outer membranes were separated at 20 µg protein/lane on SDS-PAGE (12% polyacrylamide gel), electrotransferred onto NC and probed by Western blotting with B1b.B9 mAb against woodchuck MHC class I heavy chain. The positions of the class I 43- and 39-kDa heavy-chain polypeptides and the prestained protein standards (lane 1) are indicated on the right and left side, respectively. The relative density values of the identified heavy-chain protein bands (scale from 0 to 4) were assigned based on integrated chemi-image scanning values. The heavy-chain display is augmented in HPM from animals with AH, whereas HPM from woodchucks with CH, as well as those from healthy or recovered animals, have comparably low contents.



Relative density value:

43 kDa	1.1	0.9	1.4	3.1	3.0	3.5	4.0	0.6	0.8	1.4
39 kDa	0	0	0	0.3	0	0.6	3.3	0	0	0

Figure 4-4. Expression of IFNγ mRNA in livers of woodchucks with acute or chronic hepatitis. Total liver RNA was reverse transcribed to cDNA and amplified with woodchuck IFNγ and β-actin specific primers, as described in Materials and Methods. The amplified PCR products were detected by Southern blot hybridization. The signals showed that the hepatic levels of IFNγ induction are comparable in animals with AH and CH.

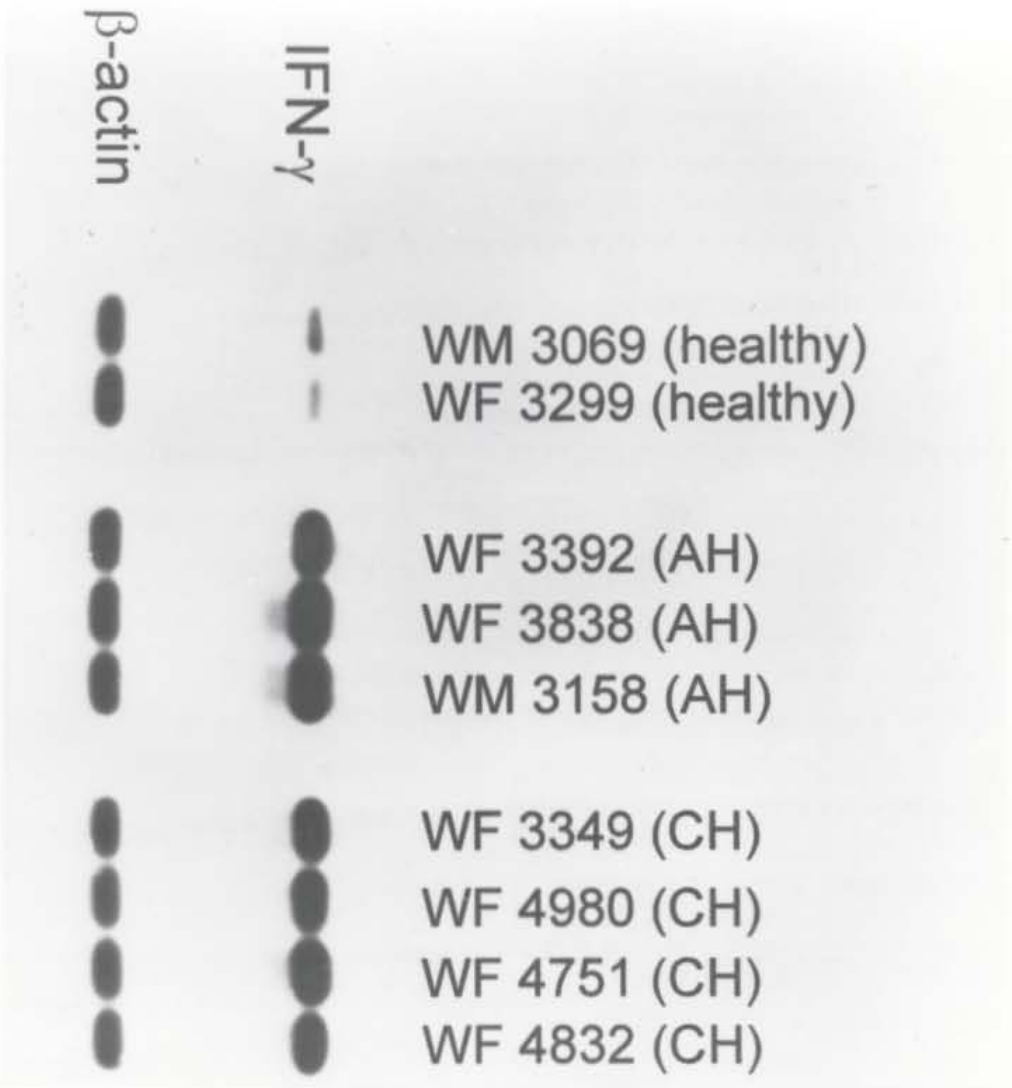
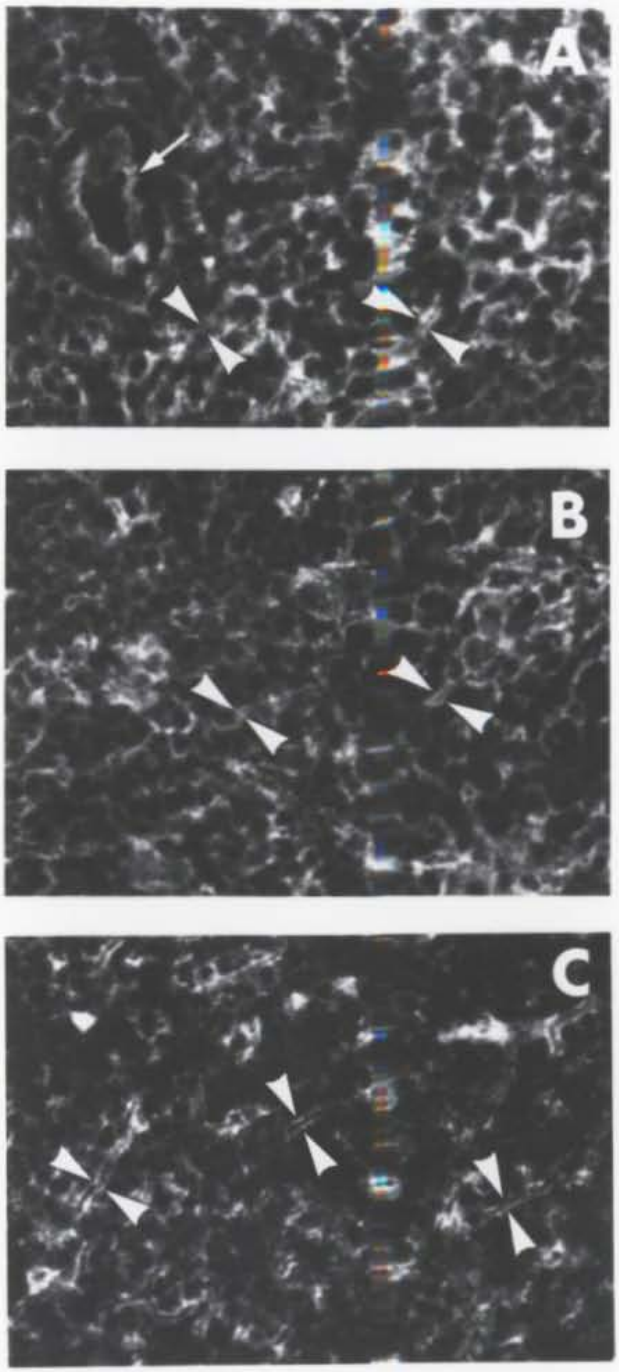

β-actin
IFN-γ
WM 3069 (healthy)
WF 3299 (healthy)
WF 3392 (AH)
WF 3838 (AH)
WM 3158 (AH)
WF 3349 (CH)
WF 4980 (CH)
WF 4751 (CH)
WF 4832 (CH)

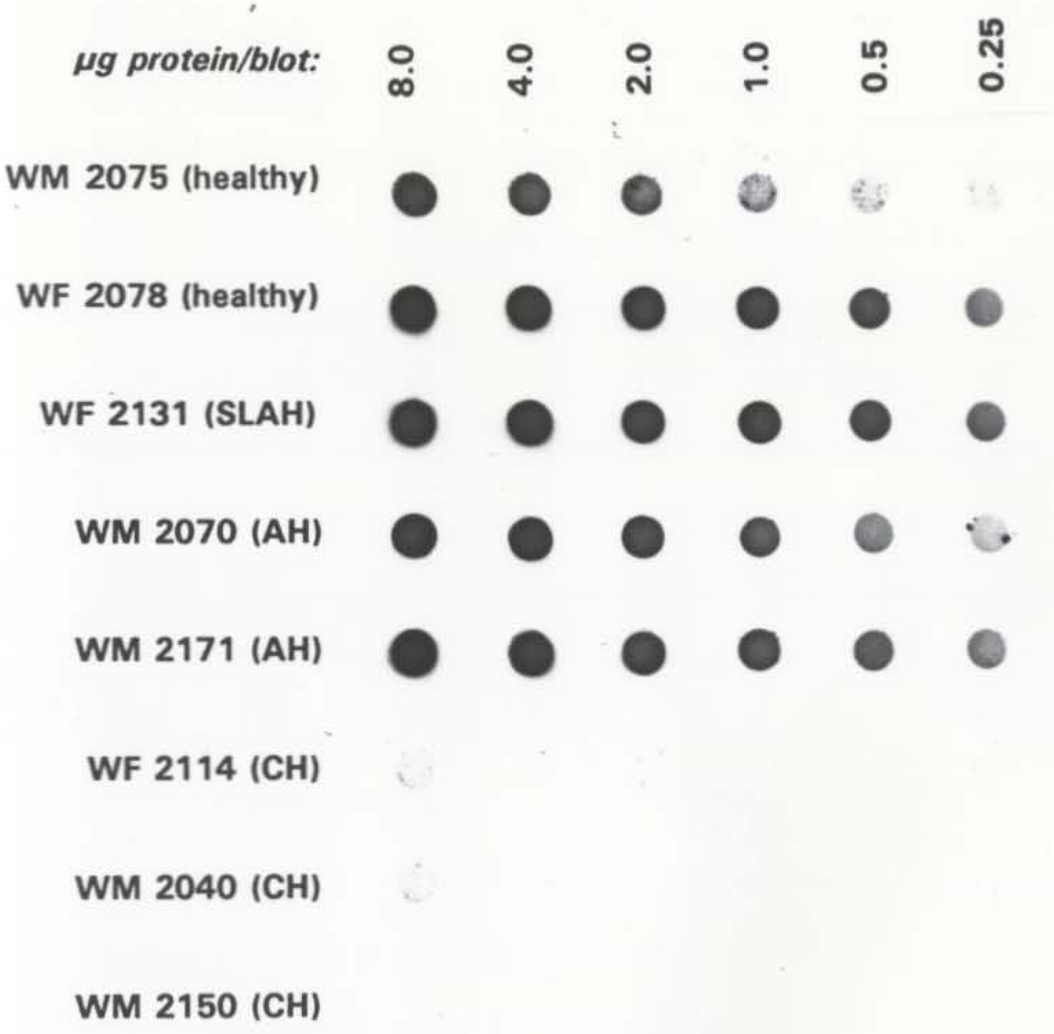
Figure 4-5. Splenic distribution of MHC class I in healthy and WHV-infected woodchucks. Sections from spleens of a healthy animal (WM 2075) (A) and from a woodchuck with AH (WM 2167) (B) incubated with B1b.B9 MAb and FITC-labeled anti-mouse IgG show immunofluorescent staining of lymphocytes as well as endothelium lining intrafollicular capillaries (arrow heads) and the central arteriole (arrow). The same staining of a spleen section from a chronically WHV-infected woodchuck (WM 2150) (C) demonstrates MHC class I expression on endothelium of blood capillaries (arrow heads) but not on lymphoid cells. Magnification. x 400.


A
B
C

periarteriolar regions in animals with CH showed recognizably less intense and sometimes almost absent staining for the class I heavy chain, despite the endothelial cells remained positive (Fig. 4-5C).

Western blotting of SPM isolated either from WHV-infected, recovered or healthy woodchucks showed both 43- and 39-kDa heavy chain polypeptide bands. The same protein bands, or only in some cases the 43-kDa species, were exhibited at lower densities in SPM from animals with CH (data not shown). Determination of the class I heavy chain display by immunodot blotting and subsequent densitometric analysis revealed closely comparable levels of class I heavy chain in SPM from healthy, convalescent and acutely infected animals, similar to the results from Western blot analysis (Table 4-3). In contrast, the MHC class I heavy chain content was evidently reduced in SPM from woodchucks with CH. Overall, SPM from chronically infected animals displayed more than a 20-fold lower level of MHC class I than SPM from woodchucks with AH or healthy controls (Table 4-3). Interestingly, identical results were obtained when the whole spleen homogenates, instead of SPM, were probed with B1b.B9 mAb, as illustrated in Fig. 4-6. This finding suggested that MHC class I expression in chronic infection is not confined to the lymphoid cell surface, as seems to be the case in WHV infected hepatocytes, but has rather a pancellular character. In contrast to splenic tissue, immunodot-blots of KPM preparations (Table 4-3), as well as whole kidney homogenates from the same animals (data not shown) did not show any variation in the MHC class I heavy chain content.

Figure 4-6. Expression of MHC class I heavy chain in spleens of woodchucks with acute or chronic hepatitis and from control animals. Serial two-fold dilutions of whole spleen homogenates prepared from woodchucks with AH or CH, healthy animals, and a woodchuck convalescent from self-limited AH (SLAH) were immobilized onto NC at the indicated protein concentrations and probed with woodchuck class I heavy-chain-specific B1b.B9 MAb. The heavy chain expression is faint in splenic tissue from animals with CH, but is intense and not altered in animals with AH and in convalescent or healthy animals.


µg protein/blot:
8.0
4.0
2.0
1.0
0.5
0.25
WM 2075 (healthy)
WF 2078 (healthy)
WF 2131 (SLAH)
WM 2070 (AH)
WM 2171 (AH)
WF 2114 (CH)
WM 2040 (CH)
WM 2150 (CH)

The WHV genome levels in the spleens of animals with AH and CH were comparable with an average viral DNA content of $8.4 \times 10^5 \pm$ SEM 3.4×10^5 vge/μg of splenic DNA for animals with AH and $1.9 \times 10^6 \pm$ SEM 1.1×10^6 vge/μg for woodchucks with CH, while WHV mRNA was not detectable by Northern blotting. Renal tissue was WHV DNA and mRNA negative by the same assays in the animals examined. In separate experiments, density of the MHC class I heavy chain display on intact, freshly isolated PBMC versus purified PBMC surface membranes was determined. Analysis of comparable numbers of PBMC by flow cytometry and the same amounts of PBMC membrane proteins by immunodot blotting with B1b.B9 mAb failed to demonstrate any consistent difference between healthy animals and those with AH or CH (data not shown). PBMC membranes probed for class I heavy chain by Western blotting displayed the 43-kDa protein only (data not shown).

4.3.4 Both acute and chronic WHV infection upregulates MHC class I-linked gene transcription in hepatic but not in splenic tissue.

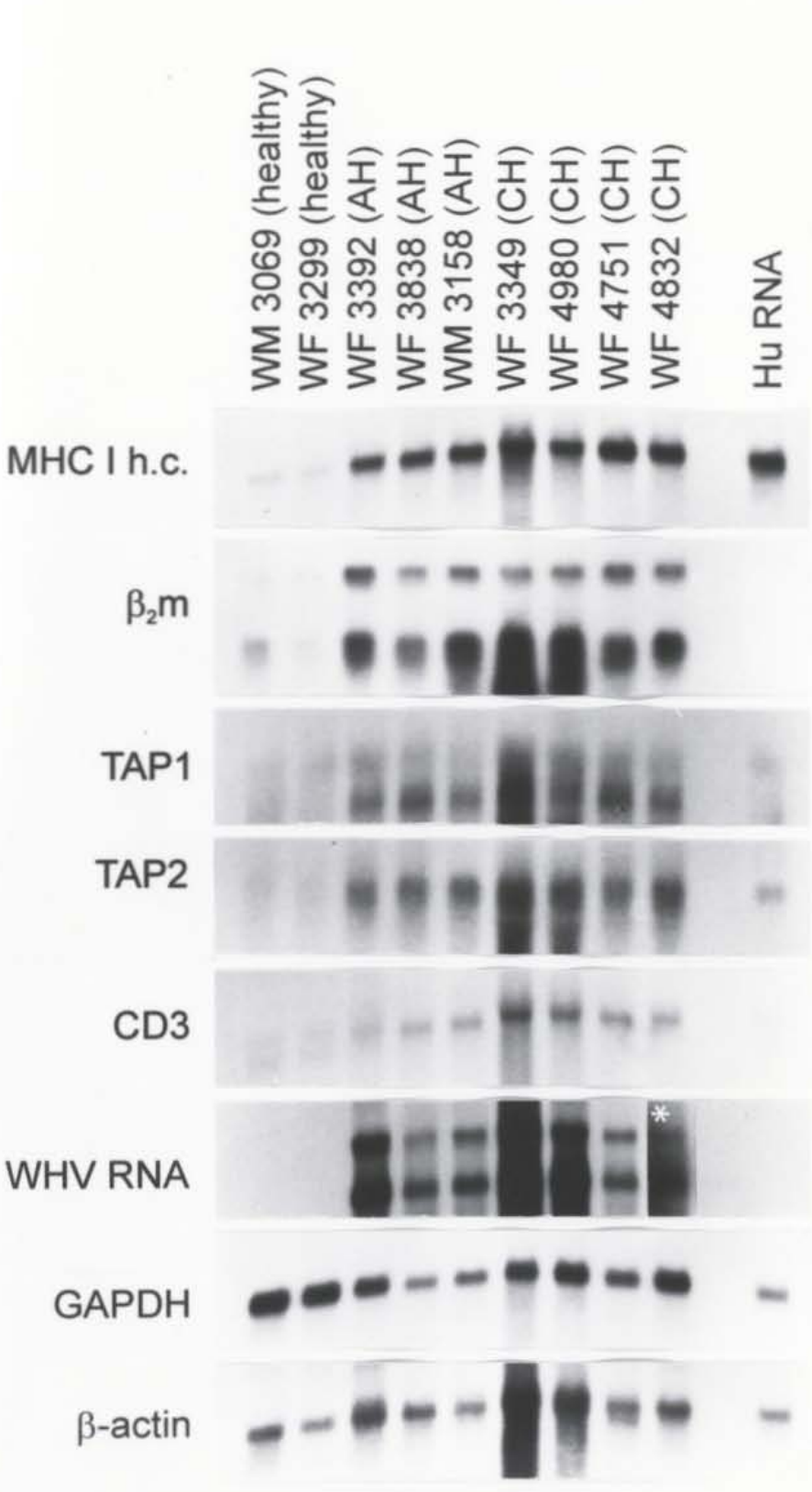
To determine whether the detected variation in the cell surface presentation of MHC class I molecules between AH and CH reflects a difference in transcriptional activity of the relevant gene loci, RNA from livers and spleens of the animals investigated were probed for MHC class I heavy and light chains, and TAP1 and TAP2 transcripts. In addition, to learn about possible differences

in the T lymphocyte contents. RNA preparations from livers and spleens of animals belonging to Study Group 2 were analyzed for expression of CD3 gene transcripts. Northern blot hybridization of hepatic RNA showed markedly elevated levels of heavy chain mRNA in both acutely and chronically infected woodchucks when compared to healthy or convalescent animals (Fig. 4-7), as well as increased expression of β2-microglobulin, TAP1 and TAP2 mRNA (Fig. 4-6B). Phospho-image quantitation of the hybridization signals, corrected to β-actin gene expression, revealed a greater than 3-fold enhanced intrahepatic transcription of the class I heavy chain gene in woodchucks with AH and a similar increase in animals with CH, when compared to normal or recovered woodchucks (Table 4-3). In general, the hepatic MHC class I-affiliated genes were upregulated to the same extent in AH and CH, indicating this augmentation was not related to the duration of WHV infection. Also, the intrahepatic levels of the CD3 RNA were the same in animals with acute and chronic liver disease, revealing that the magnitude of lymphocytic infiltrations was comparable in these two phases of WHV-induced necroinflammation in the animals studied (Fig. 4-7B). As previously presented (Fig. 4-4), the extent of induction of IFNγ was the same in livers from acutely and chronically infected woodchucks. Taken together, these results imply that the defect in presentation of these molecules in chronic infection was certainly related to posttranscriptional suppression.

Figure 4-7. Effect of acute and chronic WHV infection on hepatic expression of MHC class I affiliated genes. Total hepatic RNA was isolated from woodchucks with different histological severity of AH or CH, an animal convalescent from AH (SLAH) and healthy woodchucks in study group 1 (A) or 2 (B) (for details see Table 4-1). The RNA was probed by Northern blotting with [^{32}P]-labeled woodchuck MHC class I heavy chain (MHC I h.c.), β_2-microglobulin (β_2m), TAP1, TAP2, CD3 cDNA, complete recombinant WHV DNA or cloned woodchuck GADPH cDNA and/or human β-actin cDNA as housekeeping genes. Frog liver RNA (A) and human RNA (Hu RNA) (B) were used as species specific references. The white asterik in panel B depicts a signal that required increased exposure. The expression of MHC class I heavy and light chains, TAP1, TAP2 and CD3 RNA is augmented to the same extent in livers of both acutely and chronically infected animals.

β-actin
WHV RNA
MHC I h.c.
Frog Liver RNA
WF 2078 (healthy)
WF 2131 (SLAH)
WM 2070 (AH)
WM 2167 (AH)
WM 2121 (AH)
WM 2171 (CH)
WF 2114 (CH)
WF 2030 (CH)
WF 2112 (CH)

A


B
WM 3069 (healthy)
WF 3299 (healthy)
WF 3392 (AH)
WF 3838 (AH)
WM 3158 (AH)
WF 3349 (CH)
WF 4980 (CH)
WF 4751 (CH)
WF 4832 (CH)
Hu RNA
MHC I h.c.
β2m
TAP1
TAP2
CD3
WHV RNA
*
GAPDH
β-actin

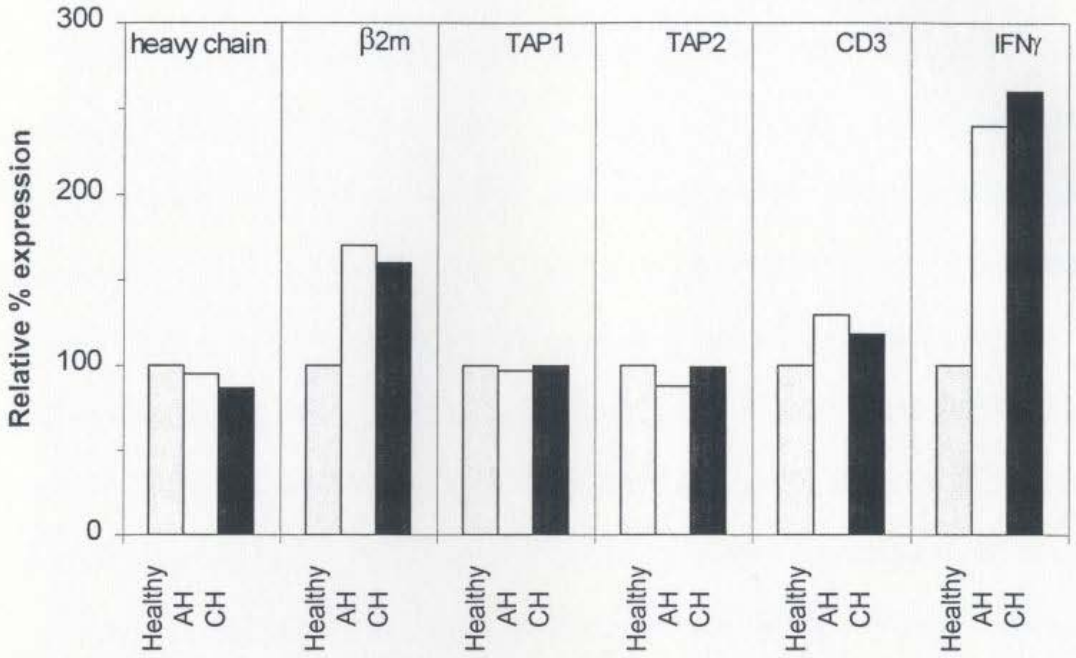


Figure 4-8. Relative expression of MHC class I heavy chain and related gene mRNA in splenic tissue from woodchucks with acute or chronic WHV hepatitis. Total RNA isolated from spleens of animals with AH (n = 3) and CH (n = 4), and from healthy animals (n =2) (study group 2) were probed with [^{32}P]-labeled woodchuck class I heavy chain, β_2-microglobulin (β_2m), TAP1, TAP2, CD3 or β-actin by Northern blotting, whereas IFNγ was assessed by RT-PCR and Southern blotting as described in Materials and Methods. Hybridization signals were quantitated by phosphoimage densitometry, normalized to the β-actin, and presented as a percentage of the average amount detected in spleens of healthy woodchucks, which were taken as 100%.

Splenic tissue showed comparable levels of the class I heavy chain, TAP1 and TAP2 RNA, as well as CD3 RNA, both in acutely or chronically infected woodchucks and in the healthy or recovered animals (Fig. 4-8). The exception was β2-microglobulin RNA that was moderately elevated in WHV-infected in comparison to healthy animals. In addition, the splenic level of IFNγ RNA was identical in acute and chronic infection but at least twice of that detected in healthy controls (Fig. 4-8). Overall, the findings showed that, in contrast to the diseased livers, neither AH or CH infection meaningfully upregulates MHC class I linked mRNA in splenic tissue. These data imply that the defective expression of MHC class I molecules in splenic lymphocytes in chronic WHV infection does not result from inhibited transcription of the relevant cellular genes and, therefore, has to occur on the posttranscriptional level.

4.4 DISCUSSION

Analysis of hepatocyte surface membranes in this study revealed a significant difference in hepatocyte presentation of MHC class I molecules in acute and chronic phases of hepadnaviral hepatitis. While acute WHV infection is accompanied by increased hepatocyte surface display of MHC class I, chronic disease, independent of severity of liver injury and duration of chronicity, is uniformly associated with a decrease in the protein level similar to that seen in healthy animals and those convalescent from AH. This defective class I antigen display in CH occurs despite of the enhanced transcription of relevant MHC

class I-affiliated hepatic genes, equal to that observed in the livers of acutely infected hosts. Considering the above findings, our study demonstrates that hepatocyte presentation of the MHC class I molecules is evidently enhanced in acute disease, but dramatically suppressed at the posttranscriptional level in chronic stage of WHV hepatitis. Importantly, this impairment in chronic infection also affects splenic lymphoid cells, another site of virus propagation. However, in contrast to the diseased livers, transcription of the MHC class I linked genes is not altered in the infected splenic tissue when compared to healthy animals. Also, the splenic display of the MHC class I molecules is essentially identical in AH as in the healthy or convalescent woodchucks. In addition, neither acute nor chronic WHV infection affects the MHC class I antigen expression in kidneys, which were found to be WHV negative. Collectively, the present findings show that:(1) the liver-restricted, augmented transcription of the MHC class I affiliated genes is an invariable characteristic of any active liver inflammation induced by WHV; (2) the severely impaired presentation of MHC class I molecules is a unique feature of chronic WHV infection; (3) the decreased cellular presentation of MHC class I in chronic disease appears to be restricted to tissues in which WHV replicates; and (4) the deficient hepatocellular and lymphoid cell display of MHC class I in CH is a consequence of virus-dependent posttranscriptional inhibition. Overall, the data from our study reveal a multifarious interplay between phases of hepadnaviral hepatitis, the status of MHC class I affiliated gene transcription in virus infected organs, and the expression of the MHC class I

molecules on cells of these organs.

The MHC class I antigen processing and presentation pathway involves formation of the trimeric complexes constituted by class I heavy chain, β2-microglobulin and a proteolytically generated peptide (Hill and Ploegh, 1995). The assembly of the stable MHC class I molecules requires translocation of cytosolically produced peptides into the lumen of endoplasmic reticulum. This process is facilitated by transmembrane transporter proteins, referred to as TAP1 and TAP2, which deliver peptides into the endoplasmic reticulum to bind to empty class I heavy-light chain heterodimers (Spies *et al.*, 1990). The created trimeric complexes are transported to the cell surface for interaction with specific CTL. The current model also includes auxiliary molecules, such as proteasomes, in the generation of peptides for loading onto class I molecules and emphasizes a role for IFNγ in the modulation of peptide processing and in upregulation of genes involved in the antigen MHC class I presentation pathway (reviewed by Früh and Yang, 1999 and Van Endert, 1999).

As uncovered in this study, progressive acute and chronic WHV hepatitis is accompanied by increased gene expression of hepatic class I heavy chain, light chain and TAP gene expression, as well as by the increased levels of hepatic T cell (CD3) and IFNγ RNA. Since lymphomononuclear cell infiltrations are an invariable feature of active liver inflammation in hepadnaviral infection and activated T lymphocytes and NK cells, secreting inflammatory cytokines including IFNγ (Guidotti *et al.* 1996; Scharton and Scott, 1993), are constituents of these

infiltrates, induction of intrahepatic IFNγ could be mainly responsible for activation of MHC class I affiliated genes in WHV hepatitis. This is supported by the fact that regulation of MHC class I genes by IFNγ occurs primarily at the level of gene transcription (Wallach *et al.*, 1982). In addition to the indirect augmentation through IFNγ, direct upregulation of the MHC class I genes by viral proteins might also be possible. It has been shown *in vitro* that the HBV X protein can transactivate the MHC class I heavy chain promoter in the virus-transfected HepG2 and related liver cell lines, leading to a 3 to 4-fold increase in the heavy chain RNA and protein levels (Zhou *et al.*, 1990). Since this event has been observed in the absence of T cells and independently of IFNγ, this may suggest that the HBV X protein can directly modulate MHC class I gene activity in cultured liver cells. It has also been postulated that HBV X, by interfering with proteasome functions, may prevent viral peptide interaction and presentation by class I molecules (Huang *et al.* 1996). Determining whether these hypothetical mechanisms operate in *in vivo* infected hepatocytes requires further studies.

In contrast to the livers, activity of MHC class I genes was not altered in the spleens of WHV-infected animals, except for β2-microglobulin RNA. The divergent effect of WHV infection on the MHC class I gene transcription in the liver and the spleen could be due to an innate difference in the levels of the gene expression between these two organs. Quantitative analysis performed in this study showed that class I heavy chain mRNA is present at 3 to 4-fold greater levels in the splenic than in hepatic tissue of healthy woodchucks. It is possible

that while viral infection is capable of upregulating class I genes in hepatocytes, where their transcriptional activities are naturally low, it is not able to exert this additive effect in lymphatic organs, like the spleen, where the gene expression is inherently high. Therefore, WHV infection may act as a conditional transcriptional inducer whose indirect or direct modulatory effect depends on the microenvironment regulating local activity of MHC class I genes. On the other hand, although perhaps less likely, the lack of class I gene upregulation in the infected spleens might be a consequence of 10 to 100-times lower WHV DNA and RNA content than that observed in the livers of these animals. Conceivably, virus replicating less efficiently or occurring at low quantities in infected cells might be unable to induce an identifiable increase in transcription of the MHC class I linked genes. This situation seems to be true for livers of woodchucks convalescent from AH, which support persistent WHV replication at low levels (Michalak *et al.*, 1999), but which do not show any noticeable change in class I heavy chain RNA or protein expression. In addition, the splenic CD3 influx and IFNγ RNA levels were identical in acutely and chronically infected animals. Therefore, a diminished T cell number or a decrease in the local activity of IFNγ cannot account for the observed class I antigen inhibition in splenocytes in chronic WHV infection.

The equally augmented expression of hepatic MHC class I-affiliated genes in AH and CH should imply enhanced synthesis of class I heterodimers and the availability of processed viral peptides for their loading. Consequently, this

should lead to the increased presentation of class I complexes on liver cells irrespective of the phase of hepatitis. However, this situation exists only in acute infection, where the HPM class I heavy chain expression was evidently higher than that detected on HPM from healthy or convalescent animals and appeared to be proportional to the elevated transcription from the respective cellular genes. In contrast, the reduced hepatocyte display of class I heavy chain in chronic infection was accompanied by an increased level of RNA compared to that observed in AH (Table 4-3). In spleens of chronically infected animals, unaltered expression of the MHC class I affiliated genes was associated with a dramatic (more than 20-fold) reduction in class I heavy chain presentation when assessed by immunoblotting. In general, the results on these quantitative analyses of class I display in isolated hepatocyte and splenocyte outer plasma membranes agreed with the immunofluorescent staining of tissue sections. In summary, the obtained data clearly document that the disparity between class I-affiliated gene activity and class I antigen display in chronic infection is not related to downregulation of gene transcription and, therefore, it has to be due to a virus-dependent posttranscriptional interference that is unique for cells supporting persistent WHV replication. However, they do not exclude that the inhibited presentation of class I molecules can be a consequence of their impaired trafficking to the cell surface or enhanced recycling (Section 1.7.6). The observed lack of heavy chain accumulation in the cytoplasm of the chronically infected hepatocytes by immunohistochemical staining has to be interpreted with

caution considering the relatively low sensitivity of this method.

Many viruses inhibit the class I surface molecules on invaded cells to avoid cytopathic or noncytopathic elimination initiated by specific CTL (reviewed by Früh *et al.*, 1999; Miller and Sedmak, 1999; Oldstone, 1997; Rinaldo, 1994). It is known that viral proteins may induce posttranscriptional inhibition of class I antigen by interfering with generation or transport of peptides predestined for interaction with class I heterodimers in endoplasmic reticulum. They may also disrupt the class I complex assembly, trafficking and cell surface presentation or increase their degradation (reviewed by Miller and Sedmak, 1999). Frequently, the same virus uses a variety of strategies mediated by more than one viral factor. In the context of WHV ability to suppress MHC class I antigen display in different cell types, it is conceivable that the virus may also utilize multifactorial mechanisms acting on the different posttranscriptional levels of the MHC class I presentation pathway.

The data collected so far reveal that the deficient expression of class I molecules on hepatocytes coincides with another unique feature of hepatocyte surface that is distinctive of chronic WHV hepatitis (Michalak and Churchill, 1988, Michalak and Lin, 1994, Michalak *et al.*, 1989). We have previously shown that HPM from chronically infected woodchucks, independent of histological severity of hepatitis and duration of chronicity, contain large quantities of WHV envelope proteins irreversibly incorporated into the membrane lipid bilayer (Michalak and Churchill, 1988; Michalak *et al.*, 1989). Assessments of the WHs antigenic

content and the binding of exogenous WHsAg to HPM from different forms of WHV-induced liver pathology revealed that HPM from CH have the greatest amounts of the integrated antigen and they are characterized by the inability to bind exogenous WHsAg (Michalak *et al.*, 1989), indicating the presence of a saturated quantity of the virus envelope. We have hypothesized that this explicit feature of hepatocyte surface membrane in chronic disease, which naturally coexists with the abundant amounts of the same viral proteins in the circulation, may constitute an important element of protection of infected hepatocytes against immunoelimination and, therefore, contributes to prolonged liver disease and virus persistence (Michalak and Churchill, 1988; Michalak and Lin, 1994; reviewed by Michalak, 1998). Since the same HPM preparations were analyzed to determine expression of MHC class I in this study (Study Group 1) and WHV envelope polypeptides in our previous work (Michalak and Lin, 1994), we can conclusively state that suppression of class I molecules on hepatocytes occurs only in the context of the massive incorporation of WHV envelope proteins into HPM. This association, coexisting with heavy deposits of viral envelope material in the endoplasmic reticulum, which are typical for hepatocytes in chronically infected livers (Michalak and Lin, 1994, Michalak *et al.*, 1989), may exert a severe constraint on intracellular assembly and transport, as well as on presentation of class I molecules at hepatocyte surface. This mechanism provides a reasonable explanation for the deficiency in class I antigen display on chronically infected hepatocytes, however, it is rather unlikely that it also

operates in lymphoid cells which, at the best, express WHsAg at minute quantities. Therefore, we speculate that class I expression in the lymphatic system is downregulated by WHV through an alternative pathway.

Independent of the mechanism involved, defective expression of MHC class I complexes on hepatocytes in chronically infected hosts has to have significant immunopathogenic consequences. As in other viral infections, the foremost could be evasion of immune surveillance by virus-specific CTL leading to suppression of cytolytic and non-cytolytic elimination of virus. This alone can contribute to perpetuation of liver disease and facilitates virus persistence. However, there could also be other implications potentially important for WHV pathogenesis during chronic infection. Since virally infected cells with reduced class I antigen expression are considered to be inherently susceptible to attack from NK cells (Brutkiewicz and Welsh, 1995; Ljunggren *et al.*, 1990), this may imply that non-MHC class I-dependent, cell-mediated cytotoxicity might play a role in both controlling virus spread and induction of hepatocellular injury in chronic hepadnaviral infection. We have recently tested this intriguing possibility by evaluating perforin and FasL-based cytotoxicity of circulating lymphoid cells from woodchucks with acute and chronic WHV hepatitis (Hodgson *et al.*, 1999; Section 3). The data from this study showed that the levels of the perforin-dependent killing, which is the principle mechanism of cell elimination by NK cells (Kagi *et al.*, 1994; Sayers *et al.*, 1998), are significantly enhanced in AH but essentially the same in chronically infected and heathy animals. Although

intrahepatic NK cells were not examined in this study, the above findings suggest that NK cell activity could be inhibited during the chronic phase of WHV hepatitis, despite suppressed hepatocyte MHC class I surface expression. In this context, of note are the past observations postulating that HBsAg depresses NK cell cytotoxicity, presumably by interfering with their binding to target cells (Azzari *et al* 1992; De Martino *et al*, 1985). If this is the case, it is conceivable that the large quantities of hepadnavirus envelope proteins inserted into hepatocyte outer membrane, coexisting with the large amounts of the same antigenic material in serum, may act as a negative modulator on intrahepatic NK cells in chronic infection. This mechanism, together with inhibition of hepatocyte surface MHC class I presentation, might constitute a strategy that the virus employs to escape elimination by both CTL and NK cells.

At least one more issue requires comment in regard to the findings of the present study. Identification of severely reduced expression of class I molecules in splenic lymphoid cells is likely reflecting a situation existing in other lymphatic organs in chronic WHV infection; although, we did not see its evidence in circulating lymphoid cells. It is known that class I molecules are involved in the elaborate network of interactions between cells of the immune system and they play key roles in regulating immune cell functions. Among them, it has been shown that interruption of the MHC class I presentation on lymphoid cells is sufficient to induce autoimmune reactions (Fu *et al.*, 1993), which are a very common consequence of WHV infection (Diao and Michalak, 1997; Diao et al.,

1998; Dzwonkowski and Michalak, 1990). Therefore, downregulation of MHC class I presentation on lymphoid cells by hepadnavirus infection could deregulate a variety of host immune reactions whose effects, although not directly apparent, may profoundly diminish overall effectiveness of anti-viral immune responses. This important issue awaits future studies. The defect in MHC class I presentation on both hepatocyte and lymphoid cells identified in this study once again exemplifies the complexity of the strategies utilized by hepadnavirus to survive within the host. Unraveling this defect and its functional consequences will be necessary to fully understand the mechanisms underlying perpetuation of liver disease and virus persistence in hepadnaviral infections.

CHAPTER 5:

AUGMENTED HEPATIC INTERFERON GAMMA EXPRESSION AND T CELL INFLUX CHARACTERIZE ACUTE HEPATITIS PROGRESSING TO RECOVERY AND RESIDUAL LIFELONG VIRUS PERSISTENCE IN EXPERIMENTAL ADULT WOODCHUCK HEPATITIS VIRUS INFECTION*

*This study was published in Hepatology. 2001 (vol. 34: pp.1049-1059). My specific contributions to this study comprises all of the molecular biology data, including cloning of woodchuck genes, Northern blot hybridization and quantitative PCR analyses, and evaluation of WHV DNA by dot blot hybridization.

5.0 SUMMARY

Woodchucks infected with WHV demonstrate profiles of liver disease and age dependent rates of progression to CH comparable to those seen in human hepatitis B. The mechanism of recovery from acute hepadnaviral infection or its evolution to chronicity remains unknown, although the local immune cell and cytokine responses in the liver are expected to play an important role. To determine the dynamics of intrahepatic cytokine expression and T cell involvement, and to assess their value in predicting the outcome of AH in the adult onset of hepadnavirus infection, we evaluated levels of liver transcription of IFNγ, TNFα and IL-2, -4 and -6, and the T cell influx in relation to histological severity of disease and virus load in serial liver biopsies collected during the lifespan of woodchucks with experimental acute WHV infection who either ceased to have hepatitis or developed chronic liver disease. Our results show that recovery from acute viral hepatitis in adult animals is preceded by a

significantly greater hepatic expression of IFNγ and CD3, an increased level of TNFα transcription, a lower hepatic WHV load and by a greater degree of liver inflammation than in acute infection with CH outcome. Furthermore, we have learned that the elevated liver IFNγ, TNFα and CD3 expression endures for years not only in the animals with evident CH but also, although to a lesser extent, in those which resolved acute infection. This is consistent with our previous findings that residual WHV replication and remnant liver inflammation continue for life after recovery from AH in adult woodchucks. The current study indicates that antiviral cytokines, in particular IFNγ, may play a central role in the long term control of occult hepadnavirus persistence in the liver.

5.1 INTRODUCTION

HBV infection in adulthood is usually followed by self limiting AH that is traced by serologically concealed persistence of small amounts of virus in the recovered hosts (Michalak *et al.*, 1994; Penna *et al.*, 1996; Rehermann *et al.*, 1996; Yotsuyanagi *et al.*, 1998). In 5-10% of adult patients, the infection advances to lifetime CH which is a frequent precursor to cirrhosis and HCC. This is in contrast to HBV infection in newborns of whom the vast majority (>90%) become serologically positive, i.e., HBsAg-reactive, chronic virus carriers. Since HBV infection of hepatocytes is essentially noncytopathic in the immunocompetent host, cell immune responses directed toward infected liver cells are considered to be a main inducer of hepatic injury and a mediator of virus

clearance (Chisari and Ferrari, 1995; Curry and Koziel, 2000). Recent evidence suggests that, in addition to virus-specific CD8+ CTL and collaborating CD4+ Th cells, non-specific cell immune responses, involving NK, NKT cells and macrophages, might be important in HBV elimination and pathogenesis of liver damage (Guidotti and Chisari, 1996; Kakimi *et al.*, 2000). It is also apparent that antiviral cytokines released by the activated effector cells of innate and adoptive immune systems in the region of their targets, such as IFNγ, IFNα and TNFα, can temporarily induce non-cytolytic suppression of HBV expression in the liver (Section 1.8.4.; Guidotti and Chisari, 1996; Guidotti *et al.*, 1996; Guidotti *et al.*, 1999).

The mechanisms underlying resolution of acute HBV infection or its progression to chronicity remain undetermined. The available data suggest, however, that a weak Th1 cell response to HBV antigens coincides with transition to chronicity and the prevalent Th1 cytokine pattern, consisting of IFNγ, TNFα and IL-2, can be associated with resolution of hepatitis B (Chisari and Ferrari, 1995; Penna *et al.*, 1997). From studies on other viral infections, it is evident that the strength of the initial antiviral response might be critical in determining the final outcome of infection. In regard to hepatitis B, only recently has data on the status of intrahepatic immunity in the initial (incubation) period of HBV infection become available from a chimpanzee model (Guidotti *et al.*, 1999) and on the peripheral blood HBV-specific T cell responses from patients examined prior to the onset of symptomatic hepatitis (Webster *et al.*, 2000). Nevertheless, there is

no information as to what extent the range and the strength of intrahepatic immunity predispose the host to either recover or become chronically infected. In general, this type of investigation is difficult to conduct in human disease due to ethical and practical problems, in particular in acquiring serial liver samples from individuals prior to HBV infection and from patients in pre-acute phases of hepatitis who ultimately recover or develop chronic liver disease.

The infection of woodchucks with HBV-related WHV has a similar disease course and age dependent rates of recovery from AH or progression to CH, and overall comparable histopathological features of liver injury as those seen in HBV-infected humans (Section 1.5; Menne and Tennant, 1999; Michalak, 1998). Similar to hepatitis B infection, acute WHV hepatitis is a transient, self resolving disease in 85% of adult woodchucks, while in the remaining animals it progresses to lifelong serologically evident (i.e., WHsAg-positive) infection and sustained liver inflammation (Michalak, 2000). This is in contrast with WHV infection induced during the perinatal period where the majority (~75%) of the animals develop CH (Cote *et al.*, 2000). In addition, a series of recent studies from our laboratory (Michalak *et al.*, 1999; Coffin and Michalak, 1999; Lew and Michalak, 2001; reviewed by Michalak, 2000), have shown that the recovery from adult onset of WHV infection is invariably followed by lifelong persistence of trace quantities of infectious virus that replicates both in the liver and the lymphatic system. This serologically concealed infection is frequently, but not always, accompanied by morphological alterations in the liver consistent with minimal to

minor residual inflammation (Michalak *et al.*, 1999). Moreover, the occult virus is transmissible to offspring in which it induces an occult, life-long virus persistence (Coffin and Michalak, 1999) and is infectious to virus naive animals (Michalak *et al.*, 1999).

In this retrospective study, we investigated the WHV-woodchuck model to determine the dynamics of the intrahepatic cytokine response, T cell infusion and liver injury in adult woodchucks who after inoculation with the same WHV infectious pool either resolved AH or developed CH. We also examined whether hepatic expression of cytokines, such as IFNγ, TNFα, IL-2, IL-4 and IL-6, in healthy animals prior to WHV inoculation or in the acute phase of WHV infection have a value in predicting the self limiting or chronic disease outcome of AH. We assessed the extent of intrahepatic T cell influx by examining CD3 mRNA and determined whether its gene expression correlates with that of any of the aforementioned cytokines, the histological severity of liver injury or hepatic WHV load. The same parameters were also analyzed in the livers of animals with progressing CH and during the lifelong follow-up after resolution of experimental AH. Our results show that acute WHV hepatitis followed by recovery is characterized by higher levels of intrahepatic IFNγ and CD3 expression, an increased transcription of TNFα, a greater severity of liver inflammation and lower liver WHV loads than those occurring in the animals who developed CH. Furthermore, our results demonstrate that the elevated transcription of IFNγ, TNFα and CD3 endured for years in the liver after termination of AH in the

context of continuing remnant replication of WHV. This raises a possibility that antiviral cytokines, in particular IFNγ, may play a pivotal role in keeping persistent residual hepadnavirus replication in the liver at the levels which are not imminently harmful to the infected host.

5.2 MATERIALS AND METHODS

5.2.1 Animals and categories of WHV infection.

Twenty-two woodchucks (8 males and 14 females) were investigated in this study. The animals were infected as young adults (yearlings or 2 years of age) with the same pool of serum-derived WHV at a dose of 1.1×10^{10} DNase-protected vge (Michalak *et al.*, 1999). All of the woodchucks developed serologically evident AH. The disease was self limiting in 15 of the animals and in the remaining 7 progressed to CH (Table 5-1). Five of the animals with a self-limited episode of AH (2/F, 3/F, 5/M, 6/M, and 7/F) were examined in our previous study aimed at determination of molecular characteristics and liver tissue alterations accompanying lifelong occult WHV persistence continuing after recovery from acute infection (Michalak *et al.*, 1999). Seven of the woodchucks which resolved acute infection (2/F, 3/F, 5/M, 6/M, 7/F, 11/M, and 12/M) and 4 that advanced to chronic disease (21/F, 22/M, 23/F, and 24/F) were followed throughout their natural lifespan. Two of the above woodchucks with transient AH (3/F and 7/F) and 3 with CH (21/F, 23/F and 24/F) had elevated GGT levels and subsequently developed hepatocellular carcinoma (HCC). Serum samples

from each animal were drawn before inoculation with WHV and, thereafter, biweekly up to 6 months, monthly until 36 months post inoculation (p.i.), and then bimonthly. Liver tissue fragments were obtained by surgical laparotomy or at autopsy, as described previously (Michalak *et al.*, 1999). Typically, the first biopsy was obtained 4 to 6 weeks prior to inoculation with WHV, the second biopsy during serologically evident acute infection, and subsequent samples were collected at approximately yearly intervals (Table 5-1). In total, 103 biopsy or autopsy liver samples were analyzed in this study (Table 5-1). The mean time interval of their acquisition was 32 weeks $\pm$ 22 SE.

The serological status of WHV infection was determined by testing sera for WHsAg, anti-WHs and anti-WHc (Section 2.3). The serum levels of WHV DNA were determined by a slot blot hybridization assay and, when negative, by PCR using WHV core gene specific primers followed by Southern blot hybridization analysis of the amplified products, as described (Section 2.9.9; Michalak *et al.*, 1999; Coffin and Michalak, 1999; Michalak *et al.*, 2000). According to serological parameters, acute phase of WHV infection was diagnosed in animals who, after inoculation with virus, became WHV DNA, WHsAg and anti-WHc positive, but in who WHs antigenemia lasted no longer than 6 months. Serological resolution of acute infection was considered when WHsAg permanently cleared from the circulation, WHV DNA dropped to levels detectable only by nested PCR/ Southern blot hybridization assays, and the animal normally, but not always, seroconverted to detectable anti-WHs.

Diagnosis of serologically evident chronic infection was made when WHsAg persisted in serum for longer than 6 months.

Histological examination of liver tissue was performed after conventional processing to paraffin (Section 2.5). Morphological assessment, normally graded on a numerical scale from 0-3 was refined by adding 0.5 grade scores to more accurately reflect alterations which, in some cases, were intermediate between those to which the full numeric grades were assigned (Section 2.5).

Based on combined serological and histological evaluations of WHV infection at the time of liver tissue acquisition, the samples were classified to one of the following categories: (1) normal (healthy) (n = 14); (2) from acute phase of infection (acute) (n=19); (3) after resolution of AH (resolved) (n = 52), and (4) during CH (chronic)(n = 18) (Table 5-1).

5.2.2 Nucleic acid preparation

Total RNA was extracted from 50-100 mg of mechanically pulverized frozen hepatic tissue or from 1 x 10^7 PBMC (Section 2.6.2). PBMC were obtained from a healthy woodchuck and stimulated with 5 µg/ml ConA (Sigma) for 72 hours before RNA isolation (Jin *et al.*, 1996). These cells were used as a source of total RNA for further identification and cloning of woodchuck cytokine gene specific sequences. After RNA extraction, DNA was separated from the non-aqueous phase of TRIzol by ethanol precipitation following Section 2.6.1.

The recovered DNA was used to evaluate WHV genome expression. Nucleic acids were quantitated by spectroscopic analysis and stored in small aliquots at -80°C (Section 2.6.3).

5.2.3 Detection of WHV DNA and RNA in tissue

The presence of WHV DNA in chronically infected animals was determined by a slot-blot hybridization assay described in Section 2.9.1. Briefly, 5 µg of hepatic DNA obtained from woodchuck biopsy or autopsy tissue samples was heat denatured and immobilized onto a nylon membrane then hybridized to a full-length, linearized, recombinant WHV DNA (rWHV DNA) labeled with ^{32}P (Section 2.10). PCR, with Southern blot hybridization, was performed to detect WHV DNA at levels below the sensitivity of dot-blot hybridization (*i.e.*, in animals recovered from AH)(Section 2.9.3.2). For estimation of the levels of WHV DNA expression, autoradiographic or phosphor images of hybridization signals were quantitated with two-fold serial dilutions of rWHV DNA using the Chemi-Imager 4000 or the Cyclone Phosphor Imaging System (Section 2.9.3.3). The estimated sensitivity of a dot blot hybridization assay was approximately 10^6 vge/ml and the nested PCR/Southern hybridization assay was between 10 to 10^2 vge/ml.

The presence of WHV liver RNA was detected after reverse transcription and PCR amplification with WHV core gene specific primers in parallel with the appropriate specificity and contamination controls. Southern blot analysis was

routinely used to detect and confirm authenticity of the amplified WHV cDNA sequences (Section 2.9.3.2; Coffin and Michalak, 1999).

5.2.4 Cloning of woodchuck cytokine genes

Woodchuck IFNγ and CD3 gene fragments were cloned and their nucleotide sequences reported in our previous study (Chapter 4: Michalak *et al.*, 2000). For cloning of TNFα, IL-2, IL-4, and IL-6, woodchuck cDNA was initially amplified by PCR using degenerate oligonucleotide primers with sequences deduced through interspecies comparison of available human, mouse, rat and rabbit sequences using PC Gene software (Intelligenetics Inc., Mountain View, CA). Briefly, total RNA (1 µg) from Con-A-stimulated woodchuck PBMC was reverse transcribed to cDNA (Section 2.7). Five µl of cDNA in a standard reagent mixture (Section 2.8.3) was denatured for 5 min at 94°C and amplified for 35 cycles using the following cycle parameters: 94°C for 1 min, 52°C for 2 min and 72°C for 3 min. These PCR amplifications were carried out in a TwinBlock Thermal cycler (Ericomp Inc.). The specificity of the PCR products was verified by Southern blot analysis using ^{32}P-end labeled oligonucleotide probes with sequences internal to those of the amplification primers. Then, the DNA amplicons were cloned into the dual promoter PCR II vector using the TA-cloning system (Invitrogen) and sequenced using the *f*mol DNA Sequencing System (Promega Corp.) or a fluorescence-based automated sequence analyzer (LI-

Table 5-1. Serological and virological characteristics of WHV infection and numbers of liver samples acquired from animals studied

Outcome of Acute Hepatitis & Animal No/Sex [a]	Observation period [b] (mo)	Serology			Duration of serum WHV DNA positivity [c] (mo)	Number of Liver Samples Analyzed in Identified Phases of WHV Infection			
		Duration of WHsAg (mo)	Duration of Anti WHs (mo)	Duration of Anti WHc (mo)		Healthy	Acute	Resolved	Chronic
Resolved Hepatitis									
6/M	60	2	neg	59	59 5	0	1	6	NA [d]
3/F	60	3	/	59	59	0	1	5	NA
11/M	57 5	1	neg	56	56 5	0	2	4	NA
12/M	53	0 5	9	51 5	52	1	0	6	NA
5/M	48	1	36	47	47 5	0	2	5	NA
2/F	47	4	6	46 5	46 5	0	2	5	NA
7/F	37	0 5	4	35 5	36	0	0	5	NA
13/M	12	0 5	2 5	11	11	1	0	3	NA
14/F	12	1	2	11	11	1	0	3	NA
15/F	12	0 5	1	11	11	1	0	3	NA
16/F	12	0 5	1 5	10 5	11	1	1	2	NA
17/M	5	0 5	neg	4	4 5	1	0	2	NA
18/F	5	1	neg	4	4	1	0	2	NA
19/F	1 5	0 5	neg	0 5	1	1	1	0	NA
20/F	1 5	1	0 5	1	1	1	1	1	NA
					subtotal	**9**	**11**	**52**	**0**
Chronic Hepatitis									
21/F	60	59	NT [e]	59 5	59 5	1	0	NA	3
22/M	40 5	39 5	NT	40	40	0	2	NA	4
23/F	34 5	33 5	NT	34	34	0	1	NA	4
24/F	26 5	24 5	NT	24 5	25 5	1	1	NA	3
25/M	18	17 5	NT	17 5	17 5	1	1	NA	1
26/F	15	14	NT	13 5	14 5	1	1	NA	2
27/F	15	14	NT	14	14 5	1	2	NA	1
					subtotal:	**5**	**8**	**0**	**18**
					total:	**14**	**19**	**52**	**18**

[a] Animals 6/M, 3/F, 5/M, 2/F and 7/F were a part of the previous study examining occult WHV persistence acquired after resolution of acute hepatitis (Michalak *et al*., 1999)
[b] From inoculation with woodchuck hepatitis virus
[c] WHV DNA detected by dot blot hybridization and when negative by PCR/ hybridization of the amplified product to recombinant WHV DNA probe
[d] NA, not applicable
[e] NT, not tested

COR)(Section 2.12; Michalak *et al.*, 2000). After sequence determination, primers spanning woodchuck specific gene introns were designed with the aid of a sequence similarity search tool (www.ncbi.nlm.nih.gov/BLAST). The exceptions were primers for woodchuck IL-2 and IL-4 which were located within one exon of each respective gene. Primers were optimized for length, GC content and melting temperatures using OMIGA software (Intelligenetics Inc.).

5.2.5 Determination of cytokine gene expression

Despite efforts, Northern blot hybridization did not achieve sufficient sensitivity even when up to 30 µg of total RNA per lane was tested. Therefore, assays based on RT-PCR were developed. Expression of each of the genes studied was analyzed in the linear PCR amplification range using 5 µl of test cDNA and serial 10-fold dilutions of the appropriate cloned gene fragment run as a quantitative standard in each PCR set-up (see example Figure 4-1). For detection of IL-2 and IL-4 sequences, since PCR primers were located within one exon of each respective gene, the samples were DNase digested for 20 min at 37°C prior to RT following the manufacturer's instruction (Ambion Inc., Austin, TX). Amplification of woodchuck IFNγ and CD3 epsilon sequences was done without DNase treatment using oligonucleotide primers and parameters previously described (Section 4.2.10). For amplification of TNFα, the sense and antisense primers 5'-ATGAGCACTGAAAGTATGATCCG and 5'-

CTCAGCAAAGTCGAGATAGC were used, respectively. The gene expression of woodchuck IL-6 was determined with plus 5'-ATCTGCCCTTCAGGAACAGCC and minus 5'-AGCTTAGATGCCCACTATGC primers. Woodchuck IL-2 was amplified with the sense primer 5'-TGGAGGAAGTGCTGAATGTACC and the antisense primer 5'-GATGTTATACACGGGAGGCACC and IL-4 with sense and anti-sense primers 5'-TTCTGTCTCCTAGTATGCCC and 5'-GGAAGTCTTTCAATGTTCTCTGC, respectively. In addition, β-actin and GAPDH were amplified as loading controls. β-actin was amplified using 1 µl of liver cDNA and woodchuck specific primers 5'-CATCCTCACCCTGAAGTACC and 5'-CATACTCCTGCTTGCTGATCC, whereas GAPDH was amplified using primers described elsewhere (Chapter 4; Michalak *et al.*, 2000; Ju *et al.*, 1995). All PCR amplifications were performed in a PTC-200 thermocycler (MJ Research, Watertown, MA), except IFNγ which was done in a TwinBlock thermal cycler (Ericomp Inc.). After gel electrophoresis, the relative band density of the PCR products was compared to plasmid quantitative standards using a computerized Chemi-Imager 4000 System (Canberra-Packard Canada Ltd.). PCR product density for each test sample was found to be in the linear range of the plasmid standard curve (r^2>0.93) that was generated in parallel with each reaction set as illustrated for IFNγ in Figure 4-1. For IL-2 and IL-4 detection, 20 µl of each amplification product was immobilized on nylon membrane (Hybond XL; Amersham) by microfiltration and subsequently hybridized with the

appropriate cloned ^{32}P-labeled probe (Section 2.9.3). Hybridized blots were exposed to a phosphor screen (Canberra-Packard Canada Ltd.) for final quantitative analysis.

5.2.6 Statistical analysis and gene sequence accession numbers

The densitometric values on WHV DNA, cytokines and CD3 expression were analyzed using the Mann-Whitney non-parametric test to determine the significance between groups. Two-tailed p values < 0.05 were considered significant. The GenBank accession numbers for the woodchuck nucleotide sequences derived in this study are as follows: IL-2, AF333964; IL-4, AF333965; IL-6, AF333966; TNFα, AF333967, and β-actin, AF232730 (see Appendix B).

5.3 RESULTS

5.3.1 Progression to chronic hepatitis is associated with a higher hepatic WHV load during acute infection

The average hepatic WHV DNA content collected from acutely infected animals in this study was not significantly different from that estimated for liver biopsies obtained during CH, as measured by dot-blot hybridization and densitometric analysis of hybridization signals (sensitivity 10^6 WHV vge/ml). For both groups, the virus loads were in the range of 1.5×10^7 to 9.5×10^7 vge/μg of liver DNA and they were comparable to those previously reported (Michalak *et*

al., 2000). However, when the average WHV DNA contents were calculated separately for liver samples acquired during acute infection from animals that ultimately resolved AH or developed CH, the WHV load was 2.7-fold greater for woodchucks which progressed to CH ($p = 0.06$) (Table 5-2). Nevertheless, there were samples in both groups for which the WHV DNA values overlapped with those from the other group (Table 5-2) and, therefore, did not provide an indication about the possible outcome of AH. In the cases examined in this study, the expression of WHV-specific mRNA was not meaningfully different between woodchucks which resolved AH or progressed to CH when assessed by Northern hybridization (data not shown) or by RT-PCR (Fig. 5-2). The reason for the apparent discrepancy between viral load and viral RNA is not known. As expected, small amounts of WHV genomes were identifiable by nested PCR/Southern blot hybridization (sensitivity $<10^2$ WHV vge/ml) in livers of animals which had recovered from AH. This result was consistent with our previous findings (Michalak *et al.*, 1999; Lew and Michalak, 2001; Michalak *et al.*, 2000). WHV-specific RNA signals were detectable by nested PCR amplification of cDNA in the majority of liver tissue samples obtained from the woodchucks with SLAH and their intensity did not vary significantly throughout the lifespan of the animals investigated (data not shown), similar to observations in our previous studies (Michalak *et al.*, 1999; Michalak *et al.*, 2000).

5.3.2 Upregulated hepatic IFNγ and CD3 transcription accompanies chronic hepatitis and continues long after resolution of acute WHV infection.

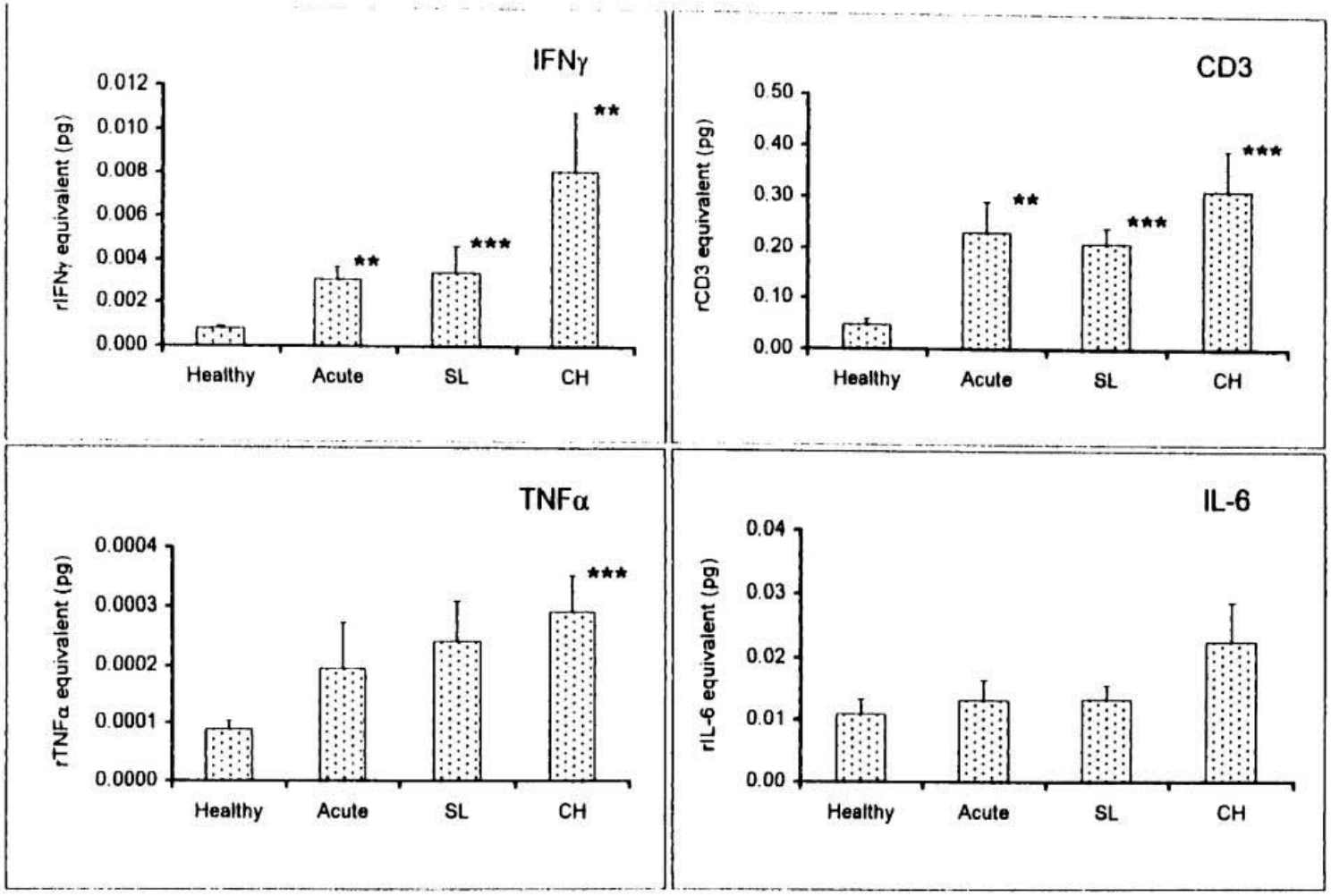
The overall IFNγ mRNA levels were found to be low in livers of healthy animals but significantly elevated in hepatic tissue samples from woodchucks with AH, reaching levels of nearly 4-times greater than those detected in healthy animals ($p < 0.005$)(Fig. 5-1). IFNγ gene expression was elevated to approximately the same levels as that seen in AH in the livers of woodchucks with CH ($p < 0.005$). Similarly, the hepatic CD3 mRNA expression, an indicator of T lymphocyte infiltration, was barely detectable in normal, WHV-uninfected animals, but upregulated (approximately by 5-fold) in hepatic tissue from woodchucks with either AH ($p < 0.005$) or CH ($p < 0.0005$). Unexpectedly, liver samples collected from woodchucks which resolved acute WHV infection and were followed, in some cases, throughout their entire natural lifespan also demonstrated statistically significantly greater IFNγ and CD3 mRNA levels than those detected in healthy animals ($p < 0.0005$). In summary, the results revealed that transcription of hepatic IFNγ and CD3 remains elevated not only in chronic WHV hepatitis but also, although at lower levels, long after apparently complete recovery from AH. TNFα mRNA was detected at significantly higher levels in hepatic tissue from animals with CH in comparison to that in healthy woodchucks ($p < 0.0005$) (Fig. 5-1). In general, transcription of TNFα tended to be elevated

Table 5-2 Hepatic WHV DNA content and histological features of liver injury in adult woodchucks with experimentally induced acute WHV hepatitis who recovered or developed chronic hepatitis.

Outcome of AH and Animal No./Sex	Time of liver biopsy (wk)[a]	WHV DNA (10^7 vge/µg liver DNA)	Morphological alterations			Histological degree of hepatitis[b]
			Hepatocellular	Extracellular intralobular	Portal	
SLAH						
6/M	8	0.6	2	2	2	2
3/F	6	4.5	1.5	1.5	1	1.5
11/M	6	1.8	1.5	2	2	2
	14	0[c]	1	1.5	1.5	1.5
5/M	4	0.5	2	2	2.5	2.5
	13	0.6	2	1.5	2	2
2/F	6	3.3	3	3	2.5	3
	13	1.7	2	2	2	2
16/F	6	0.2	1.5	1.5	2	2
19/F	2	7.4	2	2.5	2.5	2.5
mean (± SE):	**7.8 (± 1.3)**	**2.0 (±0.7)**	**1.8**	**1.9**	**2**	**2.1**
CH						
22/M	6	14.0	1	1	1	1
	14	7.3	1	1	1.5	1
23/F	3	8.0	1	1.5	1.5	1.5
24/F	7	0.9	0.5	0.5	0.5	0.5
25/M	4	7.3	0.5	0.5	0.5	0.5
26/F	6	0.9	2.5	2	2.5	2.5
27/F	1	4.4	0.5	0.5	0	0.5
	8	1.7	2	2	1.5	2
mean (± SE):	**6.1 (± 1.4)**	**5.6 (±1.6)**	**1.1**	**1.1**	**1.1**	**1.2**
***p*[d]**	**0.51**	**0.06**	**0.04**	**0.02**	**0.02**	**0.02**

[a] From the appearance of WHV surface antigen in serum.
[b] Histological degree of hepatitis from 0 to 3 reflects severity of liver injury determined on the basis of grades separately assigned for each category of hepatic tissue alterations (Section 2.5).
[c] Reactive for WHV DNA by direct PCR with WHV core gene specific primers.
[d] Statistical comparison (p) by non-parametric two tailed Mann-Whitney test.

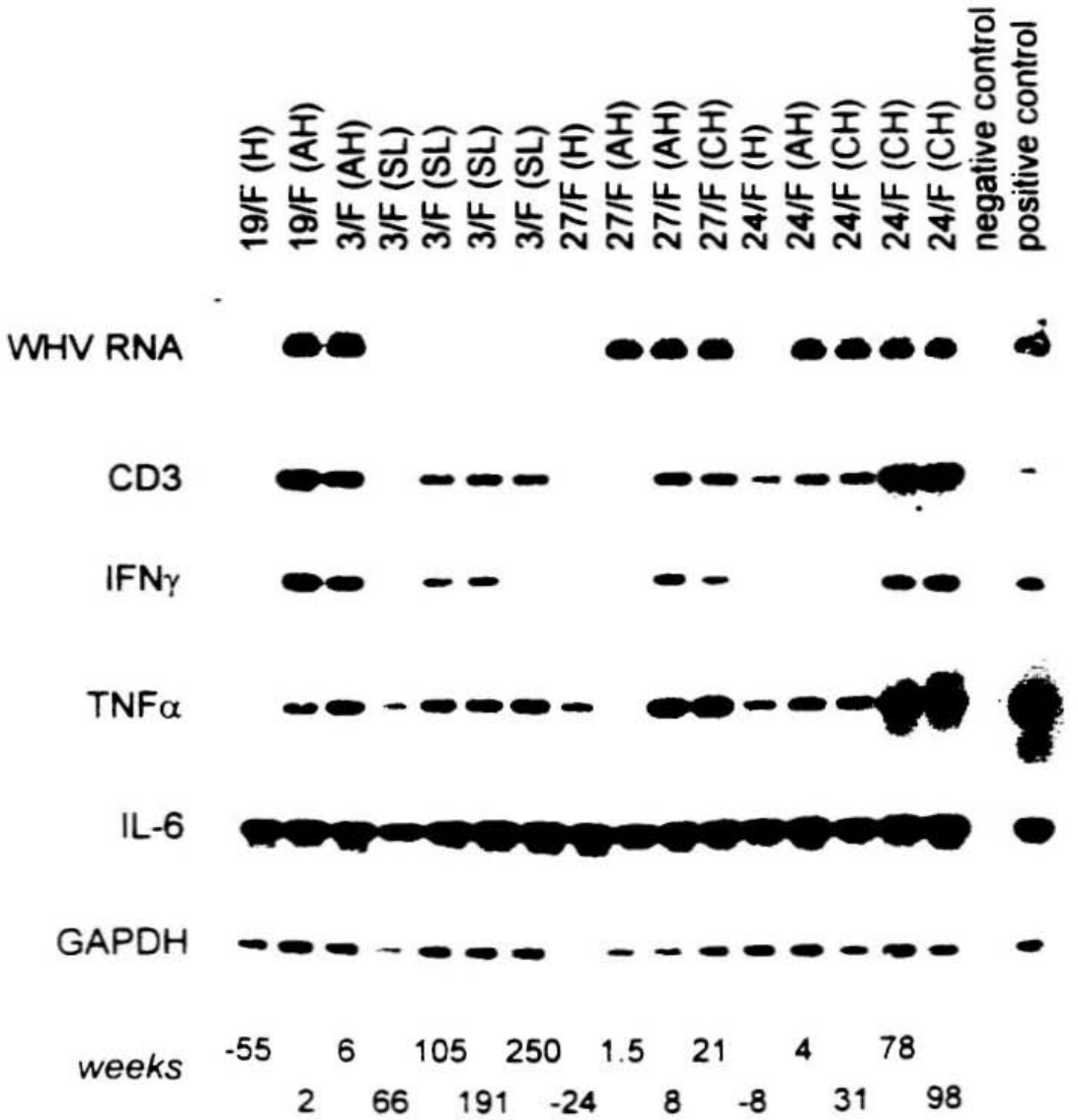
Figure 5-1. Analysis of cytokine and CD3 gene expression in liver samples obtained from adult woodchucks prior to inoculation with WHV, during acute or chronic WHV hepatitis, or after resolution of acute infection. The histograms summarize the results of IFNγ, CD3, TNFα and IL-6 mRNA levels identified by RT-PCR using total RNA (0.25 µg/reaction) isolated from liver biopsies collected from healthy animals (Healthy; n = 14), during the acute phase of WHV infection (Acute; n = 19), in convalescent period after resolution of AH (SL; n = 52), and in the course of CH (n=18). The median and range of acquisition times for biopsies obtained from AH, SL and CH phases were 11 range 5-19 weeks, 77 range 4-261 weeks, and 76 range 17-202 weeks, respectively. Results are presented as mean pg equivalent $\pm$ SE determined for each liver sample by comparison to PCR band density standard curves generated by amplification of serial dilutions of the appropriate recombinant woodchuck gene sequences. Data bars marked with ** are significant at $p < 0.005$ and those with *** at $p < 0.0005$ when compared to the levels detected in livers of healthy woodchucks.


IFNγ
rIFNγ equivalent (pg)
0.012
0.010
0.008
0.006
0.004
0.002
0.000
**
***
**
Healthy
Acute
SL
CH
CD3
rCD3 equivalent (pg)
0.50
0.40
0.30
0.20
0.10
0.00
**
***
***
Healthy
Acute
SL
CH
TNFα
rTNFα equivalent (pg)
0.0004
0.0003
0.0002
0.0001
0.0000
***
Healthy
Acute
SL
CH
IL-6
rIL-6 equivalent (pg)
0.04
0.03
0.02
0.01
0.00
Healthy
Acute
SL
CH

both during acute infection and after resolution of AH, but the differences did not reach a statistical significance. The overall hepatic IL-6 expression appeared to be slightly increased in CH, but it remained essentially at the same levels in livers of healthy woodchucks and those acquired during and after resolution of acute infection (Fig. 5-1).

Although IL-2 transcription was detectable in the majority of the tissue samples tested by dot-blot hybridization of RT-PCR products, the expression of this cytokine was not meaningfully different between healthy and recovered animals and those progressing to either AH or CH (data not shown). The transcription of IL-4 was detected only rarely in livers from both healthy and WHV-infected animals and their levels were without any noticeable relation to the status of WHV hepatitis (data not shown). Figure 5-2 illustrates a time course of the detection of the WHV, CD3 and cytokine mRNA in serial liver biopsies acquired from 4 animals who after WHV inoculation developed AH and either resolved the disease (19/F and 3/F) or advanced to CH (27/F and 24/F). As shown, the density of hybridization signals reflecting expression of IFNγ, TNFα and CD3 mRNA mirror each other in the individual liver samples. Evidently, the expression of IL-6 did not follow those of IFNγ, TNFα and CD3, as the density of hybridization signals was approximately the same in the liver samples obtained from the same woodchucks prior to WHV inoculation, during and after AH or in the course of CH.

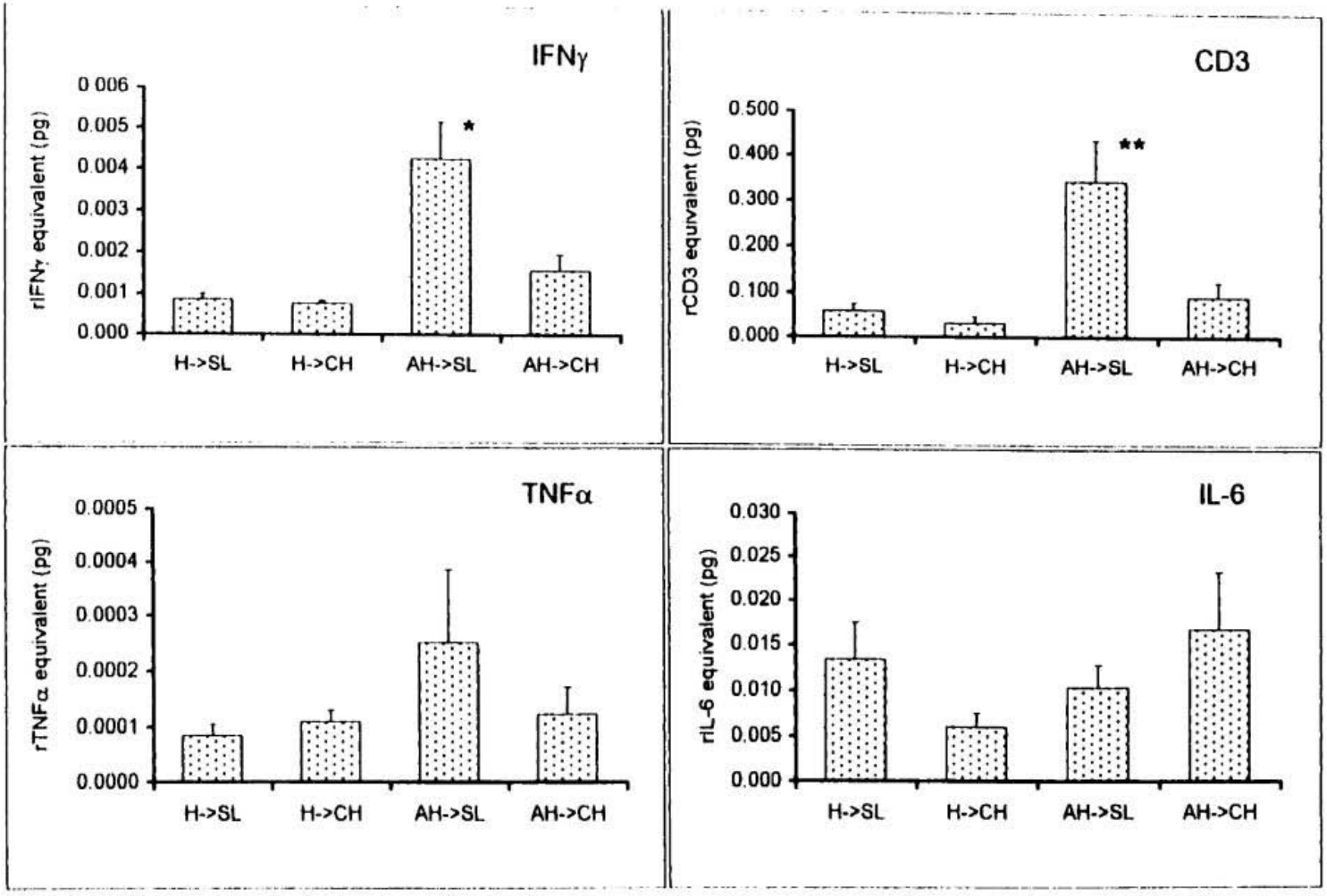
Figure 5-2. A time course of cytokine, CD3 and WHV mRNA expression in serial liver biopsies collected from woodchucks with experimentally induced WHV infection which either resolved acute hepatitis or developed chronic liver disease. Total RNA (0.25 µg/reaction) isolated from liver biopsies taken prior to WHV inoculation (H) and at the indicated time points after the appearance of WHsAg in the circulation (weeks) during acute infection (AH) which progressed to CH or after spontaneous resolution of acute infection (SL). The biopsies were obtained from 2 woodchucks (19/F and 3/F) who finally resolved AH and 2 animals (27/F and 24/F) in which disease advanced to CH. The expression of the individual genes or WHV mRNA was determined by RT-PCR under conditions described in Materials and Methods (Section 2.8) and the resulting amplicons were visualized by Southern blotting with appropriate ^{32}P-labeled cloned woodchuck probes. WHV mRNA signals were detected with ^{32}P-labeled complete recombinant WHV DNA. GADPH was used as a housekeeping gene. Mock extracted samples treated under identical conditions as test RNA was used as a negative control and appropriate recombinant woodchuck gene fragments as positive PCR controls. The intensity of CD3, IFNγ and TNFα signals approximately parallel each other in the individual samples obtained from either healthy animals or those with different stages of WHV hepatitis.


19/F (H)
19/F (AH)
3/F (AH)
3/F (SL)
3/F (SL)
3/F (SL)
3/F (SL)
27/F (H)
27/F (AH)
27/F (AH)
27/F (CH)
24/F (H)
24/F (AH)
24/F (CH)
24/F (CH)
24/F (CH)
negative control
positive control
WHV RNA
CD3
IFNγ
TNFα
IL-6
GAPDH
weeks
-55
6
105
250
1.5
21
4
78
2
66
191
-24
8
-8
31
98

5.3.3 Increased IFNγ and CD3 gene expression during acute phase of WHV infection precedes recovery from hepatitis.

To determine whether hepatic expression of IFNγ, TNFα, IL-6, and CD3 could predict the outcome of acute WHV infection, we evaluated the levels of relevant mRNA in the liver samples obtained from healthy animals before inoculation with WHV and from the same woodchucks during acute phase of WHV infection, and analyzed them according to whether the animals ultimately resolved AH or developed CH. Figure 5-3 shows that there were only minimal, statistically non-significant differences in the intrahepatic expression of IFNγ, TNFα, IL-6 or CD3 mRNA in all healthy woodchucks. However, liver samples acquired during acute infection from the animals who subsequently cleared hepatitis, but not those who developed CH, demonstrated significantly greater overall levels of IFNγ mRNA ($p < 0.05$) and CD3 expression ($p < 0.005$) when compared to the samples obtained from the same animals prior to WHV infection (Fig. 5-3). Furthermore and most importantly, expression of IFNγ and CD3 in hepatic tissue collected during acute infection from the woodchucks who prospectively resolved AH was significantly greater than in those who developed CH ($p < 0.05$ and $p < 0.005$, respectively). Although the average mRNA level of TNFα demonstrated similar trends to those identified for IFNγ and CD3 mRNA, its expression did not statistically vary between the groups analyzed (Fig. 5-3).

Figure 5-3. Comparison of intrahepatic cytokine and CD3 gene expression in healthy woodchucks and in animals during acute phase of WHV infection who either resolved hepatitis or developed chronic liver disease. The histograms summarize the results on IFNγ, CD3, TNFα and IL-6 expression evaluated by RT-PCR on RNA samples isolated from liver biopsies of healthy woodchucks that ultimately recovered from acute infection (H->SL; n=9) or developed CH (H->CH; n=5) and from animals with serologically and histologically confirmed acute WHV infection who subsequently resolved disease (AH->SL; n=10) or advanced to CH (AH->CH; n=8). The mean time of biopsy acquisition from animals who resolved AH was 7.8 ± SE 1.3 weeks, whereas that for animals who progressed to CH was 6.1 ± SE 1.4 weeks (see Table 5-2). Results are presented as means ± SE, determined as described in the legend to Figure 5-1. Data bar marked with * is significant at $p < 0.05$ and that with ** at $p < 0.005$ when compared to liver samples from healthy animals with a self limited or chronic outcome of AH, respectively.

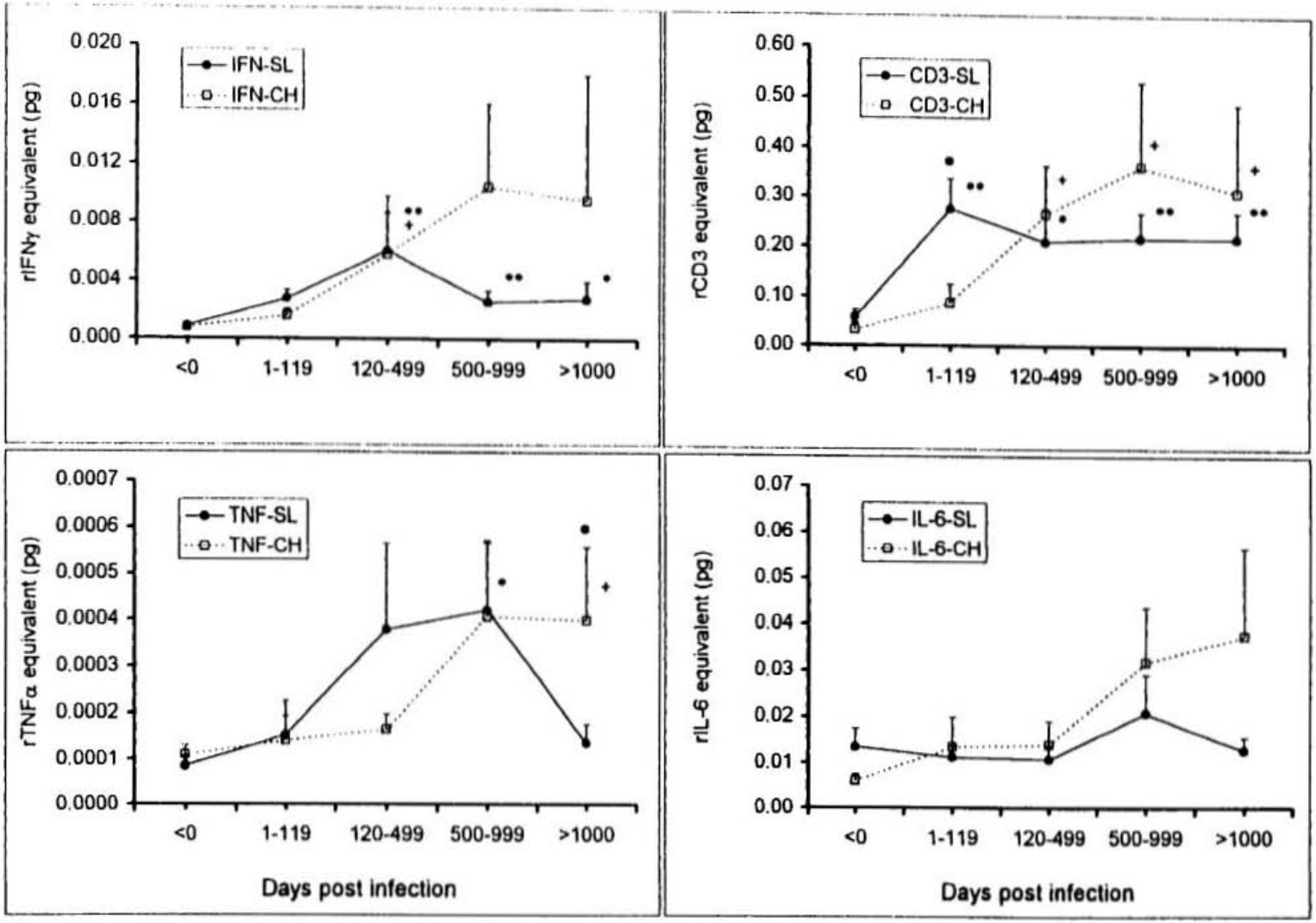

IFNγ
rIFNγ equivalent (pg)
0.006
0.005
0.004
0.003
0.002
0.001
0.000
*
H->SL
H->CH
AH->SL
AH->CH
CD3
rCD3 equivalent (pg)
0.500
0.400
0.300
0.200
0.100
0.000
**
H->SL
H->CH
AH->SL
AH->CH
TNFα
rTNFα equivalent (pg)
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
H->SL
H->CH
AH->SL
AH->CH
IL-6
rIL-6 equivalent (pg)
0.030
0.025
0.020
0.015
0.010
0.005
0.000
H->SL
H->CH
AH->SL
AH->CH

In contrast to IFNγ and CD3, the rate of IL-6 transcription remained relatively constant between all groups studied. Taken together, the results clearly showed that highly upregulated expression of hepatic IFNγ and CD3, an indicator of T cell infiltration, and an increased transcription of TNFα during acute WHV infection is an indicator of the favourable outcome of AH in adult woodchucks.

5.3.4 Intrahepatic cytokine and CD3 expression profiles in WHV infection progressing to recovery or chronic hepatitis.

To determine cytokine transcription and T cell infiltration dynamics during progression from acute WHV infection to CH, recovery from AH, and during occult lifelong WHV persistence after termination of acute infection, the results on liver expression of relevant mRNA were analyzed after grouping them according to the time period between WHV inoculation and acquisition of the liver samples. Average expression of the individual cytokines or CD3 was calculated for each of the time periods (Fig. 5-4). The results showed that only the animals which finally resolved AH showed an early (before 119 d.p.i.) increase in hepatic CD3 expression ($p<0.005$) and that this increment was significantly greater ($p<0.05$) than that for woodchucks progressing to CH (Fig. 5-4). Interestingly, the CD3 mRNA level remained higher than preinfection levels in the recovered animals for more than 3 years after resolution of acute infection. In woodchucks who developed classical CH, the average hepatic CD3

Figure 5-4. Profiles of intrahepatic cytokine and CD3 gene expression in adult woodchucks who developed acute WHV hepatitis followed by recovery and persistent occult WHV infection or progressed to chronic hepatitis. The hepatic IFNγ, CD3, TNFα and IL-6 transcription levels were analyzed before inoculation (days post infection < 0) and in liver biopsies obtained throughout the course of WHV infection from animals who ultimately resolved AH (SL) or developed CH. The data were subsequently grouped according to the time periods elapsed between WHV inoculation and acquisition of test liver samples. Each data point represents the mean (± SE) expression of the indicated mRNA examined in 9 to 19 (mean, 14) or 4 to 10 (mean, 7) individual liver biopsy samples collected from woodchucks with self limiting AH or CH, respectively. Results are presented as pg equivalents of the respective recombinant woodchuck gene fragments, as described in the legend to Figure 5-1. Data points marked with * or + are significant at $p < 0.05$ and those with ** or ++ at $p < 0.005$ when compared to the levels detected in livers from healthy woodchucks who finally recovered from hepatitis (*, SL) or progressed to chronic liver disease (+, CH). The means marked with ● are significantly different at $p < 0.05$ between animals who resolved acute infection and those who developed CH.


rIFNγ equivalent (pg)
0.020
0.016
0.012
0.008
0.004
0.000
IFN-SL
IFN-CH
<0
1-119
120-499
500-999
>1000
rCD3 equivalent (pg)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
CD3-SL
CD3-CH
rTNFα equivalent (pg)
0.0007
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
TNF-SL
TNF-CH
Days post infection
rIL-6 equivalent (pg)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
IL-6-SL
IL-6-CH
Days post infection

expression rose at a later stage of infection (120 d.p.i.) and remained elevated up to the end of follow-up (Fig. 5-4). The average IFNγ gene expression tended to increase until approximately one year into infection in animals who either recovered from AH or developed CH (Fig. 5-4). After approximately one year, the IFNγ mRNA began to decline in the woodchucks who cleared hepatitis, although the average level of IFNγ expression never returned to that observed in healthy animals (Fig. 5-4). In contrast, the mean IFNγ mRNA levels in the animals which developed CH continued to increase, reaching a plateau after approximately 3 years of infection.

Hepatic TNFα expression rose from the moment of WHV infection in animals that subsequently recovered from AH and remained elevated long after termination of acute infection, although statistical significance was found only for biopsies obtained between 500-999 d.p.i. (Fig. 5-4). In the final phase of follow-up of these animals (>1000 d.p.i.), the average TNFα mRNA returned nearly to the values detected in healthy woodchucks. In animals with CH, hepatic TNFα transcription became elevated 500 d.p.i and remained at approximately the same levels to the end of the observation period.

In contrast to the increases seen in hepatic CD3, IFNγ and TNFα expression, there were no apparent changes in the average rate of hepatic IL-6 transcription throughout the life span of the animals that ultimately recovered from AH. The level of IL-6 transcription showed a slow progressive increase during the course of CH after 500 d.p.i., although it never reached statistical

significance (Fig. 5-4).

5.3.5 Recovery from hepatitis is associated with an exacerbated episode of acute liver inflammation

Morphological severity of WHV-induced liver injury was evaluated in all liver samples examined in this study using numerical scores separately assigned for hepatocellular, extrahepatocellular intralobular and portal alterations, and by taking into consideration a global impression of the pathological picture of liver damage as a whole, according to the criteria described in detail in previous works (Michalak and Lin, 1994; Michalak *et al.*, 1990) and in Section 2.5. A particular emphasis was placed on the recognition of the extent of morphological changes in the acute phase of hepatitis in animals who ultimately recovered from AH or developed CH. Table 5-2 summarizes the data on these assessments in 7 woodchucks in which AH was resolved and in 6 animals with acute infection that progressed to CH. The results demonstrate that the grade of necroinflammation in acute phase of WHV infection, referred to as histological degree of hepatitis, was significantly greater ($p<0.05$) in the animals which ultimately recovered from AH (mean score 2.1) than in those which advanced to CH (mean score 1.2). This difference was the result of the aggravated morphological damage diagnosed in all three compartments of liver parenchyma analyzed as described in Section 2.5. Thus, the extent of hepatocellular lesions (including single cell, spotty, piecemeal and/or bridging necrosis, degenerative changes and presence

of acidophilic bodies), the range of extrahepatocellular changes (including the degree and distribution of intralobular inflammatory cell infiltrates, influx of macrophages, plasma cells and neutrophils, hyperplasia of sinusoidal Kupffer cells and lining endothelium, disorganization of reticular network), and the status of portal alterations (embodying oedema of portal areas, inflammatory infiltrations, bile duct proliferation) were more severe ($p<0.05$) in animals who subsequently recovered from AH than in those who developed CH (Table 5-2). In 5 of the woodchucks (2/F, 5/M, 11/M, 22/M and 27/F), the second liver sample collected during acute phase of WHV infection in 7 to 8 weeks after the first biopsy was also available for examination. In these auxiliary biopsies from the animals which resolved AH (2/F, 5/M and 11/M), histological alterations were generally less severe than those detected in the earlier phase of AH, suggesting that the disease already subsided in this time period (Table 5-2). Two second liver samples available from 2 woodchucks in which CH became established showed either a similar level of acute inflammation (22/M) or much more severe hepatitis (27/F) than that diagnosed in the earlier biopsy from the same animals (Table 5-2).

As expected, histological examination of the liver tissue samples acquired during serologically evident chronic infection, showed features of lingering necroinflammation with a variable degree of liver injury usually ranging from moderate (grade 2) to severe liver damage (grade 3)(Michalak, 1998; Michalak and Lin, 1994). Serial liver biopsies obtained from woodchucks recovered from

AH showed recurring, minimal to very mild inflammatory changes (not exceeding histological degree of hepatitis consistent with grade 1) that continued throughout life with periods of normal or nearly normal liver morphology, as described in the previous study from this laboratory (Michalak *et al.*, 1999). Liver biopsies from healthy animals did not show any pathological alterations.

5.4 DISCUSSION

The multiparametric analysis of serial liver samples from adult woodchucks prior to and during the course of WHV infection allowed a recognition of the lifelong intrahepatic dynamics and the interdependencies between expression of key antiviral cytokines, CD3-positive T cell infiltration, liver injury and virus load in hepadnavirus infection. The results showed that the acute phase of WHV infection in adult woodchucks significantly differs with respect to intrahepatic expression of IFNγ and TNFα, the degree of T cell infiltration, severity of liver inflammation and hepatic WHV content, depending upon whether the host recovered or had persistent viral hepatitis. Hence, while the resolution of AH is preceded by upregulated transcription of IFNγ and TNFα, augmented liver T cell influx and increased hepatic tissue damage, early WHV infection ultimately evolving to CH had the opposite characteristics and is typically associated with a higher viral load. Despite differences in viral load, there were no changes in viral gene expression during AH regardless of whether the animal recovered or progressed to CH. These results are consistent with

those obtained in neonatal woodchucks (Cote *et al.*, 2000). The authors of this study suggest the differences between DNA and RNA expression may be related to host inhibition of the reverse transcription step of pregenomic RNA into DNA or on the polymerase-mediated second-strand DNA synthesis. Our study also revealed that the serologically concealed, lifelong persistence of WHV, which is a usual consequence of recovery from an acute episode of adult WHV infection (Michalak *et al.*, 1999; Coffin and Michalak, 1999), is accompanied by an enhanced intrahepatic expression of IFNγ and TNFα and by the presence of the T-cell marker CD3. Unexpectedly, T cell influx was not readily detectable by histological examination.

In this adult model of hepadnavirus infection, acute infection induced by the same pool and dose of WHV either subsided or advanced to chronicity in the context of the fully developed immune system, in which all arms of virus-specific and innate immunological responses would be expected to be operational. This contrasts WHV infection acquired in the neonatal period where the immunological immaturity of the host at the time of the primary virus exposure may significantly influence the range and the strength of immune reactions, and predetermine the pathological outcome of the infection. Furthermore, the capacity to produce individual cytokines may change with age, as reported with increasing IFNγ production during childhood (Campbell *et al.*, 1999), making extrapolation to an adult situation difficult. Despite fundamental differences between adults and neonates in responses to microbes and exogenous antigens

(Arvin, 1997), the parameters characterizing the early phase of WHV infection progressing to recovery or chronicity uncovered in our study are, unexpectedly, similar to those delineated for neonatal WHV infection (Cote *et al.*, 2000; Nakamura *et al.*, 2001). Thus, eventual recovery from AH is associated with greater levels of hepatic IFNγ and TNFα mRNA expression, increased histological severity of hepatitis, and lower initial viral load in both adults and neonates. A similar profile of alterations has also been observed in another study in which adult woodchucks with transient WHV hepatitis were investigated (Guo *et al.*, 2000). In this work, the recovery from AH was also preceded by an influx of CD3-positive T cells and by a transient increase in CD4 and CD8-positive cells in the liver which were estimated by examining expression of respective marker mRNA. Therefore, although frequency of spontaneous recovery from WHV hepatitis and progression to CH are dramatically different in adults and neonates, the same parameters measured in the early phase of infection depict its final outcomes independent of age of the animal host.

Analysis of the liver biopsy material collected from healthy animals prior to WHV inoculation in the current study did not provide an indication in regard to the ultimate outcome of hepatitis, nor did we see noticeable variations in the indigenous expression of cytokine genes tested in the healthy woodchucks investigated. However, genetic predisposition to high or low cytokine production, including large differences in both the numbers of Th1 cytokine expressing cells and in the quantity of Th1 and Th2 cytokine proteins secreted between normal

humans or animals, have been reported (Cartwright *et al.*, 1999). These natural variations in the Th1 and Th2 responses might be an important element predisposing an infected host to a prompt and strong, or a delayed and insufficient, Th1 cytokine response (Paul and Seder, 1994; Hunter and Reiner, 2000). There are a number of microbial infections explicitly illustrating a critical role of the imbalance between Th1 and Th2 cytokine reactivity in the infection outcome, including leprosy and experimental murine leishmaniasis (Yamamura *et al.*, 1992; Sher and Coffman, 1992). The same seems to be true with respect to the readiness of innate immunity to mount a defense against invading pathogens (Biron *et al.*, 1999). In the case of WHV infection, the issue of the natural predisposition to a particular type of immune response reflected in the cytokine expression profile and its eventual association with susceptibility to CH need to be re-examined when assays measuring the cytokine proteins and markers identifying individual cell types producing these cytokines become available.

The increased intrahepatic expression of IFNγ and TNFα was accompanied by the presence of CD3-positive T cells in the liver in all stages of WHV infection analyzed in this study, including the late convalescent phase. However, as the dynamics of the hepatic transcription of these two cytokines and CD3 showed, the recovery from AH was preceded by the increased expression of CD3 mRNA followed by upregulation in IFNγ and TNFα mRNA (Fig. 5-4), implying that the liver infusion with CD3-positive T cells occurred prior to

enhancement in cytokine production. This result seems to be compatible with that reported by Guo *et al.* (2000) where the peak of liver IFNγ and TNFα expression was preceded by elevations in CD3, CD4 and CD8 mRNA in adult woodchucks progressing to recovery.

The increased levels of IFNγ and TNFα mRNA in the early WHV infection in the resolving animals does not exclude the possibility that the cytokine upregulation was, at least in part, a consequence of activation of an innate immune response coinciding with T cell influx. It is known that IFNγ and TNFα are not only produced by antigen-primed CD3/CD8-positive CTL and CD3/CD4-positive T cells of the Th1 subtype but also by activated NK cells, NKT cells and macrophages (Biron *et al.*, 1999; Bendelac *et al.*, 1997). Further, it is now evident that the liver is naturally abundant in NKT cells (Bendelac *et al.*, 1997; Doherty *et al.*, 1999), where they may account for up to 30% of intrahepatic lymphocytes, and that NKT cells are able to secondarily activate NK cells through secretion of IFNγ and they are likely to be involved in the control of intracellular pathogens and some tumors (Bendelac *et al.*, 1997; Denkers *et al.*, 1996; Cui *et al.*, 1997). Altogether, this raises the possibility that innate immune cells residing in the liver may contribute to both the pathogenesis and the resolution of acute hepadnaviral hepatitis. The recent data from transgenic mice and chimpanzee models of HBV infection support this possibility and imply that T-cell-independent induction of hepatic IFNγ, IFNα/β, and TNFα may lead to transient inhibition of HBV replication (Guidotti *et al.*, 1999; Kakimi *et al.*, 2000).

At this stage, innate immunity and its cellular components have not yet been characterized in the woodchuck. However, the available data indicate that an increase in the number of macrophages and proliferation of sinusoidal lining epithelium, including Kupffer cells, are typical features of early WHV infection in adult animals (Michalak, 1998) and that they are progressively prominent when histological severity of AH escalates. The same appears to be also true for acutely infected neonates (Nakamura *et al.*, 2001). This indicates that activation of the monocyte/macrophage lineage is a typical constituent of the early liver inflammatory response to WHV infection. Furthermore, our recent study demonstrated that increased perforin-mediated cytotoxicity by PBMC occurs selectively in the early phase of WHV infection (Chapter 3; Hodgson *et al.*, 1999). This observation supports a possible role for innate cell response, in particular NK cells and possibly NKT cells, in the recovery from hepatitis in acutely infected woodchucks. In this context, the baseline expression of intrahepatic IL-2 mRNA during acute WHV hepatitis, reported similarly for neonatal WHV infection (Nakamura *et al.*, 2001), might exclude the presence of activated CTL or CD4-positive Th1 cells and, therefore, support involvement of natural killer-like cells and macrophages. However, following a similar type of argument, the apparent absence of the upregulated IL-4 mRNA expression in the early phase of WHV infection may argue against NKT cell involvement, since these cells are producers of IL-4 in other species (Bendelac *et al.*, 1997). The inability, at the present time, to identify differences in IL-2 and IL-4 expression in sequential

phases of adult WHV infection may be overcome by the analysis of RNA derived from cells isolated from liver inflammatory infiltrates or the direct quantitation of IL-2 and IL-4 proteins *in situ* when appropriate reagents become available.

Despite serological recovery, WHV levels detectable only by PCR/Southern hybridization assays and essential histological resolution of hepatitis, liver IFNγ and CD3 mRNA continued to be elevated throughout the entire observation period lasting for up to 5 years after resolution of hepatitis, while TNFα expression remained upregulated for up to almost 3 years after AH. Previous studies from our laboratory revealed that woodchucks once infected with WHV never completely eliminate the virus (Michalak *et al.*, 1999; Coffin and Michalak, 1999; Lew and Michalak, 2001). In the recovered animals, the hepatic loads of WHV DNA and RNA remain relatively stable and very mild recurrent liver alterations consistent with minimal to moderate inflammation continue through life, as has been also observed in the present study. Considering these findings, it was postulated that the steady, low rate of WHV replication occurring in the convalescent animals could be a consequence of the dynamic equilibrium in which the persistently multiplying virus is kept under confinement through continuous immune pressure that includes, possibly, immune elimination of infected cells (Michalak *et al.*, 1999, reviewed by Michalak, 2000). Consistent with this interpretation is the existence of vigorous HBV-specific CTL reactivity in the peripheral blood of patients years after recovery from AH type B (Penna *et al.*, 1996; Rehermann *et al.*, 1996). Demonstration of the exacerbated

expression of CD3 mRNA in this study is in agreement with remnant inflammation perpetuating in livers of the recovered animals. The continuing increased expression of IFNγ and TNFα in serial liver biopsies from the same woodchucks is consistent with this finding. Taken together, our study suggests that IFNγ and TNFα, could be directly involved in the local control of hepadnavirus persistence which progresses as an asymptomatic, serologically concealed infection. IFNγ has been implicated in the control of other viral infections, including LCMV and Friend retrovirus persistence (Tishon *et al.*, 1995; Iwashiro *et al.*, 2001) and in reactivated infection with herpes simplex virus type 1 (Cantin *et al.*, 1999) in mice.

The enhanced local production of antiviral cytokines continuing long after recovery from hepadnaviral hepatitis may also have another important consequence. It may contribute to protection of the liver against reinfection. In our previous work, two of the adult woodchucks who resolved AH after administration of the WHV inoculum used in the present work and who were challenged with the same virus 25 months after recovery did not show any molecular, serological or histological evidence of re-activation of WHV infection or hepatitis (Michalak *et al.*, 1999). Therefore, we can conclusively state that the virological status and the profile of intrahepatic cytokine and T cell reactivity delineated in the recovered animals in this study is consistent with the total resistence of the animal host against WHV reinfection. This observation may hold promise for the design of novel preventive approaches aimed at protection

of the liver against infection which could be beneficial, among others, to HBV-infected liver transplant recipients.

In summary, this study documents that the combined quantitative evaluation of intrahepatic expression of antiviral cytokines, liver T cell influx, liver histology and hepatic virus load, performed in the acute phase of hepadnaviral infection, provides an insight into the final outcome of hepatitis, i.e., whether acute infection resolves or progresses to chronic liver disease. The results also show that residual asymptomatic WHV infection, continuing long after resolution of hepatitis, is associated with elevated hepatic expression of IFNγ and TNFα and with morphologically undetectable liver T cell infusion. This indicates that antiviral cytokines, in particular IFNγ, may be a key component in the control of persistent hepadnavirus infection in the liver.

CHAPTER 6:

GENERAL DISCUSSION

The purpose of the present series of studies was to investigate selected aspects of the virus-host interactions that accompany hepadnavirus infection and underlie the development of different forms of virus-induced liver disease. In particular, our studies focused on identifying host immunological factors which may contribute to recovery from hepatitis or the establishment of persistent infection and perpetuation of liver pathology. In the first study we evaluated the ability of circulating lymphoid cells to eliminate target cells by either perforin or Fas-FasL mediated pathways in both acute and chronic phases of WHV hepatitis and compared it to that found in healthy animals (Chapter 3). In the next study, we examined the surface display of MHC class I on hepatocytes and organ lymphoid cells and determined whether this expression correlated with that of related genes (Chapter 4). This lead to new findings which may be instrumental in our understanding of the mechanisms involved in the initiation and progression of chronic liver damage and evasion of the anti-viral immunological surveillance in chronic hepadnavirus infection. Lastly, we evaluated the intrahepatic gene expression profiles of the main antiviral and proinflammatory cytokines in conjunction with T cell influx into the liver and virus load during the lifetime follow-up of the adult onset of hepadnavirus infection (Chapter 5). These studies uncovered a number of important findings, including the observation that the outcome of acute viral hepatitis may depend on the strength of the initial antiviral

and inflammatory response and as such can be predicted based on analysis of the hepatic IFNγ and TNFα expression, the degree of T cell influx and the virus load in the liver. In addition, they showed that serologically undetectable virus persistence, continuing long after resolution of AH, remains associated with upregulated hepatic IFNγ and TNFα transcription, indicating that these cytokines could play a critical role in keeping the virus under tight immunological control, or that the virus continually activated the host immune response, both occurring after apparent complete resolution of viral hepatitis.

Despite the fact that the current studies were designed to gain information on different, although related, aspects of hepadnavirus infection and associated liver disease, their overall strength was enhanced by the congruity of our procedures and the uniformity of the infection model used. For example, the vast majority of the woodchucks examined were infected as adults using the same pool and dose (i.e., 1.1×10^{10} vge) of WHV. Further, identical methods and diagnostic criteria were applied in all our studies to determine the serological and molecular characteristics of WHV infection and to assess histological features of liver disease. In addition, we took advantage of the fact that tissue materials and sera derived from the same animals could be utilized in different study protocols. Altogether, these common elements minimized the influence of extraneous factors, such as virus dose or variations in virus nucleotide sequence, on the progression of infection and liver disease outcomes and increased the integrity of our findings.

Overall, our studies revealed that there are significant differences in the host's molecular and immunological response to hepadnavirus in the acute and chronic phases of WHV hepatitis and that specific features of this response also characterizes silent WHV infection progressing throughout the life span after the recovery from hepatitis. The differences are also evident during the acute stage of WHV infection between animals who either resolve hepatitis or develop chronic liver disease. Thus, based on the results of our studies, AH is characterized by elevations in perforin mediated killing and the intrahepatic expression of IFNγ and TNFα, increased transcription of TAP-1, TAP-2 and β_2m, as well as by upregulated MHC class I heavy chain gene and protein expression (Fig. 6-1). To what extent the innate or virus-specific immune response contributes to the increased expression of antiviral cytokines during AH remains unknown. In support of the involvement of the innate immune response is our findings, discussed in Chapter 3, that the antiviral cytokine increase coincided with a selective elevation in peripheral lymphoid cell-mediated perforin-based cytotoxicity, which is a recognized marker of NK cell activity (Kagi *et al.*, 1994; Sayers *et al.*, 1998). Hepatic influx of CD3-positive lymphomononuclear cells seen in AH could be also interpreted, at least in a part, in support of the involvement of the innate cell immune response, since CD3 expression characterizes NKT cells in addition to T cells. However, at this moment we are not able to differentiate between T-cells or NKT cells types. This would be in the agreement with several previous reports which suggested that innate immune

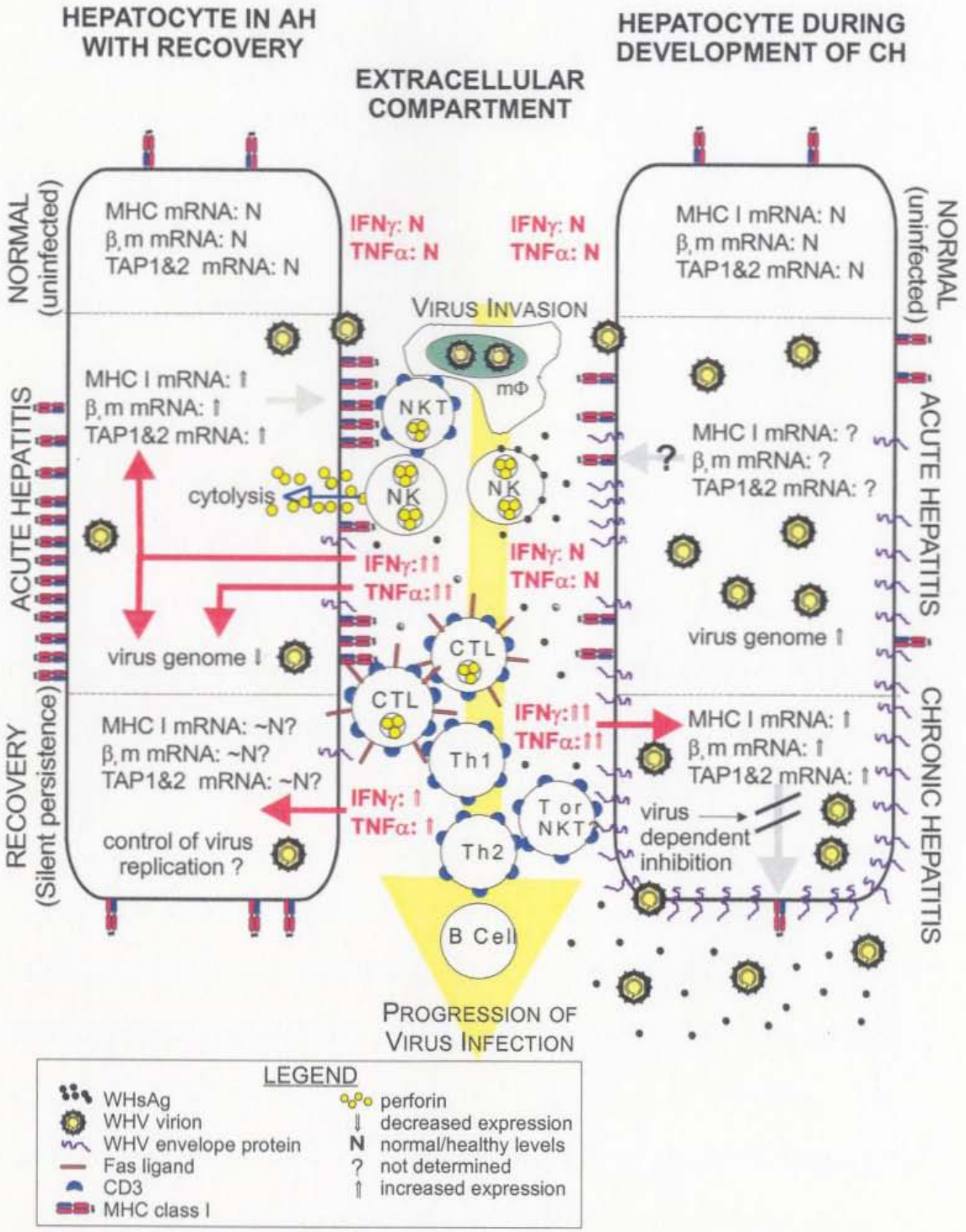
cells could be the main source of antiviral cytokines during AH. Specifically, the observations that monocyte/macrophage activation is a typical constituent of the early liver inflammatory response to WHV infection (Kajino *et al.*, 1994, Michalak, 1998, Nakamura et al., 2001), that NK cell cytolytic activity and proliferation occurs shortly after viral invasion (reviewed by Biron et al., 1999), and that a high proportion of NK cells in the liver bear the CD3 marker (Bendelac *et al.*, 1997; Doherty *et al.*, 1999). The recent demonstration that NKT and subsequent NK cell activation are able to transiently control hepadnavirus replication in HBV transgenic mice through type I and type II IFN release (Kakimi *et al.*, 2000) further supports this notion. Overall, these results suggest that, just as in other types of viral and microbial infections, the innate cell immune response, like the adaptive immune cell response, may play a pivotal role in determining the outcome of hepadnaviral infection (see Chapter 5: Biron *et al.*, 1999, Yamamura *et al.*, 1992; Sher and Coffman, 1992).

In contrast to animals that recover from AH, woodchucks who ultimately develop CH had significantly lower elevations in intrahepatic IFNγ, TNFα and CD3 mRNA during the acute phase of hepatitis (Fig. 6-1). At this point, we cannot comment on the levels of PBMC mediated cytotoxicity in this group of animals, however due to the increased viral load and decreased level of liver injury, we would predict a decrease in the host's immune responsiveness to the virus. Additionally, we cannot comment on the levels of gene expression of TAP or β_2m, nor the transcription or translation of MHC class I in animals with AH that

progress to chronicity. These issues will require further investigation. However, since IFNγ is the main inducer of the transcription of these molecules (Wallach *et al.*, 1982), it would be unlikely they are upregulated during acute infection leading to CH. Since the level of gene transcription is the primary regulator of protein translation (Lewin B, 1997), it is unlikely that MHC class I display on hepatocytes would be elevated in this stage of WHV infection (Figure 6-1).

Interestingly, once woodchucks develop CH, the levels of IFNγ, TNFα, IL-6 and CD3 mRNA all increase, coinciding with elevated MHC class I, β_2m and TAP1 and 2 gene expression. However, there is no associated increase in the hepatocyte surface presentation of class I antigen. This important finding suggests there might be virus dependent posttranscriptional inhibition of this protein expression. Additionally, in animals with CH the perforin-based PBMC mediated cytotoxicity is comparable to that seen in healthy animals. This occurs despite upregulated intrahepatic IFNγ which is known to be a potent activator of NK cells (Carnaud *et al.*, 1999), and inhibited MHC class I protein presentation on hepatocytes and organ lymphoid cells. This suggests that an active suppression of the NK cell response may be occurring. Additionally, the altered viral peptide/MHC class I presentation would interfere with virus-specific CTL-mediated elimination of infected hepatocytes and aid in hepadnavirus persistence (see Section 1.7.6 and Chapter 4). Although viruses, including WHV, may utilize several mechanisms to interfere with MHC class I presentation

Figure 6-1. Hypothetical mechanisms of hepadnaviral hepatitis progressing to recovery or chronic disease constructed through the results obtained during the course of the present studies. The resolution of hepadnaviral induced acute liver disease or its progression to CH is a complex process. There are inherent differences between the levels of antiviral cytokines and viral replication levels during the acute phase of disease correlating with the outcome of infection. Those animals with strong innate and specific cellular immunity have elevated anti-viral cytokines, increased MHC expression and reduced viral loads and ultimately recover. In the animals that recover from acute infection the antiviral cytokines continue to remain elevated, perhaps controlling the lifelong occult virus presence. Those animals with an initially weak innate and specific response after hepadnaviral infection have minimal antiviral cytokine release, increased viral load and progress to chronic infection. It should be noted that this diagram is not meant to illustrate all possible mechanisms that may contribute to the pathogenesis of hepadnaviral infection or its resolution.


HEPATOCYTE IN AH WITH RECOVERY
EXTRACELLULAR COMPARTMENT
HEPATOCYTE DURING DEVELOPMENT OF CH
NORMAL (uninfected)
ACUTE HEPATITIS
RECOVERY (Silent persistence)
CHRONIC HEPATITIS
MHC mRNA: N
β2m mRNA: N
TAP1&2 mRNA: N
IFNγ: N
TNFα: N
IFNγ: N
TNFα: N
MHC I mRNA: N
β2m mRNA: N
TAP1&2 mRNA: N
VIRUS INVASION
mΦ
MHC I mRNA: ↑
β2m mRNA: ↑
TAP1&2 mRNA: ↑
NKT
NK
NK
cytolysis
MHC I mRNA: ?
β2m mRNA: ?
TAP1&2 mRNA: ?
IFNγ:↑↑
TNFα:↑↑
IFNγ: N
TNFα: N
virus genome ↓
virus genome ↑
CTL
CTL
IFNγ:↑↑
TNFα:↑↑
MHC I mRNA: ~N?
β2m mRNA: ~N?
TAP1&2 mRNA: ~N?
Th1
MHC I mRNA: ↑
β2m mRNA: ↑
TAP1&2 mRNA: ↑
IFNγ: ↑
TNFα: ↑
T or NKT
virus dependent inhibition
control of virus replication ?
Th2
B Cell
PROGRESSION OF VIRUS INFECTION
LEGEND
WHsAg
WHV virion
WHV envelope protein
Fas ligand
CD3
MHC class I
perforin
↓ decreased expression
N normal/healthy levels
? not determined
↑ increased expression

(reviewed by Früh *et al.*, 1999), we have noted that the impaired class I antigen expression on hepatocytes in chronic WHV infection occurs only in the context of extensive virus envelope proteins integration into the HPM (Chapter 4; reviewed by Michalak, 1998). The pathogenic role of the large amount of virus envelope material produced during chronic hepadnavirus infection is unknown. Some reports have suggested that it may function as an immunomodulator that overwhelms the host's humoral immune response against the virus (reviewed by Milich, 1995). Additionally, it has be shown that HBsAg can inhibit NK cell-mediated cytotoxicity *in vitro* (Azzari *et al* 1992; De Martino *et al*, 1985) and that the absence of the innate cell immune response can tolerize antigen-specific lymphocytes (Kos and Engleman, 1995; Medzhitov and Janeway, 1998). In this respect, the lack of a virus-specific cellular immune response is a characteristic of both human and woodchuck chronic hepadnaviral infection (reviewed by Chisari, 2000; Menne *et al.*, 1997 and personal communication). If the specific CTL response against virally infected hepatocytes was prevented because of decreased MHC class I presentation and the NK cell-mediated elimination of hepatocytes not expressing MHC class I protein was suppressed by WHsAg (as is the case with HBsAg), then this complimentary effect of impeding both the innate and specific cellular immune response might be an important element of virus escape from immunosurveillance in chronic WHV infection (discussed in Chapters 3 and 4). Consistent with the above hypothesis are the findings that: (1) the level of WHV envelope proteins integrated into the HPM of animals with

CH are substantially greater than during AH (reviewed by Michalak, 1998), (2) serum (or circulating) WHsAg during AH in neonatal woodchucks who progressed to chronicity was found to be significantly greater than in animals who resolved hepatitis (Wang et al, in preparation), and (3) hepatic WHV DNA levels in neonatal (Nakamura *et al.*, 2001) and adult woodchucks (Chapter 5) during AH progressing to chronicity are significantly elevated in comparison to those found in AH followed by recovery. Since all of our animals were infected intravenously with identical doses of the same WHV pool, the differences in viral replication levels and intrahepatic cytokine production are most likely related to host dependent factors.

Following recovery from WHV hepatitis, hepatic antiviral cytokine levels (IFNγ and TNFα) and T cell infiltration drop, but still remain significantly elevated compared to uninfected healthy animals (Fig. 6-1 and Chapter 5). This suggests that the occult low level virus replication which is known to continue for life after recovery from AH in woodchucks (Michalak *et al.*, 1999) may continually stimulate the host's immune system. In consequence, this may contribute to the control of virus replication, or even protect against reinfection. This is supported by the fact that strong polyclonal HBV-specific T cell reactivity continues in patients years after recovery from AH in conjunction with occult HBV presence (Penna *et al.*, 1996; Rehermann *et al.*, 1996). The virus may also be responsible for the development of diseases not traditionally associated with hepadnavirus infection (e.g., HCC)(reviewed by Michalak, 2000).

The results of the present studies suggest that WHV has the ability to circumvent both specific and innate immune cell surveillance, and influence the elimination of infected cells. It appears that the main potential mechanism of hepadnavirus persistence might be related to immunologically unopposed viral replication early in infection and the subsequent excessive production of virus envelope material. Although the intrahepatic NK/NKT cell response was not specifically examined, our findings suggest that innate immunity may contribute to both the pathogenesis and resolution of AH. This observation may hold promise for the design of novel preventive approaches aimed at activating innate cell immunity early in hepadnavirus infection and lead to recovery from AH and prevent progression to CH. It could also be particularly beneficial to HBV-infected liver transplant recipients who are renowned for rapid re-infection of the donor liver.

CHAPTER 7:

FUTURE DIRECTIONS

The results obtained during the course of these studies have raised several important questions which should be investigated further to help clarify the contribution of the anti-WHV host immune response in the pathogenesis of hepadnavirus induced liver injury. They are as follows:

1. Woodchuck cellular markers for the innate immune response (e.g., NK, NKT and macrophages) must be developed and the immune response immediately following virus invasion should be examined to determine whether this system plays a role in WHV elimination and recovery from hepatitis.
2. The issue of whether the immune response and cytokine profile predisposes an animal to CH must be reexamined when methods to determine the protein levels of the anti-viral cytokines and individual cell types are established.
3. The influence of the Th1/Th2-related cytokines on the outcome of hepadnavirus infection and virus-induced liver disease should be investigated by taking advantage of exogenous immunomodulatory agents which modify intrahepatic cytokine and NK/NKT cell responses.
4. The development of markers to identify cell subsets of the adaptive immune response (e.g., CD4+ and CD8+ T cells) will allow the examination of T cell responses during the course of viral hepatitis

progression or recovery.

5. The molecular mechanisms underlying the downregulation of the MHC class I heavy chain protein on hepatocytes and lymphoid cells must still be determined.

6. A detailed characterization of host immune reactions would determine if the downregulation of MHC class I presentation on lymphoid cells by hepadnavirus infection influences the overall effectiveness of anti-hepadnaviral immune responses.

CHAPTER 8:

SUMMARY AND CONCLUSIONS

In the course of the current studies, we examined selected mechanisms of the immunopathogenesis of chronic liver injury and hepadnaviral persistence using the woodchuck model of hepatitis B. To facilitate this research we sequenced several relevant woodchuck genes and generated specific molecular reagents which were not available prior to the initiation of this work. In addition, we adapted assays for evaluating *in vitro* woodchuck lymphoid cell cytotoxicity and developed molecular methods for the quantitative detection of woodchuck specific cytokine gene transcription in normal and virus-invaded tissues. The use, in our studies, of biological materials collected from animals during the lifelong follow-up of adult hepadnaviral infection provided a significant investigative advantage not available when examining HBV infected patients. Overall the results obtained can be summarized as follows:

1. We have cloned and sequenced several (14) woodchuck genes and gene fragments relevant to the host's immune responses, including FasL, perforin, MHC class I heavy chain, β_2-microglobulin, TAP1, TAP2, the epsilon subunit of CD3, IFNγ, TNFα, IL-2, IL-4, IL-6, GAPDH and β-actin. These nucleotide sequences were submitted to the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, USA (GenBank) and are available to interested investigators in the scientific community. This should aid comparative

genetic studies and foster future research on the immunopathogenesis of hepadnaviral infection in the woodchuck-WHV system.

2. By analyzing lymphoid cells from WHV-infected and healthy woodchucks, we have demonstrated that these cells kill cell targets through both the Fas/FasL and perforin-granzyme pathways. We have found that their cytopathic capacity can be examined using heterologous cells, such as mouse P815 (which constitutively express Fas) and human K562 (which are Fas negative) as targets. Comparative analysis revealed that lymphoid cells from animals with acute WHV hepatitis have an augmented capacity to elicit perforin-dependent killing. In contrast, lymphoid cells from animals with chronic WHV hepatitis, independent of the severity of liver disease and duration of chronicity, had the same or lower cytotoxic potential as those from healthy controls. Since perforin mediated killing is the primary mechanism of NK cell cytotoxicity, these findings suggest that nonspecific cellular immunity might be important in the early control of WHV infection. This also suggests that decreased perforin-granzyme mediated killing can contribute to the establishment of CH in hepadnaviral infection.

3. We have discovered that, in contrast to woodchucks with AH, chronic WHV hepatitis is characterized by a profound deficiency in the expression of MHC class I molecules on the surface of infected hepatocytes and, notably, organ lymphoid cells. This inhibition occurs despite upregulated

hepatic gene expression of MHC class I heavy chain, β_2-microglobulin, TAP1 and TAP2. Further, the decrease in class I antigen display is not related to severity of liver injury, the extent of lymphocytic infiltrations, the level of intrahepatic IFNγ expression or WHV load in the liver. Therefore, our results imply that the defective presentation of MHC class I molecules on cells supporting WHV replication in chronic infection is due to virus initiated posttranscriptional interference. This event may diminish the susceptibility of infected hepatocytes to immunocytolysis by virus-specific T lymphocytes, hinder virus clearance, and deregulate the class I MHC-dependent functions of the host's immune system and, therefore, may support hepadnavirus persistence.

4. Analysis of cytokine gene expression and CD3 mRNA, a marker of T-cell infiltration, during the course of WHV infection in adult animals revealed that hepatic IFNγ and CD3 mRNA levels were significantly upregulated during the acute phase of infection only in animals who ultimately resolve viral hepatitis. Further, the results suggest that hepatic TNFα gene expression follows a similar trend, but IL-6 levels remain relatively constant. Animals that would develop chronic WHV hepatitis had minimally elevated hepatic levels of IFNγ and TNFα transcription, increased hepatic WHV load and a lesser degree of liver inflammation during acute infection, when compared to animals with self limiting hepatitis. These findings suggest that the outcome of AH may be decided

very early in the course of hepadnavirus infection by the strength and swiftness of the local cytokine and cellular immune responses. Therefore, it is conceivable that an enhanced innate and/or specific response to the invading virus favours recovery from AH, whereas a diminished or delayed response and increased viral load predispose to the development of CH. Furthermore, the hepatic levels of IFNγ and CD3 mRNA remained significantly elevated in animals years after recovery from a self limiting episode of WHV hepatitis, suggesting that stimulation of host immunity continues long after resolution of acute infection. This is in agreement with our previous observations that traces of replicating WHV persist for life in livers and lymphatic system of convalescent, apparently healthy animals. This finding also points to the potential significance of antiviral cytokines, in particular IFNγ, in the control of long term occult hepadnavirus persistence in the liver

Overall, our studies on the mechanisms by which immune effector cells mediate cytotoxicity and the characteristics of the cytokine response suggests that an early and strong local immune response permits the termination of acute hepadnaviral hepatitis, presumably due to the innate immune response and adequate priming of specific anti-viral reactivity. In contrast, weak or delayed immune responses may allow enhanced viral replication leading to CH that is sustained, by the posttranscriptional inhibition of MHC class I expression on infected hepatocytes and lymphoid cells. This permits WHV to evade virus

specific CTL, compromises the host's entire immune system and allows perpetuation of chronic hepadnavirus infection and continuation of liver disease.

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APPENDIX A:

MOLECULAR BIOLOGY BUFFERS, REAGENTS AND PROTOCOLS

ACK buffer: 0.15 M NH_4Cl, 1 mM $KHCO_3$, pH 7.3, in 0.1 mM EDTA.

Agar plates: LB medium containing 1.5% Bacto-Agar (Difco Laboratories, Detroit MI) was autoclaved. The solution was allowed to cool to 50°C and antibiotic (kanamycin or tetracycline) was added to a final concentration of 50 µg/ml.

Ammonium actetate (10 M): 77.08 g ammonium acetate in water to 100 ml.

Denhardt's solution (50X): 1% Ficoll 400 (Sigma), 1% polyvinylpyrrolidone (Sigma) and 1% bovine serum albumin.

Denaturing solution (DNA): 1.5 M NaCl and 0.5 M NaOH.

Denaturing solution (RNA): 100 µl of 10X MOPS, 350 µl of 12.2 M formaldehyde (Fisher), and 1.0 µl of formamide (Fisher) in 1.5 ml total volume.

EDTA (0.5 M): 18.61 g Na2EDTA*2 H_2O in 70 ml water, adjusted to pH 8.0 with 10 M NaOH (~50 ml), then water added to 100 ml.

Formamide stop solution: 10 mM NaOH in 95% formamide with 0.05% bromophenol blue and 0.05% xylene cyanole.

Forward reaction buffer (5X): 350 mM Tris pH 7.6 with 50 mM $MgCl_2$, 500 mM KCl and 5 mM β-mercaptoethanol.

GTE (glucose/Tris/EDTA): 50 mM glucose and 10 mM EDTA in 10 mM Tris-HCl buffer, pH 8.0

Hybridization buffer (DNA): 5X SSPE (see below), 5X Denhardt's (see above), 1% SDS, and 100 µg/ml denatured salmon sperm DNA.

Hybridization buffer (RNA): 50% formamide (Fisher), 5X SSPE, 5X Denhardt's, 1% SDS, and 100 µg/ml denatured salmon sperm DNA.

LB medium: 1% (w/v) peptone 140, 0.5% (w/v) yeast extract, 100 mM NaCl (ICN Biomedical Inc.), and 0.1% glucose (Fisher Scientific). Adjust to pH 7.0.

Lysis buffer (DNA): 100 mM NaCl, 10 mM Tris-HCl buffer, pH 8.0 with 25 mM

EDTA, pH 8.0, 0.5 % SDS and 0.1 mg/ml proteinase K (freshly prepared).

Loading buffer (RNA): 50% glycerol, 1 mM EDTA, pH 8.0, with 0.15% bromophenol blue, 0.15% xylene cyanol and 1 µl of a 1 µg/µl ethidium bromide added to each sample.

Loading dye (DNA): 0.25% bromophenol blue, 0.25% xylene cyanol and 15% Ficoll 400 in ddd water.

MOPS buffer: 0.4 M 3-(N-morpholino)-propanesulfonic acid, 0.1 M sodium acetate and 0.01 M EDTA (Sigma).

Neutralizing solution (DNA): 1.5 M NaCl in 1 M Tris-HCl buffer, pH 8.0.

PEG solution: 30% (w/v) polyethylene glycol (PEG) 8000 (Sigma), 1.6 M NaCl (Fisher). Filter through a 0.45 µm filter.

Phenol (buffered): Add 0.1% (w/v) of 8-hydroxyquinoline (Sigma) to 25 ml of melted phenol and adjust pH with 25 ml of 50 mM Tris base (Sigma). Mix for 10 min and allow phases to separate. Discard the excess Tris base. Add an equal volume of 50 mM Tris-HCl buffer, pH 8.0, to the remaining phenol. Mix for 10 min, allow the phases to separate, and discard aqueous layer. Repeat 3 times and store at 4°C in 50 ml tubes (< 2 months).

REact 3 buffer (10X): 500 mM Tris, pH 8.0, with 100 mM $MgCl_2$ and 1 M NaCl.

RNase decontamination:

Treament of water: To prepare RNase-free water dddH_20 was treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC; Sigma) at 37°C overnight and then autoclaved for 45 min.

Treatment of equipment: The electrophoresis tank, gel casting trays and combs were decontaminated using 0.1 M NaOH containing 0.01 mM EDTA and then rinsing several times with DEPC-treated water. Disposable Eppendorf homogenizers (Fisher Scientific) were rinsed overnight at 37°C in 0.1% DEPC and then autoclaved.

SDS (10%): 10 g sodium dodecyl (lauryl) sulphate in 100 ml water, filter through a 0.2 µm filter.

SOC medium: 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl (Fisher Scientific), 2.5 mM KCl, 10 mM $MgCl_2$, 10mM $MgSO_4$ and 20 mM glucose (Sigma).

Sodium Acetate (3 M; pH 5.2): 40.8 g sodium acetate*3 H_2O in 80 ml water, add water to 100 ml and adjust pH to 5.2 with 3M acetic acid.

SSC (sodium chloride/sodium citrate; 20X): 3 M NaCl (Fisher) in 0.3 M C_6H_5O-Na_3*2 H_20, adjusted to pH 7.0 with 1 M HCl.

SSPE (standard sodium phosphate EDTA; 20X): 3 M NaCl, 0.2 M NaH_2PO_4*H_2O, and 20 mM EDTA.

TAE (10X): 10 mM EDTA in 400 mM Tris-HCl buffer, pH 8.0.

TBE (10X): 108 g Tris base, 55 g boric acid, 40 ml 0.5 M EDTA (pH 8.0), add ddd water to 1L.

TE Buffer: 10 mM Tris*Cl (pH 8.0), in 1 mM EDTA (pH 8.0).

X-Gal: 40 mg of 5-bromo-4-chloro-3-indolyl β-D-galacto-pyranoside resuspended in 1 ml of dimethylformamide (DMFO).

All reagents were obtained from Gibco BRL unless otherwise noted.

APPENDIX B:

WOODCHUCK GENE SEQUENCES CLONED DURING THE STUDY

Appendix	Sequence Name	Genbank Accession Number	Release Date
B.1	Fas Ligand	AF152368	Feb 7, 2000
B.2	Perforin	AF298158	Oct 22, 2000
B.3	MHC class I	AF232723	May 9, 2000
B.4	TAP-1	AF232724	May 9, 2000
B.5	TAP-2	AF232725	May 9, 2000
B.6	β2-microglobulin	AF232726	May 9, 2000
B.7	CD3 ε	AF232727	May 9, 2000
B.8	IFNγ	AF232728	May 9, 2000
B.9	GAPDH	AF232729	Aug 17, 2000
B.10	β-actin	AF232730	Aug 22, 2000
B.11	IL-2	AF333964	May 12, 2001
B.12	IL-4	AF333965	May 12, 2001
B.13	IL-6	AF333966	May 12, 2001
B.14	TNFα	AF333967	May 12, 2001

The following appendices document woodchuck sequences that were cloned in this laboratory during the course of the present study. These sequences are also available by their accession number in a similar format at National Institute of Health Genbank, accessible at www.ncbi.nlm.nih.gov.

Appendix B.1

```
LOCUS       AF152368    508 bp   mRNA        ROD     07-FEB-2000
DEFINITION  Marmota monax Fas ligand mRNA, partial cds.
ACCESSION   AF152368
VERSION     AF152368.1  GI:5051980
SOURCE      woodchuck
FEATURES    Location/Qualifiers
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            /organism="Marmota monax"
            /db_xref="taxon:9995"
            /tissue_type="healthy liver"
   CDS              <1..>508
            /note="apoptosis inducing protein, CD95 ligand, APO-1ligand"
            /codon_start=2
            /product="Fas ligand"
            /protein_id="AAD38387.1"
            /db_xref="GI:5051981"
/translation="LFHLQKELPELRESINQRNTEPSLEKQIGHPSSPSDKKALRRAAHLTGKPNSRSSPL
EWEDTYGISLISGVKYQKGGLVINDTGLYFVYSKIYFRGQSCNNQPLSHKVYVKNSKYPQDLVLM
EGKMMNYCTTGQMWARSSYLGAVFNFTSNDHLYVNVSELSLINFEES"

BASE COUNT    142 a    130 c    122 g    114 t
ORIGIN
        1 gctcttccac ctgcagaagg aactgccaga actccgcgag tcaatcaatc aaagaaatac
       61 agaaccatct ttggagaagc aaataggcca ccccagttca ccctctgata aaaaggcact
      121 gaggagagcg gcccatttaa caggtaagcc caactcaagg tccagcccgc tggaatggga
      181 agacacctac ggaatttccc tgatctctgg agtgaagtat cagaagggtg gccttgtgat
      241 caatgacact gggctgtact ttgtgtattc caaaatatac ttccggggtc agtcctgcaa
      301 caaccagccc ctgagccaca aggtctacgt gaagaactct aagtatcccc aggacctggt
      361 gctgatggag ggcaagatga tgaactactg cactactggc cagatgtggg cccgcagcag
      421 ctatctgggg gctgtgttca acttcaccag taatgaccat ttatatgtca acgtatctga
      481 gctctctctg atcaattttg aggaatct//
```

Appendix B.2

```
LOCUS       AF298158    718 bp    mRNA        ROD     22-OCT-2000
DEFINITION  Marmota monax perforin mRNA, partial cds.
ACCESSION   AF298158
VERSION     AF298158.1  GI:10945604
SOURCE      woodchuck.
FEATURES    Location/Qualifiers
   source   1..718
            /organism="Marmota monax"
            /db_xref="taxon:9995"
   CDS      <1..>718
            /function="forms pores in membranes"
            /codon_start=1
            /product="perforin"
            /protein_id="AAG24611.1"
            /db_xref="GI:10945605"

/translation="FPVDTQRFLRPDGTCTLCKNPLQKGALQRLPLALTHWRGQGSSCRRQVAKAKISS
TEEVAREAASSINNDWRVGLDVTPPEPSSKVHMSVAGSHSKAADFAAQKTHQDQYSFSTDTVEC
HLYSFHVVHKPPLHPDFKRALGDLPPHLNTSTEPDYLRLIHNYGTHFIRSVELGGRVSALTALRTC
ALALDGLTADEVGDCLAVEAQVSIGGHAESSSEFKACEEKKQRHKMITSFHQTY"

BASE COUNT    149 a   252 c   205 g   112 t
ORIGIN
        1 ttcccagtgg acacacagag gttcctgcgg cccgacggca cctgcaccct ctgtaaaaac
       61 cccctgcaga agggcgccct gcagcgtctg cccctggcgc tcacccactg gcgtgggcag
      121 ggctccagct gcaggcgcca agtagccaag gccaaaatca gctccaccga ggaggtggcc
      181 cgggaggcgg cctccagcat caacaatgac tggcgggtgg ggctggacgt gactcccccc
      241 gagcccagca gcaaggtgca catgtctgtg gctggctcgc actccaaggc agcggacttc
      301 gcagcccaaa agacccacca ggaccagtac agcttcagca cggacacggt ggagtgtcac
      361 ctctacagtt tccatgtggt gcacaaaccc ccactgcacc ctgatttcaa gagggcactt
      421 ggggacctgc ccccccacct caacacctcc accgagcctg actacctcag gctcatccac
      481 aactacggca cccacttcat ccggtccgtg gagctgggtg gccgagtctc agccctcacg
      541 gccctacgca cctgtgcact ggccttggat gggctcacgg ccgacgaggt gggggactgc
      601 ctggctgtgg aggcccaggt gagcatcggt ggccatgccg agtcctcatc cgagttcaag
      661 gcctgcgagg agaagaagca gcggcacaag atgataacct ctttccacca aacctacc
```

Appendix B.3

```
LOCUS          AF232723   276 bp   mRNA        ROD   09-MAY-2000
DEFINITION     Marmota monax major histocompatibility complex class I heavy chain mRNA,
               partial cds.
ACCESSION      AF232723
VERSION        AF232723.1  GI:7739665
SOURCE         woodchuck.
FEATURES       Location/Qualifiers
   source      1..276
               /organism="Marmota monax"
               /db_xref="taxon:9995"
   CDS         <1..>276
               /note="alpha 3 region"
               /codon_start=3
               /product="major histocompatibility complex class I heavy chain"
               /protein_id="AAF68955.1"
               /db_xref="GI:7739666"
/translation="PPKTHVTHHPSPEGEVTLRCWALGFYPKEITLTWRRDGEDQTQEMELVETRPSGD
GNFQKWAAVVVPAGEEQRYTCRVHHEGLPEPLTLRW"

BASE COUNT     65 a    75 c    87 g    49 t
ORIGIN
      1 atcctccaaa gacccatgtg actcaccacc ctagccctga aggagaggtc actctaaggt
     61 gctgggccct gggcttctac cctaaggaga tcaccctaac ttggcgacga gatggggagg
    121 accagaccca ggagatggaa cttgtggaga ccagaccttc tggggatgga aacttccaga
    181 aatgggcagc tgtggtggtg cctgctggag aggagcagag atacacctgc cgtgtgcacc
    241 atgaggggct gcctgagccc ctcaccctga gatggg
```

Appendix B.4

LOCUS AF232724 1359 bp mRNA ROD 09-MAY-2000
DEFINITION Marmota monax transporter associated with antigen processing TAP-1 mRNA, partial cds.
ACCESSION AF232724
VERSION AF232724.1 GI:7739667
SOURCE woodchuck.
FEATURES Location/Qualifiers
source 1..1359
/organism="Marmota monax"
/db_xref="taxon:9995"
CDS <1..>1359
/codon_start=1
/product="transporter associated with antigen processing TAP-1"
/protein_id="AAF68956.1"
/db_xref="GI:7739668"
/translation="WLQDKTATTLTRNITLMCILTIASALLEFVGDGIYNSTMGRVHSHFQGKVFQAVLRQ
ETEFFQQNQTGNITSRVTDDTSTLCESLSEELSILMWYLVRGLCLLGLMLWGSLSLTLVTLAALPL
LFLLPKKLGKWCQSLGVQVRDSLAEASQVAIEALSAMPTVRSFANEEGEAQKFRQKLEEMKTLN
QKEALAYAVNLCTTDVSGLLLKVGILYIGGQMVITGTISSGNLVAFVLYQIQFTVAVKVLLSAYPRVQ
KAIGSSEKIFEYLDRTPRCPPRGLLIPSNMKSLVQFQDVSFAYPNDPDVLVLQGLTFTLYPGKVTAL
VGPNGSGKSTVAALLQNLYQPTMGQLLLDGEPLPQYEHRYLHRQVAAVGQEPQLFGRSIQENIA
YGLIQKPTMEEIKSAAIQSGAHSFISGLPQGYDTEVGEAGGQLSGGQRQAVALARALIRKPRVLIL"

BASE COUNT 325 a 345 c 373 g 316 t
ORIGIN
1 tggattctac aagataagac agctactact ttaacccgaa acatcactct tatgtgcatt
61 ctcaccatag ccagtgcact gctggagttc gtgggcgatg ggatctataa cagcacaatg
121 gggcgtgtac atagtcactt tcagggaaag gtgtttcagg ctgtcctgcg ccaggagacg
181 gagtttttcc agcagaacca aacaggtaac atcacctctc gagtaacaga tgacacttcc
241 accctgtgtg agtctctgag tgaggagctg agcatattga tgtggtacct ggtgcgagga
301 ctgtgtctct tggggctcat gctctggggg tcactgtccc tcaccctggt caccctggct
361 gccctgcctc tgcttttcct tctgcctaag aagctgggaa aatggtgcca gtcactggga
421 gtacaggtgc gggactctct ggcagaggcc agccaggtgg ccatcgaggc cctgtcagct
481 atgcctacgg tccggagctt tgccaatgag gaaggcgagg cccagaagtt taggcagaag
541 ctggaggaaa tgaagacact caaccaaaag gaggccctgg cctatgcagt caacctgtgc
601 accactgatg tctcagggct gctgctgaag gtgggaatcc tatacattgg tgggcagatg
661 gtgataacag ggactataag cagtgggaac cttgttgcat ttgttctcta ccagatacag
721 ttcaccgttg ctgtcaaggt gctgctttct gcctatcccc gggtacagaa ggctataggc
781 tcctcagaga aaatatttga atatctggac cggacccctc gctgcccacc caggggtcta
841 ttgattccct caaacatgaa gagccttgtc cagttccaag atgtctcctt tgcctatcct
901 aatgatccag acgtcctagt gctacagggg ctgacattca ccctatatcc tggtaaagtg
961 acagcactag tgggacccaa tggatcaggg aagagcactg tggctgccct gctgcagaat
1021 ctttaccagc ccaccatggg ccagctgctg ctggatgggg agccccttcc tcaatatgaa
1081 catcgctacc tgcacagaca ggtggctgca gtgggacaag aaccacagct gtttggaaga
1141 agtattcaag aaaatattgc ctatggtctg atccagaagc caactatgga ggagataaaa
1201 agtgctgcaa tacagtctgg agcccatagt ttcatttctg ggctccccca gggctatgac
1261 acagaggtag gtgaggctgg gggccagctg tcaggaggtc agcgacaggc agtggccttg
1321 gcccgagcat tgattcggaa gccacgtgta ctcattcta

Appendix B.5

LOCUS AF232725 1121 bp mRNA ROD 09-MAY-2000
DEFINITION Marmota monax transporter associated with antigen processing TAP-2 mRNA, partial cds.
ACCESSION AF232725
VERSION AF232725.1 GI:7739669
SOURCE woodchuck.
FEATURES Location/Qualifiers
source 1..1121
/organism="Marmota monax"
/db_xref="taxon:9995"
CDS <1..>1121
/codon_start=3
/product="transporter associated with antigen processing TAP-2"
/protein_id="AAF68957.1"
/db_xref="GI:7739670"
/translation="GGDFDPDAFVSAIFFMCLFSVGSSLSAGCRGGSFTFIMSRINLRTREQLFSSLLRQD
LSFFQEIKTGELNSRLSSDTTLMSRWLPLNANVILRSLVKVIGLYGFMLSVSPRLTFLSMLEMPLLIA
VEKLYNMRHQAVLLEIQDEVAKAGQVVREAVGGLQTVRSFGAEEDEVCLYKKAVERCRQLWWR
RDLERALYLLIRRVLTLGVQVLVLSCGLQQILAGEVTRGGLLSFLLDQEDMGNYVRALVFGFGDML
SNVGAAEKVFRYLDRKPNLPEPGTLAPPTLQGIVEFQDVSFAYPNRPDQPVLKGLTFTLHAGEMT
ALVGPNGSGKSTVAALLQNLYQPTGGRLLLDGEPISNYEHHYLHSQVALVGQEP"

BASE COUNT 211 a 291 c 358 g 261 t
ORIGIN
1 tgggcggtga ttttgaccct gatgcttttg tcagcgccat atttttcatg tgcctgttct
61 ccgttgggag ctcgctgtct gcaggttgcc gaggggctc ctttaccttc atcatgtcca
121 gaatcaactt gcggacccgg gagcagcttt tctcctccct gctgcgtcag gacctcagtt
181 tcttccagga gattaagaca ggggagttga actcacggct gagctcagat accaccctga
241 tgagccgctg gcttccttta aatgccaatg taatcttgcg gagcctggtg aaagtgatcg
301 ggctgtatgg cttcatgctg agcgtgtcac cgcgactcac cttcctctcc atgctcgaaa
361 tgcccctcct aatagcagtg gaaaagctgt acaatatgcg ccatcaggct gtgctgctgg
421 agatccagga tgaagtggcg aaggcagggc aggtggtgcg ggaggcagtt ggcgggctgc
481 agaccgtgcg cagttttggg gccgaggagg atgaggtctg cctctataag aaggctgtgg
541 agcggtgccg gcagttgtgg tggcgacggg acctggaacg tgctctgtac ctgcttatac
601 ggagggtgct gaccttgggt gtgcaggtgc tggtgctgag ctgcgggctg cagcagatcc
661 tggctggcga ggtcacgagg ggcgggctgc tctcctttct gctcgaccag gaggacatgg
721 ggaactacgt tcgagctctg gtatttggtt ttggtgatat gctgagcaac gtgggagctg
781 cagagaaggt tttcagatac ctggacagaa agcccaacct gcctgagcca gggactctgg
841 cccctcccac actgcagggg attgtggaat tccaagacgt ctcatttgca tatcccaatc
901 gccctgacca acctgtgctc aaggggctga cgttcaccct ccacgctgga gagatgacag
961 cgctggtggg acccaatggt tctgggaaga gcactgtggc tgccctgctg cagaatctgt
1021 accagcccac aggcgggcgg ctgctgttgg atggggagcc catctcaaac tatgagcacc
1081 actacctgca cagccaggtg gctttggttg ggcaggagcc c

Appendix B.6

LOCUS AF232726 331 bp mRNA ROD 09-MAY-2000
DEFINITION Marmota monax beta-2-microglobulin mRNA, partial cds.
ACCESSION AF232726
VERSION AF232726.1 GI:7739671
SOURCE woodchuck.
FEATURES Location/Qualifiers
source 1..331
/organism="Marmota monax"
/db_xref="taxon:9995"
CDS <1..>331
/note="MHC class I light chain"
/codon_start=3
/product="beta-2-microglobulin"
/protein_id="AAF68958.1"
/db_xref="GI:7739672"
/translation="VALALFMLLFLTGLDADPRSPKIQVYTRHPAENGKPNFLNCYVSGFHPPQIQIDLLKN
GQKIEKVEQSDLSFSKDWSFYLLVHTEFTPNDKDEYACRVTHETLKEPKIV"

BASE COUNT 95 a 84 c 72 g 80 t
ORIGIN
1 cggtggcgtt ggccttgttc atgctactct ttctgaccgg tctggacgct gacccgcgtt
61 ctccgaaaat tcaagtttat acacggcacc cagctgagaa tggaaaaccg aacttcctca
121 actgctacgt atctgggttt catccacccc agattcaaat agatctgttg aaaaacggac
181 agaagataga aaaagtcgag cagtcggacc tctctttcag caaggactgg tccttctatc
241 ttctggtgca cactgaattc acccccaatg ataaagatga atacgcatgc agagttacac
301 atgaaactct gaaggagccc aagatagtga a

Appendix B.7

```
LOCUS          AF232727   535 bp   mRNA      ROD   09-MAY-2000
DEFINITION     Marmota monax CD3 epsilon chain mRNA, partial cds.
ACCESSION      AF232727
VERSION        AF232727.1  GI:7739673
KEYWORDS
SOURCE         woodchuck.
FEATURES       Location/Qualifiers
   source      1..535
               /organism="Marmota monax"
               /db_xref="taxon:9995"
   CDS         <1..>535
               /note="TCR accessory molecule"
               /codon_start=1
               /product="CD3 epsilon chain"
               /protein_id="AAF68959.1"
               /db_xref="GI:7739674"
/translation="LGLCLLSVGAWGQEDDEENDDLTQIQYKVSISGTDVMLTCPPKALQGTINWERNDK
KLEGENDEQLILKNFSEMDNSGYYACYTTPRQKENIHFLYLRARVCENCVEVDLTAVATVIVVDIIV
TLGLLMLVYYWSKNRKAKSKPVTRGAGAGGRPRGQKKERPPPVPNPDYEPIRKGQ"

BASE COUNT     168 a   117 c   141 g   109 t
ORIGIN
      1 ctgggactct gcctcttatc agttggtgct tgggggcagg aagatgatga agaaaatgat
     61 gacctaacac agatacaata caaagtctcc atctcgggaa ctgatgtgat gctgacatgc
    121 cctccaaaag ctctgcaggg cacaataaat tgggaaagaa atgacaaaaa actagaaggc
    181 gaaaatgacg aacaactgat actgaagaat ttttcagaaa tggataacag tggttattac
    241 gcctgctaca caaccccaag acaaaaagag aatatccatt ttctgtacct gagagctaga
    301 gtgtgtgaga actgcgtaga ggtggatctg acggctgtgg ccacagtcat cgtagtcgac
    361 atcattgtca ctctgggctt gctgatgctg gtttattact ggagcaagaa tagaaaggcc
    421 aagtccaaac ctgtgacacg tggagcaggc gctggtggca ggcccagggg acaaaagaag
    481 gagaggccac cacctgttcc caacccggac tatgagccca tccggaaagg ccagc
```

Appendix B.8

LOCUS AF232728 530 bp mRNA ROD 09-MAY-2000
DEFINITION Marmota monax interferon gamma mRNA, complete cds.
ACCESSION AF232728
VERSION AF232728.1 GI:7739675
SOURCE woodchuck.
FEATURES Location/Qualifiers
source 1..530
/organism="Marmota monax"
/db_xref="taxon:9995"
CDS 22..522
/note="IFN gamma"
/codon_start=1
/product="interferon gamma"
/protein_id="AAF68960.1"
/db_xref="GI:7739676"
/translation="MKYTSYFLAFQLCIILGSSSCYSQDTVNKEIEDLKGYFNASNSNVSDGGSLFLDILDK
WKEESDKKVIQSQIVSFYFKLFEHLKDNKIIQRSMDTIKGDLFAKFFNSSTNKLQDFLKVSQVQVN
DLKIQRKAVSELKKVMNDLLPHSTLRKRKRSQSSIRGRRASK"

BASE COUNT 176 a 104 c 109 g 141 t
ORIGIN
1 ggcctaactc tctctgaaac gatgaaatac acaagttatt tcttggcttt tcagctctgc
61 atcattttgg gttcttctag ctgttactcc caggacacag ttaataaaga aatagaagat
121 ttaaaaggat atttcaatgc aagtaattca aatgtatcag atggcgggtc tctcttcttg
181 gatattttgg ataaatggaa agaggagagt gacaaaaaag taatccagag ccaaattgtc
241 tctttctact tcaaactctt tgaacactta aaagacaaca agatcatcca aaggagcatg
301 gacaccatca agggggatct ttttgctaag ttcttcaaca gcagtaccaa taagctgcag
361 gacttcctaa aggtgtctca agttcaggta aatgacctga agatccagcg taaagcagtg
421 agtgaactca agaaagtgat gaatgatctg ttaccacact ctaccctaag gaagcgaaaa
481 aggagtcagt cttcgattcg gggtcggaga gcatccaaat aacagtcctc

Appendix B.9

LOCUS AF232729 535 bp mRNA ROD 17-AUG-2000
DEFINITION Marmota monax glyceraldehyde 3-phosphate dehydrogenase mRNA, partial cds.
ACCESSION AF232729
VERSION AF232729.2 GI:9838357
SOURCE woodchuck.
FEATURES Location/Qualifiers
source 1..535
/organism="Marmota monax"
/db_xref="taxon:9995"
CDS <1..>535
/codon_start=3
/product="glyceraldehyde 3-phosphate dehydrogenase; GAPDH"
/protein_id="AAF68961.2"
/db_xref="GI:9838358"
/translation="DPANIKWGDAGAEYVVESTGVFTTMEKAGAHLKGGAKRVIISAPSADAPMFVMGV
NHEKYDNSLKIVSNASCTTNCLAPLAKVIHDNFGIVEGLMTTVHAITATQKTVDGPSGKLWRDGR
GAAQNIIPASTGAAKAVGKVIPELNGKLTGMAFRVPTPNVSVVDLTCRLEKAAKYDD"

BASE COUNT 120 a 144 c 144 g 127 t
ORIGIN
1 gagatcccgc caacatcaaa tggggtgatg ctggtgctga atatgttgtg gagtccactg
61 gtgtcttcac taccatggag aaagccgggg ctcatttgaa gggtggtgcc aaaagggtca
121 tcatttctgc accttctgct gatgccccca tgtttgtgat gggcgtgaac catgagaagt
181 atgacaactc cctcaagatt gtcagcaatg cctcctgtac caccaactgc ttagcccccc
241 tggccaaggt catccatgac aactttggca ttgtggaagg actcatgacc acagtccatg
301 ccatcactgc tactcagaag actgtggatg gcccctctgg gaaactgtgg cgtgatggcc
361 gtggggctgc ccagaatatc atccctgcat ccactggtgc tgccaaggct gtgggcaagg
421 tcatccctga actgaatggg aagctcactg gcatggcttt ccgtgtgccc actcccaatg
481 tgtcagttgt ggatctgacc tgccgcctgg agaaagctgc caaatacgat gacat

Appendix B.10

```
LOCUS       AF232730    855 bp   mRNA        ROD     22-AUG-2000
DEFINITION  Marmota monax beta-actin mRNA, partial cds.
ACCESSION   AF232730
VERSION     AF232730.3  GI:9864779
SOURCE      woodchuck.
FEATURES    Location/Qualifiers
   source   1..855
            /organism="Marmota monax"
            /db_xref="taxon:9995"
   CDS              <1..>855
            /codon_start=1
            /product="beta-actin"
            /protein_id="AAF68962.2"
            /db_xref="GI:9864780"
/translation="IEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKMTQIMFETF
NTPAMYVAIQAVLSLYASGRTTGIVMDSGDGVTHTVPIYEGYALPHAILRLDLAGRDLTDYLMKILT
ERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSSLEKSYELPDGQVITIGNERFRCPEAL
FQPSFLGMESCGIHETTFNSIMKCDVDIRKDLYANTVLSGGTTMYPGIADRMQKEITALAPSTMKI
KIIAPPERKYSVWIGGSILASLSTFQQM"
BASE COUNT     187 a    262 c    223 g    183 t
ORIGIN
      1 attgagcatg gcatcgtcac caactgggac gacatggaga agatttggca ccacaccttc
     61 tacaacgagc tgcgtgtggc tcctgaggag caccctgtgc tgctgaccga ggctcccctg
    121 aaccctaagg ccaaccgtga gaagatgacc cagatcatgt ttgagacctt caacacccca
    181 gccatgtatg tggccatcca ggctgtgctg tccctgtatg cctctggccg taccactggc
    241 attgtgatgg actccggtga tggggtcacc cacacagtgc ccatctatga ggggtatgcc
    301 cttccccacg ccatcctgcg tctggacctg gctggccggg acctgacaga ctacctcatg
    361 aagatcctga ctgagcgtgg ctacagcttc accaccacag ccgagcggga aatcgtgcgt
    421 gacatcaagg agaagctgtg ctacgttgcc ctggacttcg agcaggagat ggccactgca
    481 gcctctagct cctccctgga gaagagctac gagctgcctg atggtcaggt gatcaccatt
    541 ggcaatgagc gattccgctg ccctgaggca ctcttccagc cttctttcct gggcatggaa
    601 tcctgtggca tccatgaaac tactttcaac tccatcatga agtgtgacgt tgacattcgc
    661 aaggacctct atgccaacac agtgctgtct ggtggcacca ccatgtaccc aggcattgct
    721 gacaggatgc agaaggagat cacagccctg gcacccagca caatgaagat caagatcatc
    781 gctccccctg agcgcaagta ctctgtgtgg attggcggct ccatcctggc ctctctgtcc
    841 accttccagc agatg
```

Appendix B.11

```
LOCUS          AF333964   237 bp   mRNA      ROD   12-MAY-2001
DEFINITION     Marmota monax interleukin-2 mRNA, partial cds.
ACCESSION      AF333964
SOURCE         woodchuck.
FEATURES       Location/Qualifiers
   source      1..237
               /organism="Marmota monax"
               /db_xref="taxon:9995"
   CDS         <1..209
               /note="IL-2"
               /codon_start=3
               /product="interleukin-2"
/translation="EEVLNVPQSKNFHLKDTRNFISNINVTVLKLKGSATTFTCEYAQETANIVEFLNTWIT
FCQSIISKLT"

BASE COUNT     75 a    57 c    49 g    56 t
ORIGIN
      1 tggaggaagt gctgaatgta cctcaaagca aaaactttca cttgaaagat accaggaact
     61 tcatcagcaa catcaacgtg actgttctga aactaaaggg atccgccacg acgttcactt
    121 gtgagtacgc ccaggagaca gcgaacattg tagaatttct gaacacatgg atcacctttt
    181 gccaaagcat catctcgaag ctcacttgag aattaggtgc ctcccgtgta taacatc
```

Appendix B.12

LOCUS AF333965 417 bp mRNA ROD 12-MAY-2001
DEFINITION Marmota monax interleukin-4 mRNA, partial cds.
ACCESSION AF333965
SOURCE woodchuck.
FEATURES Location/Qualifiers
source 1..417
/organism="Marmota monax"
/db_xref="taxon:9995"
CDS 1..>417
/note="IL-4"
/codon_start=1
/product="interleukin-4"
/translation="MGLSSQLIATLFCLLVCPGNFTHGCNVTLEEIIKTLNTLSGKKLVPKTTCMEVMVADV
FAVPKNTTEKEILCTATTVLRQTYQDHPVSRCLNKNGKLDILKLLRGLYRNLRSMAQLHNCPVSES
RQRTLKDFLESLKRI"

variation 130..141 / note="insertion relative to other interleukin-4 genes"

BASE COUNT 124 a 111 c 92 g 90 t
ORIGIN
1 atgggtctca gctcccagct gattgccact ctcttctgtc tcctagtatg ccctggcaac
61 ttcacccacg gatgcaacgt taccttagaa gagatcatca aaactttgaa cacactctca
121 gggaaaaagc ttgtgccaaa gactacatgc atggaggtga tggtagcaga cgtctttgct
181 gtccccaaga acacaaccga gaaggaaatc ctctgcacgg ctacaactgt gcttcggcag
241 acctatcaag accacccggt gtctaggtgt ttgaacaaaa atggaaaact tgacattctc
301 aaactcctga gaggactcta caggaacctc cgaagcatgg cccagttgca caactgtccc
361 gtgagtgaat ccaggcagag aacattgaaa gacttcctgg aaagcctaaa aaggatc

Appendix B.13

LOCUS AF333966 654 bp mRNA ROD 12-MAY-2001
DEFINITION Marmota monax interleukin-6 mRNA, partial cds.
ACCESSION AF333966
SOURCE woodchuck.
FEATURES Location/Qualifiers
source 1..654
/organism="Marmota monax"
/db_xref="taxon:9995"
CDS 22..645
/note="IL-6"
/codon_start=1
/product="interleukin-6"
/translation="MKFFSIASLGLLLVVATAFPASELQREDGENSVTRNKPTRASSGKTAGQISYLIKEVF
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LSNFEDFLQFTLRAVRKA"

BASE COUNT 198 a 153 c 153 g 150 t
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121 actcgaaata aaccaacacg tgcctcttct ggaaaaaccg caggacagat ttcctacctc
181 atcaaggaag tcttcgaaat gagaaaagag ctgtgcaaga atgatgagac ctgtatcaag
241 agccatgtgg cagtgtccga aaacaatctg aaccttccaa agatgactga aaaagatgga
301 tgcttccaaa ctggatacaa tcgggacaac tgccttgtgc gaatcacctc tgggcttctg
361 gagtttcagg tctacctgag gtacatccgg aacaagtttc aggaaggcaa taacagggac
421 agagctgaac atgtgcagtt cagttccaaa gccctgattg agatcctgaa acaagaggtg
481 aaggatccca ataaaatagt cttccctagc ccaactgcaa atatcaacct attggcaaaa
541 ctggagtcac agaatgattg gcagaaggtc atgaccatgc aactcatctt gagcaacttt
601 gaggatttcc tgcagttcac cctgagagct gttcggaaag catagtgggc atct

Appendix B.14

LOCUS AF333967 712 bp mRNA ROD 12-MAY-2001
DEFINITION Marmota monax tumor necrosis factor alpha mRNA, complete cds.
ACCESSION AF333967
SOURCE woodchuck.
FEATURES Location/Qualifiers
source 1..712
/organism="Marmota monax"
/db_xref="taxon:9995"
CDS 1..702
/note="TNF"
/codon_start=1
/product="tumor necrosis factor alpha"
/translation="MSTESMIRDVELAEEALPKEAWGPQGSSRCLCLSLFSFLLVAGATTLFCLLHFGVIG
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DNQLVVPANGLYLVYSQVLFKGQGCPSYVLLTHTVSRFAVSYQDKVNLLSAIKSPCPKESLEGAE
FKPWYEPIYLGGVFELQKGDRLSAEVNLPSYLDFAESGQVYFGVIAL"

BASE COUNT 150 a 211 c 195 g 156 t
ORIGIN
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61 gcatgggggc cccagggctc cagccggtgc ctgtgcctca gcctcttctc cttcctgctt
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421 ttcaagggcc aaggctgccc ctcctacgtg ctcctcaccc acactgtcag ccgctttgct
481 gtctcttatc aggacaaggt caacctcctc tctgccatca agagcccttg cccaaaggag
541 agcctggagg gggctgagtt caagccctgg tatgaaccca tctatctagg aggggtcttc
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661 gctgagtccg ggcaggtcta ctttggggtc attgctctgt gaagggaatg ga