MECHANISMS OF HEPADNAVIRUS PERSISTENCE AND ROLE OF INTERFERON GAMMA IN MODULATION OF ANTI-VIRAL IMMUNE RESPONSE

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by

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ABSTRACT

Hepatitis B virus (HBV) is a major pathogen that induces chronic hepatitis in approximately 400 million people worldwide frequently leading to liver cirrhosis and hepatocellular carcinoma. Woodchucks infected with woodchuck hepatitis virus (WHV) demonstrate virological and immunological profiles of infection and liver disease patterns highly comparable to those seen in human hepatitis B. Many attributes of HBV and the immune system interactions remain unknown, particularly how HBV persists and causes chronic liver disease. In the current studies, we investigated several aspects of the hepadnavirus-host immune system interaction and examined a role in these interactions of interferon gamma (IFNγ), which is known to be a potent antiviral and immune system regulatory cytokine. The specific objectives of our studies were to: (1) clone and express woodchuck IFNγ and tumor necrosis factor alpha (TNFα) in the baculovirus and *Escherichia coli (E. coli)* expression systems; (2) establish an *in vitro* model in which the effect of WHV genome expression on the hepatocyte surface presentation of the class I major histocompatibility complex (MHC) molecules can be investigated and, by using this system, to determine which WHV translation product interferes with the class I antigen display and whether this interference can be reversed by hepatocyte treatment with IFNγ, and (3) investigate the modulator effect of IFNγ on a DNA vaccine in preventing WHV infection. In the course of these studies, we have successfully produced biologically active recombinant woodchuck IFNγ and TNFα. The data showed that the baculovirus-derived IFNγ and TNFα had considerably

greater biological potency than the same cytokines produced in *E. coli*. In a subsequent study, we established an *in vitro* model to examine hepadnavirus interference with hepatocyte class I MHC expression. The results revealed that the class I antigen presentation was significantly inhibited on hepatocytes transcribing the complete WHV genome, similarly as it was previously reported by this laboratory for hepatocytes in chronic WHV hepatitis. WHV envelope preS2 protein was found to be responsible for the class I MHC antigen suppression, while transcription of WHV X gene significantly augmented the class I antigen display on the hepatocytes surface. This defect in class I MHC presentation may diminish the susceptibility of hepatocytes to virus-specific T cell-mediated elimination, hamper hepadnavirus clearance, and contribute to the establishment of chronic hepatitis. Importantly, we also demonstrated that IFNγ, a stimulator of class I MHC expression, can reverse this WHV-induced inhibitory effect and restore class I MHC presentation on hepatocytes, suggesting that the same could be true for hepatocytes in chronic hepadnaviral hepatitis. Further, our results demonstrated that DNA vaccination with combined WHV core gene and IFNγ DNA provided significantly better protection against serologically evident WHV infection and hepatitis than WHV core DNA vaccine alone. However, our study revealed that protection against the virus is never complete. The data also showed that IFNγ can serve as an adjuvant and can augment immune response against hepadnavirus, as well as possibly clear infection in a non-cytopathic manner. In summary, the results and the investigative tools generated should advance our understanding of the

complexity of molecular interactions occurring between hepadnavirus, hepatocytes and the host immune system which govern development of HBV persistence and chronic hepatitis. They may also aid in the development of novel more effective therapies against HBV.

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I dedicate this thesis to my wife, Zhili Kang and lovely daughter, Amy. The hugs and kisses given by my wife and daughter have always lit up my day and have helped me live through frustrations and stresses. I also wish to thank my parents, Xuebin Wang and Xianxu Lu and my parents in-law, Jishan Kang and Xiugai Yang. Their unconditional support and love were instrumental in the completion of this work.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

aa	amino acid
AH	acute hepatitis
ALT	alanine aminotransaminase
anti-HBc	antibodies to hepatitis B virus core antigen
anti-HBs	antibodies to hepatitis B virus surface antigen
anti-WHc	antibodies to woodchuck hepatitis virus core antigen
anti-WHe	antibodies to woodchuck hepatitis virus e antigen
anti-WHs	antibodies to woodchuck hepatitis virus surface antigen
AST	aspartate aminotransferase
β2m	β2-microglobulin
bp	base pairs
BSA	bovine serum albumin
C	core gene of HBV or WHV
cDNA	complementary DNA
cccDNA	covalently closed circular DNA
CD	cluster of differentiation
CH	chronic hepatitis
CMV	cytomegalovirus
ConA	Concanavalin A
cpm	counts per minute
CTL	cytotoxic T lymphocyte
DC	dendritic cells
DEPC	diethylpyrocarbonate
DHBV	duck hepatitis B virus
DMSO	dimethyl sulfoxide
DMEM	Dulbecco's modified Eagle medium
dNTP	deoxynucleotide triphosphate
DR	direct repeat
EB	ethidium bromide
EBV	Epstein-Barr virus
ECMV	encephalomycocarditis virus

E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Enh	enhancer
ER	endoplasmic reticulum
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
g	gravity units of force (9.8 m/s^2)
GM-CSF	granulocyte-monocyte colony-stimulating factor
GSHV	ground squirrel hepatitis virus
h	hour
HBcAg	hepatitis B virus core antigen
HBeAg	hepatitis B virus e antigen
HBsAg	hepatitis B virus surface antigen
HBSS	Hanks' balanced salt solution
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HSV	herpes simplex virus
IB	inclusion bodies
IFNα	interferon alpha
IFNβ	interferon beta
IFNγ	interferon gamma
Ig	immunoglobulin
IL	interleukin
IPTG	isopropyl-beta-D-thiogalactopyranoside
IRES	internal ribosome entry site
kb	kilobase pairs
kDa	kiloDaltons
LCMV	lymphocytic choriomeningitis virus
LMP2	low molecular mass protein 2
LMP7	low molecular mass protein 7
mAb	monoclonal antibody

MCMV	murine cytomegalovirus
MECL1	multicatalytic endopeptidase complex-like 1
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
min	minute
MMLV-RT	Moloney murine leukemia virus- reverse transcriptase
mo	months
MTT	microculture tetrazolium assay
NK	natural killer cell
NK T	natural killer T cell
nm	nanometres
nt	nucleotide
OAS	2',5'-oligoadenylate synthetase
OD	optical density
ORF	open reading frame
P	polymerase gene of HBV or WHV
PA28α	proteasome activator 28 alpha
PA28β	proteasome activator 28 beta
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming unit
p.i.	post-infection
preC	pre-core
preS1	large surface protein
preS2	middle surface protein
pro	promoter
rcDNA	relaxed circular DNA
rpm	revolutions per minute
RT	reverse transcriptase
rWHV DNA	recombinant woodchuck hepatitis virus DNA
S	surface/envelope gene of HBV or WHV
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

S.I.	stimulation index
SOI	secondary occult infection
SSC	standard saline citrate
ss	single stranded
TAP	transporters associated with antigen processing
Taq	Thermus aquaticus
TCR	T-cell receptor
TE	Tris-EDTA
Th	helper T cell
Th1	T helper type 1
Th2	T helper type 2
TNFα	tumor necrosis factor alpha
U	units
UV	ultraviolet
vge	virus 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
wk	weeks
X	X gene of HBV or WHV
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

LIST OF APPENDICES

CHAPTER 1. INTRODUCTION

1.1 Epidemiology of hepatitis B virus infection

Viral hepatitis is a liver inflammatory disease caused by hepatotropic DNA or RNA viruses. The disease is characterized by intrahepatic lymphomononuclear cell infiltrations, hepatocellular injury, liver regeneration and it may, in consequence, compromise liver functions. There are at least six known hepatitis viruses, named from A to G which appear to primarily target the liver. Other viruses, such as Epstein-Barr virus, cytomegalovirus and yellow fever virus, can also induce hepatitis-like illness. Among these six main types of viruses causing hepatitis in humans, only the B, C and D viruses would lead to life long disease, *i.e.* chronic hepatitis (CH).

It has been well recognized that hepatitis B is one of the most common and serious human infectious diseases worldwide, although the availability of protective vaccines, as well as the introduction of vigorous blood screening programs, have dramatically lowered the risk of hepatitis B virus (HBV) infection in the last two decades. Nevertheless, It is estimated that about 30% of the world population, or an estimated 1.8 billion people, have been exposed to HBV (Fact Sheet WHO/204, 2000). Among these people, approximately 350 million have serologically evident, *i.e.*, HBV surface antigen (HBsAg)–positive, chronic infection and at least 1 million chronically infected persons die each year from HBV-associated cirrhosis, hepatocellular carcinoma (HCC) or liver failure (Block *et al.*, 2003). However, the geographical distribution of HBV infection varies

greatly across the world. In western countries, although HBV incidence in the general population is less than 1%, certain high-risk groups, such as drug abusers and sexually promiscuous persons, have higher rates of HBV infection (Hollinger and Liang, 2001). In endemic areas, such as Subsaharan Africa and South-East Asia, up to 10% of the population are serum HBsAg-positive chronic HBV carriers (Chisari and Ferrari, 1997). In these regions, the vertical transmission from mother to baby and close contact within a family are major routes of HBV transmission. In Canada, incidence of serologically diagnosable acute hepatitis B (AH) is calculated as 2.3 per 100 000 (Zhang *et al.*, 2001a). Chronic HBV carriage rate in Canada varies due to heterogeneity of the population. It has been reported to be 6.9% in the Innuit population (Baikie *et al.*, 1989), 7.4% in immigrants from Asia and Africa (Delage *et al.*, 1986), and 0.2-0.5% in non-immigrants (Zhang *et al.*, 2001a).

1.2 HBV structure and molecular organization

HBV is a 42-nm diameter enveloped DNA virus, also known as Dane particle named after the discoverer of the complete virion by electron microscopy (Dane *et al.*, 1970). This virus has an envelope composed of three related surface proteins named as large (L), middle (M), and small (S) or major surface proteins. These surface proteins are inserted into a lipid bi-layer derived from the host cell. Inside the virion, the 21-nm diameter core is constituted by an icosahedral nucleocapsid containing a copy of the HBV genome, which occurs in

the form of partially double-stranded DNA molecule (Onodera *et al.*, 1982). In addition, the nucleocapsid contains HBV DNA polymerase which is attached to the HBV genome. In the circulation of HBV-infected individuals, there are large quantities of subviral spherical and filamentous particles, 22 nm in diameter, which are constituted by envelope proteins. The amount of these subviral particles is usually 100-fold greater than that of Dane particles and could be as high as 200 μg/ml or more (Hollinger and Liang, 2001; Zoulim *et al.*, 1992). This large excess of virus envelope material carrying HBs antigenic reactivity can bind and exhaust antibodies directed against HBsAg (anti-HBs) which are considered to be able to neutralize the virus.

1.2.1 Genome organization

The HBV genome contains about 3.2 kilo-base pairs (Kb) of nucleotides forming a partially double-stranded circular form (see Figure 1.1). The minus-strand DNA is a full size virus genome with the HBV DNA polymerase covalently linked to its 5'-end. The positive strand has a variable length and is always shorter than the negative strand. At the 5'-end of the positive strand, there is a capped short oligoribonucleotide which was generated during HBV genome replication. The circularity of HBV DNA is maintained by cohesive and complementary 5'-ends. The HBV genome has four overlapping open reading frames (ORF) encoding both structural proteins (core and envelope proteins) and non-structural proteins (viral DNA polymerase and X protein) (Figure 1.1).

Figure 1.1. Schematic presentation of HBV and WHV genome structures. Partially double stranded DNA (black bold-line circles) which encodes viral proteins are shown as black arrows. The name, length, and corresponding nucleotide positions of each viral protein are marked. These viral proteins are translated from 4 viral mRNA transcripts (colored lines at outer circle) which were generated during viral replication by corresponding viral endogenous promoters presented as colored-filled eclipses. Other regulatory viral sequences such as enhancers (open color eclipse) and direct repeats (DR1 and DR2, purple rectangles) are presented in the diagram as well. All nucleotide positions are relative to the unique E*coR*I site which was arbitrarily set as 1.

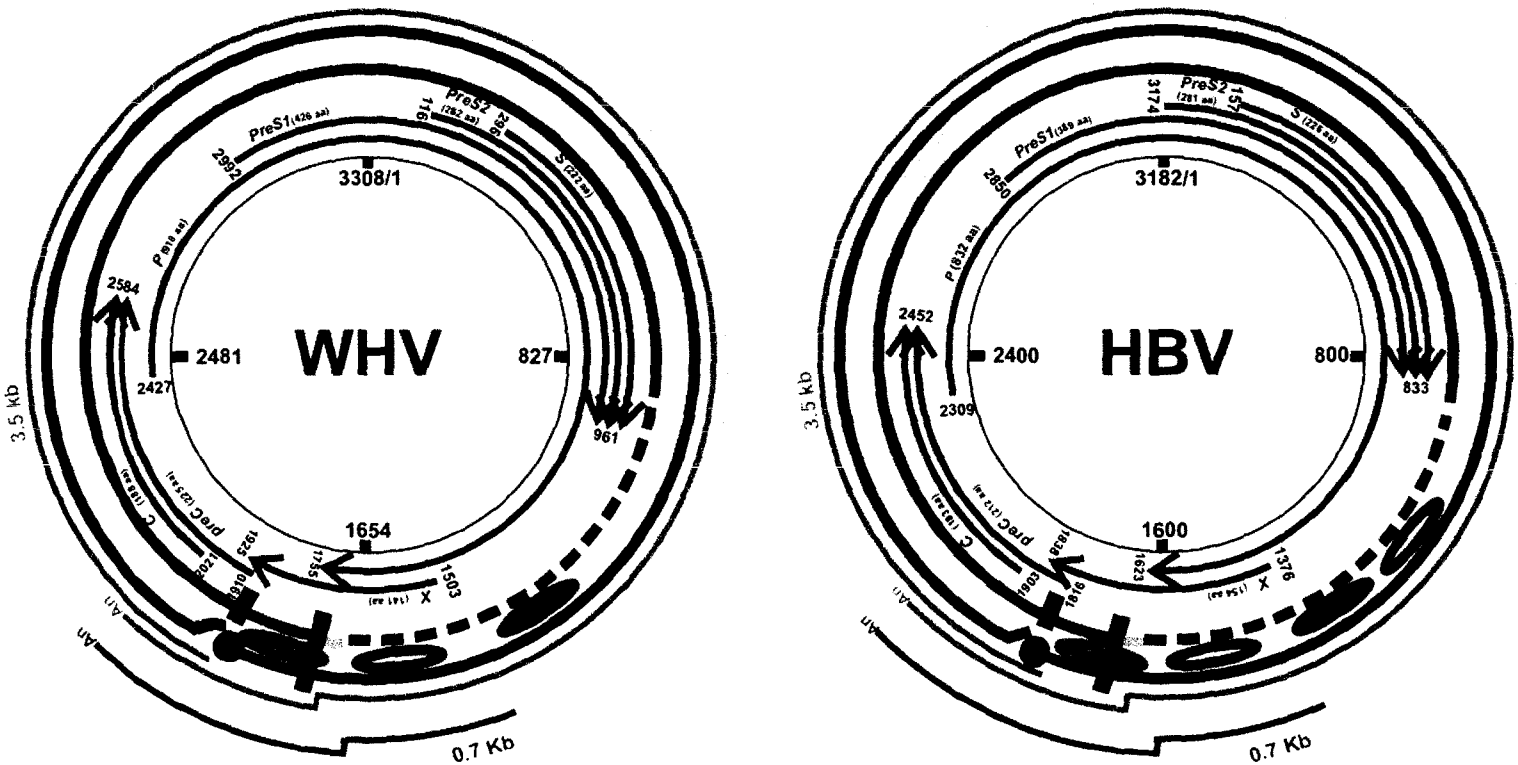
WHV
3308/1
2481
827
1654
2584
2427
961
1503
2982
PreS1(426 aa)
PreS2
(282 aa)
S (226 aa)
P(832 aa)
preC (234 aa)
C (183 aa)
X (154 aa)
3.5 kb
0.7 Kb
AAAAA
HBV
3182/1
2400
800
1600
2452
2309
833
1623
3174
2850
PreS1(389 aa)
PreS2
(281 aa)
S (225 aa)
P(832 aa)
preC (212 aa)
C (183 aa)
X (154 aa)
3.5 kb
0.7 Kb
AAAAA

Figure 1-1

Besides the ORFs, the HBV genome also contains regulatory DNA sequences to control the transcription of viral RNAs. Four promoters (*i.e.*, preC promoter, preS promoter, S promoter and X promoter) and two enhancers (enh1 and enh2) have been identified within HBV genome (Figure 1.1). In addition, a polyadenylation sequence located within the core gene provides the transcription termination signal.

1.2.2 Proteins and their functions

Depending on the size, the HBV transcripts can be divided into two classes: a genome-size transcript (3.5 Kb) and subgenomic transcripts (2.4, 2.1 and 0.7 Kb). The subgenomic transcripts serve as mRNA templates for the translation of viral proteins including envelope proteins (L, M, S) and the X protein; whereas the genomic transcripts function not only as mRNA for the translation of pre-core (preC), core (C) and polymerase (Couillin *et al.*), but also as the template for reverse transcription of the HBV minus-strand DNA.

The three envelope proteins (L, M, S) are type II transmembrane proteins that share the same carboxy-terminus but have different amino-terminal extensions due to the different use of a within frame start codon. L protein is the largest envelope protein and is probably responsible for binding to the cellular, not yet identified, receptor/s. It also interacts with the nucleocapsid to facilitate the enveloping of the core particles and formation of virions (Bruss *et al.*, 1996a). The function of M protein is not clear and it seems dispensable for HBV. S

protein, the major component of both Dane particles and subviral, freely circulating envelope particles, is the smallest and also the most abundant envelope protein. Although both glycosylated and non-glycosylated enveloped proteins exist, HBV infectivity seems to be independent of glycosylation, but requires myristylation of L protein (Bruss *et al.*, 1996b).

Core protein is encoded by a 3.5-Kb genome size transcript. Its arginine-rich domain, located at the carboxyl terminus, can bind to the negatively charged HBV nucleotides, whereas its amino-terminal domain can mediate assembly of the core particles. After forming the core particle, reverse transcription is initiated by the attached HBV polymerase using the 3.5-Kb genome size HBV RNA as the template. Using the same ORF but an upstream start codon, the preC can be translated into a protein. The protein, referred as HBV e antigen (HBeAg), is a form of the preC product protein which is truncated at both signal peptide, as well as the arginine-rich domain at the carboxyl terminal. HBeAg is secreted and may serve as a tolerogen to the host immune system and is dispensable for HBV replication and infection (Milich and Liang, 2003). Clinically, HBeAg has been used as a serological marker of actively progressing HBV infection. Clearance of HBeAg and the appearance of antibodies to HBeAg (anti-HBe) are considered to be an indicator of suppressed HBV replication (Milich and Liang, 2003).

The largest protein of HBV is HBV DNA polymerase, which is encoded by the 3.5-Kb pregenomic RNA. It overlaps with C, S, and X ORFs. The HBV polymerase has three enzyme activities including: DNA-dependent DNA

polymerase, reverse transcriptase, and RNase H. Its amino-terminal domain can also serve as a protein primer for the initiation of reverse transcription of HBV negative strand DNA. Heat shock protein 90, a cellular chaperon molecule, is also believed to facilitate reverse transcription by mediating the formation of a ribonucleoprotein complex between HBV polymerase and an RNA ligand (Hu and Seeger, 1996).

The HBV X is a multifunctional protein that can interact with transcriptional factors and tumor suppressor p53 protein, as well as influence a variety of signal transduction pathways (Elmore *et al.*, 1997). Not only viral genes, but also host genes can be regulated by the X protein. Recent evidence suggested that X may regulate HBV replication through targeting mitochondria calcium release (Bouchard *et al.*, 2001). In addition, it can activate proliferation and induce apoptosis of the infected cells (Gottlob *et al.*, 1998). The development of HCC due to chronic HBV infection is considered to be, at least to some degree, a consequence of the activity of X protein (Diao *et al.*, 2001; Wang *et al.*, 1990). The requirement of X for HBV replication is disputable due to the contradictory results reported by different groups, all using woodchucks infected with woodchuck hepatitis virus (WHV) as the animal model of hepatitis B (Zhang *et al.*, 2001b; Zoulim *et al.*, 1994).

1.3 HBV replication cycle

Although the liver is the site of the most active virus replication, HBV also propagates in the lymphatic system (Michalak, 2000). In the liver, the infectious Dane particles first bind to the yet unknown receptor on hepatocytes. Subsequently, HBV enters the cell and the nucleocapsid delivers virus-relaxed circular DNA (rcDNA) into the nucleus. Within the nucleus, the synthesis of HBV DNA positive strand is completed by the attached HBV polymerase using the genome-size HBV DNA minus-strand as the template. The gaps of both strands are then repaired, yielding a covalently closed circular double-stranded DNA, referred to as cccDNA. Using the RNA polymerase from the infected cell, four different lengths of HBV mRNA are transcribed from the HBV cccDNA template (as indicated in Section 1.2.2). These transcripts are then polyadenylated and transported into the cytoplasm. HBV proteins are translated from the respective viral transcripts as described in Section 1.2.2. Subsequently, the genome-size HBV RNA and the associated HBV DNA polymerase are packaged into the assembled virus nucleocapsids through the interaction between the core protein and the polymerase. The reverse transcription of the negative strand HBV DNA starts within the capsid and leads to the synthesis of the less than full-length positive DNA strand. The mature capsid can follow two intracellular pathways. One pathway leads to assembly of Dane particles, which bud into the lumen of the endoplasmic reticulum (ER) and are then secreted from the cell through the Golgi complex. In another pathway, matured HBV capsids can re-enter the

nucleus and initiate a new round of virion replication without superinfecting the cell (Seeger and Mason, 2000).

1.4 Natural history of HBV infection

The course and symptoms of HBV infection can be extremely variable. It depends on the age of the host at the time of infection, the dose of virus at its entry, as well as the status of the host's immune system. Clinically, liver disease caused by HBV infection can be divided into several categories: acute hepatitis (AH), fulminant hepatitis, CH and a "healthy" serum HBsAg-positive chronic carrier state (Hollinger and Liang, 2001). In addition, subclinical, serologically evident HBV infection and occult, asymptomatic, serological undetectable infections are also recognized (Michalak *et al.*, 2004).

The incubation time of hepatitis B is between 6 to 24 weeks (wks). There are no specific symptoms in this period other than fatigue, nausea, anorexia and slight fever. Some patients may develop jaundice and some may not feel any obvious unwellness. The diagnosis is based on the detection of HBV-specific serological markers, such as HBsAg, HBeAg, antibodies to hepatitis B core (anti-HBc), and molecular markers, *i.e.* HBV DNA. Also, biochemical indicators of liver injury are tested, such as elevated levels of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and bilirubin.

Approximately 1% of acute HBV infections will progress to a usually fatal, severe (fulminant) hepatitis. This form is due to the massive, rapid destruction of

hepatocytes and loss of liver function, and is characterized by the development of hepatic encephalopathy associated with coma.

Usually, more than 90% of adult patients with AH type B will recover from the disease and become serum-negative for HBsAg. However, 5-10% of the acutely infected patients progress to CH. This form of hepatitis is diagnosed when serum HBsAg-positive infection lasts more than 6 months (mo). In contrast to HBV infection in adults, more than 90% of neonatally acquired HBV infection will result in CH with mild symptoms or in asympomatic HBV carriage (Hollinger and Liang, 2001). In general, the frequency of clinically evident acute disease increases, whereas the percentage of chronic HBV carriers decreases with age at infection.

There is a clear correlation between chronic HBV infection and the development of HCC. The risk of developing HCC is almost 100-times greater in chronic HBV carriers than in uninfected individuals (Beasley *et al.*, 1981). The persistence of liver inflammation and continued hepatocyte regeneration may induce the malignant transformation of hepatocytes. Also, the postulated oncogenic potential of HBV X protein may contribute to HCC development through either modulating cells' susceptibility to apoptosis or inducing uncontrolled cell growth (Chirillo *et al.*, 1997; Gottlob *et al.*, 1998). Integration of HBV genome or its fragments into the host hepatocyte genome could be another important factor for the increased incidence of HCC. This integration may either

disrupt the function of cell cycle regulatory proteins or activate pre-oncogenes, such as N-myc2 oncogene (Bruni *et al.*, 1999).

HBV can also induce extrahepatic disorders through the deposition of the immune complexes of viral antigens and their specific antibodies. The pathogenic role of HBsAg-anti-HBs immune complexes has been documented in glomerulonephritis, polyarteritis nodosa, acute vasculitis and arthritis (Gocke, 1975; Michalak, 1978; Michalak *et al.*, 1976; Slusarczyk *et al.*, 1980).

1.5 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). Key members of the mammalian genus are HBV, WHV (Summers *et al.*, 1978), 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). The following common features are shared by viruses belonging to this family: (1) Similar genome organization, viral RNA transcripts, and replication strategy; (2) Enveloped virions contain 3-3.3 Kb partially double-stranded rcDNA; (3) Virion contains a viral DNA polymerase which is able to repair the DNA gap; (4) Large amounts of circulating genome-free lipoprotein particles constituted by viral envelope proteins; (5) Narrow host range; (6) Frequently occurring life-long

persistent infection, and (7) Induction of similar courses of infection and pathological forms of virus-induced liver disease (Tiollais *et al.*, 1985).

However, mammalian and avian hepadnaviruses also display important differences. The avian hepadnavirus genome does not contain the X gene and it encodes two instead of three envelope proteins (pre-S and S). Furthermore, liver disease induced by avian hepadnaviruses seemingly does not progress to HCC (Cova *et al.*, 1993).

1.6 Animal models of HBV infection

1.6.1 Woodchuck hepatitis model

WHV was the first non-human hepadnavirus that was identified. It was originally discovered in a woodchuck colony at the Philadelphia Zoological Garden (Summers *et al.*, 1978). WHV is morphologically indistinguishable from HBV. Viral genome analysis indicates overall more than 60% homology in nucleotide sequence between HBV and WHV (Galibert *et al.*, 1982). Among all the members of the hepadnavirus family, infection of woodchucks with WHV is accepted as the most suitable model to study HBV pathobiology. This is based on the fact that WHV and HBV share significant similarities with regards to ultrastructure, genomic organization, antigenic cross-reactivity of gene translation products, range of targeted organs (liver and lymphatic system), course of infection, the immunovirological and pathological features of the virus-induced liver disease, and the development of HCC (Michalak, 1998a).

Like HBV infection in humans, persistent WHV infection is common in some woodchuck populations, where it is frequently associated with CH and development of HCC. Similar to HBV, neonatal WHV infection causes serum WHsAg-positive infection in >90% of animals, while the same form of the infection is observed only in 5-10% of adult woodchucks. Vertical transmission of WHV from mother to babies has also been verified with the woodchuck model (Coffin and Michalak, 1999).

Woodchucks chronically infected with WHV have been extensively used for pre-clinical evaluations of antiviral drugs. The woodchuck model also provides valuable pharmacodynamic and pharmacokinetic information on novel anti-HBV agents before clinical trials. Toxicity and hepatic injuries have been shown in woodchucks treated with certain test antiviral nucleotide analogues to be similar to those observed in treated humans (Korba *et al.*, 2000; Le Guerhier *et al.*, 2001). In summary, using the woodchuck-WHV model it is possible to study almost any aspect of the pathogenesis of HBV infection, anti-HBV therapeutic and preventive strategies.

1.6.2 Other natural models

Due to the narrow host range, chimpanzees and some higher primates are the only animals able to be infected with HBV. However, the chimpanzee is an endangered species and its availability, cost, and breeding are the major obstacles in laboratory usage. Certain strains of ducks, such as Peking ducks,

are susceptible to DHBV. Although DHBV lacks the X gene and it encodes only two surface proteins (as mentioned in Section 1.5), the ability of DHBV to propagate in primary duck hepatocyte cultures significantly contributed to the recognition of the replication cycle of hepadnaviruses. Also, these animals are easy and inexpensive to breed. Viral hepatitis can be induced after inoculation with DHBV, but it remains inconclusive whether chronic DHBV infection is associated with HCC development. Extrahepatic DHBV replication, such as in the lymphatic system and pancreas, has also been documented (Hosoda *et al.*, 1990; Walter *et al.*, 1991). However, the avian immune system considerably differs from the mammalian counterparts. Therefore, ducks infected with DHBV are not being frequently used as a model to study the immunopathogenesis of HBV.

Advances in molecular biology led to the discovery of new members of the *Hepadnaviridae* family. They have been found in a variety of animals, such as Beechey ground squirrels (Ganem *et al.*, 1982), arctic ground squirrels (Tennant *et al.*, 1991), wild herons (Sprengel *et al.*, 1988), tree squirrels (Feitelson *et al.*, 1986), wild and domestic geese, marsupials, orangutans (Marion *et al.*, 1987), and woolly monkeys (Lanford *et al.*, 1998). So far, no detailed immunological or molecular studies have been carried out using these species.

1.6.3 HBV transgenic mice

Because of the absence of an inbred animal model susceptible to HBV infection, and the inability of HBV to efficiently propagate in *in vitro* culture systems, a number of researchers have generated transgenic mice that express a single virus gene or the complete genome of HBV. The well characterized mouse immune system, homogenous genetic background, low cost, as well as the availability of commercial reagents and assays facilitate effective usage of HBV transgenic mouse models (Chisari and Ferrari, 1995).

One of the first HBV transgenic mice expressing the HBV envelope proteins was created using constructs containing liver-specific promoters or the endogenous virus promoters (Babinet *et al.*, 1985; Burk *et al.*, 1988; Chisari *et al.*, 1985). It has been demonstrated that the liver, as well as other organs (*i.e.*, kidney) are the place of HBV envelope protein production driven by an endogenous viral promoter (DeLoia *et al.*, 1989). In the case when the L envelope protein expression was controlled by the exogenous albumin promoter, the intracellular accumulation of HBsAg has been found within hepatocytes. This defect in secretion resulted in the expansion of ER and induction of hepatocyte injury, which is similar to 'ground glass' hepatocytes observed in patients chronically infected with HBV (Chisari *et al.*, 1987). Moreover, adoptive transfer of HBsAg-specific, cloned CD8+ cytotoxic T lymphocytes (CTL) induced an acute necroinflammatory liver disease in these mice, similar to that occurring in HBV infection in humans (Ando *et al.*, 1994; Moriyama *et al.*, 1990). A three-step

process has been proposed to cause liver damage due to this adoptive transfer. The first step supposedly involves the flush of HBsAg-specific CTL into the liver, whereby apoptosis of hepatocytes expressing envelope protein is induced by direct contact with transferred CTL. The second step occurs between 4 and 12 h after CTL injection. The CTL recruit host non-specific inflammatory cells to the liver and these cells amplify the effects of CTL. An extensive necrosis of liver parenchyma beyond the site of CTL infiltration has been observed, suggesting that hepatocytes are killed by host-derived inflammatory cells other than CTL. The third step, histologically comparable to fulminant hepatitis in human, happened within 24-72 h of CTL transfer to half of the transgenic mice with a high-level HBsAg display in hepatocytes. At this stage, liver failure causes death of the host, which is marked by diffuse infiltration of inflammatory cells, massive destruction of liver structure, and hyperplasia of Kupffer cells (Ando *et al.*, 1994; Ando *et al.*, 1993; Moriyama *et al.*, 1990).

HBV nucleocapsid protein and HBeAg have also been successfully expressed in transgenic mice. HBcAg was accumulated in the nucleus and HBeAg was found in the circulation (Milich *et al.*, 1994; Reifenberg *et al.*, 1997; Takashima *et al.*, 1992). Adoptive transfer of HBe/HBcAg-specific CD4+ T helper (Th) cells has been shown to induce liver injury in mice expressing HBe/HBcAg (Chen *et al.*, 2000). It has also been demonstrated that HBeAg is able to pass through the placenta and that in *utero* exposure to HBeAg can lead to T cell tolerance to HBe/HBcAg in the neonates (Milich *et al.*, 1990). It appears

that the circulating HBeAg can also modify the host immune response by depletion of HBeAg-specific Th1 cells, but not HBeAg-specific Th2 population, through a Fas-mediated mechanism (Milich *et al.*, 1998).

The nonstructural HBV X protein has also been expressed in transgenic mice (Koike *et al.*, 1994; Lee *et al.*, 1990). Because of its transactivation property, it has been suggested that expression of X protein might induce HCC. In support of this hypothesis, two groups have shown the development of HCC in some strains of X gene transgenic mice (Kim *et al.*, 1991; Koike *et al.*, 1994). However, other investigators have not observed this association in independently derived X transgenic mice (Lee *et al.*, 1990).

Several groups have expressed the entire HBV genome in the mice under the control of endogenous viral promoters (Araki *et al.*, 1989; Burk *et al.*, 1988; Choo *et al.*, 1991; Farza *et al.*, 1988; Guidotti *et al.*, 1995). Similar to CH in humans, large amounts of HBV virions were found in the circulation of these mice. Liver, as well as kidney, were the sites of production of the engineered viral particles (Araki *et al.*, 1989; Burk *et al.*, 1988; Choo *et al.*, 1991; Farza *et al.*, 1988; Guidotti *et al.*, 1995). However, HBV cccDNA, the replication form of HBV genome, was not found in these mice. Furthermore, the high-level HBV production is not associated with liver pathology. On the other hand, one group has successfully infected chimpanzees with viral particles derived from HBV transgenic mice (Heise *et al.*, 1999), proving their biological competency. By using the HBV transgenic mouse models, a number of factors, such as HBV-

specific CTL (Guidotti *et al.*, 1996b), cytokines (Gilles *et al.*, 1992; Guidotti *et al.*, 1994a; Guidotti *et al.*, 1994b), and co-infection with lymphocytic choriomeningitis virus (LCMV) (Guidotti *et al.*, 1996a), have been shown to inhibit HBV replication in these mice. Based on these studies, several mechanisms of HBV elimination from the infected liver have been proposed. The potential mechanisms include elimination of HBV nucleocapsid particles in the cytoplasm of infected cells and post-transcriptional degradation of cytoplasmic HBV mRNA (Guilhot *et al.*, 1993; Heise *et al.*, 1999).

HBV transgenic mice provided a significant amount of data regarding HBV biology, host immune responses against HBV proteins, and HBV oncogenic potential. However, there are also numerous limitations of this model. First, this is not an infective model of HBV-induced liver disease and transgenic mice support only HBV replication and virus assembly, but not the pre-transcription stages of the virus infection. Second, the replication scheme of HBV in transgenic mice is different from that occurring in nature where episomal HBV genome, but not the integrated HBV genome, is the main template. Third, HBV cccDNA is absent. Fourth, the HBV nucleocapsids assembled within the mouse hepatocytes were unable to re-enter into the nucleus. Fifth, the virus is not able to spread through re-infection of hepatocytes. Recenly, the development of chimeric mice transplanted with human liver provide a new tool to study HBV in mice (Mercer *et al.*, 2001; Petersen *et al.*, 2004).

1.7 Cell cultures for hepadnaviral propagation

One of the obstacles to study HBV is the lack of an *in vitro* cell culture system able to support efficient propagation of HBV. So far, using primary hepatocytes as targets, *de novo* hepadnavirus infection has been successful only in duck primary hepatocytes (Tuttleman *et al.*, 1986b). It was estimated that around 10% of primary duck hepatocytes are usually being infected. DHBV cccDNA and single-strand DHBV DNA were detected in these cells. Newly synthesized viral structural antigens were also identified in the infected duck hepatocytes. Interestingly, the susceptibility window for *in vitro* DHBV infection was only within 4 days after isolation of hepatocytes. Nevertheless, with the help of this cell culture system, it was found that hepadnaviral cccDNA is amplified within the infected hepatocytes from rcDNA prior to virion assembly (Tuttleman *et al.*, 1986a). Therefore, amplification of virus does not require the newly produced virions to re-infect liver cells or integration of virus DNA into the host genome. Although no conclusion regarding the receptors for HBV has been drawn, several candidate receptors for DHBV have been identified (reviewed in Chisari, 2000).

Establishment of equivalent culture systems for the propagation of other mammalian hepadnaviruses has proven to be much more difficult. This is mainly because of difficulties in maintaining differentiated mammalian hepatocytes in culture. In general, using primary human hepatocytes and infectious HBV inocula derived from chronically infected patients, only very limited propagation of HBV has been documented in some studies (Gripon *et al.*, 1988; Rumin *et al.*,

1996). It has been shown that chemical agents, such as dimethyl sulfoxide (DMSO) (Gripon *et al.*, 1988; Paran *et al.*, 2001; Rumin *et al.*, 1996) and a mixture of DMSO and polyethylene glycol (PEG) (Gripon *et al.*, 1993) can enhance HBV synthesis in cultured primary human hepatocytes. However, the underlying mechanisms for DMSO treatment are not clear.

Due to the difficulties in obtaining primary human hepatocytes, the human hepatoma HepG2 cells have been widely used as a HBV infection target. It has been demonstrated that this cell line is susceptible, to some degree, to infection with serum-derived HBV (Bchini *et al.*, 1990). These cells were kept for several months after inoculation with HBV and were able to express low levels of HBV cccDNA and mRNA, as well as HBsAg. Furthermore, complete viral particles containing HBV DNA and DNA polymerase activity were secreted. By infecting HepG2 cells with serum-derived HBV, a cell line, which secretes a high level of HBsAg due to the integration of HBV genome into the HepG2 chromosome, was derived (Mabit *et al.*, 1994). Recently, a human hepatocyte cell line HepaRG has been generated. It has been shown that these cells after treatment with hydrocortisone and DMSO become susceptible to HBV infection (Gripon *et al.*, 2002).

With advances in molecular biology techniques, cloned HBV sequences have been introduced into HepG2 and Huh7 human hepatocarcinoma cell lines. Various constructs containing more than full-length HBV genome have been transfected into these cells (Bouchard *et al.*, 2001). This led to the release of

Dane-like particles in the culture medium (Chang *et al.*, 1987; Sureau *et al.*, 1986; Yaginuma *et al.*, 1987). Besides HBV virions, HBV replicative intermediates, as well as viral proteins were detected in these cell culture systems. This suggests that human hepatoma cells transfected with more than full-length HBV genomes are able to support replication of HBV in spite of low susceptibility to infection with serum-derived HBV particles. In the latter situation, poor binding or entry of HBV into the hepatoma cells could be a possible explanation.

Similar to HBV, WHV and GSHV were found to infect, with limited success, primary woodchuck hepatocytes (Aldrich *et al.*, 1989). In our laboratory, the WCM-260 woodchuck hepatocyte cell line derived from a healthy animal was established a few years ago (Diao *et al.*, 1998). These cells have been shown to be susceptible to infection with both serum and lymphoid cell-derived WHV inocula (Lew and Michalak, 2001). Expression of WHsAg and WHcAg has been detected in *in vitro* infected WCM-260 cells by flow cytometry. The presence of WHV cccDNA was also identified beginning from 3 days post infection. WHV serially transmitted by *de novo* infection in WCM-260 hepatocytes proved to be infectious to healthy woodchucks and able to induce classical acute hepatitis (Lew and Michalak, 2001). These data clearly documented that WCM-260 hepatocytes can support the complete cycle of replication of biologically competent WHV.

Cell culture systems capable of supporting replication of HBV or other hepadnaviruses would provide a simple model to examine virus-target cell

interactions without involvement of other cell types or soluble factors, as it happens *in vivo*. By studying HBV in cell cultures, the replication strategy of HBV and identification of potential receptors could eventually be achieved. Furthermore, the cell culture system may provide useful information regarding mechanisms of antiviral function of cytokines, as well as help in evaluating and screening of new antiviral agents (Deres *et al.*, 2003; Lu *et al.*, 2002; Pasquetto *et al.*, 2002; Suri *et al.*, 2001).

However, the common shortfall of all current *in vitro* infection systems is a relatively low level of virus propagation. As a consequence, the use of highly sensitive detection methods to assess virus presence and its replication status is required.

1.8 Host immune responses against HBV infection.

It is generally accepted that HBV is essentially not cytopathic to the infected liver cells (Chisari and Ferrari, 1995; Lee, 1997). In fact, the immune responses mounted against HBV antigens are mainly responsible for the liver damage as well as the virus clearance. There are two major types of immune activities against any microbial infection, referred to as innate immunity and adaptive immunity. Within the innate immune response, which is the first line of host defense against pathogens, both cellular and soluble factors are involved. Cellular constituents of this system include: monocytes, macrophages, dendritic cells (DC), natural killer cells (NK), natural killer T cells (NK T), and

polymorphonuclear cells. Activation of these cells can lead to the production of anti-viral cytokines which may directly inhibit viral replication, *i.e.*, interferon α/β (IFNα/β), interferon γ (IFNγ), and tumor necrosis factor α (TNFα). Furthermore, secretion of chemokines by these cells contributes to the recruiting of inflammatory cells to the site of infection. More importantly, the processing and presentation of viral antigens by macrophages and DCs are key steps in the activation of the host's adaptive immunity (Bohm *et al.*, 1995; Freigang *et al.*, 2005).

Adaptive immunity involves two types of specific responses, humoral response, which normally targets extracellular pathogens, and cellular immune response, which deals with intracellular infections. 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 cells. In many infections with noncytopathic viruses, the clinical symptoms are a result of the host's immune responses against virally infected cells and the subsequent destruction of these cells.

1.8.1 Antibody responses

Antibodies to HBsAg (anti-HBs) are considered to be virus neutralizing and their generation is a T cell-dependent process (Milich and McLachlan, 1986). It is presumed that these antibodies are able to inhibit the attachment and entry

of HBV to target cells, and to facilitate removal of the virus by macrophages. Induction of anti-HBs alone due to prophylactic vaccination, using either serum-derived HBsAg or yeast-produced recombinant HBsAg, is normally sufficient to protect the host from HBV infection (Stephenne, 1988). Also, the recovery from acute HBV infection is usually accompanied by the seroconversion from HBsAg to anti-HBs.

Unlike antibodies to HBsAg, antibodies against HBV core antigen, *i.e.*, anti-HBc, have been normally explored as a diagnostic marker of progressing or past HBV infection. The presence of a high titer anti-HBc of IgM class can be used to distinguish between a recent onset of HBV infection and chronic HBV infection. However, in some patients with CH, IgM anti-HBc is detectable due to reactivation of HBV infection (Hollinger and Liang, 2001).

On the other hand, there is substantial experimental evidence indicating that induction of anti-core antibodies is associated with protection against hepadnavirus infection (see Chapter 6). In this regard, it was reported that immunization of two chimpanzees with liver-derived or recombinant HBcAg led to the generation of anti-HBc and protection of the animals from HBV infection (Iwarson *et al.*, 1985). In the same study, a third chimpanzee which was immunized with liver-derived HBcAg developed hepatitis following HBV challenge. A similar observation was reported in the woodchuck model of hepatitis B (Schodel *et al.*, 1993). Thus, it has been shown that woodchucks immunized with recombinant WHcAg produced anti-core antibodies and were apparently not

susceptible to infection after challenge with an infectious WHV dose (Schodel *et al.*, 1993). In the same study, when woodchucks were immunized with HBcAg, 4 out of 6 woodchucks were protected from challenge with WHV. Since the elicited anti-HBc antibodies showed little cross-reactivity with WHcAg, it was hypothesized that the T cell responses, rather than anti-HBc antibodies, were playing a pivotal role in the protection of the woodchucks immunized with HBcAg (Schodel *et al.*, 1993). However, in the above studies, the presence of virus post challenge was not examined by sensitive detection techniques, making the conclusions less convincing. Therefore, it is not clear whether such an immunization strategy in fact is capable of rendering a sterile immunity against the virus, *i.e.*, completely protect the host from viral infection. If this would be the case, there is also the question whether protective immunity induced by immunization with hepadnavirus core antigen can be enhanced.

The anti-HBe has also been routinely monitored in clinical situations. Similar to anti-HBc, the anti-viral and pathogenic significance is not clear. Induction of anti-HBe alone does not protect from HBV infection (Milich and Liang, 2003). However, the seroconversion from HBeAg to the anti-HBe-positive status has been associated with a decrease in activity of HBV replication and a milder progression of liver disease (Maruyama *et al.*, 1993).

Although it has been reported that antibodies to the carboxyl-end of HBV polymerase, especially the RNaseH domain, can be used as an indicator of HBV infection (Weimer *et al.*, 1989), little is known about biological significance of

these antibodies. The origin of these antibodies is unknown and they are not tested in clinical situations.

1.8.2 Cellular responses

1.8.2.1 Innate responses

The first line of immune defense is innate immunity, which provides rapid, non-specific protection against a variety of pathogens without establishing long-term memory. This innate immunity not only renders the host immediate protection but also initiates the development of pathogen-specific, adaptive immune responses. As mentioned at the beginning of Section 1.8, the cells maintaining the innate response include monocytes/macrophages, DC, NK, NK T and polymorphonuclear cells (reviewed by Basset *et al.*, 2003).

Macrophages can eliminate pathogenic invaders by phagocytosis before they can spread, and stimulate adaptive immune responses. After activation of these cells, cytokines, such as interleukin-1β (IL-1β), TNFα, IFNγ and chemokines are released into the environment. The recruitment of other inflammatory cells, such as neutrophils, can also contribute to elimination of pathogens, as well as to the local tissue damage *via* oxidative and non-oxidative stress mechanisms (Burg and Pillinger, 2001). Interestingly, when neutrophils were depleted in the HBV transgenic mice before the adoptive transfer of HBV-specific CTL, the degree of liver damage was significantly reduced, although the anti-viral effect of CTL was not influenced (Sitia *et al.*, 2002). Recently, it was

found that the activation of toll-like receptors is able to initiate IFNα/β-mediated suppression of HBV in transgenic mice (Isogawa *et al.*, 2005).

DCs arise from the bone marrow and are widely distributed in both lymphoid and non-lymphoid tissues. They guard the sites against pathogen entry and are able to capture and process invading microbes. Upon recognition and appropriate activation, which includes processing and loading of peptides into major histocompatibilty complex (MHC) molecules, up-regulating co-stimulatory molecules, and secreting of cytokines, DC maturation is triggered. The cells migrate to lymphoid organs where they stimulate a primary or memory immune response. Interference with DC antigen presenting may, therefore, benefit virus by inhibiting induction of anti-viral immunity. It has been reported that HBV can inhibit function of monocyte-derived DCs by compromising antigen presentation and by reducing the production of Th1 cytokines (Beckebaum *et al.*, 2003). In addition, DC derived from patients with CH type B showed impaired abilities to stimulate HBV-specific T cells compared to DCs from patients who resolved AH and to those from healthy individuals (Lohr *et al.*, 2002). Addition of IL-12 was able to restore function of DCs derived from patients chronically infected with HBV (Lohr *et al.*, 2002). Furthermore, activation of intrahepatic antigen presenting cells (APCs), which may include DC, by injection of anti-CD40 antibodies has been shown to inhibit HBV replication in HBV transgenic mice (Kimura *et al.*, 2002a). This effect is mediated by IL-12 and TNFα produced by

activated APCs and by NK cells secreting IFNγ. The NK cells are triggered by IL-12 which is secreted by activated APCs (Kimura *et al.*, 2002a).

NK cells are derived from the lymphoid cell precursors residing in the bone marrow (Biron *et al.*, 1996). Unlike T cells, NK cells lack antigen specificity and they do not have immunological memory. They contain perforin and granzymes, which are released following cell to cell contact, and are able to kill virally infected cells or tumor cells (Biron *et al.*, 1999). Their rapid response (0-5 days) after infection with pathogenic micro-organisms provides immediate protection before the induction of an adaptive immune response (reviewed by Biron *et al.*, 1996). The function of NK cells is determined by the balance between their activation and inhibition signals transduced through NK cell surface receptors. These receptors include CD94/NKG2 heterodimers, NKG2D, Ly49H, NKp30, NKp44, NKp46 and CD16, which belong to two distinct receptor categories, killer Ig-like receptors (KIR) and lectin-like receptors (reviewed by Mandelboim and Porgador, 2001). Although the natural ligands for the activation of these receptors are not well recognized, it has been shown that NKp46 is able to bind hemagglutinin of influenza virus and hemagglutinin-neuraminidase of parainfluenza virus and render resistance to these viruses (Mandelboim *et al.*, 2001). In addition to a direct stimulation through the activation receptors, NK cells can be activated during the initial stages of viral infection by cytokines and chemokines. For example, IFNα/β, IL-12, IL-15 and IL-18, produced by viral infected cells or by macrophages and DCs, can stimulate NK cells to secrete

IFNγ, TNFα and macrophage inflammatory protein (MIP) (reviewed by Biron *et al.*, 1999). Therefore, NK cells can control the virus infection both in a non-cytopathic manner and by killing virus-infected cells.

It has been suggested that cytokines secreted by NK cells (*i.e.*, IFNγ and TNFα) might inhibit HBV replication through a non-cytopathic mechanism (Kimura *et al.*, 2002a), which possibly involves disruption of assembly of virial nucleocapsid (Biermer *et al.*, 2003). However, the exact mechanism by which NK cells may control HBV infection is still not clear. HBV constituents that potentially activate NK cells have not yet been identified.

NK T cells are a sub-population of T cells expressing T cell receptor (TCR) αβ that share some characteristics with NK cells (Bendelac *et al.*, 1997). They are CD4-positive or CD4/8-negative cells which possess some surface receptors (*i.e.*, NK1.1 and Ly49A) found on NK cells. They have a restricted TCR repertoire, which use Vα14Jα28.1 and Vβ8 in mice. TCR of NK T cells can interact with glycolipids presented in the context of non-polymorphic class I MHC molecules which are composed of CD1d and beta-2-microglobulin (β2M) (reviewed by Bendelac *et al.*, 1997). In mice, NK T cells can be detected wherever conventional T cells are found, although the ratio of NK T to T cells varies widely in a tissue-specific manner, *i.e.*, 30-50% of mature T cells in liver, 20-30% in bone marrow, and 10-20% in thymus are NK T cells (Bendelac *et al.*, 1997). In other tissues and organs, they represent a smaller percentage of T cells, such as in spleen (3%) and blood (4%) (Eberl *et al.*, 1999). Without

knowing the natural ligands which activate NK T cells, it has been found that α-galactosylceramide, derived from a marine sponge, binds CD1d and strongly stimulates both CD4+ and CD4- NK T cells (Kawano *et al.*, 1997). Once activated, NK T cells can produce a high level of immunoregulatory cytokines, such as IL-4, IFNγ and TNFα. In addition to the cytokine production, NK T cells can exhibit a potent lytic activity against CD4/CD8-double positive thymocytes through FasL-Fas pathway (Arase *et al.*, 1994) or kill tumor cells in a perforin-dependent manner (Smyth *et al.*, 2000).

Regarding a role of NK T cells in the control of HBV replication, it has been reported that a single injection of α-galactosylceramide was able to strongly inhibit HBV replication in the liver of transgenic mice within 24 h (Kakimi *et al.*, 2000). This effect was preceded by rapid production of IFNα/β and IFNγ, and followed by recruitment of NK cells to the liver. IFNα/β or IFNγ receptor deficiency, but not depletion of CD4/CD8+ T cells, interfered with α-galactosylceramide-mediated HBV inhibition (Kakimi *et al.*, 2001). Activated NK T cells can also stimulate hepatocytes to produce CXC chemokines and recruit lymphomononuclear inflammatory cells into the liver (Kakimi *et al.*, 2001). The inflammatory cells infiltrating the liver seem not to be responsible for the inhibition of HBV replication in transgenic mice, but they may contribute to the severity of hepatitis by damaging hepatocytes in a virus non-specific manner (Kakimi *et al.*, 2001). Furthermore, there is also some evidence indicating that some populations of NK T cells in the liver can be activated by HBV envelope antigens

but not by α-galactosylceramide in HBV S gene transgenic mice generated on the recombinase-activated gene deficient (RAG-$1^{-/-}$) background (Baron *et al.*, 2002).

1.8.2.2 Adapative immune responses:

1.8.2.2.1 CD8+ T cell response

CD8+ T cells recognize pathogen-derived peptides presented by class I MHC molecules on the surface of the infected cells (see Fig. 1.2). Peptides generated by proteasome-mediated protein degradation are transported to the ER, bound by newly synthesized class I molecules MHC and transported to the cell surface. Since most peptides bound by class I MHC are derived from endogenously synthesized, cytosolic proteins, CD8+ T cells principally defend against microbes that replicate within infected cells (Pamer and Cresswell, 1998).

Classically, class I MHC-bound peptides are generated in the cytosol through a stepwise process involving the ubiquitin-proteasome system (Gromme and Neefjes, 2002). In this pathway, newly synthesized proteins are released from ER and tagged with ubiquitin by E3 ubiquitin ligase. Then, the ubiquitin tagged protein is directed to the barrel-like proteasome which is composed of α and β subunits. The peptidase activity resides in β subunits. The proteasome's constitutive β subunits can be replaced by IFNγ-induced β subunits, *i.e.*, low molecular weight protein 2 (LMP2), LMP7 and multicatalytic endopeptidase

Figure 1.2. Class I MHC antigen processing and presentation pathway. Several steps are involved in the class I MHC antigen processing and presentation. The majority of class I MHC binding peptides are generated by proteasome-mediated degradation. The peptides are transported into the lumen of ER by the TAP1 and TAP2 complex. Then, translocated peptides are selectively loaded to the class I MHC heavy chain (MHCI-hc)-b2m complex within the ER with the help of chaperones such as, tapasin, calreticulin and the thiol oxidoreductase ERp57. Finally, the peptide-MHCI-hc-β2m complex is transported to the cell surface for recognition of CD8+ T cells via the Golgi apparatus.

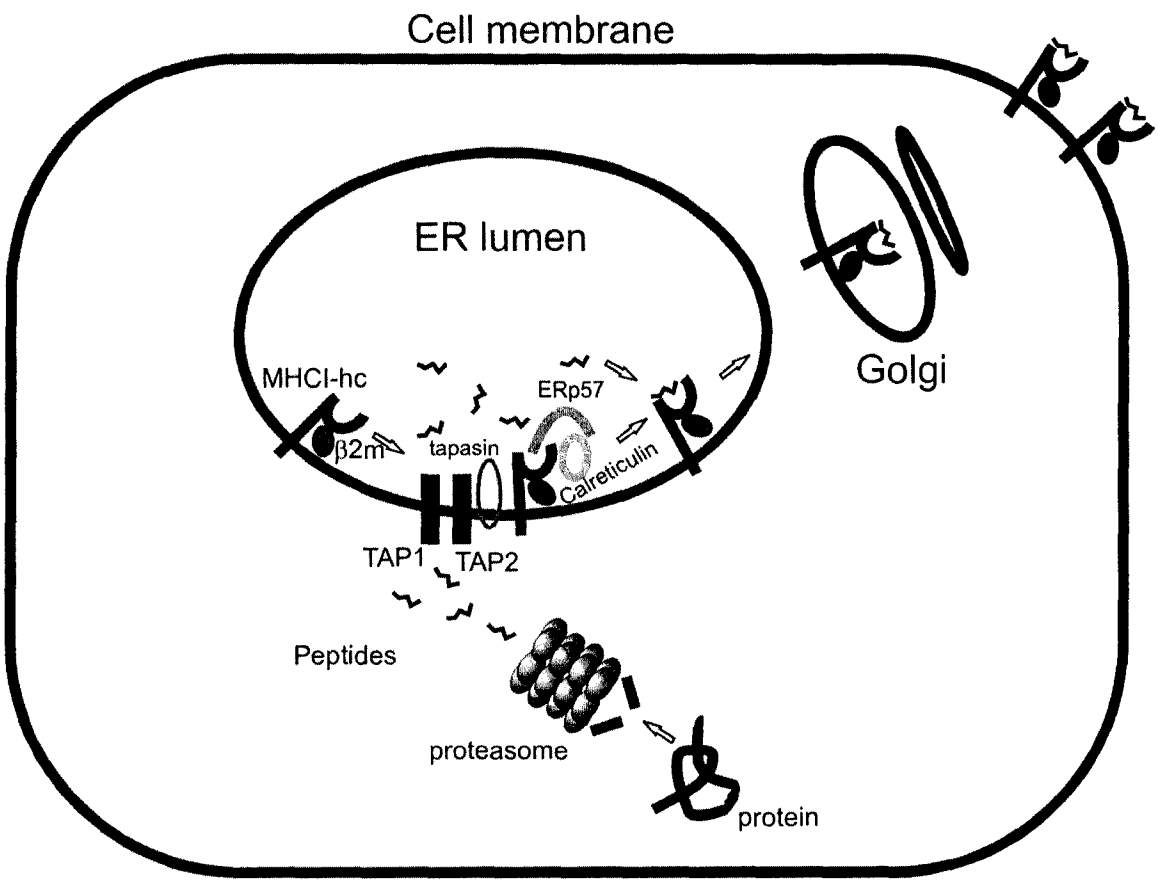
Cell membrane
ER lumen
Golgi
MHCI-hc
β2m
tapasin
ERp57
calreticulin
TAP1
TAP2
Peptides
proteasome
protein

Figure 1.2

complex 1 (MECL1) subunits. Such a newly formed proteasome with new β subunits is called immuno-proteasome. This results in different peptidase activity which cleaves the poly-peptides differently from the proteasome with constitutive β subunits. The proteasome-generated peptides are trimmed further to fit into the class I MHC complex by aminopeptidases which reside in either cytosol or in the ER. In order to be loaded to the class I MHC complex, peptides need to be translocated from cytosol to the lumen of the ER, where the actual loading happens. This process is mediated by transporters associated with antigen presentation 1 (TAP1) and TAP2 complex located on the membrane of the ER and requires ATP (Androlewicz *et al.*, 1993). Subsequently, with the help of chaperon molecules, such as tapasin, peptides are loaded to the class I MHC heavy chain which has been pre-complexed with β2m. The final complex leaves the ER *via* the Golgi apparatus and is displayed on the cell surface, as schematically shown in Fig. 1.2.

In some situations, an exogenous protein can be phagocytosed and processed in the lysosome to generate class I MHC-bound peptides which then enter the ER by the TAP-dependent transport mechanisms. Although its physiological significance remains unknown, this non-classical pathway, referred to as cross presentation, adds a broader peptide spectrum to the class I MHC presentation.

Several mechanisms are proposed to explain the anti-viral function of CD8+ T cells (reviewed by Harty *et al.*, 2000). First, upon activation of CD8+ T

cells by interaction of TCR with MHC class I-peptide complex, granules containing perforin and granzyme B are released toward the target cell. Assembled perforin can punch holes in the membrane of the target cell and deliver granzyme B, an apoptosis inducing enzyme, and cause death of target cells. Second, Fas ligand (CD95L)/Fas(CD95) interaction is also able to cause cell apoptosis. The FasL protein, expressed on the activated CD8+ T cells, interacts with Fas protein on the target cell, initiating an intracellular signaling cascade which leads to apoptosis of target cells. Third, in addition to production of chemokines attracting other inflammatory cells to the site of infection, CD8+ T cells can also secrete cytokines, such as INFγ and TNFα, which can be cytotoxic as well as having direct anti-viral effects without killing target cells (Nakamoto *et al.*, 1997).

The anti-HBV effect of CD8+ T cells has been extensively studied in the HBV transgenic model. Adoptive transfer of HBV-specific CD8+ T cells into HBV transgenic mice resulted in a transient, mild hepatitis and in a significant drop in the HBV genome transcripts in the liver (Tsui *et al.*, 1995). It has been estimated that no more than 10% of hepatocytes had been lysed by transferred CTL in a perforin and FasL-dependent manner (Guidotti *et al.*, 1996b; Nakamoto *et al.*, 1997). Therefore, other factors have to contribute to the elimination of HBV in the liver, since almost all hepatocytes are infected in advanced CH. It has been suggested that cytokines, such as IFNγ and TNFα actually mediate inhibition of HBV replication (Guidotti *et al.*, 1996b).

In HBV-infected patients, multi-specific antiviral CD4+ and CD8+ T cell responses were found in the PBMC from patients with a self-limited AH (Bertoletti *et al.*, 1991; Ferrari *et al.*, 1990; Jung *et al.*, 1991; Rehermann *et al.*, 1995). These responses were significantly stronger than those found in patients with CH (Rehermann *et al.*, 1996b). A recent study of patients with well-defined time of exposure to HBV has indicated that HBV-specific CD8+ and CD4+ T cell responses occur before symptomatic disease (Webster *et al.*, 2000). Consequently, maximal reduction of HBV DNA load has been observed before the appearance of hepatitis (Webster *et al.*, 2000). Quantification of the liver infiltrating HBV-specific CD8+ T cells using tetramer staining and flow cytometry has shown an equivalent number of HBV-specific CD8+ T cells in the liver of patients with active chronic HBV hepatitis accompanied by high serum ALT levels as in those with low HBV replication without hepatitis and with normal serum ALT levels. This may suggest that inhibition of virus replication by CD8+ T cells is independent of the severity of liver damage (Maini *et al.*, 2000). Antibody-mediated depletion of CD8+ or CD4+ T cells after HBV infection in chimpanzees indicated that HBV-specific CD8+ T cells are a major contributor to inhibition of HBV replication in the liver (Thimme *et al.*, 2003).

1.8.2.2.2 CD4+ T cell response

CD4+ T cells recognize peptide in the context of class II MHC on the surface of APC. The major function of CD4+ T cells is helping CTL immunity or in production of antibodies by B cells. There are two subsets of CD4+ T cells,

referred to as T helper 1 (Th1) and T helper 2 (Th2). Th1 CD4+ T cells are characterized by producing cytokines such as IL-2, TNFα and IFNγ, which mainly stimulate cellular immune response. Th2 cells are able to secrete IL-4, IL-5, IL-6, and IL-10 to favour the humoral immune responses. Although some CD4+ T cells are able to kill target cells through cell contact (Hahn *et al.*, 1995), cytokine production and immunoregulation are the main functions of these cells. Therefore, the antiviral effect of CD4+ T cells is achieved by either helping other immune effector cells to proliferate and differentiate or by releasing cytokines to inhibit directly virus replication.

In contrast to the relatively well-recognized role of CD8+ T cells in HBV infection, CD4+ T cell contribution is much less characterized. In the transgenic mouse model, it has been found that adoptive transfer of a HBV envelope-specific CD4+ Th1 cell line induces transient liver injury and inhibition of HBV replication (Franco *et al.*, 1997). This effect is mediated by production of IFNγ, but not by the direct killing of hepatocytes by CD4+ T cells (Mancini *et al.*, 1998). Consistent with the finding that a higher number of HBV-specific CD8+ CTL accompanies AH but not CH, a stronger Th1 CD4+ T cell response against HBV was observed in AH, but not in CH (Lohr *et al.*, 1998). Long-lasting CD4+ T memory cells specific for HBV nucleocapsid protein were found in patients with a past history of resolved acute HBV infection (Penna *et al.*, 1996; Rehermann *et al.*, 1996a). Surprisingly, in these convalescent patients, the strength and breadth of T cell responses directed against HBV epitopes are similar to those

observed in the acute phase of HBV infection. Since low levels of HBV DNA and HBV RNA are detectable in individuals who recovered from AH type B by sensitive polymerase chain reaction (PCR) assays (Michalak *et al.*, 1994), it has been suggested that trace amounts of replicating HBV might help maintain the virus-specific T cells responses. These active HBV-specific T cell reactivities are believed to play an important role in restricting the recurrence of HBV infection (Penna *et al.*, 1996; Rehermann *et al.*, 1996a). On the other hand, in spite of the presence of anti-HBV T cell responses, continued low level of HBV replication seems persistent for life.

In chronically HBV infected patients, it has been found that Th1 type CD4+ T cells directed against the virus envelope proteins can be induced by vaccination with HBsAg and they may, to some extent, control the level of HBV viremia (Couillin *et al.*, 1999). This suggests that the HBV induced immune tolerance in CH can be broken if an efficient immunization strategy can be applied. In general, HBV-specific CD4+ T cells can, to some degree, inhibit HBV replication directly and, more importantly, provide the necessary help for the generation of a strong anti-HBV CTL response.

In the woodchuck model, it has been found that there is a strong T cell proliferative response to the WHV core antigen (WHcAg), as measured using PBMC from acutely infected animals (Menne *et al.*, 1997b). Immunization with a synthetic dominant T cell epitope of WHcAg, constituted by WHcAg amino acids located in the positions 97-110 (WHc 97-110), can apparently completely protect

woodchucks from challenge with WHV, suggesting that a specific T cell response was playing an important role for defense against WHV (Menne *et al.*, 1997b). Deficiency in T cell response to WHcAg, as well as to other WHV antigens in the acute phase of hepatitis was associated with the progression to CH in woodchucks infected in the neonatal period (Menne *et al.*, 2002b).

1.9 Cytokines and HBV replication

Cytokines are small soluble proteins that can alter the function of the cells which display appropriate receptors. Most cytokines perform multiple biological functions, and some of the functions can also be mediated by several different cytokines. Many cytokines are directly or indirectly involved in the host's antiviral immunity. In this section, I will mainly focus on the group of antiviral cytokines designated as interferons and will only briefly describe TNFα, whose antiviral activity is much less understood.

1.9.1 Interferons

Interferons are a family of functionally related cytokines that induce a range of molecular and cellular responses, including antiviral, antiproliferative, antitumor and immunomodulatory activities (Pestka *et al.*, 1987). According to the structural and functional properties, they are classified as type I (IFNα/β, κ, ω *etc.*) and type II (IFNγ) interferons. IFNα/β are produced by many types of cells

upon virus infection, whereas IFNγ is mainly secreted by immune cells, such as Th1 CD4+ T cells, CTL, NK and NK T cells. Although there are many members of the type I IFN family, they bind to the same receptor and signal through the same signal transducer and activator of transcription (STAT1/2) complex (Smith *et al.*, 2005). They activate transcription of a series of genes containing interferon stimulated regulatory element (ISRE) (Stark *et al.*, 1998), while IFNγ, the only member in the type II family, binds to a distinct receptor and signals through STAT1 homodimers. Once dimerized, the STAT1 translocates to the nucleus and binds to the gamma-activated sequence (GAS) element and initiates nearby gene transcription (Stark *et al.*, 1998).

Several IFN-induced antiviral effector molecules have been identified. One of them is the virus double strand RNA (ds-RNA)-activated, IFN-induced protein kinase R (PKR), which can phosphorylate the translation initiation factor eIF-2α. This results in the rapid inhibition of protein translation and leads to inhibition of viral replication (Williams, 1999). Other molecules are the IFN-inducible 2'-5' oligoadenylate synthetases (OAS), which are stimulated by virus ds-RNA and lead to the generation of 2',5'-oligoadenylates. Subsequently, latent RNase (RNase L) is activated by 2',5'-oligoadenylates and mediates degradation of RNA and inhibition of viral replication (Rebouillat and Hovanessian, 1999). Another molecule is the IFN-inducible Mx protein, a member of the GTPase superfamily. It has been suggested that Mx protein interferes with replication of the influenza virus and bunyavirus family *via* direct binding to viral

ribonucleoprotein complex and by blocking their entry into the nucleus (Haller *et al.*, 1998). Other genes induced by IFNs include: (1) adenosine deaminases that act on RNA (ADAR), an RNA editing enzyme which modifies the sequence of viral and cellular RNA through binding to ds-RNA, leading to the generation of mutant proteins in virus-infected cells (Liu and Samuel, 1996; Saunders and Barber, 2003); (2) P56, an inhibitor of protein translation due to its ability to bind the translation factor eIF-3 (Guo *et al.*, 2000), and (3) inducible nitric oxide synthase (iNOS), which has been shown to inhibit the growth of ectromelia, vaccinia, and herpes simplex virus 1 (HSV-1) in mouse macrophages (Karupiah *et al.*, 1993). Among these effector molecules, PKR, ADAR and Mx genes are induced by type I IFNs, whereas 2'-5' OAS and RNase L by both types of IFNs. iNOS is induced by IFNγ, but not by type I IFNs (Stark *et al.*, 1998). Mice triply deficient for RNase L, Mx1, and PKR are still partially resistant to encephalomyocarditis virus (ECMV), indicating that multiple, functionally overlapping anti-viral molecules are responsible for antiviral activities (Zhou *et al.*, 1999).

1.9.2 Immunomodulatory effect of IFNs

Besides the fact that IFNs can activate intracellular antiviral molecules which directly inhibit virus replication, they also play key roles in the regulating of immune responses against viruses. First, IFNs are able to upregulate expression of class I MHC and enhance the effectiveness of the endogenous antigen

presentation pathway (Fruh and Yang, 1999; Kloetzel and Ossendorp, 2004). As a result, the virus-infected cells might be able to better process and present viral peptides in the context of class I MHC complexes on the cell surface, which are then recognized by specific CD8+ CTL. In consequence, the activated virus-specific CD8+ CTL could either eliminate infected cells by inducing apoptosis or inhibit virus replication in a non-cytopathic manner. Second, IFNγ is a strong stimulator of gene transcription for class II MHC as well as co-stimulatory molecules in APCs (Boehm *et al.*, 1997). The activated APCs could further promote anti-viral humoral and cellular immunity (see Section 1.8.2.2). Third, IFNγ is secreted by CD4+ Th1 T cells and is necessary for the skewing of the immune response toward the antiviral favorable CTL immunity (Farrar and Schreiber, 1993).

It was first found in HBV transgenic mice that adoptive transfer of HBV-specific CTL could inhibit virus replication through secreting IFNγ and TNFα without killing the infected hepatocytes (Guidotti *et al.*, 1996b). It was proposed that two independent antiviral pathways are involved in this noncytopathic process. One is related to the disruption of HBV nucleocapsid structure and another to the degradation of HBV RNA transcripts (Guidotti *et al.*, 1996b). Recent data indicated that nitric oxide (NO) is one of the antiviral mediators of IFNγ activity (Guidotti *et al.*, 2000). Further experiments suggested that even induction of IFNα/β and INFγ in the liver of HBV transgenic mice through infection with unrelated hepatotropic LCMV (Guidotti *et al.*, 1996a), adenovirus or

mouse cytomegalovirus (MCMV) (Cavanaugh *et al.*, 1998) can inhibit HBV replication. Systemic administration of IFNγ stimulating cytokines, such as IL-12 and IL-18, which can activate IFNγ production by APCs, NK, and T cells, has shown a similar HBV inhibitory effect in transgenic mice (Cavanaugh *et al.*, 1997; Kimura *et al.*, 2002b). A single dose of α-galactosylceramide, which can be presented by non-classical CD1d molecules to the NK T cells, is able to stimulate the rapid secretion of IFNγ by NK T cells (as mentioned in Section 1.8.2.1). Consequently, replication of HBV in the transgenic mice is inhibited by degradation of viral RNA. A similar HBV susceptibility has also been observed after induction of IFNα/β by injection of HBV transgenic mice with polyinosinic-polycytidylic acid complex, which mimics ds-RNA (Guidotti *et al.*, 2000). In spite of the transient viral inhibition at the RNA level, HBsAg and HBV DNA in the circulation, as well as in the liver, did not decrease. Moreover, there is no evidence that an anti-HBV T cell immune response was induced in these transgenic mice.

Finally, investigations of the natural course of experimental AH in chimpanzees have validated the findings in HBV transgenic mice described above (Guidotti *et al.*, 1999). It has been found that the appearance of intra-hepatic IFNγ is associated with the apparent complete elimination of HBV replication intermediates and cccDNA before the onset of liver injury. As a result, the level of HBV in serum and liver was greatly reduced prior to the appearance of hepatitis in these chimpanzees (Guidotti *et al.*, 1999).

1.9.3 Tumor necrosis factor alpha

TNF-related cytokines, including TNFα and lymphotoxin α/β etc, are type II transmembrane glycoproteins that signal through cognate receptors. The trimeric ligands cluster the receptor, initiating signal tranduction which regulate cell survival and death (Beutler and Cerami, 1989; Locksley *et al.*, 2001). Although apoptosis induced by TNFα can be an effective strategy for the elimination of viral-infected cells, it does not seem to be beneficial due to the severe cost to the host, especially in HBV infection, where the whole liver is infected by the virus. Unlike IFNs, the effector molecules responsible for TNF's noncytopathic inhibition of virus replication have not been identified. Indeed, synergized with IFNγ, TNFα secreted by CTL has been shown to inhibit HBV replication without apparent destruction of hepatocytes in both mouse (Guidotti *et al.*, 1996b) and chimpanzee models of HBV infection (Guidotti *et al.*, 1999). It has been suggested that disruption of HBV nucleocapsid formation is probably one of the strategies used by TNFα to noncytopathically inhibit HBV replication (Biermer *et al.*, 2003). Recent data also suggest that TNFα might be required to elicit and maintain HBV-specific CTL induced by DNA immunizations (Kasahara *et al.*, 2003). In addition, since TNFα is also involved in promoting NK and lymphocyte differentiation (Locksley *et al.*, 2001), this cytokine can indirectly assist immunological defense against viral and other pathogen infections.

1.10 Strategies of virus escape from host immune responses

The host's immune system employs a variety of strategies to eliminate the invading virus. On the other hand, the virus has developed multiple evasion mechanisms to avoid being cleared by the host's immune system. The balance between the strength of immune response and the virus escape strategies determine the outcome of infection, *i.e.*, virus clearance or its persistence. This section will briefly outline the mechanisms employed by virus to evade the host's immune system. However, particular emphasis will be placed on delineations of the class I MHC-related escape mechanisms.

1.10.1 General considerations

1.10.1.1 Infection of immune-privileged sites

For the benefit of the host, certain areas of the body are not subject to immune surveillance. One such place is the central nervous system (CNS) which has a blood-brain barrier and, under normal circumstances, antibodies and T cells can not penetrate this barrier. Infection of the CNS with viruses, such as HSV, LCMV, varicella zoster virus (VZV) and measles virus, will usually prevent the virus from being seen and cleared by the immune system (Oldstone and Rall, 1993).

Another example is the infection of lymphocytes which themselves contribute to clearance of virus. By using this strategy, virus may compromise

the function of cells of the immune system and, sometimes, lead to a general immune suppression. For example, human immunodeficiency virus (HIV), which mainly infects CD4+ T cells and macrophages, leads to decline of CD4+ T cells and, in consequence, to severe cellular immune deficiency (Embretson *et al.*, 1993). Another example is the measles virus which targets lymphoid tissues and causes general immuno-suppression. Although the detailed mechanism is not clear, primary and secondary T cell proliferation has been found to be inhibited in measles virus infected hosts (Niewiesk *et al.*, 1997).

1.10.1.2 Antigenic variation

Due to the lack of proof-reading capabilities of viral polymerases, genomes of certain viruses, such as HIV, hepatitis C virus (HCV) and HBV, are highly prone to mutations. This may lead to variations in their proteins. As a result, the newly developed virus may no longer be recognized by the parental virus-specific B and T cell-mediated responses.

For example, influenza virus is an enveloped virus with a protein shell composed of hemagglutinin (HA) and neuraminidase (N). These envelope proteins are responsible for the virus attachment and entry into cells. The host is able to mount humoral immunity characterized by production of neutralizing antibodies targeting specific HA and N epitopes. Upon binding to virus, the antibodies can block the viruses' attachment to the cells. However, subtle changes in the HA and N protein sequences resulting from mutations in the virus

genome can lead to the alteration of antigenic B epitopes on the surface of virion particles, while preserving its infectivity. In consequence, the pre-existing neutralizing antibodies cannot recognize the mutated epitopes (Treanor, 2004).

Virus antigenic variation also has an impact on the cellular immune responses. Presentation of endogenous peptides to .class I MHC is influenced not only by the amino acid constitution of the epitope, but also by the surrounding amino acid residues. Therefore, variation in the viral protein can lead to an alteration of the CTL-recognized epitope, causing diminished antigenicity or reduction in the affinity of peptide-MHC interaction. It has been shown in HIV-infected patients, that novel epitopes generated from mutated variants can function as antagonists to silence HIV-specific T lymphocytes (Klenerman *et al.*, 1995).

1.10.1.3 Inhibition of class II MHC antigen presentation

CD4+ T cells play a key role in the activation of CD8+ CTL, differentiation and development of B cells, and secretion of antiviral cytokines. The activation of CD4+ T cells requires the interaction between the TCR on T cells and the peptide-MHC class II complex presented on the APCs. Viruses have been using different mechanisms to avoid CD4+ T cell activation by interfering with the class II MHC presentation pathway.

Signalling through the JAK-STAT pathway, IFNγ (as indicated in Section 1.9.1) can upregulate the transcription of class II MHC molecules. It was found

that cells infected with human cytomegalovirus (HCMV) did not respond to IFNγ stimulation as shown by failure of class II MHC up-regulation (Miller *et al.*, 1998). This effect is due to the rapid degradation of Janus kinase 1 (JAK1) by the virus (Miller *et al.*, 1998). Similarly, vaccinia and HSV have evolved mechanisms to modulate phosphorylation and dephosphorylation of STAT1 to impede its entry into the nucleus or the recycling of STAT1 back to the cytoplasm (reviewed by Goodbourn *et al.*, 2000; Gotoh *et al.*, 2002). Consequently, class II MHC expression failed to increase upon IFNγ stimulation.

MHC class II αβ dimers assemble with the invariant chain (Ii) in the ER and the complexes are sorted to the MHC class II-loading compartment, where loading of peptides occurs with the aid of DM molecules. The expression of HCMV protein US2 causes rapid proteasome–mediated degradation of two essential proteins in the class II presentation pathway, *i.e.*, the HLA-DM-α and HLA-DR-α chains, preventing antigen presentation to CD4+ T cells (Tomazin *et al.*, 1999).

1.10.1.4 Inhibition of class I MHC pathway

The CD8+ CTL response is believed to be responsible for the clearance of the majority of virus infections (Wong and Pamer, 2003). The interaction between TCR on CD8+ CTL and virus peptide-MHC class I complex on the infected cells provides the primary activation signal to the CTL. Therefore, interfering with the MHC class I presentation pathway may facilitate the

persistence of viral infection. As many reported studies have shown, the viral interference may happen at almost any step of the class I MHC presentation pathway (see Section 1.8.2.2).

1.10.1.4.1 Interference with proteasome degradation

Most class I peptides are generated by proteasomes in the cytosol (review by Gromme and Neefjes, 2002) (see Fig. 1.2). Proteasomal degradation is dependent on the recognition and cleavage of specific peptide sequences within a protein. Modification of viral protein can interfere with proteasomal processing and lead to deficiency in generation of certain peptides. It has been reported that a single amino acid change in a proteolytic cleavage site of a murine leukemia virus has abolished productive processing of an immunodominant CTL epitope (Ossendorp *et al.*, 1996). On the other hand, some viral proteins can deliberately inhibit activity of the proteasome by interfering with transcription of its subunits. For example, LMP2 and LMP7, two subunits of the proteasome, are transcriptionally inhibited in cell lines transformed by adenovirus 12 (Rotem-Yehudar *et al.*, 1996). As a result, class I MHC display is compromised.

1.10.1.4.2 Inhibition of TAP1 and TAP2-mediated peptide transport

Peptides generated in the cytosol are loaded to the MHC class I complex in the ER *via* TAP1 and TAP2 molecules located on the ER (see Section 1.8.2.2).

HCMV encoded US6 protein binds the TAP1 molecule and disables its ability to bind ATP, which is a prerequisite for active peptide transport (Hewitt *et al.*, 2001). In another example, the ICP47 protein produced by HSV type 1 and 2 has also been found to inhibit the formation of the peptide-MHC class I complex by competitive blocking of the peptide-TAP1/2 binding sites (Ahn *et al.*, 1996).

1.10.1.4.3 Retention and degradation of MHC class I molecules

The HCMV encoded US3 protein is able to bind to the β2m-class I heavy chain complex prior to peptide loading in the ER. Although it does not prevent peptide binding, the peptide-MHC class I complex is unable to leave the ER, resulting in the absence of class I MHC display on the cell surface (Jones *et al.*, 1996). In the presence of HCMV US2 or US11 protein, it has been found that the class I MHC molecules were transported from the ER back into the cytosol, where they were degraded by the proteasome with a half-life less than 1 min (Wiertz *et al.*, 1996). Gp48, a protein of MCMV, binds class I MHC molecules and redirects their transport into lysosomes, where the class I MHC complexes are degraded (Reusch *et al.*, 1999). Another example is the Nef protein encoded by HIV. After binding to class I MHC displayed on the cell surface, Nef is able to trigger the endocytosis process which leads to the intracellular degradation of class I MHC complexes (Mangasarian and Trono, 1997; Schwartz *et al.*, 1996).

1.10.1.4.4 Downregualtion of MHC class I-related gene transcription

By reducing the mRNA transcription of class I MHC and related genes, virus may suppress the display of class I MHC antigens on the cell surface. For example, the level of class I MHC-related (*i.e.*, TAP1, TAP2, class I heavy chain and β2m) mRNA levels were found to have a 2-100 fold reduction in Ad12-transformed cell lines compared to non-transformed cells (Rotem-Yehudar *et al.*, 1996). This inhibition of transcription by Ad12 was due to the reduction in formation of active transcription complex (Hou *et al.*, 2002).

1.10.1.4.5 Inhibition of MHC class I chaperone molecules

Adenoviral gene product E19 has been found to bind to TAP and class I MHC heavy chain independently, acting as a competitive inhibitor for tapasin, a chaperone molecule which helps in transporting the peptide from TAP to the class I MHC complex in the ER (Bennett *et al.*, 1999). Therefore, E19 interrupts the relay of peptides from TAP to class I MHC complex and delays the presentation of MHC class I-peptide complexes on the cell surface.

1.10.1.5 Inhibition of NK cell-mediated killing

In accordance with the "missing self" hypothesis, down regulation of class I MHC molecules may render the virus infected cells susceptible to NK cell-mediated killing (Ljunggren and Karre, 1990). In order to avoid NK killing, one of

the mechanisms used by virus is the expression of viral gene encoded MHC class I heavy chain analogs. UL18 and m144, proteins of HCMV and mouse CMV (MCMV), respectively, share tertiary homology to class I MHC heavy chain. Consequently, it has been shown that these proteins can replace the heavy chain of class I MHC and assemble with β2m to form surrogate, class I MHC-like complexes on the surface of the infected cells. Accordingly, an inhibitory signal is delivered to the NK cells, preventing the killing of virus-infected cells by these effector cells (Farrell *et al.*, 1997). Another strategy used by virus to avoid activation of NK cells is the up-regulation of non-classical class I MHC. For example, HLA-E, an inhibitor of NK-mediated cytolysis, is up-regulated by HCMV (Tomasec *et al.*, 2000). Furthermore, down-regulation of ligands capable of activating NK cells has also been discovered in MCMV infection. A MCMV gene product, m152/gp40, is able to suppresses the expression of heat shock protein (Hsp)-60, which is a high-affinity ligand for the NKG2D receptor. This prevents activation of NK cells (Karre, 2002; Krmpotic *et al.*, 2002).

1.10.1.6 Inhibition of cytokine function

Cytokines are mediators which regulate immune responses and some of them can directly inhibit virus replication. IL-10, a Th2 cytokine, which is able to inhibit T cell proliferation and IL-2 production, is a negative regulator of antiviral T cell responsiveness. Virus can express a biologically functional IL-10 analogue and inhibit the host's antiviral responses. For example, EBV synthesizes the

BCRF1 protein, which is the homologue of IL-10 (Zdanov *et al.*, 1997). A similar IL-10-like protein has also been identified in HCMV (Kotenko *et al.*, 2000). Additional interference with cytokine functioning may occur at the level of cytokine receptors. For instance, a secreted protein, M-T7, encoded by myxoma virus, was found to function as a soluble IFNγ receptor (Upton *et al.*, 1992). Release of the receptor homologues in a soluble form seems to compete with binding of IFNγ to the cell surface, which compromises the cytokine function. Viral products can also interfere with cytokine activity by inhibiting the downstream signal transduction pathway, as discussed above in Section 1.9.1.

1.10.2 Immune escape mechanisms by hepadnavirus

As indicated in Section 1.10.1.1, although the liver is the main target of HBV infection, HBV also replicates in lymphoid tissue, which serves as a reservoir of persistent HBV infection (Feray *et al.*, 1990; Lew and Michalak, 2001; Michalak, 2000; Michalak *et al.*, 1994). Hiding of HBV in the lymphatic system may also compromise immunological functions of the infected cells. Under certain conditions, like organ transplantation and chemotherapy, it is possible that the virus residing in the lymphatic system may re-establish symptomatic infection in the context of weakened immune surveillance (Abdelmalek *et al.*, 2003; Berger *et al.*, 2005; Knoll *et al.*, 2004; Lau *et al.*, 1997b).

HBV DNA and RNA have been detected in monocyte-derived DCs obtained from patients with CH (Tavakoli *et al.*, 2004). In addition, these HBV

infected DCs have shown reduced capacity to stimulate lymphocytes in allogenic mixed lymphocyte reactions (Beckebaum *et al.*, 2003). The level of IL-12 secreted by these DCs was also significantly lower than that secreted by DC from healthy controls (Arima *et al.*, 2003). This suggests that not only DCs were infected by HBV, but also that their functions were impaired.

HBV may also persist by generation of immune escape mutants. It has been reported that a naturally occurring mutant within HBcAg located between residues 18-27 in patients with CH can serve as antagonists for TCR recognition of the wild-type virus epitope (Bertoletti *et al.*, 1994). The presence of the mutated epitope was able to inhibit the HBV-specific CTL response against the wild-type epitope. Therefore, in this situation, not only the mutated virus, but also the wild-type HBV can evade specific CTL recognition due to the active suppression of anti-HBV CTL.

It has been reported that HBV pol gene transfection resulted in the non-responsiveness of transfected cells to IFNα (Foster *et al.*, 1991). The precise mechanism is not clear, but it seems that the binding of IFNα to the target cells was not influenced (Foster *et al.*, 1991). This finding may partially explain why the majority of CH patients do not respond to IFNα therapy. It also suggests that HBV might use this strategy to avoid being eliminated by the innate immune response.

In the woodchuck model of HBV infection, the down-regulation of class I MHC heavy chain presentation on hepatocytes has been demonstrated in

animals chronically infected with WHV but not in acute WHV hepatitis (Michalak *et al.*, 2000). In spite of the difference in class I MHC protein display between CH and AH, the level of class I MHC-related gene expression, such as class I MHC heavy chain, β2m, TAP1 and TAP2, in the liver was found to be similar in CH and AH, and transcription of all these genes was elevated compared to the levels detected in healthy animals. Furthermore, the level of IFNγ mRNA, the cytokine which is a potent stimulator of class I MHC expression, was also found to be up-regulated in hepatic tissues to a similar extent in both CH and AH. Based on the above findings, a post-transcriptional interference with hepatocyte class I MHC presentation in chronic WHV hepatitis has been proposed. However, a molecular mechanism of this interference was not investigated. It was postulated that a difference in class I MHC protein display may contribute to the persistence of WHV infection, since a decreased class I MHC expression could be associated with inhibition of HBV-specific CTL recognition of infected hepatocytes and lead to failure of eliminating this virus.

1.11 HBV Vaccines and antiviral therapy

1.11.1 Current anti-HBV agents

For the treatment of chronic hepatitis B, three drugs are currently licensed by Food and Drug Administration (FDA), USA, *i.e.,* IFNα, lamivudine (3TC), and adefovir dipivoxil. Clinical studies indicated that IFNα monotherapy is associated with loss of serum HBeAg in 30-40% of the patients (van Nunen *et al.*, 2001) and

clearance of serum HBsAg in 5-10% (Perrillo *et al.*, 1990). It was also concluded that the patients with elevated transaminases, low serum HBV DNA level, and active liver histology without cirrhosis benefit the most from IFNα therapy (Lau *et al.*, 1997a; Wong *et al.*, 1993). However, the permanent loss of serum HBsAg is only approximately 6% greater in IFNα-treated than in non-treated patients, and even in the IFNα-susceptible patients, serum HBV DNA is still detectable by sensitive PCR assays (Wong *et al.*, 1993). This suggested that IFNα therapy is able to suppress HBV replication only to some degree and in a limited number of patients, and is unable to eliminate HBV completely. Systemic administration of comparable doses of mouse IFNα into the HBV transgenic mice did not show any effect on HBV replication in the liver (Guidotti and Chisari, 2001). However, induction of IFNα/β/γ production in the liver of HBV transgenic mice was effective in HBV inhibition (Guidotti and Chisari, 2001). This suggests that a local high concentration of IFNα probably has a stronger anti-viral effect than a high level of IFNα in the circulation. Side effects of interferon treatment are frequent and include constitutional symptoms such as: bone marrow suppression; neuropsychiatric effects; and the occurrence of autoimmune disease, especially thyroiditis (Hollinger and Liang, 2001). Subcutaneous or parental injection of IFNα is a major inconvenience to the treated patients. Nevertheless, patients responding to the therapy have a survival advantage over nonresponders.

One type of promising anti-HBV agents are nucleoside or nucleotide analogs that target the virus-encoded reverse transcriptase, inhibiting its activity

and terminating DNA chain extension. Lamivudine is a cytosine nucleoside analog that is phosphorylated by intracellular kinases to the active compound (Hanazaki, 2004). Treatment of HBV patients with lamivudine is followed by a rapid and profound reduction of serum HBV DNA load and aminotransferase levels (Dienstag *et al.*, 1995; Lai *et al.*, 1998). After a one year treatment period with lamivudine, HBeAg seroconversion occurs in about 20% of the patients *versus* 6-7% in controls (Dienstag *et al.*, 1999; Lai *et al.*, 1998). Similarly to IFNα therapy, patients with elevated ALT and a greater histological activity index of liver injury before treatment are prone to have a higher HBeAg conversion rate (Chien *et al.*, 1999; Perrillo *et al.*, 1999). Lamivudine is given orally and is well tolerated with few side effects. Therefore, long-term therapy is possible with this drug and it can be given in situations where IFNα is not appropriate, such as decompensated cirrhosis, after liver transplantation, and during immunosuppressed states (Bain *et al.*, 1996; Perrillo *et al.*, 1999). The major problem with lamivudine treatment is the induction of drug-resistant HBV mutants due to the selection pressure on the virus reverse transcriptase. One of the most common lamivudine-resistant HBV mutations is in the YMDD motif which is part of the catalytic site of the reverse transcriptase (Allen *et al.*, 1998). The mutation usually happens with 14-39% of the patients after 8-10 months lamivudine therapy (Honkoop *et al.*, 1997) and can reach up to 66% after 4 years of therapy (Leung, 2000). The appearance of the YMDD mutant can be associated with elevation of serum HBV DNA and liver enzymes (Liaw *et al.*, 1999). Termination

of lammivudine treatment is usually accompanied by recurrence of wild-type HBV and a flare-up of hepatitis (Liaw *et al.*, 1999).

Another newly approved acyclic nucleotide analog is adefovir dipivoxil, which exhibits a broad-spectrum of antiviral activity in HBeAg-positive, HBeAg-negative and HIV-HBV coinfected patients (Rivkin, 2004). More importantly, adefovir dipivoxil alone appears to be able to inhibit HBV lamivudine-resistant YMDD mutants (Peters *et al.*, 1999). As with lamivudine, it is an oral agent with even fewer side effects in a short-term study (reviewed by Marcellin, 2002). Recent clinical observations indicate that about 1.6% of patients develop HBV mutants within 2 years of adefovir dipivoxil treatment (Dando and Plosker, 2003). If lamivudine and adefovir dipivoxil were given to patients in sequence or combined together, this could avoid mutant generation to each drug (Villeneuve *et al.*, 2003).

1.11.2 Current HBV vaccines

In spite of the relative limited success in treatment of CH type B discussed above (see Section 1.11.1), the effective prevention of HBV infection can be achieved by administration of HBV subunit vaccines consisting of 22-nm subviral particles composed solely of HBV envelope proteins. Initially these particles were purified from individuals chronically infected with HBV, but their usage was restricted by safety concerns. Most HBV vaccines used today are recombinant yeast-derived HBsAg vaccines which include only major S envelope proteins

(McAleer *et al.*, 1984). Although a small percentage of healthy individuals do not produce high titer protective antibodies, triple immunization with this vaccine is generally sufficient to render apparently life-long protection against HBV (Lemon and Thomas, 1997). Unfortunately, this protein-based HBV vaccine is not able to exert a therapeutic effect in already established HBV infection. This is probably because it induces almost entirely humoral immune responses whose presumed action is to bind and block the cell entry of HBV particles. In the case of already established HBV infection, a CTL response is required to recognize and to eliminate intracellular viruses, as discussed in Sections 1.8 and 1.9.

1.11.3 Experimental HBV vaccines

It has been found that clearance of HBV infection in AH is normally accompanied by a strong, multi HBV-specific CTL response, which is barely detectable in chronic HBV infection (see Section 1.8.2.2). Therefore, strategies to alter the quality and quantity of HBV-specific cellular immune responses might be a promising avenue for treatment of chronic HBV infection.

DNA immunization has been shown to elicit both humoral and cellular immunity against virus-encoded proteins in various animal models (review by Encke *et al.*, 1999; Wands *et al.*, 1997). DNA immunization with HBV C and HBV S DNA plasmids have been studied in mice (Geissler *et al.*, 1997). It was shown that after 1-4 times repeated intramuscular DNA injection, high titers of anti-HBc and anti-HBs were readily detectable, respectively. More importantly,

immunized-mice also developed an antigen-specific, class II MHC-restricted CD4 T cell response (Sallberg *et al.*, 1997). Similar to what was observed in patients acutely infected with HBV, production of Th1 cytokines was seen with HBsAg-stimulated T cells from DNA-immunized mice (Whalen *et al.*, 1995). Furthermore, HBV-specific, long lasting CTL response against HBsAg was also found after DNA immunization with plasmids containing the HBV S gene (Whalen *et al.*, 1995). Similar effects were observed in the duck model of hepatitis B (Triyatni *et al.*, 1998) and in woodchucks infected with WHV (Lu *et al.*, 1999; Menne *et al.*, 2002a).

The therapeutical potential of genetic immunization has been validated with the HBV transgenic mouse model (Mancini *et al.*, 1996). The clearance of HBsAg as well as the inhibition of S gene transcription were observed after immunization with plasmids encoding the HBV S gene. It has been shown that these effects were mediated by both neutralizing antibodies and T cell responses. These experiments suggest that DNA immunization can break the immune tolerance which accompanies chronic HBV infection.

One of the concerns with the treatment of chronic HBV infection by DNA immunization is that the elicited HBV-specific CTL might kill the hepatocytes presenting HBV epitopes and lead to exacerbation of hepatitis due to the immune attack. In HBV transgenic mice, it has been shown that adoptive transfer of HBsAg-specific CTL can cause liver damage if the mouse liver has been engineered to express high level HBsAg (Chisari, 1995) (see Section 1.6.3).

However, in another study, where HBsAg-specific CTL were transferred into mice with progressive HBV replication, no severe inflammation was observed (Sette *et al.*, 2001). In woodchucks chronically infected with WHV, WHsAg immunization was able to induce antibodies to WHsAg (anti-WHs) and resulted in severe damage of the livers expressing WHsAg (Hervas-Stubbs *et al.*, 1997). The discrepancy between these studies needs to be further investigated before this strategy can be tested in a human disease situation. At the present time, it is assumed that the best regime for the treatment of chronic HBV patients might be a combination therapy, where a potent antiviral agent is combined with immunization with HBV antigens to elicit a strong anti-HBV cellular and humoral immunity, as has been seen in natural acute HBV infection progressing to recovery.

CHAPTER 2. GENERAL MATERIALS AND METHODS

2.1 Animals

2.1.1 Woodchucks

All woodchucks (*Marmota monax*) used in this study were maintained by the Molecular Virology and Hepatology Research Laboratory at the Health Sciences Centre, Memorial University, 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*, as reported previously (Diao *et al.*, 1998; Michalak and Bolger, 1989; Michalak and Churchill, 1988; Michalak *et al.*, 1990; Michalak *et al.*, 1994).

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

The beginning of WHV infection was considered to be at the time of the first appearance of detectable WHV DNA in the circulation (*i.e.*, serum and/or PBMC) when assayed by PCR/Southern blot hybridization assays. 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 (see Section 2.5). Recovery from AH was diagnosed when serum WHsAg permanently cleared and anti-WHs usually, but not always, appeared.

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

2.1.2 Other species

Other animal species were used for control experiments or to produce polyclonal antibodies in this study. These species included rabbit, mouse and guinea pig. 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). The blood was collected into tubes with no additives (red top Vacutainer; Becton Dickinson,

Franklin Lakes, NJ) for serum isolation (see Section 2.2.2) or into tubes containing sodium ethylenediamine tetra-acetic acid (EDTA; lavender top Vacutainer; Becton Dickinson) for plasma and PBMC isolation (see Section 2.2.3).

2.2.2 Serum isolation.

Blood was allowed to clot at room temperature for approximately 1 h. The tube was then centrifuged at 720 x *g* for 10 min. The isolated serum was aseptically aliquoted in small volumes and stored at –70 °C until use.

2.2.3 Isolation of peripheral blood mononuclear cells

Approximately 4 ml of EDTA-treated blood was gently overlaid on 4 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, pH 7.4, with EDTA (PBS-EDTA; Appendix A), and centrifuged at 330 x *g* for 10 min. Left over red blood cells remaining in the PBMC pellet were lysed with 3 ml of buffered ammonium chloride solution (ACK; Appendix A) for 10 min at room temperature with occasional shaking, 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 under the conditions described above and frozen in a mixture of 90% heat-inactivated (at 56 °C for 1 h) fetal calf serum (FCS-HI; Gibco BRL, Gaithersburg, MD) and 10% DMSO (Sigma Chemical Co., St. Louis, MO) at –70 °C. The cells were transferred to liquid nitrogen the next day for long-term storage. Frozen cells were used to isolate nucleic acid (see Section 2.6).

2.2.4 Liver biopsies

Liver biopsies were obtained by surgical laparotomy under sterile conditions, as described elsewhere (Michalak *et al.*, 1999). Each animal was sedated by an intramuscular injection of ketamine (23 mg/kg; Ketaset; CDMV Inc., St. Hyacinthe, Quebec) and xylazine (10 mg/kg; Lloyd Laboratories, Shenandoah, Iowa), and then anaesthetised with 2-4% isofluorane (CDMV Inc.). Each biopsy was divided aseptically into several portions. Some of these fragments (1-2 mm^3 each) were immediately frozen in liquid nitrogen for future nucleic acid analyses. Other tissue fragments (about 5 mm^3 each) were fixed in 10% buffered formalin (Fisher Scientific Ltd., Nepean, Ontario) for histological examination or embedded in HistoPrep (Fisher Scientific Ltd.) and frozen in isopentane alcohol cooled in liquid nitrogen for immunofluorescent examinations.

2.2.5 Collection of specimens at autopsy

For autopsy, animals were injected with an overdose of ketamine:xylazine mixture. 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 2.2.4.

2.3 Cell cultures

2.3.1 Woodchuck WCM-260 hepatocytes

The WCM-260 hepatocyte cell line used in this study was established previously in our laboratory (Diao *et al.*, 1998; Lew and Michalak, 2001). The hepatocyte culture medium for WCM-260 cells consists of 80% (vol/vol) Hepato-STIM™ medium (Becton Dickinson) supplemented with 10 ng/ml epidermal growth factor (EGF; Becton Dickinson), 2 mM L-glutamine (ICN Pharmaceuticals, Montreal, Quebec), 50 units/ml penicillin (Gibco BRL) and 50 µg/ml streptomycin sulfate (Gibco BRL). The medium also contained 20% (vol/vol) of filtered, culture supernatant obtained from 90% confluent HepG2 cells (see Section 2.3.2). This enriched medium was called a conditioned hepatocyte medium (Diao *et al.*, 1998). WCM-260 hepatocytes were either seeded in T-25 culture flask (Corning; Corning Costar Corp., Cambridge, MA) or in the wells of a 12-well culture plate

(Corning; Corning Costar Corp.). They were maintained in a humidified tissue culture incubator with 5% CO_2 (Forma Scientific, Marietta, Ohio) at 37 °C. The medium was replaced every 2 to 3 days until cells become confluent. WCM-260 cells were detached by trypsinization. For this purpose, cells were incubated with 2 ml trypsin solution consisting of 0.25% (wt/vol) trypsin and 0.02% (wt/vol) EDTA (Trypsin-EDTA; Gibco BRL) at 37 °C for 5 min. Then, 10 ml of Hank's balanced salt solution (HBSS) was added and the cells were transferred to a 15-ml centrifuge tube and pelleted at 45 x *g* for 5 min at room temperature. Recovered hepatocytes were resuspended in 1 ml of fresh, conditioned hepatocyte medium. Ten µl of this mixture was diluted 1:2 with 0.1% trypan blue and cells counted in a hematocytometer. Samples containing approximately 1 x 10^6 hepatocytes in 1 ml of 90% FCS-HI/10% DMSO were frozen in liquid nitrogen for long-term storage. Hepatocytes were maintained in culture through repeated passage by re-seeding at 6 x 10^4 cells/ml at approximately weekly intervals.

2.3.2 Other cells

HepG2 cell line (ATCC number HB-8065; American Tissue Culture Collection, Rockville, MD) and L cells (ATCC number CCL-1) were cultured in Dulbecco's-modified Eagle medium (D-MEM; Gibco BRL) supplemented with 1.5 g/l sodium bicarbonate, 1 mM sodium pyruvate (Gibco BRL), 0.1 mM non-essential amino acids (Gibco BRL), 50 U/ml penicillin, 50 µg/ml streptomycin sulfate (Gibco BRL), and 10% FCS-HI (Gibco BRL), under conditions

recommended by the supplier, *i.e.* ATCC. To detach and passage these two cell lines, they were treated in a similar way as WCM-260 hepatocytes (described in Section 2.3.1).

2.4 Nucleic acid isolations

2.4.1 DNA isolation

DNA was extracted from approximately 5 x 10^6 hepatocytes or frozen PBMCs, or from 100 µl of serum or culture supernatant, or from 50 mg of homogenized tissues. All tissue fragments predestined for DNA isolation were washed with HBSS by centrifugation at 1,000 x *g* prior to homogenization in a final volume of 100 µl of HBSS. Cells used for DNA extraction were resuspended in 100 µl of HBSS. Then, the samples were incubated with 200 µl of lysis buffer (Appendix A) containing 50 µg of proteinase K (Sigma Chemical Co.) at 42 °C for 16 h. The digest was supplemented with 300 µl of phenol equilibrated in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.1% (wt/vol) hydroxyquinilone (Sigma Chemical Co.), mixed for 15 min at ambient temperature, and centrifuged at 18,000 x *g* for 5 min to separate the aqueous and organic phases. The upper aqueous layer was removed and transferred to a new 1.5-ml tube containing 300 µl chloroform:isoamyl alcohol (24:1, vol/vol; Sigma Chemical Co.). The mixture was rotated for 10 min and then centrifuged for 5 min at 10,000 x *g* at room temperature. The aqueous layer containing DNA was transferred to a 1.5-ml Eppendorf tube and precipitated with 750 µl of cold

100% ethanol (Sigma Chemical Co.) with 30 µl of 3 M sodium acetate (pH 5.2) and kept for 16 h at –20 °C. DNA was recovered by centrifugation at 20,000 x *g* for 30 min at 4 °C. The resultant pellet was washed by centrifugation with 600 µl of room temperature 70% ethanol. The DNA was then air-dried for 5 min and resuspended in 40 µl (cell or tissue DNA) or in 10 µl (DNA from serum, culture supernatant or cell washes) of either TE buffer (1 mM EDTA in 10 mM Tris-HCl buffer, pH 8.0) or autoclaved double-distilled water.

2.4.2 RNA isolation

Total RNA was isolated from tissue samples, frozen cells or cultured cells, as well as from naive and mitogen-stimulated PBMC using TRIzol reagent (Gibco BRL), according to the manufacturer's instruction. Briefly, tissues or cells were homogenized in 1 ml of TRIzol reagent (Gibco BRL) 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 Chemical Co.) 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 prepared by treatment with diethyl pyrocarbonate (DEPC; Sigma Chemical Co.). RNA was quantitated as described in Section 2.4.3 and used immediately for reverse transcription reaction or stored at –70 °C.

2.4.3 Quantitation of nucleic acids

DNA, RNA and synthetic oligonucleotide primers or probes used in these studies 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 a 1:50 dilution in TE buffer. The concentration of RNA was estimated assuming an optical density of 1 corresponded to 40 mg RNA/ml. 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. The concentration of oligonucleotides was determined using the specific weight per OD obtained in the certificate of analysis from the manufacturer (Gibco BRL). The estimation of the purity of the nucleic acid preparations was based on the 260:280 nm absorbance ratio. Only RNA preparations with a $OD_{260\ nm}$ to $OD_{280\ nm}$ ratio of greater than 1.8 and DNA greater than 1.6 were used.

2.5 Reverse trancription reaction

The reverse transcription (RT) reaction was applied to convert total RNA to single stranded cDNA. Possible genomic DNA contamination during RNA extraction was eliminated by digestion with DNAse I (2 U/20 µl; Ambion, Auxtin,

TX) at 37 °C in the presence of 10 mM Tris-HCl buffer, at pH 7.5 with 2.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$. The inactivation of DNAse I was achieved by adding 5 µl of a chelate reagent from a DNAse-*free* kit (Ambion). After reacting for 2 min, samples were briefly spun down and RNA present in supernatant was transferred to another tube as DNA-free RNA sample. One to two µg of RNA in 10 µl of RNase-free water was added to 1.5 µl of random primers (100 ng/µl) and incubated for 4 min at 75 °C, then chilled on ice. RT reaction was completed in a 20 µl reaction volume by mixing RNA random primer mixture, 4 µl of 5 x RT buffer (375 mM KCl, 15 mM $MgCl_2$ in 250 mM Tris-HCl buffer, pH 8.3; Gibco BRL), 2 µl of dithiothreitol (DTT; 0.1 M; Gibco BRL), 2 µl of deoxynucleotide triphosphate (dNTP) mixture (10 mM of each nucleotide; Promega Corp., Madison, WI), 10 U of RNase inhibitor (RNasin; 40 U/µl; Promega Corp.) and 200 U of reverse transcriptase from Moloney murine leukemia virus (Gibco BRL). In a control reaction, reverse transcriptase was replaced with RNase-free distilled water. After incubation at 37 °C for 1 h and then at 95 °C for 5 min, the samples were stored at -70 °C for future PCR amplification (see Section 2.6).

2.6 Polymerase chain reaction

In this study, nucleic acid amplifications by PCR were used for two different purposes. First, to detect the presence of WHV DNA in serum, PBMC, and liver of WHV-infected animals and, secondly, to amplify cDNAs for cloning woodchuck gene sequences not identified prior to this work.

2.6.1 General criteria for designing oligonucleotide primers

Since many of the woodchuck cDNA sequences required in this study were never determined previously, the PCR primers initially used for amplification were based on the consensus of the respective published human, mouse and rat 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 by using 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 commercially synthesized by Gibco BRL (Gaithersburg, MD), IDT DNA Technologies (Coralville, IA) or Qiagen (Mississauga, Ontario). Primers used for the initial amplification and cloning of particular woodchuck genes are outlined in the respective chapters.

2.6.2 Standard PCR conditions for WHV DNA detection

Three sets of primers specific for non-overlapping regions of WHV DNA, *i.e.*, C, S and X genes, were used for the detection of virus genome by direct PCR. In addition, 3 other primers 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, 1 X reaction buffer comprising 1.5 mM $MgCl_2$ with 50 mM KCl in 20 mM Tris-HCl buffer, pH 8.4, and 2.5 U of *Taq* DNA polymerase (all from Gibco BRL) in a 100 µl total volume. The reaction mixture was layered with 100 µl of mineral oil (Sigma Chemical Co.) 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, recombinant WHV DNA (Galibert *et al.*, 1982; Pardoe and Michalak, 1995) 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

but not test DNA, 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 with nested primers specific for appropriate WHV genes. PCR amplifications were performed under conditions limiting the possibility of contamination, as outlined in detail in our previous studies (Coffin and Michalak, 1999; Michalak *et al.*, 1999).

2.6.3 Amplification of cDNA for cloning of woodchuck genes

All PCR using degenerate primers were performed under lenient PCR conditions and using 1-5 µl of cDNA (see Section 2.5) as the amplification template. The 100-µl final reaction mixture was the same as for WHV DNA amplification (see Section 2.6.2) except it contained 40 pmol of each synthetic oligonucleotide primer and high fidelity *Taq* polymerase Pwo (Boehringer Mannheim, Laval, Quebec) with exonuclease activity. The following program was employed for PCR using degenerate primer pairs: 94 °C for 10 sec, 52 °C for 10 sec, and 72 °C for 2 min for 35 cycles. The products were analyzed by agarose gel electrophoresis. If the size of the product matched the deduced size from relevant human, mouse and rat gene fragments, gel purification was performed which was followed by cloning (see Section 2.8).

2.7 Detection of amplified DNA

2.7.1 Agarose gel electrophoresis

Ten µl final PCR product was mixed with 2 µl of 6 x blue/orange loading dye (Promega Corp.) prior to loading on a 1% agarose gel containing 0.5 µg/ml ethidium bromide (EB). In addition, 0.25 µg of φX174 DNA/*Hae* III molecular weight standards (Promega Corp.) was also loaded in a parallel well. The gel was run at 120 V for 30 min in TAE buffer (1 mM EDTA in 40 mM Tris-HCl buffer, pH 8.0). DNA bands were visualized by UV and the images were preserved using GeneGenius, a gel documenting system (Syngene, Cambridge, UK).

2.7.2 Southern blot hybridization

2.7.2.1 Blotting of DNA onto nylon membrane

Following electrophoresis and documentation of the UV-visualized DNA bands, the gel predestined for Southern blot analysis was soaked in denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 30 min and then rocked in neutralizing buffer (1.5 M NaCl in 1 M Tris-HCl buffer, pH 8.0) for 1 h.

Transfer of DNA from the gel to a nylon membrane was done using capillary transfer. A glass platform was set up in a dish containing 10 x standard saline citrate (SSC, diluted from 20 x SSC constituted by 3 M NaCl in 0.3 M $Na_2Cit._2H_2O$, pH 7.0). A Whatman (3MM) filter paper wick was soaked in 10 x SSC and mounted on this platform. The gel was placed upside down on the wick

and parafilm was used to surround the gel. A piece of nylon membrane (Hybond-N; Amersham, Piscataway, NJ) and 2 pieces of filter paper cut to size and briefly soaked in 6 x SSC were laid on top of the gel, followed by 5 cm high stack of paper towel trimmed to size. A glass weight of approximately 500 g was placed on top to facilitate the 16 h-capillary transferring. The next day, the membrane was dried between sheets of filter paper for 30 min at room temperature, and then baked at 80°C in a vacuum oven for 2 h.

2.7.2.2 Hybridization

The baked nylon membrane with blotted test DNA was put into a hybridization tube with 6 ml of hybridization buffer (6 x SSC, 0.5% SDS, 5 x Denhardts' solution made from 50 x Denhardts' solution (1% [wt/vol] Ficoll, 1% [wt/vol] polyvinylpyrrolidone, 1% [wt/vol] bovine serum albumin), and 100 µg/ml sonicated salmon sperm DNA). The membrane was prehybridized in a 65 °C hybridization oven for 1.5 h. Subsequently, 6 x 10^6 counts per minute (cpm) of ^{32}P-labelled DNA probe, which was previously boiled for 5 min and then chilled on ice for 2 min, was added into the hybridization tube. After hybridization at 65 °C for 16 h, the buffer containing probe was removed and the membrane was washed twice at 65 °C in 50 ml of pre-warmed 2 x SSC with 0.1% SDS for 10 min each. This was followed by 2 washes with 50 ml of 0.1 x SSC, 0.05% SDS for 15 min each at ambient temperature. The membrane was then rinsed in 0.1 x SSC and dried briefly on paper towels. It was then covered in plastic wrap and

exposed on a phosphor screen for phosphorimaging (Cyclone™; Canberra-Packard, Meriden, CT) or on an X-ray film (XRP-1; Kodak, Toronto, Ontario). For autoradiography, cassettes equipped with intensifying screens were exposed at –70 °C.

2.7.2.3 Recombinant WHV DNA probe

Recombinant WHV DNA used for the Southern blot detection of amplified WHV DNA sequence was derived from the pSP65 plasmid vector containing the *Eco*RI fragment of the full WHV genome (Galibert *et al.*, 1982). This plasmid was kindly provided by Dr. J. Summers from the University of New Mexico, Albuquerque, New Mexico (Pardoe and Michalak, 1995). The construct was digested with *Eco*RI and separated by electrophoresis in 1% low melting point, ethidium bromide-agarose (Gibco BRL). The band containing the linear, cloned WHV DNA (rWHV DNA) was excised from the gel and isolated using the Wizard™ PCR Prep DNA Purification System (Promega Corp.), as per manufacturer's instruction. DNA concentration was determined based on its absorbance at 260 nm, as described above (see Section 2.4.3).

This rWHV DNA was labelled with ^{32}P-dCTP using the *Redi*prime™ DNA labelling system (Amersham) following manufacturer's instructions. Briefly, 25 ng of rWHV DNA was made up to 45 µl in TE buffer, boiled for 5 min, and chilled on ice for about 2 min. The DNA mixture was added to the *Redi*prime™ reaction tube. Then, 5 µl of α-^{32}P-dCTP (10 µCi/µl; Amersham) was added and the tube

was flicked a few times to resuspend the lyophilized mixture which consisted of dATP, dGTP, dTTP, Klenow enzyme, and 9-mer random primers. The labelling reaction was incubated for 1 h at 37 °C.

The ^{32}P-labelled probe was purified from unincorporated ^{32}P-dCTP by fractionation on a NICK™ column (Amersham), as described in the manufacturer's instruction sheet. The column was washed with 3 ml of TE buffer and inserted in a 15-ml disposable centrifuge tube. Fifty µl of the probe mixture was applied onto the column, followed by 400 µl of TE buffer. The eluent was discarded and 450 µl of TE buffer was applied to the column. This eluted fraction containing the labelled probe was collected and a 2-µl sample was used for radioactivity counting in a scintillation counter. The cpm per µl of the aliquot was determined. The probe was stored at 4 °C prior to being used for Southern blot hybridization analysis (see Section 2.7.2.2).

2.8 DNA cloning

2.8.1 Purification of PCR-amplified DNA

PCR products destined for cloning were purified using the Wizard PCR preps DNA purification system (Promega Corp.). 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 micro-tube 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 80%

isopropanol, and then centrifuged at 12,000 x *g* for 20 sec. The collected isopropanol was discarded. TE buffer (50 µl) prewarmed to 65 °C was added to the column and left for 1 min at room temperature. 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 at 1:10 dilution in TE buffer, as described in Section 2.4.3.

2.8.2 Cloning

Gel purified DNA was cloned using a dual promoter TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA). The final cloning mixture consisted of 4 µl purified PCR product, 1 µl NaCl and 1 µl of TOPO pCRII vector in a total volume of 6 µl. After incubating at room temperature for 30 min, 2 µl of the reaction mixture was added to 50 µl of ice-cold, competent TOP10 bacterial cells (Invitrogen). The mixture was further chilled for 30 min on ice, heat shocked for 30 sec at 42 °C, and placed on ice for 2 min. Subsequently, 500 µl of SOC medium (Appendix A) was added and the cells were incubated at 37 °C for 1 h in a rotary shaker at 200 rpm. A 100-µl aliquot of each transformation reaction mixture was then spread onto 1.5 % Bacto-Agar (Difco Laboratories, Detroit, MI) coated with 40 µl of X-Gal (Sigma Chemical Co.; Appendix A) in a 10-cm Petri dish. Cells were then incubated at 37 °C for 16 h and white colonies obtained were screened further (see Section 2.8.3).

2.8.3 Plasmid DNA preparation

Individual bacterial colonies were collected and inoculated into 5 ml of sterile LB medium supplemented with 50 µg/ml kanamycin (Sigma Chemical Co.). Bacteria were grown overnight at 37 °C with 250 rpm shaking. For preparation of small amounts of plasmid DNA predestined for preliminary testing, 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 100 µl of freshly prepared solution of 0.2 N NaOH/1% SDS for 5 min at room temperature. To neutralize the solution and aid in the removal of chromosomal DNA and proteins, 100 µl of prechilled 3 M potassium acetate, pH 5.5, was added to the tube, and the mixture was kept on ice for 5 min. Then, the mixture was centrifuged at 20,000 x *g* for 5 min to pellet cell debris and chromosomal DNA. The supernatant was transferred to a 1.5-ml Eppendorf tube and the nucleic acids were precipitated with 1 ml of 95% ethanol for 10 min at room temperature. Plasmid DNA was pelleted by centrifugation at 15,000 x *g* for 5 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 2.8.4.

A large-scale plasmid DNA preparation (maxi or midi preparations) was produced by using a Maxi/Midi plasmid preparation kit from Qiagen, according to manufacture's instruction. Briefly, 50 µl bacterial aliquot containing the proper

plasmid insert, as judged by miniprep analysis described above, was grown to saturation in 200 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 15 min at 4 °C. The pellet was resuspended in 10 ml of P1 solution (Appendix A) supplemented with 100 µg/ml RNase A. Bacteria were lysed with 10 ml of P2 solution (Appendix A) for 5 min at room temperature followed by neutralizing with 10 ml of ice-old P3 solution (Appendix A) on ice for 30 min. Chromosomal DNA and bacterial protein were removed by passing through a filter cartridge provided with the kit. The cleared solution was then applied to the column filled with DNA binding resin pre-equilibrated with QBT buffer (Appendix A). After twice washing with QC buffer (Appendix A), DNA was eluted with 15 ml of QF buffer (Appendix A). Plasmid DNA was precipitated by adding 10.5 ml of isopropanol (Sigma Chemical Co.) and incubation at room temperature for 5 min followed by centrifugation at 20,000 x *g* for 30 min. The pellet was washed with 70% ethanol and resuspended in 500 µl of TE buffer. The DNA was quantitated, as described in Section 2.4.3, and stored at –20 °C.

2.8.4 Digestion with restriction enzymes

For the purpose of either releasing the DNA fragment of interest from the vector or to examine the specificity of the DNA sequence, restriction enzyme digestion was performed with appropriate enzymes, following the instruction provided by the manufacture of a particular enzyme. Briefly, for single restriction

enzyme digestion, a 20-µl reaction mixture with 0.5-1 µg plasmid DNA, 10 U enzyme and the appropriate buffer was incubated at 37 °C for 1-2 h. Digestions with two enzymes were done in a 30-µl reaction containing 0.5-1 µg plasmid DNA, 10 U of each restriction enzyme, and the appropriate buffer compatible with both enzymes. Incubation was performed at 37 °C for 2-4 h. Agarose gel electrophoresis (see Section 2.7.1) was used to determine the size of the inserts obtained after the digestion.

2.9 DNA sequencing

2.9.1 End-labelling of oligonucleotide probes

Synthetic oligonucleotides were labelled with ^{32}P-ATP using T4-kinase (Gibco BRL) following manufacturer's instruction. The final reaction mixture consisted of 10 pmol of template, 3 µl of ^{32}P-ATP (Amersham), 1 x forward reaction buffer (Appendix A), and 10 U of T4 kinase in a total volume of 10 µl. The tube contents were mixed gently and incubated for 1 h at 37 °C.

2.9.2 PCR amplification

Final confirmation of the identity of a particular DNA fragment 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 or a PCR product, 1.5 pmol of a sequencing primer

end-labelled with ^{32}P-ATP (Amersham) (Section 2.9.1), 2 mM of $MgCl_2$ in 50 mM Tris-HCl buffer, pH 9.0 (supplied as a 5 x buffer), and 5 U 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 tubes were stored on ice until electrophoresis in a sequencing polyacrylamide gel (Jung *et al.*) (see Section 2.9.3).

2.9.3 Sequencing PAGE

Prior to loading on the sequencing gel each sample was heat-denatured for 5 min at 70 °C, then chilled on ice. The DNA samples were separated on an 8% denaturing polyacrylamide gel containing 7 M urea (Gibco BRL). After electrophoresis at 50 °C (monitored by a thermal probe) at 50 W, 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 performed 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 GenBank library (National Centre for Biotechnology Information, National Library of Medicine, National Institute of Health, Bethesda, MD) using the BLAST search engine software (http://www.ncbi.nlm.nih.gov /BLAST). Once confirmed, the complete sequences of the cloned woodchuck DNAs were determined by employing a commercial fluorescence-based automated DNA sequence analyzer (LI-COR; LiCor Inc., Lincoln, NB) (service provided by Department of Genetics, Hospital for Sick Children, Toronto, Ontario).

2.10 Expression and purification of woodchuck recombinant proteins

2.10.1 Expression of protein in *E. coli* system

An *E. coli* expression system, pET41 (Novagen, Madison, WI), was used to express the following WHV proteins, *i.e.,* recombinant preS(1-176) protein encoded by nucleotides from 2992 to 211 (rPreS1), recombinant WHcAg coded by nucleotides from 2021 to 2584 (rWHcAg), recombinant truncated WHcAg (1-149) encoded by nucleotides 2021 to 2467 (rtWHcAg), and recombinant X protein encoded by nucleotides 1503-1928 (rWHxAg). All nucleotide positions refer to WHV/tm3 sequence (Genbank AY334075) (Michalak *et al.*, 2004). In addition, the recombinant mature form of woodchuck IFNγ (*ec*-rwIFNγ) and the recombinant mature form of woodchuck TNFα (*ec*-rwTNFα) were expressed (see Chapter 4). Briefly, each gene sequence of interest was directionally inserted into the pET41b vector at *Nde*I and *Xho*I restriction enzyme sites by a PCR-

based strategy similar to that described in Chapter 4. Final constructs were engineered to have an 8-mer poly-histidine (His) tag at the carboxyl-end to facilitate their purification. After transforming with the pET41 plasmid containing the exogenous gene of interest into *E. coli* cells BL21(DE3) or BL21 (DE3/PlysS), which encodes T7 lysozyme for a tighter control of protein expression, individual colonies were grown in LB medium supplemented with 50 µg/ml kanamycin and 1% glucose at 37 °C with shaking (see Section 2.8.2). When the *E. coli* culture reached a density reading of 0.4-1 at OD_{600} nm, it was split into two parts. Isopropyl-beta-D-thiogalactopyranoside (IPTG) (Appendix A), the induction reagent for pET system, was added to one part at the final concentration of 1 mM. The other tube was left as un-induced control. Both cultures were incubated for an additional 2-3 h at 37 °C with shaking. Bacteria were pelleted by centrifugation at 15,000 x *g* for 1 min and lysed with BugBuster (Novagen) reagent in the presence of 25 U/ml benzonase (Novagen). Following 10-20 min lysing, insoluble inclusion bodies (IB) were separated from the soluble proteins by centrifugation at 16,000 x *g* for 20 min at 4 °C. Subsequently, IB was treated with lysozyme at a final concentration of 200 µg/ml for 5 min at room temperature, followed by three washings with 1:10 diluted BugBuster reagent. Finally, the IB were suspended in a denaturing buffer containing 8 M urea (Appendix A). All the samples, soluble and IB from both IPTG-induced and non-induced controls, were then examined by SDS-PAGE to determine the presence of the recombinant proteins (see Section 2.12).

2.10.2 Affinity purification of recombinant proteins

Due to the presence of 8 consecutive histidine residues at the carboxyl-end of the recombinant proteins produced, their purification was achieved by nickel-charged resin, as proposed in the supplier's instruction. Briefly, His-Bind resin (Novagen) was washed 2 times with binding buffer (Appendix A) and then incubated with bacterial lysate in BugBuster reagent for 20-60 min at room temperature. Subsequently, the resin was washed 2 times with binding buffer and the resulting washout buffer saved for later SDS-PAGE analysis. After another two washings with washing buffer (Appendix A), 5-times resin bed volume elution buffer (Appendix A) was added. Then, 100-µl fractions were collected. For the purification of recombinant protein from IB, a similar procedure was employed except that urea at a final concentration of 8 M was added into each buffer used in the process. Finally, the purity of the recombinant protein was determined by SDS-PAGE gel analysis

2.11 Antibodies

2.11.1 Production of polyclonal antibodies against recombinant proteins

Antisera against purified recombinant proteins, such as WHV rpreS1 and *ec*-rwIFNγ were raised in rabbits and guinea pigs. Animals were subcutaneously injected at multiple sites with 10-50 µg of recombinant protein emulsified in

complete Freund's adjuvant (Sigma Chemical Co.). Four weeks later, each animal was boosted with a subcutaneous injection of 10-50 µg of the respective protein with incomplete Freund's adjuvant (Sigma Chemical Co.). Blood samples were collected before the first immunization (pre-immune serum) and 2 weeks after booster injection. Anti-sera were analysed by Western immunoblotting (see Section 2.12) using recombinant proteins as substrates. Pre-immune sera as well as non-related recombinant proteins were used as negative controls. In some cases, where animals did not produce high-titer antibodies, a second or even third booster injection with 10-50 µg purified recombinant protein was given intramuscularly at 4-5 weeks intervals until a satisfactory level of antibodies was generated.

2.11.2 Monoclonal antibody to woodchuck class I MHC 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 detail in a previous study from this laboratory (Michalak *et al.*, 1995). This antibody recognizes two polypeptide species of woodchuck class I heavy chains with molecular masses of 43- and 39-kDa. This mAb was used previously to document that chronic WHV hepatitis is associated with an inhibited display of class I MHC antigen on infected hepatocytes (Michalak *et al.*, 2000).

2.12 Western blotting

A 12% acrylamide gel in 37.5 mM Tris-HCl buffer, pH 8.8, with 0.1% (wt/vol) SDS along with a 3% acrylamide upper gel in 12.5 mM Tris-HCl buffer, pH 6.8, with 0.1% (wt/vol) SDS were prepared in a mini-gel apparatus (Mini-Protean II dual slab cell; Bio-Rad Laboratories, Hercules, CA). Before analysis, test samples were denatured for 5 min at 95 °C in buffer containing 3% SDS, 5% 2-mercaptothanol, 10% glycerol in 62.5 mM Tris-HC buffer, pH 6.8. After electrophoresis at constant voltage (200 V) for about 45 min in reservoir buffer (20 mM glycine, 2.5 mM Tris base), the proteins were electrotransferred to nitrocellulose (NC) membrane (Amersham) by semi-dry transfer using a Bio-Trans unit (Gelman Inc., Ann Arbor, MI) with semi-dry transfer buffer (Appendix A). In parallel, gels containing the same electrophorectically separated proteins were directly stained with 0.5% Coomassie blue for identification of the complete protein band pattern. Efficiency of protein transfer was determined using pre-stained molecular weight marker (Invitrogen). The same markers were used to estimate molecular mass of the separated proteins. To perform Western blot analysis, the NC blots were soaked with PBS containing 3% bovine serum albumin (BSA) and 0.05% Tween-20 for 2 h at ambient temperature. The blots were then incubated overnight at 4 °C with antibodies specific for the target protein at pre-tested dilutions in PBST (Appendix A). After 3 times washing with PBST, NC blots were incubated with affinity-purified goat anti-rabbit IgG or goat anti-guinea pig IgG antibodies conjugated with alkaline phosphatase (Jackson

Immunoresearch Labs Inc., West Grove, PA) for 2 h at room temperature. This was followed by an other 3 washes with TBST (Appendix A). Then, the reaction was developed in a solution containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, as described in supplier's instructions (Sigma Chemical Co.) (Michalak *et al.*, 1994).

2.13 Immunoflorescent methods

2.13.1 Immunostaining for class I MHC antigen

Cultured WCM-260 hepatocytes (see Section 2.3) were harvested by trypsinization, divided into 100-µl aliquots and placed in 12 x 75 mm tubes (Fisher Scientific Ltd.) on ice. B1b.B9 mAb specific for woodchuck class I MHC heavy chain (Section 2.11.2) was added at 1:10 dilution and cells left on ice for 45 min. Cells were then washed twice with 1 ml of PBS by pelleting at 45 x *g* for 5 min. The washed cell pellets were resuspended in 100 µl of PBS containing goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC; Jackson Immunoreserach Laboratories Inc.) and kept on ice for 30 min. As controls, unstained cells and cells incubated in the first instance with PBS and then FITC-conjugated anti-mouse IgG were included. Subsequently, hepatocytes were washed twice by centrifugation and the cell pellets were fixed in 200 µl of 1% (wt/vol) paraformaldehyde (Marivac Ltd, Halifax, NS) in PBS for 15 min at room temperature in the dark.

2.13.2 Immunoflurescence microscopy

Stained cells (Section 2.13.1) were mounted in 50% glycerol buffered in PBS and examined using a Leitz-Diaplan epifluorescence microscope equipped with a mercury lamp. In each set of experiments, unstained cells and cells incubated with a relevant secondary antibody were included to determine the background fluorescence.

2.12.3 Confocal microcopy

Cells grown on clover slips were subjected to immuno-staining and then mounted in 50% glycerol buffered in PBS. Nail polish was applied to seal the edge of the cover slip for long-term storage or left unsealed for immediate examination. An Olympus FluoView FV300 confocal system (Olympus America Inc. Melville, NY) equipped with a 488-nm blue argon laser, a 543-nm green helium neon laser and a 633-nm red helium neon laser and Olympus BX50WI microscope (Olympus America Inc) were used for the confocal fluorescent microscopy analysis. Digital images were obtained with FluoView application software (Ver. 3.0; Olympus America Inc.). Dual channel scanning was applied when samples were stained with two different fluorescent dyes (see Chapter 5). In each set of experiments, unstained cells, cells stained with secondary antibody alone, and cells exposed to preimmune sera and then incubated with the second layer antibodies were routinely included to determine the background and specificity of fluorescence.

2.13.4 Flow cytometry

Stained cells were examined with a FACS Star™-Plus flow cytometer (Becton Dickinson). The FACS data were analysed using the CellQuest software (Becton Dickinson) or WinMDI (version 2.8) software (The Scripps Research Institute, La Jolla, CA). In each experiment, unstained and cells stained with secondary antibody were included as controls. The geometrical mean fluorescence intensity was calculated by the software to compare the level of expression between different samples.

2.14 Statistic analysis

Data were analyzed, when required, with GraphPad Prism software (GraphPad Software, Inc. San Diego, CA). Appropriate statistical analyses were performed where applicable.

CHAPTER 3. PURPOSE OF THE STUDY

Chronic infection with HBV is an important cause of mortality due to the induction of severe liver diseases, including chronic active hepatitis, cirrhosis and HCC. Both virus and host factors are involved in the pathogenesis of liver damage and, most likely, in the establishment of long-term hepadnavirus persistence. These processes remain only partially elucidated. WHV infection in woodchucks (*Marmota monax*) closely resembles HBV infection in humans with respect to virological, immunological and pathological characteristics, as well as the features and outcomes of liver disease. In the present studies, the woodchuck model of HBV infection was applied to investigate the nature of selected molecular mechanisms potentially utilized by hepadnavirus to persist and to examine how antiviral cytokines, particularly IFNγ, may reverse a hepadnavirus-caused hindrance relevant to its escape from immunological surveillance. We also tested the adjuvant action of IFNγ in induction of preventive, hepadnavirus-specific immunoresponse using combined immunization with viral gene and IFNγ DNA. To perform these studies, a number of woodchuck cellular genes, biologically active recombinant cytokines and WHV-specific assays, which were not previously available, were identified, produced or developed.

The specific objectives of the studies were as follows:

1. To produce functionally active recombinant woodchuck IFNγ and TNFα in the baculovirus expression system and compare their biophysical features, antiviral or cytolytic properties, and class I MHC inducing activities with those of the same cytokines generated in the E. *coli* expression system.

2. To investigate the molecular nature of the previously made *in vivo* observation that chronic WHV infection is invariably associated with a severe suppression of the class I MHC antigen on infected hepatocytes. For this purpose, create a hepatocyte culture system in which the effect of the expression of the complete WHV genome, WHV genes encoding individual viral proteins or WHV DNA mutants on the surface display of class I MHC antigen can be analyzed. Further, to establish which one of the WHV proteins exerts the class I MHC inhibitory effect and what could be a possible underlying mechanism. Also, to examine whether treatment with an exogenous IFNγ can restore the virus-induced suppressed class I MHC antigen presentation on hepatocytes in vitro.

3. To study whether IFNγ DNA can act as an adjuvant and enhance protection against hepadnavirus in the woodchuck model of hepatitis B when administered as a component of a bicistronic DNA vaccine carrying virus nucleocapisd and IFNγ gene encoding sequences. To investigate the nature of immunological responses induced by this DNA vaccine, examine whether this vaccination strategy can protect from the development of serologically and

histologically evident hepatitis, and whether it can establish complete (sterilizing) immunity against hepadnavirus.

CHAPTER 4. COMPARISON OF BIOLOGICAL ACTIVITY OF RECOMBINANT WOODCHUCK INTERFERON GAMMA AND TUMOR NECROSIS FACTOR ALPHA PRODUCED IN BACULOVIRUS AND *E. COLI* EXPRESSION SYSTEMS*

4.1 Summary

To create biologically active recombinant woodchuck interferon gamma (rwIFNγ) and woodchuck tumor necrosis factor alpha (rwTNFα), the full-length cDNAs of these cytokine genes were cloned into baculovirus transfer vectors and expressed in insect Sf9 cells. The recombinant proteins secreted by the insect cells, *bac*-rwIFNγ and *bac*-rwTNFα, were found to be functionally competent. Their biological activities were compared to those of rwIFNγ and rwTNFα produced in the *E. coli.* expression system. The *bac*-rwIFNγ demonstrated a 4.5-fold greater protective activity against encephalomyocarditis virus (ECMV)-induced cytolysis of woodchuck hepatocytes and that of class I MHC antigen presentation on the hepatocytes than rwIFNγ derived from *E. coli* (*ec*-rwIFNγ). The *bac*-rwTNFα was cytotoxic towards murine fibroblasts and able to upregulate class I MHC antigen display and these effects were about 18-fold greater that those triggered by rwTNFα from *E. coli* (*ec*-TNFα) at a comparable protein level. In addition, the antiviral activity of *bac*-rwIFNγ was inhibited by anti-wIFNγ

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antibodies and the cytotoxicity of *bac*-rwTNFα neutralized by cross-reactive antibodies to murine TNFα. The study showed that expression of rwIFNγ and rwTNFα in the baculovirus system generated biologically active cytokines whose potency was considerably greater than those produced in *E. coli*.

4.2 Introduction

IFNγ and TNFα are potent regulators of both systemic and local immune responses, which also inhibit replication of a number of viruses (Rosenblum and Donato, 1989; Beutler and Cerami, 1989; Rook *et al.*, 1991; Baron and Dianzani, 1994; Landolfo *et al.*, 1995; Benedict, 2003). In the liver, they are secreted by the residing lymphoid cells and by the cells infiltrating hepatic tissue due to inflammatory processes. An upregulated intrahepatic transcription of IFNγ and TNFα is a hallmark of hepadnaviral hepatitis, particularly in patients and animals who ultimately recover from acute disease (Guidotti *et al.*, 1999; Guo *et al.*, 2000; Nakamura *et al.*, 2001; Hodgson and Michalak, 2001).

IFNγ is a pleiotropic cytokine produced by activated T lymphocytes, NK cells and NK T cells. It favors T helper type 1 lymphocyte immune responses and exerts a potent antiviral action against certain viruses, including HBV and HCV (Baron and Dianzani, 1994; Landolfo *et al.*, 1995; Chisari, 2000; Frese *et al.*, 2002). IFNγ also plays a main role in augmentation of immunoproteosome formation (Van den Eynde and Morel, 2001; Kloetzel and Ossendorp, 2004) and presentation of class I and class II MHC (Boehm *et al.*, 1997) leading to

enhancement in the cell surface display of antigenic epitopes recognized by specific effector T cells. Due to its important immunomodulatory functions and direct antiviral activity, IFNγ is considered to be a suitable vaccine adjuvant and as a possible modality in viral infections (Chow *et al.*, 1998; Kim *et al.*, 2000). IFNγ activity is highly species specific and studies in animal or cell culture models require its homologous origin.

TNFα is mainly secreted by activated macrophages and T cells. It performs a wide range of functions, including modulation of host immune responses, mediation of inflammation, induction of tumor cell death, and interference with virus replication (Rosenblum and Donato, 1989; Beutler and Cerami, 1989; Rook *et al.*, 1991; Benedict, 2003). TNFα appears to be highly conserved between different species. Nevertheless, the availability of a species-specific TNFα is required to unambiguously define its biological functions in test animal or culture systems.

It has been demonstrated that both IFNγ and TNFα have potent anti-HBV action in *in vivo* models of hepatitis B (see Section 1.9). Briefly, these cytokines, particularly IFNγ, can reduce HBV gene expression in the liver prior to the appearance of the virus-specific CTL and destruction of hepatocytes, implying that the cytokine-mediated, non-cytolytic inhibition of virus replication may occur in the pre-acute phase of hepatitis B (Guidotti *et al.*, 1999). The mechanism of this direct antiviral action remains unrecognized (Guidotti and Chisari, 2001; Wieland *et al.*, 2003). The release of these cytokines by HBV-specific CTL also

is important in activation of other immune effector cells which contribute to recovery from hepatitis and their high intrahepatic levels are strongly connected with cessation of the acute disease (Guidotti *et al.*, 1999; Nakamura *et al.*, 2001; Hodgson and Michalak, 2001). However, a precise role of these cytokines in the development and resolution of hepadnaviral infection is not yet solved.

The baculovirus system is one of the most powerful expression tools by virtue of its high efficiency, eukaryotic processing of recombinant proteins and availability of abundant vectors (Luckow and Summers, 1988). Various biologically active cytokines (Smith *et al.*, 1983; Maeda *et al.*, 1985) and growth factors (Chiou and Wu, 1990) have been successfully produced in this system. Interestingly, insect cells seem able to recognize and cleave human (Chiou and Wu, 1990; Churgay *et al.*, 1997), equine (Wu *et al.*, 2002) and bovine (Murakami *et al.*, 2001; Nagata *et al.*, 2003) signal peptides. The matured form of recombinant polypeptides was therefore released into the culture supernatant and this makes it easy for downstream purification, especially in serum free insect cell cultures. Although post translational modification of recombinant proteins in insects is not identical to its in human counterpart, certain strategies have been used to engineer insects cells as well as transfer vectors to produce humanized glycoproteins (Jarvis, 2003).

The production of rwIFNγ and rwTNFα in the *E. coli* expression system has been recently reported (Lohrengel *et al.*, 2000; Lu *et al.*, 2002). However, the commonly acknowledged difficulties in the removal of bacterial

contaminations, especially traces of a highly pathogenic lipopolysaccharide (Gao and Tsan, 2003), make the use of the *E. coli*-derived rwIFNγ and rwTNFα not well suitable for in vivo and cell culture experimentations. To overcome this problem and, at the same time, to generate cytokines which structurally and functionally should be more compatible with those of the woodchuck, we expressed the full-length rwIFNγ and rwTNFα cDNAs in the baculovirus system. We have found that the insect cells infected with the respective baculoviral constructs were able to secrete the woodchuck cytokines and that the cytokines were functionally competent. Further, we compared the biological activities of rwIFNγ and rwTNFα produced in the baculovirus and *E. coli* expression systems.

4.3 Materials and Methods

4.3.1 Cloning of full-length rwIFNγ and rwTNFα cDNA

PBMCs isolated from healthy adult woodchucks (see Section 2.2.3) were cultured in the presence of 10 μg/ml concanavalin A (ConA; Sigma Chemical Co.) for 72 h in RPMI 1640 medium with 50 U/ml penicillin, 50 μg/ml streptomycin sulfate and 10% heat-inactivated fetal calf serum (HI-FCS) (all from Invitrogen). Then, the cells were collected and RNA extracted with Trizol reagent (Section 2.4.2). Approximately 2 μg of total RNA was treated with 2 U of DNase (DNA-free kit, Ambion) for 30 min at 37 °C to remove potential DNA contaminations. The enzyme was inactivated with a chelating reagent from the DNA-free kit

(Ambion). cDNA was generated using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and hexamer random primers, as described before (Hodgson and Michalak, 2001).

Specific primers, spanning the full-length of wIFNγ and wTNFα cDNAs, were designed based on homology in the woodchuck sequences reported previously by this and other laboratories (Michalak *et al.*, 2000; Lohrengel *et al.*, 2000; Guo *et al.*, 2000). The GenBank accession numbers for the sequences used were: AF08152, Y14138 and AF232728 for wIFNγ, and AF082491, Y14137 and AF333967 for wTNFα. For PCR amplification of wIFNγ, the sense primer IFNp 5'-GGCCTAACTCTCTCTGAAACG and antisense primer IFNm 5'-GAGGACTGTTATTTGGATGC were applied. wTNFα was amplified with sense TNFp 5'-ATGAGCACTGAAAGYATGATCCG and antisense TNFm 5'-CCATTCCCTTCACAGAGCAATGAC primers. PCR was performed using 50 ng cDNA from ConA-stimulated PBMC under cycling conditions established before (Michalak *et al.*, 2000). After purification of the PCR products from low-melting point agarose using Wizard PCR Preps DNA purification system (Promega Corp.), the amplicons were cloned into TOPO-PCRII vector using a TA cloning kit as described in Section 2.8.2. The specificity and orientation of the sequences cloned were confirmed by DNA sequencing, as described previously in Section 2.9.

4.3.2 Baculovirus vectors and rwIFNγ and rwTNFα production in insect cells

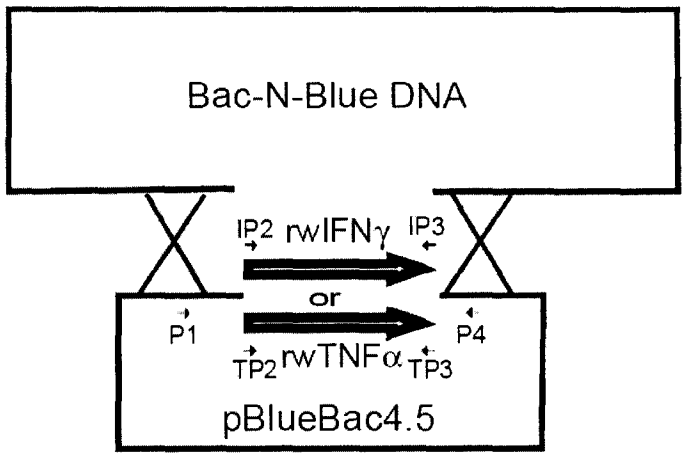
To directionally insert rwIFNγ and rwTNFα sequences into the baculovirus transfer vector pBlueBac 4.5 (Invitrogen), specific PCR primers with introduced *BamH*I and *EcoR*I restriction sites (marked by low case letters) were designed and synthesized (see Figure 4.1). Primers for the insertion of rwIFNγ cDNA were IP2, 5'-GCGCggatccATGAAATACACAAGTTATTT and IP3, 5'-GCGCgaattcTTATTTGGATGCTCTCCGAC, whereas those for rwTNFα cDNA were TP2, 5'-GCGCggatccATGAGCACTGAAAGTATGAT' and TP3, 5'-GCGCgaattcTCACAGAGCAATGACCCCAA. The PCR, gel purification, and subcloning were carried out as outlined before (see Sections 2.6 and 2.8).

Spodoptera frugiperda cells (Sf9; Invitrogen) were maintained in complete Grace's insect medium (Invitrogen) supplemented with 10% HI-FCS, 50 U/ml penicillin and 50 μg/ml streptomycin sulfate at 27 °C. Following the Bac-N-Blue transfection kit's procedure (Invitrogen), *Autographa californica* Multiple-embedded Nuclear Polyhedrosis Virus (AcMNPV) DNA (0.5 μg) and the baculovirus vector containing rwIFNγ or rwTNFα (4 μg) were co-transfected into Sf9 cells using a cationic liposome reagent (InsectinPlus Liposome; Invitrogen). Four days later, the culture supernatant was collected and a plaque assay performed.

Figure 4.1. Vector construction and screening strategies.

(A) Schematic presentation of the baculoviral constructs containing rwIFNγ or rwTNFα cDNA, showing relative locations of the vector primers P1:P4 flanking the inserted cytokine cDNAs and IP2:IP3 and TP2:TP3 primer pairs used for identification of rwIFNγ and rwTNFα cDNA inserts, respectively. (B) Identification and purity screening of three randomly selected Sf9 cell plaques transfected with recombinant baculovirus carrying either rwIFNγ or rwTNFα cDNA. Lanes: culture supernantants from plaques1 to 3; WT, supernatant form Sf9 cells infected with wild-type baculovirus; M, molecular marker ladder. Molecular sizes (bp) of PCR amplicons indicated on sides of the panels.

A


Bac-N-Blue DNA
IP2 rwIFNγ IP3
or
P1
P4
TP2 rwTNFα TP3
pBlueBac4.5

B

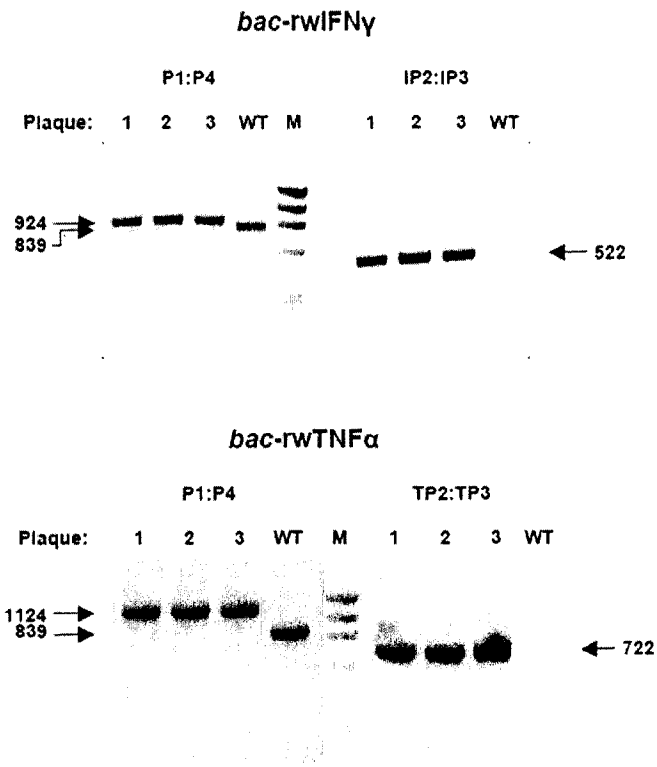

bac-rwIFNγ
P1:P4
IP2:IP3
Plaque: 1 2 3 WT M 1 2 3 WT
924
839
522
bac-rwTNFα
P1:P4
TP2:TP3
Plaque: 1 2 3 WT M 1 2 3 WT
1124
839
722

Figure 4.1

The plaque positivity and discrimination between colonies carrying the recombinant vectors and those expressing the recombinant and the wild type (WT) baculovirus were determined by a PCR assay with primers P1 and P4 complementary to the baculovirus vector sequences flanking the cytokine cDNA inserts (see Fig. 4.1). To produce large amounts of the recombinant virus, infected Sf9 cells were cultured for 10 days post-infection. Based on the results of the plaque assay with serial 10-fold dilutions of the final supernatants, the titer of the recombinant virus carrying rwIFNγ or rwTNFα was 8 x 10^5 or 6 x 10^5 plaque forming units (PFU)/ml, respectively.

For protein production, Sf9 cells were infected with rwIFNγ or rwTNFα recombinant baculovirus at multiplicity of infection of 1-2. Since sequences encoding both a secretion signal or a membrane binding domain and leader peptides were included in the cytokine cDNAs, the secretion of their proteins was expected. After 7-10 day culture at 27 °C, the cell supernatant was collected and biological activity of wIFNγ or rwTNFα, designated as *bac*-rwIFNγ or *bac*-rwTNFα, was tested without further purification. In parallel, supernatant from Sf9 cells infected with a WT baculovirus (*bac*-WT) was prepared.

4.3.3 Expression of rwIFNγ and rwTNFα in *E. coli*

A fragment of cDNA encoding the mature form of wIFNγ was subcloned into pET41b *E. coli* expression vector (Novagen, Darmstadt, Germany) by PCR

using primers 5'-ATAGCGCcatatgCAGGACACAGTTAATAAGG and 5'-GCGCctcgagTTTGGATGCTCTCCGACC with introduced *Nde*I and *Xho*I restriction sites, respectively. Similarly, cDNA encoding wTNFα fragment spanning nucleotides 232 and 699 (GenBank accession number AF333967) (Hodgson and Michalak, 2001) and likely representing the mature protein of wTNFα for which size was deduced based on the location of the cleavage site predicted through interspecies comparison of available human, mouse and rat TNFα sequences, was subcloned into pET41b vector using primers 5'-ATAGCGCcatatgCTCAGATCATCTTCTCAAA and 5'-GCGCctcgagCAGAGCAATGACCCCAAAG.

E. coli strain BL21(DE3)pLysS was transformed with the above constructs following the procedure suggested by the supplier (Novagen) and outlined in Section 2.8.2. A single colony of the transformed cells was inoculated into LB medium containing 50 μg/ml kanamycin and cells incubated at 200 rpm at 37 °C until the OD_{600} reached 0.6. Then, 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) was added and the incubation continued for an additional 3 h. In parallel, an aliquot of the same cell suspension was incubated in the absence of IPTG, as an un-induced cell control. Subsequently, culture medium was removed by centrifugation and the cell pellet immediately frozen at -70 °C. The next day, the cells were lysed with BugBuster reagent (Novagen) with 20 U/ml benzonase (Novagen). Both the soluble fraction and the inclusion bodies (insoluble fraction) suspended in 8 M urea were examined by SDS-PAGE to verify the presence of

the cytokine proteins by comparing lysates from IPTG-induced and not induced cells.

Since a polyhistidine was tagged to the carboxy-terminus of each cytokine protein, the final purification of the *E. coli*-derived rwIFNγ and rwTNFα, designated as *ec*-rwIFNγ and *ec*-rwTNFα, was done by IMAC. For this purpose, crude *ec*-rwIFNγ and *ec*-rwTNFα preparations denatured with 8 M urea were incubated for 30 min with pre-charged, pre-washed TALON cell-thru resin (Clontech, Palo Alto, CA). After centrifugation at 700 x *g* for 2 min, supernatant was removed and the resin washed 3 times with 8 M urea buffer. The protein bound was eluted with 5-bed volumes of elution buffer and examined by SDS-PAGE. The proteins were extensively dialyzed against phosphate-buffered saline, pH 7.4 (PBS) and stored at 4 °C. Using the same approach, unrelated recombinant proteins, *i.e.,* WHV e antigen (*ec*-rWHe) and recombinant woodchuck CD3 (*ec*-rwCD3), were produced in *E. coli* and used as specificity controls.

4.3.4 IFNγ antiviral protection and antibody neutralization assays

An ECMV protection assay was employed to assess antiviral activity of *bac*-rwIFNγ and *ec*-rwIFNγ. For this purpose, 3 x 10^4 woodchuck WCM-260 hepatocytes cultured in conditioned Hepato-Stim medium (see Section 2.3.1) or human HepG2 cells maintained in DMEM (see Section 2.3.2) were seeded 24 h prior to the assay at 100 µl/well in a flat-bottom 96-well plate (Fisher Scientific

Limited, Nepean, Ontario). Then, the cell supernatant was replaced with 100 µl of fresh medium containing serial 2-fold dilutions of *bac*-rwIFNγ or *ec*-rwIFNγ, or with equivalent dilutions of *bac*-WT supernatant, as a control. Triplicate wells were tested for each dilution. After 24-h incubation, an additional 100 µl of medium containing cytopathic ECMV (Jin *et al.*, 1994) was added and the plate incubated for 24 h.

The number of viable cells was determined by a microculture tetrazolium (MTT) assay (Mosmann, 1983; Alley *et al.*, 1988). Briefly, each well was supplemented with 50 µl of MTT (3-[4-,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide; 1 mg/ml in PBS; Sigma) and the plate incubated for 4 h at 37 °C. The supernatant was removed and 100 µl of dimethylsulfoxide added to dissolve MTT metabolites. The reaction intensity was read at $A_{570\ nm}$ and at $A_{630\ nm}$ (Mosmann, 1983; Alley *et al.*, 1988). One unit of wIFNγ was defined as the ability to protect 50% of WCM-260 cells from cytolysis induced by ECMV infection, as deduced from the slope of the $OD_{570\text{-}630}$ vs. rwIFNγ dilution plots (see Figure 4.4).

For neutralization of *bac*-rwIFNγ antiviral protective activity, supernatant containing the cytokine at 10 or 100 U/ml was pre-incubated with rabbit anti-rwIFNγ (prepared as described below) at 1:20 or 1:100 dilution for 4 h at 37 °C prior to performing the ECMV protection assay. The same dilutions of pre-immune rabbit serum were used as controls. $OD_{570\text{-}630}$ readings from triplicate wells after the MTT assay were used to determine mean values. The percentage

of OD reduction was calculated as the difference between mean OD from wells containing pre-immune serum and those containing anti-rwIFNγ divided by mean OD from wells containing pre-immune serum.

4.3.5 TNFα cytopathic and neutralization assays

A mouse fibroblast line L929 (see Section 2.3.2) was used as target cells to measure cytotoxicity of *bac*-rwTNFα and *ec*-rwTNFα (Meager *et al.*, 1989; Lohrengel *et al.*, 2000). After seeding L929 cells at 2 x 10^4 cells in 50 µl/well into 96-well flat-bottom plates 24 h prior to the assay, two-fold serial dilutions of *bac*-rwTNFα or *ec*-rwTNFα in 50 µl of DMEM containing 2 µg/ml Actinomycin D (Sigma Chemical Co.) were added in triplicate. Comparable dilutions of *bac*-WT supernatant were added to control wells. After incubation for 24 h, cell viability was determined by the MTT assay described above. Actinomycin D was used to increase sensitivity of the assay, as reported by others (Meager *et al.*, 1989; Baarsch *et al.*, 1991). One laboratory unit of rwTNFα was defined as the ability to kill 50% of L929 cells in test triplicate wells, as deduced from the $OD_{570\text{-}630}$ vs. rwTNFα dilution plots (Meager *et al.*, 1989).

For neutralization of *bac*-rwTNFα-mediated cytotoxicity, the supernatant containing 10 or 100 U/ml of *bac*-rwTNFα was preincubated with affinity-purified immunoglobulin G (IgG) fraction of rabbit anti-mouse TNFα serum (Cedarlane Laboratory Ltd, Ontario, Canada) at 2, 1 or 0.5 µg/ml for 4 h at 37 °C prior to performing the cytotoxicity assay with L929 cells described above. The same

concentrations of IgG purified from normal rabbit serum were used as controls. The percentage of cell survival was determined by comparing the mean OD readings from triplicate test wells to mean OD of control wells without rwTNFα from an MTT assay.

4.3.6 Class I MHC antigen display enhancement assay

To test the ability of rwIFNγ and rwTNFα to upregulate the cell surface presentation of class I MHC antigen, 5 x 10^5 WCM-260 hepatocytes, kept at 2 ml/well in conditioned Hepato-Stim medium in a 6-well plate (Fisher Scientific Ltd.), were exposed for 18 h to predetermined concentrations of the recombinant cytokines derived from the baculovirus and *E. coli* systems. Then, the cells were trypsinized, washed twice with PBS, and exposed for 30 min on ice to B1bB9 mAb against a woodchuck class I MHC heavy chain (Section 2.11.2). After three washes with PBS, cells were stained with FITC-conjugated goat anti mouse IgG/M (Jackson Immunoresearch Labs, Inc.) for 30 min on ice, washed, and fixed in 2% paraformaldehyde. Cells stained with B1bB9 but not pre-incubated with the cytokines or incubated with *bac*-WT supernatant, and cells stimulated with the cytokines but stained with the secondary layer antibodies only were used as controls. The cells were analyzed in a FACScalibur flow cytometer (Becton Dickinson) and the data interpreted with appropriate softwares (Section 2.13.4).

4.3.7 Production of antibodies and Western blot analysis

Polyclonal antibodies against *ec*-rwIFNγ were raised in rabbits as described in Section 2.11.1. Antibody specificity and titer were determined by Western blot analysis using 10-fold serial dilutions of the IMAC-purified *ec*-rwIFNγ and unrelated *ec*-rWHe and *ec*-rwCD3 proteins as controls (see Section 2.12).

To identify rwTNFα proteins, affinity- purified rabbit IgG against murine TNFα (Cedarlane Laboratory Ltd.) at 0.15 ng/ml were applied in Western blotting (see Section 2.12). Reactions were detected with goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) and a home-made enhanced chemiluminescence (ECL) reagent (Yakunin and Hallenbeck, 1998). The optical density of the protein bands was quantified by densitometry using ChemiGenius bio-imaging system (SYNGENE, Frederick, MD). The *bac*-rwIFNγ or *bac*-rwTNFα protein content in the insect cell supernatants was estimated by comparing their band densities with those of serial dilutions of the IMAC-purified *ec*-rwIFNγ or *ec*-rwTNFα containing known protein concentrations, which were probed in parallel on the same immunoblots.

4.4 Results

4.4.1 Expression of recombinant baculovirus-rwIFNγ and -rwTNFα constructs

Figure 4.1A shows the relative locations of the PCR primers specific for the baculovirus transfer vector and wIFNγ or wTNFα which were used to test the specificity and purity of the recombinant baculoviruses carrying rwIFNγ or rwTNFα cDNA, which were propagated in Sf9 cells (see Section 4.3.2). It also illustrates detection of the recombinant baculoviral DNA and rwIFNγ or rwTNFα fragments in the supernatants from culture of three randomly selected insect cell plaques infected with the constructs (Figure. 4.1B). As shown by PCR, all supernatants gave a single DNA band with the size which corresponded to that of the recombinant virus carrying either rwIFNγ (924 bp) or rwTNFα (1124 bp) cDNA, in the absence of the WT virus DNA fragment (839 bp). Further, PCR with primers specific for wIFNγ or wTNFα gave, as expected, products with molecular sizes of 522 bp and 722 bp, respectively, while the WT baculovirus DNA was not amplified. This screening procedure ensured that only cell plagues expressing the recombinant baculovirus were selected for further cytokine production.

4.4.2 SDS-PAGE and Western blot analyses

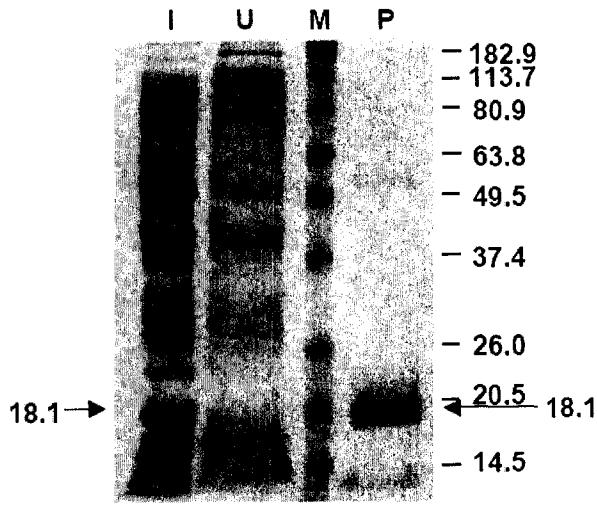
In parallel with production of rwIFNγ or rwTNFα in the baculovirus system, the sequences encoding matured proteins of the same cytokines were also expressed in *E. coli*. The *ec*-rwIFNγ and *ec*-rwTNFα amino acid sequences were tagged with the octamer polyhistidine at the carboxy-terminus to facilitate purification by IMAC, as described in Section 2.10.2. Upon induction of the transfected *E. coli* cells with IPTG, *ec*-rwIFNγ protein of 18.1 kDa was detected in the cell insoluble fraction by SDS-PAGE (Figure 4.2A). This protein was purified by IMAC to more than 99% purity, as assessed by a densitometric analysis of the protein bands separated by SDS-PAGE. The estimated concentration of the affinity purified *ec*-rwIFNγ was 80 μg/ml. This preparation was subsequently used to produce anti-rwIFNγ antibodies, which in turn were applied to determine protein profiles of *ec*-rwIFNγ and *bac*-rwIFNγ by Western blotting (Figure 4.2B).

Immunoblot analysis of the purified *ec*-rwIFNγ revealed two bands of 18.1 and 36 kDa, corresponding to a monomer and a dimer of wIFNγ, respectively (Figure 4.2B). The molecular size of the monomer was identical to that predicted from the sequence of rwIFNγ with a methionine residue attached to the N-terminus and the *Xho*I restriction site plus polyhistidine to the C-terminus of the recombinant cytokine. Unrelated proteins, such as *ec*-rWHe and *ec*- rwCD3, produced by the same expression procedure in *E. coli*, were non-reactive with anti-rwIFNγ antibodies (Figure 4.2B).

Figure 4.2. SDS-PAGE and Western blot analyses of *ec*-rwIFNγ and *bac*-rwIFNγ.

(A) SDS-PAGE monitoring of the expression and affinity purification of *ec*-rwIFNγ. Lanes: I, inclusion bodies from *E. coli* cell lysate after IPTG induction; U, uninduced *E. coli* cell lysate; M, pre-stained protein marker ladder; P, IMAC-purified *ec*-rwIFNγ of 18.1 kDa. (B) Western blot analysis of *ec*-rwIFNγ and *bac*-rwIFNγ with rabbit anti-rwIFNγ antibodies. Lanes: 1, IMAC-purified *ec*-rwIFNγ (100 ng) showing 18.1 and 36-kDa protein bands; 2, IMAC-purified *ec*-rWHe (100 ng); 3, IMAC-purified *ec*-rwCD3 (100 ng); 4 and 5, 5 and 10 μl of *bac*-rwIFNγ supernatant showing protein bands of 16.5, 17.4 and 34 kDa; 6 and 7, 5 and 10 μl of *bac*-WT supernatant.

A


I
U
M
P
182.9
113.7
80.9
63.8
49.5
37.4
26.0
20.5
14.5
18.1
18.1

B

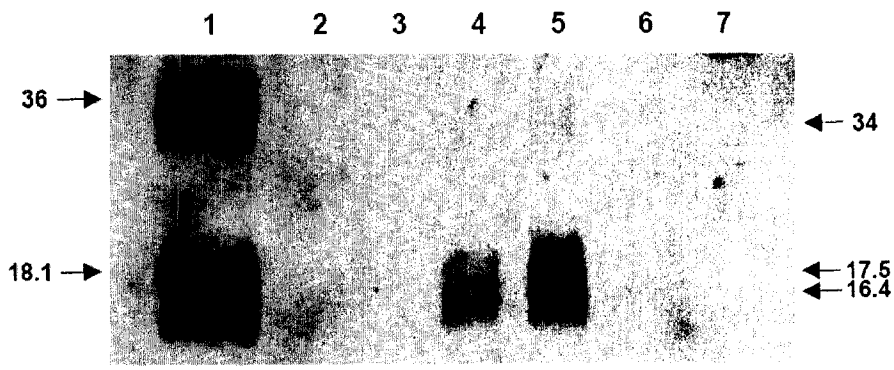

1
2
3
4
5
6
7
36
18.1
34
17.5
16.4

Figure 4.2

SDS-PAGE analysis of *bac*-rwIFNγ did not yield protein signals, even when a relatively large volume (20 μl) of the insect cell supernatant containing the cytokine was examined. However, when *bac*-rwIFNγ was analyzed by Western blotting, three protein bands of 16.5, 17.4 and 34 kDa were detected (Figure 4.2B). The 17.4-kDa protein had a slightly greater molecular size than that deduced from the nucleotide sequence of the full-length rwIFNγ (*i.e.*, 16.5 kDa) and most likely represented a glycosylated form of the 16.5-kDa protein. The supernatant from Sf9 cells infected with *bac*-WT was not reactive with anti-rwIFNγ antibodies (Figure 4.2B), confirming *bac*-rwIFNγ specificity of the identified proteins.

Upon induction of *E. coli* with IPTG, the rwTNFα cDNA fragment gave an 18.8-kDa protein species, which was subsequently purified to homogeneity (98%) by IMAC (Figure 4.3A). The final concentration of the affinity-purified *ec*-rwTNFα was 60 μg/ml. Western blot analysis of *ec*-rwTNFα with cross-reactive anti-murine TNFα antibodies (see Section 4.3.7) showed two protein bands, 18.5 and 37 kDa (Figure 4.3B), corresponding to wTNFα monomer and its dimer, respectively. The specificity of this identification was confirmed by probing unrelated proteins, *ec*-rWHe and *ec*-rwCD3, which did not yield any visible signals (Figure 4.3B). The same analysis of the *bac*-rwTNFα supernatant showed a 17-kDa protein band, while an equivalent amount of the *bac*-WT supernatant was nonreactive (Figure 4.3B). The 17-kDa protein has a molecular

size compatible with that deduced from the nucleotide sequence for mature wTNFα (17.3 kDa).

4.4.3 Antiviral effect of rwIFNγ

Two-fold serial dilutions of the insect cell culture supernatants containing *bac*-rwIFNγ and the affinity-purified *ec*-rwIFNγ, starting from 1.6 μg/ml, were assayed for antiviral activity using woodchuck WCM-260 hepatocytes and human HepG2 cells infected with ECMV, as described in Section 4.3.4. As shown in Figure 4.4A, *bac*-rwIFNγ was able to protect woodchuck hepatocytes, but not human liver cells from the virus-induced lysis. Similarly, *ec*-rwIFNγ protected woodchuck, but not human liver cells (Figure 4.4B). Interestingly, this antiviral effect was seen only when WCM-260 cells were exposed to *bac*-rwIFNγ or *ec*-rwIFNγ prior to infection, but not when the cytokine was added at the time or after exposure of the cells to ECMV (data not shown). In control experiments, *bac*-WT (Figure 4.4A) and an unrelated protein derived from *E. coli* (*i.e.*, *ec*-rWHe) (Figure 4.4B) did not exert an antiviral effect.

Figure 4.3. SDS-PAGE and immunoblot analyses of *ec*-rwTNFα and *bac*-rwTNFα.

(A) SDS-PAGE monitoring of the expression and affinity purification of *ec*-rwTNFα. Lanes: M, pre-stained protein marker ladder; I, *E. coli* cell lysate after IPTG induction; U, lysate of uninduced cells; P, IMAC-purified *ec*-rwTNFα of 18.8 kDa. (B) Western blot analysis of *ec*-rwIFNγ and *bac*-rwIFNγ with anti-murine TNFα antibodies. Lanes: 1, IMAC-purified *ec*-rwTNFα (40 ng) showing protein bands of 18.5 and 37 kDa; 2, IMAC-purified *ec*-rWHe (40 ng); 3, IMAC-purified *ec*-rwCD3 (40 ng); 4, 20 μl of *bac*-rwTNFα supernatant showing 17-kDa protein band; 5. 20 μl of *bac*-WT supernatant.

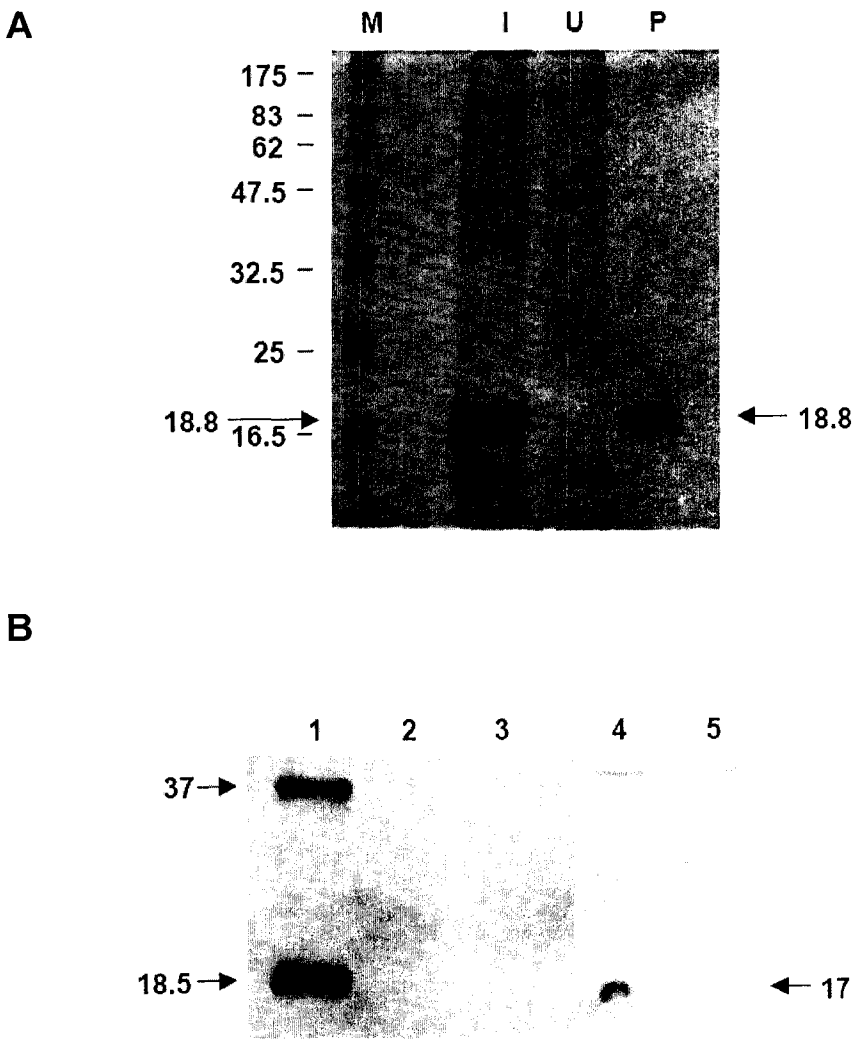


Figure 4.3

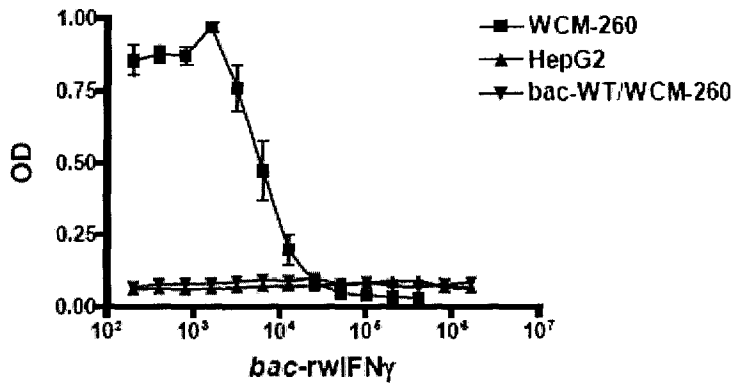
Based on the definition of an IFNγ unit as the ability to protect 50% of cells from ECMV-induced lysis, it was calculated that the *bac*-rwIFNγ concentration in the insect cell supernatant was 7.5 x 10^4 U/ml, whereas that of the affinity-purified *ec*-rwIFNγ was 900 U/μg. As shown in Figure 4.4, *bac*-rwIFNγ and *ec*-rwIFNγ produced comparable profiles of anti-ECMV activity at tested serial dilutions. Taking into account this observation and by estimating the *bac*-rwIFNγ protein content from the band densities detected on immunoblots, using serial dilutions of the purified *ec*-rwIFNγ as quantitative standards, it was estimated that *bac*-rwIFNγ has approximately 4.5-fold greater antiviral potency than *ec*-rwIFNγ tested at a comparable protein level.

Specificity of the *bac*-rwIFNγ antiviral effect was confirmed by neutralization with rabbit anti-rwIFNγ antibodies which were raised during this study using the affinity- purified *ec*-rwIFNγ as an immunogen. As shown in Figure 4.4C, inhibition of the ECMV-induced hepatocytolysis by *bac*-rwIFNγ was almost completely (90.3%) reversed when the cytokine at 10 U/ml was preincubated with anti-rwIFNγ serum at 1:20, but not with preimmune serum at the same dilution. However, this inhibitory effect was not observed when *bac*-rwIFNγ at 100 U/ml or anti-rwIFNγ at a dilution greater than 1:20 were tested (data not shown), indicating that the antibodies neutralization capacity was relatively low.

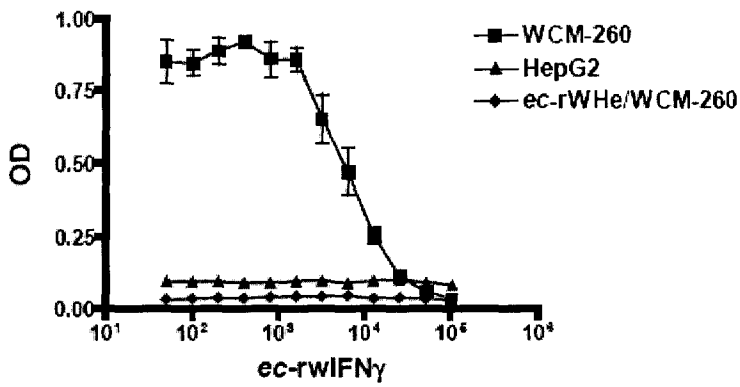
Figure 4.4. rwIFNγ virus protection assay.

WCM-260 hepatocytes and human HepG2 cells were incubated 24 h prior to infection with ECMV with two-fold serial dilutions of: (A) *bac*-rwIFNγ supernatant or (B) IMAC-purified *ec*-rwIFNγ. As controls, *bac*-WT supernatant (*bac*-WT/WCM-260) or IMAC-purified *ec*-rWHe (*ec*-rWHe/WCM-260) were added at comparable dilutions to WCM-260 cells. (C) Neutralization of *bac*-rwIFNγ-induced antiviral activity by rabbit anti-rwIFNγ antibodies. Supernatant containing *bac*-rwIFNγ at 10 U/ml was preincubated with anti-rwIFNγ or with preimmune rabbit serum, both at 1:20 dilution, which was then added to woodchuck WCM-260 hepatocytes and the cells infected with ECMV 24 h later. Anti-rwIFNγ antibodies inhibited *bac*-rwIFNγ antiviral effect by ~90% comparing to preimmune serum. In all assays, cell survival was monitored by the MTT test performed 24 h post-infection with ECMV. Results shown are mean values of OD_{570} -OD_{630} readings from triplicate wells and vertical bars are standard deviations of means.

A



B



C

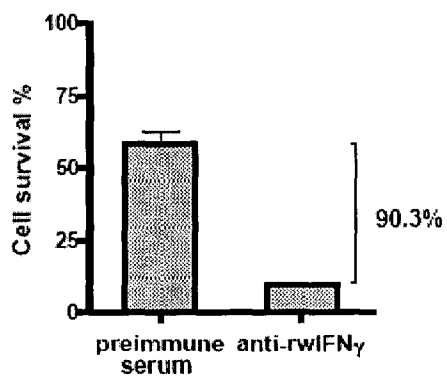


Figure 4.4

4.4.4 Cell killing by rwTNFα

The cytotoxicity of rwTNFα was assayed with murine L929 fibroblasts by a MTT assay. In this assay, the culture medium was supplemented with Actinomycin D, following reports that the presence of this transcription inhibitor enhances detection of TNFα-induced cytotoxicity (Ruddle, 1992). As shown in Figure 4.5, serial two-fold dilutions of the insect cell culture supernatant containing *bac*-rwTNFα and the affinity-purified *ec*-rwTNFα, starting from 60 ng/ml, showed a progressively decreasing ability to kill L929 cells. In contrast, *bac*-WT supernatant (Figure 4.5A) and an unrelated *ec*-rWHe protein, at comparable concentrations, displayed only marginal or no cytotoxicity, respectively (Figure 4.5B). Based on these results, it was estimated that the activity of *bac*-rwTNFα in the Sf9 cell supernatant was $3.2x10^4$ U/ml, whereas that of the purified *ec*-rwTNFα was $5.3x10^3$ U/μg protein. Testing *bac*-rwTNFα and *ec*-rwTNFα at concentrations giving comparable cytotoxic results and taking under consideration the estimated *bac*-rwTNFα protein content in the supernatant, determined as described for *bac*-rwIFNγ, it was approximated that *bac*-rwTNFα was about 18-fold more cytotoxic than *ec*-rwTNFα at a comparable protein level. The *bac*-rwTNFα cytopathic effect was neutralized by rabbit anti-murine TNFα antibodies. As illustrated in Figure 4.5C, the *bac*-rwTNFα-induced killing of L929 cells was progressively blocked when the cytokine at 100 U/ml was preincubated

with increasing concentrations of anti-TNFα antibodies. The inhibition of more than 80% was achieved when anti-TNFα IgG was used at 2 μg/ml.

4.4.5 Cytokine-induced class I MHC upregulation

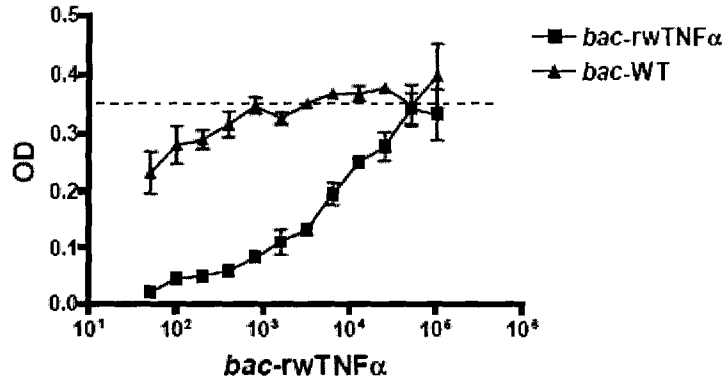
As shown in Figure 4.6A, incubation of WCM-260 hepatocytes with the supernatant containing 150 U/ml (~30 ng/ml) of *bac*-rwIFNγ led to a display of class I MHC antigen on the vast majority (88.1%) of the cells, indicating that this recombinant cytokine was highly potent in inducing MHC expression. Similarly, 150 U/ml (135 ng/ml) of *ec*-rwIFNγ greatly enhanced (91.5%) the cell surface presentation of the antigen (Figure 4.6B). Incubation of WCM-260 cells with the supernatant containing 320 U/ml (~3.4 ng/ml) of *bac*-rwTNFα also enhanced class I MHC display (Figure 4.6C), although to a lesser extent (17.8%) than the test concentration of *bac*-rwIFNγ. The affinity purified *ec*-rwTNFα, used at 320 U/ml (60 ng/ml), increased class I MHC presentation to a similar level (21.1%) as *bac*-rwTNFα (Figure 4.6D).

In summary, the woodchuck cytokines generated in the baculovirus expression system demonstrated a biological potency that was superior over that displayed by the cytokines produced in the *E. coli* system. This functional potency was to a similar degree greater when either the antiviral or cytotoxic activity or the ability to upregulate class I MHC antigen presentation was assessed.

Figure 4.5. rwTNFα cytotoxicity assay.

L929 cells were incubated in the presence of 1 μg/ml Actinomycin D with serial two-fold dilutions of: (A) *bac*-rwTNFα or *bac*-WT supernatant or (B) IMAC-purified *ec*-rwTNFα or IMAC-purified *ec*-rWHe protein. Survival of L929 was measured by the MTT assay 24 h later. Mean value of OD_{570} -OD_{630} readings from triplicate wells with SD were plotted against dilutions of rwTNFα. The dashed line marked 100% survival of L929 in DMEM medium supplemented with 1 μg/ml Actinomycin D. (C) Neutralization of *bac*-rwTNFα with rabbit anti-murine TNFα antibodies. Supernatant containing *bac*-rwTNFα at 100 U/ml was pre-incubated with anti-TNFα IgG at 0.5, 1 or 2 μg/ml or with normal rabbit serum IgG at the same protein concentrations and then added to L929 cells, as described in Materials and Methods. Cell survival was plotted as mean ± SD from triplicate wells in the MTT assay. Percentage values indicate the extent to which a particular concentration of anti-TNFα IgG inhibited L929 cytotoxicity compared to normal serum IgG.

A



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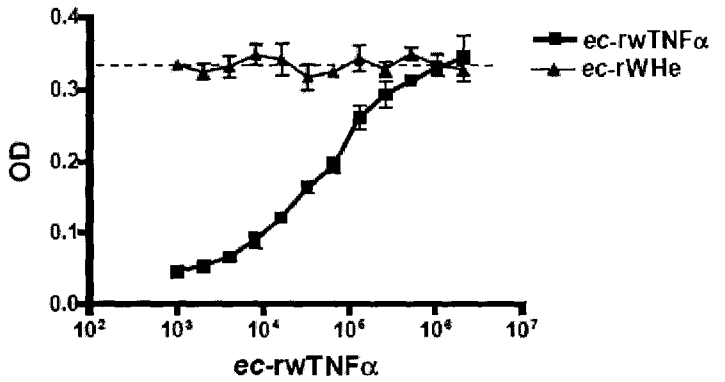

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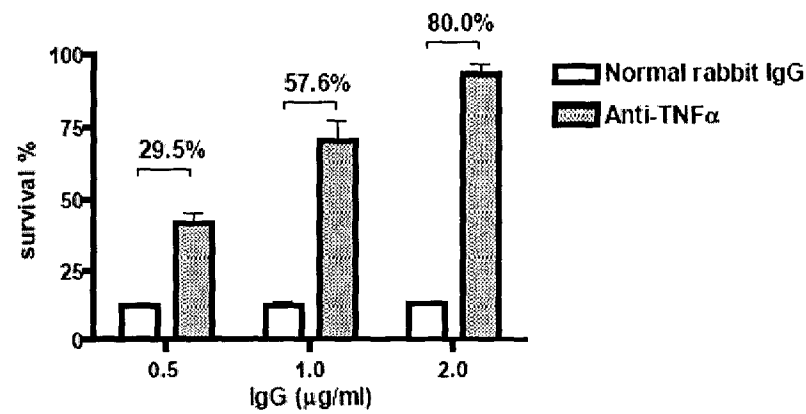


Figure 4.5

Figure 4.6. Flow cytometric evaluation of class I MHC upregulation.

Analysis of class I MHC antigen display on woodchuck WCM-260 hepatocytes treated for 18 h with: (A) 150 U/ml of *bac*-rwIFNγ (~30 ng/ml); (B) 150 U/ml of IMAC-purified *ec*-rwIFNγ (135 ng/ml); (C) 320 U/ml of *bac*-rwTNFα (~3.4 ng/ml) or (D) 320 U/ml of IMAC-purified *ec*-TNFα (60 ng/ml). Cytokine unstimulated WCM-260 cells and WCM-260 exposed to *bac*-WT supernatant or IMAC-purified *ec*-rWHe protein were used as controls. Cells were stained with mouse anti-woodchuck class I MHC heavy chain B1bB9 monoclonal antibody. Percentages of positive cells (gate M1) are indicated on histograms.

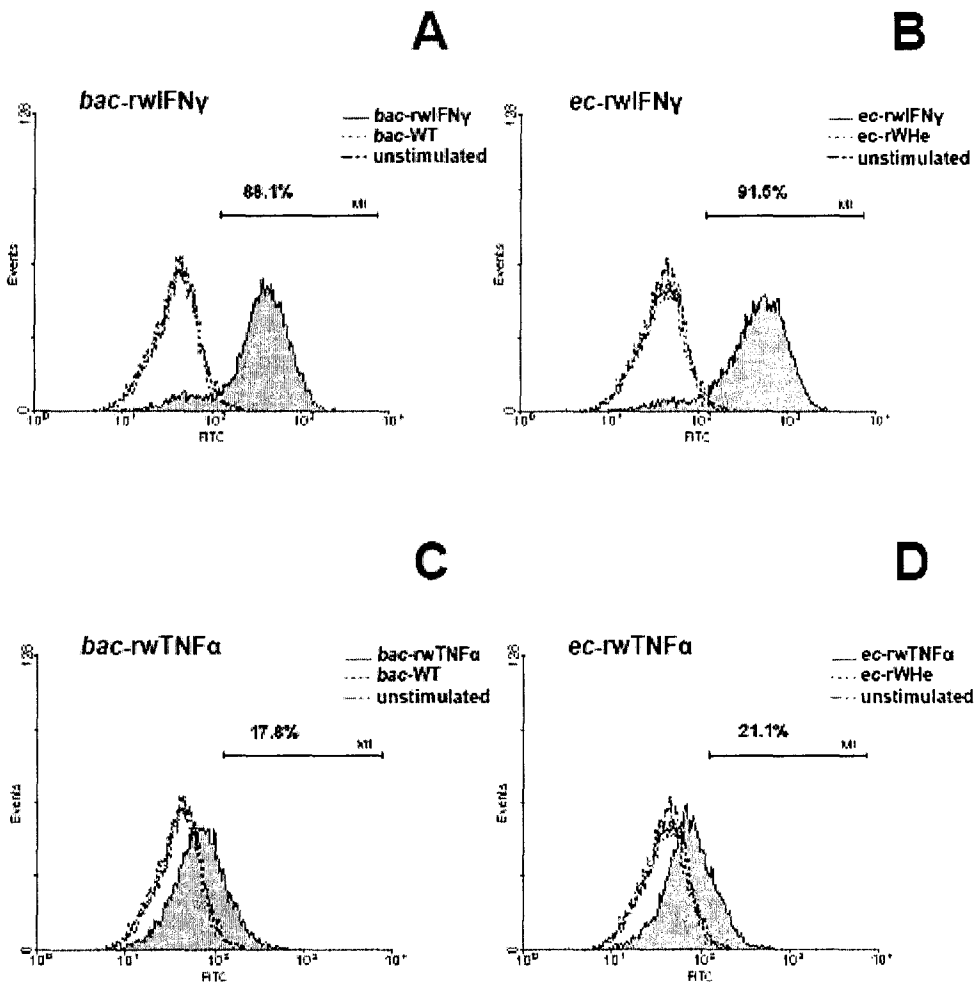

A
B
C
D
bac-rwIFNγ
bac-rwIFNγ
bac-WT
unstimulated
88.1%
M1
Events
FITC
ec-rwIFNγ
ec-rwIFNγ
ec-rWHe
unstimulated
91.6%
M1
Events
FITC
bac-rwTNFα
bac-rwTNFα
bac-WT
unstimulated
17.8%
M1
Events
FITC
ec-rwTNFα
ec-rwTNFα
ec-rWHe
unstimulated
21.1%
M1
Events
FITC

Figure 4.6

4.5 Discussion

We demonstrated that full-length rwIFNγ and rwTNFα cDNAs, encoding both the leader proteins and the predicted secretion signal of IFNγ or a membrane-bound domain of TNFα, were successfully cloned and expressed in insect Sf9 cells transfected with recombinant baculovirus constructs. Both cytokines were secreted to the cell culture supernatant, suggesting that the insect cells correctly recognized and cleaved these woodchuck proteins. The deduced mature forms of rwIFNγ and rwTNFα were also expressed in *E. coli* as polyhistidine-tagged proteins and affinity purified. Polyclonal antibodies to rwIFNγ and to murine TNFα specifically recognized *bac*-rwIFNγ and *bac*-rwTNFα proteins, respectively, confirming their antigenic competence.

Since *bac*-rwIFNγ was not detectable by SDS-PAGE, Western blot analysis was applied to identify the constituent proteins. Using this approach, *bac*-rwIFNγ showed three protein bands of 16.4, 17.5 and 34 kDa, suggesting that a non-glycosylated monomer, a glycosylated monomer and a dimer of wIFNγ were secreted by the infected insect cells. This was in contrast to *ec*-rwIFNγ which displayed on immunoblots two protein bands with molecular sizes corresponding to rwIFNγ non-glycosylated monomer and its dimer. It has been reported that glycosylation of IFNγ is not necessary for its biological activity, but it likely influences the cytokine circulatory half-life (Kelker *et al.*, 1983; Rutenfranz and Kirchner, 1988).

The functional capacity of *bac*-rwIFNγ and *ec*-rwIFNγ was examined by two independent methods, *i.e.,* by measuring the antiviral activity and by assessing the capacity of enhancing the cell surface display of class I MHC antigen. It was found that both *bac*-rwIFNγ and *ec*-rwIFNγ protected woodchuck, but not human, liver cells from lysis with ECMV, indicating that wIFNγ may function in a species-specific manner, similarly as it has been reported for human and murine IFNγ (Devos *et al.*, 1982; Gray and Goeddel, 1983; Celada, 1988). Furthermore, *bac*-rwIFNγ was approximately 4.5-fold more effective than *ec*-rwIFNγ in protecting woodchuck hepatocytes from ECMV lysis, as well as in enhancing class I MHC antigen presentation, when the extent of these biological effects was adjusted to a comparable concentration of the cytokine protein. Overall, it became evident that rwIFNγ derived from the baculovirus expression system was significantly more potent than that produced in *E. coli*. This finding was not surprising considering that the results from Western blot analysis suggested that *bac*-rwIFNγ, not *ec*-rwIFNγ, encompassed a glycoslylated protein.

It is of note that pre-incubation of hepatocytes with rwIFNγ was required to render protection against ECMV-induced lysis. Addition of rwIFNγ at the time of inoculation with ECMV or after ECMV infection did not inhibit virus causing hepatocytolysis, even when relatively high amounts (*i.e.,* 5000 U/ml) of the cytokine were tested (data not shown). This may suggest that to induce the most pronounced antiviral effect *in vivo*, hepatocytes may have to be exposed to this cytokine prior to virus infection.

The antiviral activity of *bac*-rwIFNγ was neutralized with antibodies raised by immunization of rabbits with the affinity-purified *ec*-rwIFNγ. Although these antibodies were inhibitory only under certain experimental conditions, *i.e.*, *bac*-rwIFNγ at 10 U/ml and the antibody dilution 1:20, the blockage was almost complete (90%). This ultimately confirmed specificity of the protective effect exerted by *bac*-rwIFNγ.

The protein profile of *bac*-rwTNFα determined by Western blot staining showed a single protein band with a molecular size which corresponded to a non-glycosylated monomer of mature wTNFα. This suggested that insect cells were able to cleave a membrane-bound domain of rwTNFα in a manner similar to that occurring in mammalian cells (Moss *et al.*, 1997).

The biological activity of *bac*-rwTNFα was tested and compared to that of *ec*-rwTNFα using two complementary approaches, *i.e.*, by assessing the cytotoxic effect against L929 cells and by measuring the capacity to increase presentation of class I MHC antigen on woodchuck hepatocytes. Using murine L929 fibroblasts, which are typically applied as targets for assessment of TNFα functionality (Meager *et al.*, 1989; Ruddle, 1992; Lohrengel *et al.*, 2000), we established that *bac*-rwTNFα was at least an 18-fold stronger inducer of cell killing and class I MHC upregulation than *ec*-rwTNFα when examined at a comparable protein level.

In a separate study, it was established that the deduced amino acid sequence of the complete wTNFα is similar by 83% to mouse, 82% to rat and

78% to human (Wang and Michalak, unpublished). Considering this high interspecies homology, we used rabbit anti-murine TNFα antibodies in an attempt to inhibit the cytotoxic effect exerted by *bac*-rwTNFα. We have found that these antibodies were remarkably effective in neutralizing the *bac*-rwTNFα-mediated cytotoxicity and under optimal conditions, *i.e.*, *bac*-rwTNFα at 100 U/ml and anti-TNFα IgG concentration of 2 μg/ml, they inhibited the killing by as much as 80%.

Recombinant proteins produced in the baculovirus expression system have shown a number of advantages over those expressed in the *E. coli* systems. These benefits are now well recognized (Miller, 1988; Luckow and Summers, 1988; Kidd and Emery, 1993; Geisse *et al.*, 1996). They are mainly related to the ability to create proteins of a superior biological efficacy which are free from bacterial endotoxin or mitogen contaminations (Stacey *et al.*, 1992; Lambrecht *et al.*, 1999). The presence of bacterial impurities is of particular concern due to their interference with the experimental outcomes, making correct interpretation of the results frequently impossible (Gao and Tsan, 2003). In the present study, we have established efficient baculoviral expression systems for production of rwIFNγ and rwTNFα and we have documented by different testing approaches the functional competence of the recombinant cytokines produced. Their availability should facilitate further studies in the woodchuck model of hepadnaviral infection on elucidation of their antiviral action and their contribution to the immunopathogenesis of liver injury in hepatitis B.

CHAPTER 5. WOODCHUCK HEPATITIS VIRUS INHIBITION OF CLASS I MAJOR HISTOCOMPATIBILITY COMPLEX PRESENTATION ON HEPATOCYTES IS MEDIATED BY VIRUS ENVELOPE PreS2 PROTEIN AND CAN BE REVERSED BY TREATMENT WITH GAMMA INTERFERON

5.1 Summary

Presentation of class I major histocompatibility complex (MHC) is severely down-regulated on hepatocytes in woodchucks chronically infected with woodchuck hepatitis virus (WHV), as our previous study documented (Michalak *et al.*, 2000). In an attempt to recognize the underlying mechanism, cultured normal woodchuck hepatocytes were transfected with the complete WHV genome, sequences encoding for individual viral proteins or with whole virus genomes in which transcription of selected proteins was disabled by site-specific mutagenesis. Hepatocyte presentation of class I MHC antigen was significantly suppressed after cell transfection with complete WHV genome or with virus subgenomic fragments encoding for envelope preS2 protein or preS1 protein which naturally encompasses the preS2 amino acid sequence. In contrast, hepatocytes transfected with WHV X gene alone demonstrated a profound enhancement in the class I antigen display, whereas those expressing virus major S protein or core protein were not different from naive hepatocytes or those transfected with empty vector. Analysis of the mutated WHV sequences confirmed that the envelope preS2 protein was responsible for inhibition of the class I MHC antigen display. Interestingly, treatment with recombinant

woodchuck interferon gamma (rwIFN-γ) restored the inhibited presentation of the class I antigen on hepatocytes. Moreover, the class I antigen suppression was not associated with down-regulation of hepatocyte genes for class I MHC heavy chain, β_2-microglobulin, transporters associated with antigen processing (TAP1 and TAP2) or proteasome subunits. These findings suggest that the defective presentation of class I MHC antigen on hepatocytes transcribing WHV is a consequence of posttranscriptional suppression exerted by virus preS2 protein and that this hindrance can be completely reversed by IFNγ.

5.2 Introduction

The hepatitis B virus (HBV) is a member of the hepadnavirus family which includes, among several mammalian and avian viruses, woodchuck hepatitis virus (WHV) naturally infecting eastern North American woodchucks (*Marmota monax*). Woodchucks infected with WHV represent a highly valuable natural model of hepatitis B in which molecular, immunological and histological sequella of virus-induced liver inflammation closely resemble those encountered in humans, including development of chronic hepatitis (Michalak, 1998b; Tennant and Gerin, 2001). It is now also evident that HBV and WHV not only invade and replicate in hepatic tissue but also in the lymphatic system, although under most circumstances, less vigorously than in the liver (Michalak *et al.*, 2004; Michalak *et al.*, 1999; Michalak *et al.*, 1994). The host cellular immune responses directed against hepadnavirus protein epitopes displayed on infected hepatocytes,

particularly those mediated by cytotoxic T lymphocytes (CTL), are regarded to be crucial in the recovery from acute hepatitis as well as in the induction of hepatocellular damage in actively progressing infection (Chisari, 2000). In contrast to acute hepatitis, which is characterized by a strong and specific CTL response directed towards multiple virus epitopes, a weak and narrowly focused CTL antiviral reactivity is associated with chronic hepatitis B. It is assumed that this hindrance is one of the main pathogenic factors underlying the development of chronic hepadnaviral infection and protracted liver damage.

Because triggering and strength of antiviral CTL responsiveness depend to a significant degree on the efficient presentation of viral peptides by class I major histocompatibility complex (MHC) molecules on the surface of infected cells, we have previously investigated characteristics of the class I MHC antigen display on hepatocytes and lymphoid cells in woodchucks acutely and chronically infected with WHV (Michalak *et al.*, 2000). Among others, we have uncovered that chronic, but not acute WHV hepatitis is associated with a profound suppression in class I antigen on the surface of infected hepatocytes. Thus, while acute hepatitis was accompanied by enhanced hepatocyte presentation of the class I antigen, inhibition of the antigen was consistently detected on these cells in chronic hepatitis. This occurred despite the fact that intrahepatic expression of class I MHC-affiliated genes encoding woodchuck class I heavy (α) and β_2-microglobulin (β_2m) chains and transporters associated with antigen processing (TAP1 and TAP2), as well as interferon gamma (IFNγ), which is one

of the most powerful simulators of the class I antigen expression (Boehm *et al.*, 1997), was augmented to the same degree in both acute and chronic hepatitis. Furthermore, the hepatocyte class I antigen suppression was not linked to the histological severity of hepatocellular injury, the extent of lymphocytic infiltrations or the hepatic load of WHV. It was concluded that the deficiency in hepatocyte class I antigen display is a uniform hallmark of chronic WHV hepatitis and is caused by a virus-dependent posttranscriptional interference. However, the basis of this interference, particularly which of the WHV genome translation products might be responsible for this impediment, was not investigated.

The presentation of peptides by the class I MHC molecules on the cell surface accessible for CTL recognition is a multi-step process involving the generation of peptides, their transport, loading onto class I heavy chain-β2m complexes in the endoplasmic reticulum (ER), and trafficking the complexes to the cell surface via the Golgi apparatus (Shastri *et al.*, 2002). The generation of short length peptides in the cytosol is achieved by the ubiquitin-proteasome system. Briefly, the endogenously synthesized proteins are first conjugated with poly-ubiquitin by ubiquitin ligase (Jentsch, 1992). Then, the ubiquitin-tagged proteins are unfolded by the 19S cap of the proteasome, which is composed of proteasome activator 28 alpha (PA28a) and proteasome activator 28 beta (PA28β) and subsequently degraded into short peptides within the proteasome 20S core (Kloetzel, 2001). The peptidase activity in the 20S proteasome resides in constitutive β subunits, termed X, Y and Z, which can be readily substituted by

IFNγ-inducible β subunits, such as multicatalytic endopeptidase complex-like 1 (MECL-1), low molecular mass protein 2 (LMP2) and low molecular mass protein 7 (LMP7), which display catalytic activity. The generated peptides are either further degraded into amino acids for recycling by cytosolic peptidase or transported to the ER by TAP1/TAP2 heterodimer complexes (Reits *et al.*, 2000). An interaction between the TAP molecules and class I MHC-β2m is required for the assembly of class I MHC-peptide complexes (Sadasivan *et al.*, 1996). Upon loading a peptide, the class I MHC complex is exported from the ER to the Golgi apparatus, and finally transported to the cell surface where it can be identified by the peptide-specific CTL.

In the present study, to investigate the nature of the molecular hindrance responsible for hepadnavirus-induced inhibition of the class I MHC antigen on hepatocytes observed in chronic WHV hepatitis (Michalak *et al.*, 2000), we established a panel of woodchuck hepatocyte transfectants transiently or stably expressing the whole genome of WHV, subgenomic fragments encoding individual virus proteins, or complete WHV sequences in which expression of individual translation products was disabled by premature stop codons introduced through site-specific mutagenesis. The display of class I MHC antigen on these hepatocytes was measured and compared to the control naive hepatocytes and those transfected with empty vector. It was found that the expression of the complete WHV genome profoundly downregulated presentation of the class I antigen on hepatocytes in vitro and, through the

differential analysis of transfected hepatocyte lines, we determined that the virus envelope preS2 protein mediated for this effect. In parallel experiments, it was also found that the inhibited presentation of the class I antigen can be fully restored by exposure of hepatocytes to IFNγ, implying that the virus-induced suppression was not tenacious but can be reversed by treatment with an appropriate exogenous agent.

5.3 Materials and Methods

5.3.1 WHV plasmids.

A plasmid containing approximately 1.1-length (exactly 1.127) of WHV genome was constructed by sequentially inserting into the eukaryotic expression vector pcDNA3.1 (Invitrogen, Carlsbad, Calif.) two fragments (A and B) derived from a wild-type WHV/tm3 DNA sequence (GenBank accession number AY334075) (Michalak *et al.*, 2004). Fragment A encompassed nucleotides (nt) from position 1 to 2120 (numbers denote the position in relation to the endogenous *EcoR*I site in the WHV/tm3 genome), whereas fragment B encompassed nt 1698 to 3308/1 (Fig. 5.1). Fragment A was generated by PCR with sense primer 5'-TTCGAAATCAAACCTGGGCC (3286 to 3305), which was homologous to the sequence located upstream from the *EcoR*I site (3308 to 5) (Fig. 5.1), and antisense primer 5'-CGC*GGTACC*CAGTGTCCACCAAAGCATTA (2101 to 2120). The produced amplicon had the *Kpn*I site incorporated at the 3'-end (indicated in underlined italic letters) and the *EcoR*I site at the 5'-terminus to facilitate cloning.

Fragment B was amplified with primers 5'-CGC*CTCGAG*TCCGGTCCGTGTTGC (1698 to 1712) containing an incorporated *Xho*I site and 5'-CGTGGTATGTCCCGAATTCC (3307 to 18) that contained the endogenous *EcoR*I site (indicated in underlined letters). The PCR cycling conditions used for amplification of both fragments were as follows: denaturation at 94 °C for 3 min, 30 cycles at 94 °C for 30 seconds, 52 °C for 30 seconds and 72 °C for 2 min at each step, and final elongation at 72 °C for 3 min. The reaction was performed in a Peltier Thermal Cycler PTC-200 (MJ Research Inc., Waltham, MA) using a standard reagent mixture described previously (Michalak *et al.*, 1999). The resulting amplicons were purified from low-melting-point (LMP) agarose using the Wizard PCR Preps DNA purification system (Promega Corp., Madison, WI).

The construction of the plasmid containing more than full-length WHV genome was accomplished by a two-step procedure. In the first step, digestion of fragment A amplicon with *EcoR*I and *Kpn*I enzymes was performed. In parallel, the pcDNA3.1 vector was linearized with the same restriction enzymes. The ligation of the excised PCR fragment and the linearized vector was done at an estimated molar ratio 2:1 at 14 °C for 16 h in a 10-µl reaction mixture containing 1 U T4 DNA ligase (Invitrogen) and 1 X ligation buffer (10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT and 5% (w/v) polyethylene glycol-8000 in 50 mM Tris-HCl buffer, pH 7.6). TOP10 competent *Escherichia coli (E. coli)* cells (Invitrogen) were

Figure 5.1. Schematic presentations of p-WHV containing ~1.1-length WHV genome and expression vectors carrying WHV sequences encoding individual virus proteins which were generated and examined in the course of this study. The nucleotide 5'-end and 3'-end positions for each WHV ORF and for the p-WHV construct, as well as location of the endogenous *EcoR*I site within p-WHV are marked. Relative positions for endogenous WHV promoters (Pro) and an enhancer (Enh) are indicated along the p-WHV sketch, while the location of pC-DNA3.1-derived cytomegalovirus promotor (CMV Pro) is indicated at the beginning of each WHV sequence insert. All numbers denote the nucleotide positions according to WHV/tm3 genome sequence (GenBank accession number AY334075; (Michalak *et al.*, 2004).

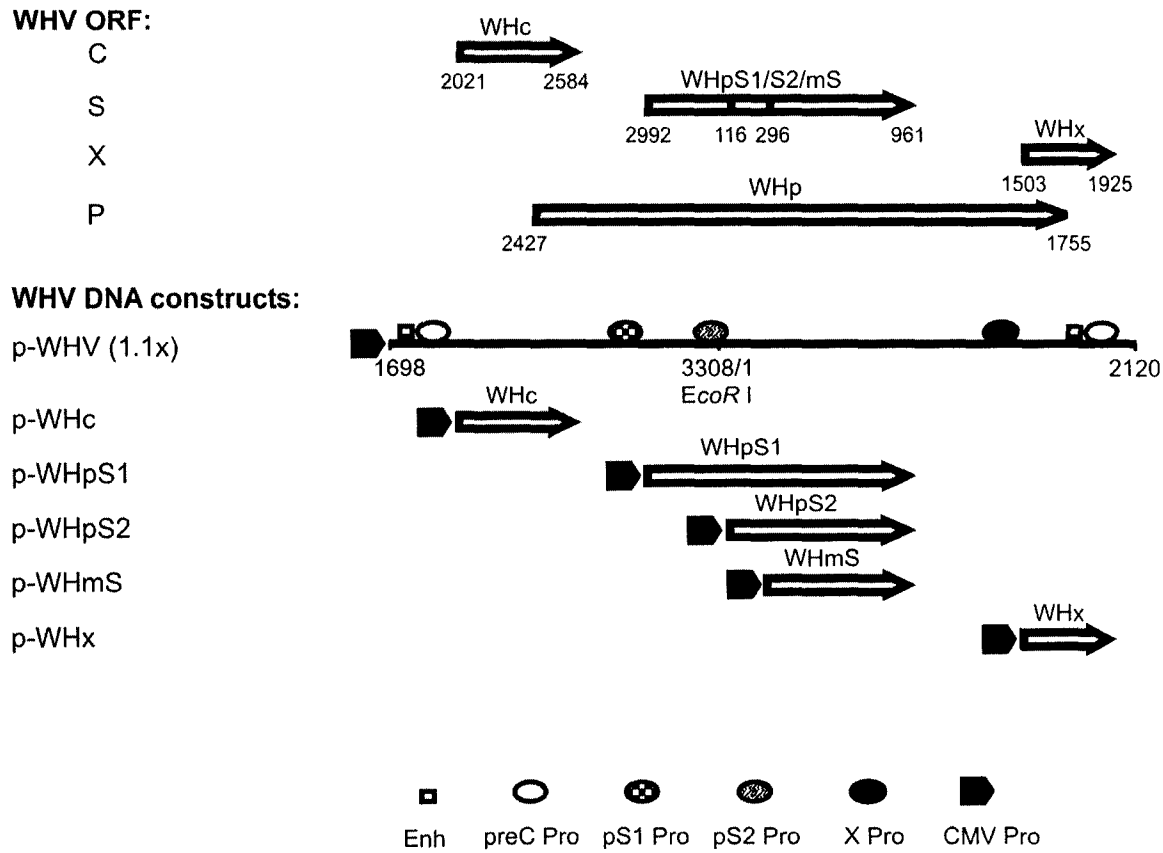

WHV ORF:
C
S
X
P
WHc
2021
2584
WHpS1/S2/mS
2992
116 296
961
WHx
1503
1925
WHp
2427
1755
WHV DNA constructs:
p-WHV (1.1x)
1698
3308/1
EcoR I
2120
p-WHc
WHc
p-WHpS1
WHpS1
p-WHpS2
WHpS2
p-WHmS
WHmS
p-WHx
WHx
Enh
preC Pro
pS1 Pro
pS2 Pro
X Pro
CMV Pro

Figure 5.1

transformed following the supplier's instructions. Screening of positive colonies was based on a standard alkaline method for mini-preps of recombinant plasmid DNA, followed by digestion with *EcoR*I and *Kpn*I enzymes, and agarose gel analysis of the fragments obtained. The selected positive clone was designated as p-WHV(0-2120).

In the next step, WHV genome fragment B was cloned into p-WHV(0-2120) using the same strategy as above. Thus, the fragment B amplicon and p-WHV(0-2120) were treated with *Xho*I and *EcoR*I enzymes and the resulting products ligated. After transformation of *E. coli*, a positive clone was identified by restriction enzyme mapping. The final construct, designated as p-WHV, contained approximately 1.1-length WHV genome starting from nt 1698, located upstream of the enhancer and preC promoter and direct repeats 1 and 2 regions (DR1 and DR2, respectively), and ending at nt position 2120 located downstream of the WHV polyadenylation signal sequence (Di *et al.*, 1997)(Fig. 5.1). The assessment of the correct design of the construct was confirmed by restriction digest analysis and DNA sequencing (data not shown).

In order to examine whether expression of individual WHV proteins can influence presentation of the class I MHC antigen, plasmids containing WHV genomic fragments encoding for the virus envelope preS1 protein (WHpS1), envelope preS2 protein (WHpS2), envelope major S protein (WHmS), core protein (WHc) or X protein (WHx) were constructed using pcDNA3.1 as backbone. Briefly, WHV sequences encoding for the respective full-length

proteins were amplified by PCR using the sequence specific oligonucleotide primers containing appropriate restriction enzyme recognition sequences. Then, the PCR products were treated with restriction enzymes and directionally inserted into pcDNA3.1 vector. The integrity of the constructs was confirmed by DNA sequencing. The names of the plasmids encoding for individual WHV proteins are delineated in Figure 5.1.

5.3.2 WHV mutants.

To study the effect of individual WHV proteins expressed in the context of the whole WHV genome on hepatocyte transcription of the class I MHC-related genes and on the surface display of the class I antigen, a series of mutants containing premature stop codons within subgenomic sequences encoding for individual WHV proteins were produced by site-directed mutagenesis using p-WHV as the backbone and an approach reported by others (Ho *et al.*, 1989). Thus, WHV genomic regions encoding for WHpS1, WHmS, WHc, WHx, and WHV polymerase (WHp) were mutated to selectively eliminate expression of a given protein without introducing amino acid changes to other translation products of the virus genome. For generation of each mutant (Fig. 5.2), a pair of specific primers was designed to introduce mutating nucleotide/s at the desired position (indicated by underlined italic letters in the primer sequences shown below). Thus, the WHpS1 mutant (ΔWHpS1) was generated using 5'-CAAAATAGCAGCGTGGT*A*GCCTGCAGTGGGCACT (3027-3060) and 5'-AGTGCCCACTGCAGGC*T*ACCACGCTGCTATTTTG (3060-3027) which

Figure 5.2. Schematic presentation of the strategy applied for generation of p-WHV mutated sequences by introducing premature stop codons. Designated names of the constructs and the introduced nucleotide modifications are identified on the left side of the panel. Relative locations of the stop codons inserted into WHV ORFs (open arrows) are marked by crosses. Detailed information on the site-specific mutagenesis leading to creation of each mutant are shown under respective ORF sketches. The bold numbers at the 5'- and 3'-ends of the outlined sequences show nucleotide positions in relation to the *EcoR*I site in the WHV/tm3 genome (see legend to Fig. 5.1). Bold and blue letters in the nucleotide sequences stand for introduced nucleotides, whereas the underlined triple nucleotide sequences represent the stop codons created. The amino acid (aa) sequences of interest and their relative positions (numbers after aa) within respective viral proteins are shown above the nucleotide sequences, while the aa sequences naturally overlapping the sequence of interest are depicted under the nucleotide sequences to indicate that no aa alterations were introduced. * marks termination of amino acid translation.

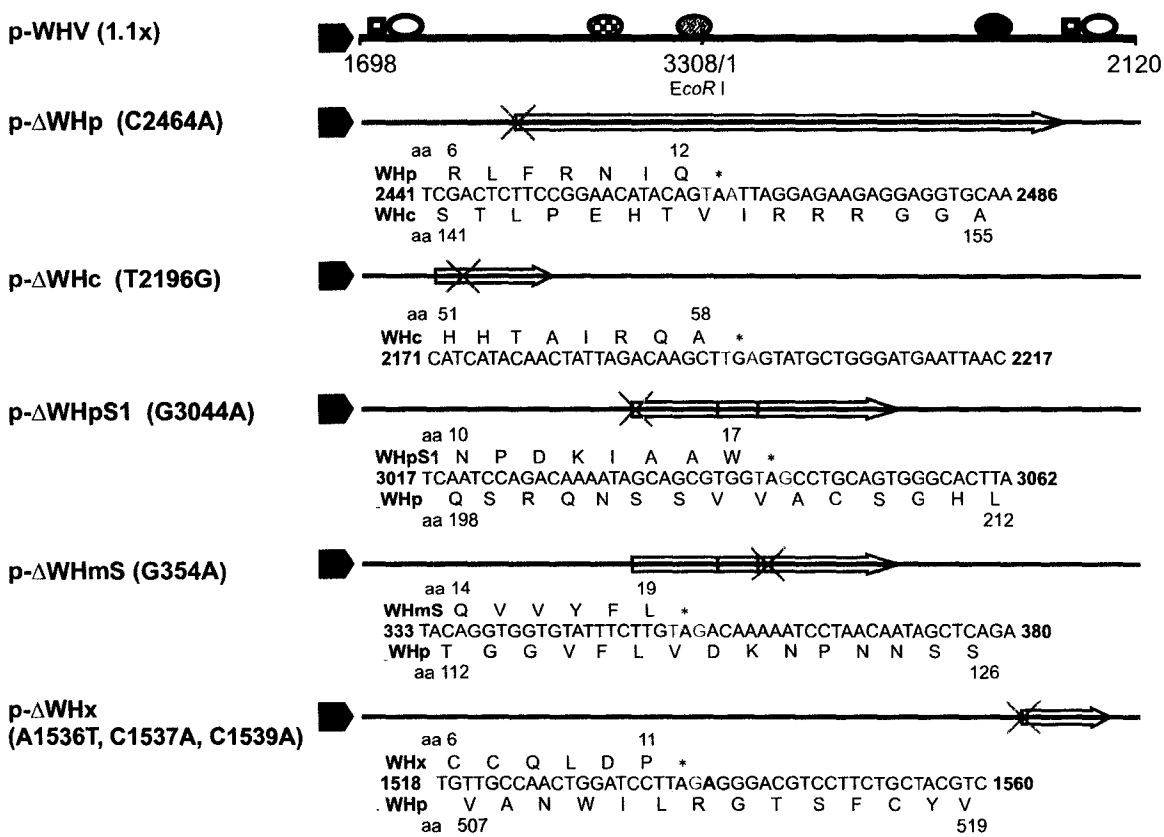

p-WHV (1.1x)
1698
3308/1
EcoR I
2120
p-ΔWHp (C2464A)
aa 6
12
WHp R L F R N I Q *
2441 TCGACTCTTCCGGAACATACAGTAATTAGGAGAAGAGGAGGTGCAA 2486
WHc S T L P E H T V I R R R G G A
aa 141
155
p-ΔWHc (T2196G)
aa 51
58
WHc H H T A I R Q A *
2171 CATCATACAACTATTAGACAAGCTTGAGTATGCTGGGATGAATTAAC 2217
p-ΔWHpS1 (G3044A)
aa 10
17
WHpS1 N P D K I A A W *
3017 TCAATCCAGACAAAATAGCAGCGTGGTAGCCTGCAGTGGGCACTTA 3062
WHp Q S R Q N S S V V A C S G H L
aa 198
212
p-ΔWHmS (G354A)
aa 14
19
WHmS Q V V Y F L *
333 TACAGGTGGTGTATTTCTTGTAGACAAAAATCCTAACAATAGCTCAGA 380
WHp T G G V F L V D K N P N N S S
aa 112
126
p-ΔWHx
(A1536T, C1537A, C1539A)
aa 6
11
WHx C C Q L D P *
1518 TGTTGCCAACTGGATCCTTAGAGGGACGTCCTTCTGCTACGTC 1560
WHp V A N W I L R G T S F C Y V
aa 507
519

Figure 5.2

changed TGG to TAG, i.e., from tryptophan to a stop codon at position 18 in WHpreS1 amino acid (aa) sequence. The WHmS mutant (ΔWHmS) was created using sense primer 5'-GGTGGTGTATTTCTTGT*A*GACAAAAATCCTAAC (337 to 369) and antisense primer 5'-GTTAGGATTTTTGTC*T*ACAAGAAATACACCACC (369 to 337) which changed TGG to TAG, *i.e.,* from tryptophan to a stop codon at aa 20 of WHmS. The WHc mutant (ΔWHc) was produced with primers 5'-CTATTAGACAAGCTT*G*AGTATGCTGGGATGAA (2181-2212) and 5'-TTCATCCCAGCATACT*C*AAGCTTGTCTAATAG (2212-2181) which modified TTA to TGA, i.e., from leucine to a stop codon at aa position 59. The WHx mutant (ΔWHx).was obtained with primers 5'-GTTGCCAACTGGATCCT*TA*G*A*GGGACGTCCTTCTGC (1519-1554) and 5'-GCAGAAGGACGTCCC*T*C*TA*AGGATCCAGTTGGCAAC (1554-1519) that changed ACG to TAG, i.e., from threonine to a stop codon at aa position 12. The WHp mutant (ΔWHp) was created with primers 5'-CTTCCGGAACATACAGT*A*ATTAGGAGAAGAGGAGG (2447-2481) and 5'-CCTCCTCTTCTCCTAAT*T*ACTGTATGTTCCGGAAG (2481-2447) which changed TCA to TAA, i.e., from serine to a stop codon at aa position 13. For generation of each mutant, two rounds of PCR were performed. The first round included two reactions, one with a forward primer and a mutant reverse primer and the second a mutant forward primer and a reverse primer. Both reactions were carried for 15 cycles of 94 °C for 20 sec, 52 °C for 20 sec and 72 °C for 2 min using 100 ng of p-WHV as template. PCR products from these two reactions

were gel purified, combined at an equal molar ratio, and used as the template for the second round PCR. The primers for the second round were the same forward and reverse primers as those used in the first round. This amplification was carried out at 94 °C for 20 sec, 52 °C for 20 sec and 72 °C for 2 min for 35 cycles with a final elongation step of 72 °C for 5 min. The product from the second round PCR was gel purified and cloned into an appropriate vector. The presence of the created stop codons were always verified by DNA sequencing (data not shown).

5.3.3 Hepatocyte cultures.

Woodchuck hepatocyte line WCM-260 was established from a liver biopsy obtained from an adult, healthy animal and was characterized in detail previously (Churchill and Michalak, 2004; Diao *et al.*, 1998; Lew and Michalak, 2001). WCM-260 were grown in a conditioned hepatocyte culture medium consisting of 80% (vol/vol) Hepato-STIM medium (Becton Dickinson, Bedford, Mass.) and 20% (vol/vol) culture supernatant from HepG2 cells (ATCC No. HB-8065). The medium was supplemented with 10 ng/ml epithelial growth factor, 2 mM L-glutamine, 50 µg/ml penicillin, and 50 µg/ml streptomycin (all from Becton Dickinson). The conditions for WCM-260 maintenance, detachment, passage, and storage were reported before (Churchill and Michalak, 2004; Diao *et al.*, 1998).

5.3.4 Hepatocyte transfections.

The transfection of WCM-260 hepatocytes with p-WHV, plasmids carrying sequences encoding individual WHV proteins or its mutants was done using LipofectAMINE 2000 (LF2000) reagent (Gibco BRL), following the supplier's instruction. The day before the transfection, cells were seeded at 3 x 10^5 per well in a 12-well culture plate (Fisher Scientific Limited, Nepean, Ontario). Immediately before transfection, the attached cells were washed 3 times with Dulbecco's modified Eagle's medium (DMEM; Invitrogen) without supplements and then 1 ml of the same medium was added to each well. At the same time, 2 µg of plasmid DNA and 2.5 µl of LF2000 per transfection were suspended in 100 µl of DMEM each, combined, and incubated for 20 min at ambient temperature. The plasmid DNA-LF2000 mixture was then added to cells and incubated for 8 h at 37 °C. Subsequently, the DMEM medium was replaced with conditioned hepatocyte culture medium and cells cultured for time periods indicated. Thus, the transient expression of a given transgene was evaluated 48-72 h later. For stable transfection, G418 reagent (Invitrogen) was added 3 days post-transfection at 900 µg/ml and hepatocytes maintained for at least a 4-week period, changing culture medium every 3-4 days. WCM-260 cells transiently or stably transfected with pcDNA3.1 vector alone (empty vector) were used as controls.

5.3.5 Hepatocyte stimulation with IFN-γ.

To assess whether the inhibition of class I MHC presentation on hepatocytes found after expression of the complete WHV genome or some of its subgenomic fragments was reversible, recombinant woodchuck IFN-γ (rwIFNγ), the cytokine which is one of the most potent naturally occurring class I MHC inducers, was produced in the baculovirus expression system, as described recently (Wang and Michalak, 2005). Briefly, the full-length IFN-γ cDNA derived from woodchuck peripheral blood lymphoid cells stimulated with Concanavalin A (5 μg/ml; Sigma Chemical Co., St. Louis, Mo) was cloned and expressed in insect Sf9 cells using the MaxBac 2.0 expression system (Invitrogen). The final concentration of rwIFNγ in Sf9 culture supernatant was 150 U/μl, as determined by inhibition of WCM-260 hepatocyte lysis caused by infection with encephalomyocarditis virus (ECMV) (Wang and Michalak, 2005). For stimulation with rwIFNγ of WCM-260 transfected with p-WHV, plasmids encoding for individual viral proteins or p-WHV mutants, 150 U/ml of rwIFNγ was added to the hepatocyte culture medium 18 h prior to examination of the class I MHC expression. Naive WCM-260 cells exposed under the same conditions to culture supernatant from Sf9 cells infected with wild-type (empty) baculovirus vector were used as controls.

5.3.6 RNA isolation and reverse transcription.

Total cellular RNA was extracted with TRIzol reagent (Invitrogen), as described previously (Michalak *et al.*, 2000; Michalak *et al.*, 1999). Possible

genomic DNA contamination was eliminated by treatment with DNAse I using DNAse-free kit (Ambion, Austin, TX), as per the supplier's instruction. One to 2 μg of total RNA was reverse transcribed into cDNA with random primers using 200 U of reverse transcriptase (RT) from Moloney murine leukemia virus (Gibco BRL), as reported (Coffin and Michalak, 1999; Michalak *et al.*, 1999). Each reaction was set up in parallel with a negative control that had all ingredients except RT.

5.3.7 Detection of WHV mRNA.

RNA extracted from transiently or stably transfected hepatocytes were reverse transcribed into cDNA and examined by PCR under conditions previously established (Coffin and Michalak, 1999; Michalak *et al.*, 1999) using primers specific for nonoverlapping regions of WHV core (C), surface (S) and X genes. Primer sequences of the X gene were reported before (Michalak *et al.*, 1999), whereas those for amplification of the C and S gene sequences were as follows: sense 5'-CTAACAGGTAGGGAACATTGC and antisense 5'-GACCTAGAAGCTCTTGCACC for C gene, and a common antisense primer 5'-GCGAAGCTTAATGTATACCCAAATC with sense primer 5'-GCGCTCGAGGCAACATAATGGGCAAC for WHpS1, or sense primer 5'-CGCCTCGAGACTATGAAAAATCAGACT for WHpS2 or sense primer 5'-CGCCTCGAGAGATGTCACCATCAAGTC for WHmS. As controls, cDNA prepared from WCM-260 transiently or stably transfected with pcDNA3.1 alone (empty vector) or from naïve (nontransfected) WCM-260 hepatocytes were

included. In addition, samples of RNA isolated during the same extractions, but not reversely transcribed, were used as DNA contamination controls (Coffin and Michalak, 1999). Southern blot hybridization analysis of the PCR product with ^{32}P-labeled complete recombinant WHV DNA (rWHV DNA) as a probe was routinely used to verify the specificity of viral sequence detected and the validity of controls, as described elsewhere (Michalak *et al.*, 1999).

5.3.8 Cloning of woodchuck proteasome subunit cDNAs.

Since sequences of woodchuck genes encoding for immunoproteasome subunits were unknown prior to this study, we have made an effort to determine their partial sequences to investigate the potential influence of WHV expression on transcriptional activity of these genes in WCM-260 hepatocytes. For this purpose, total RNA isolated from WCM-260 hepatocytes was treated with DNase and then reverse transcribed to cDNA, as described above. Amplification of woodchuck LMP2, LMP7 and MECL-1, PA28α, and PA28β cDNA fragments was done using primers deduced by inter-species homology comparison of human, mouse and rat cDNA sequences using PC Gene software (IntelliGenetics Inc., Mountain View, Calif.). The LMP2 was amplified with primers 5'-CGGRAGAAGTCCACACCGGG and 5'-CTCATCRTAGAATTTTGGCAG, LMP7 with 5'-GCCTTCAARTTCCAGCATGG and 5'-CCTCCAGAATAGYTGTCTCTGTG, MECL1 with 5'-CTTCTCYTTCGAGAACTGCC and 5'-TCCACCTCCATRGCCTGCAC, PA28α with 5'-GGGAGCTATTTYCCCAAGAA and 5'-GGGCTTCTTGCGCTTCTC, and

PA28β with 5'-ATGGCCAAGCCKTGTGGG and 5'-GATGGCTTTTCTTCACCCTT. PCR amplification was performed in a PTC-200 cycler using 50 ng of template cDNA and the standard reagent mixture described before (Michalak *et al.*, 1999). Cycling conditions were: 30 cycles of 94 °C for 20 sec, 52 °C for 20 sec and 72 °C for 1 min, followed by an elongation step at 72 °C for 3 min. The DNA amplicons of the expected sizes were purified from LMP agarose and cloned into the pCRII vector using a TOPO-TA cloning kit (Invitrogen). Plasmids with cloned fragments were examined by DNA sequencing. The nucleotide sequences obtained were compared to those of human, mouse and rat. This comparison showed that the woodchuck sequences were more compatible with those of human (91.5% to 98.5%) than with those of mouse (87.1% to 94.2%) or rat (87.9% to 93.9%).

5.3.9 Quantitation of transcriptional activity of class I MHC- and proteasome-affiliated genes.

The quantitation of transcription of class I MHC heavy chain, β_2-microglobulin (β_2m), transporter associated with antigen processing type 1 (TAP1) and type 2 (TAP2), LMP2, LMP7, MECL-1, PA28α, PA28β, and β-actin in WHV-transfected and control WCM-260 hepatocytes was done by real-time PCR using a Light Cycler (Roche, Laval, Quebec, Canada). cDNA samples from WCM-260 hepatocytes transfected with p-WHV or with plasmids encoding for individual WHV proteins or for p-WHV mutants were prepared as described above. Real-time PCR was carried out in capillary tubes with 0.2 µl of cDNA (equivalent of 25-

50 ng of total RNA) in a 20-µl volume using FASTstart SYBR reagent kit (Roche Diagnostics, Laval, Quebec). Primers designed to specifically amplify the sequences of interest are listed in Table 5.1. It is of note that primer sequences for woodchuck class I heavy chain, β_2m, TAP1 and TAP2 were designed based on the partial gene sequences previously determined in this laboratory (Michalak *et al.*, 2000). The copy number of each gene was extrapolated from a standard curve generated using 10-fold serial dilutions of a relevant cloned gene fragment, which were run in parallel in each reaction. Specificity of the products was determined by melting curve analysis. Negative controls included water instead of cDNA and samples treated exactly as test RNA samples but in the absence of RT. The house-keeping gene, β-actin, was used to standardize the expression of the genes tested.

5.3.10 Flow cytometry and confocal microscopy.

To determine presence of WHV envelope and core proteins in WCM-260 hepatocytes transfected with p-WHV or plasmids encoding for WHV proteins and selected protein mutants, the cells were grown on cover slips, fixed with cold ethyl ether for 3 min, and incubated with the respective specific antibodies. The WHs antigenic reactivity was detected with guinea pig anti-preS antibodies raised by immunization with a recombinant WHV preS1/preS2 protein produced in an *E. coli* expression system (J. Wang and T.I. Michalak, unpublished data) or

Table 5.1. Primer sequences for real-time RT-PCR quantitation of woodchuck class I MHC- and proteasome-affiliated genes expression.

Gene		Primer sequence	Size of amplicon (bp)
Heavy chain	Sense Anti-sense	AGATGGGGAGGACCAGACC GTGAGAGACACATCAGAGCC	348
β_2M	Sense Anti-sense	TTCATGCTACTCTTTCTGACC CGACTTTTTCTATCTTCTGTCC	181
TAP-1	Sense Anti-sense	GGAAATGAAGACACTCAACC TCCAGACTGTATTGCAGC	676
TAP-2	Sense Anti-sense	TTCCAGGAGATTAAGACAGG TATAAGCAGGTACAGAGCACG	417
LMP2	Sense Anti-sense	ATAGGGAAGACTTGTCGGC ACATAACCGTAAATGTAAGTGCT	145
LMP7	Sense Anti-sense	TGCCTCCAAACTGCTCTCC ATGAACAATAGCCCTGCGACC	253
MECL1	Sense Anti-sense	GGGCTTCGGGTTCCTCATG CGTGTAGTCATTTCGGCGT	203
PA28α	Sense Anti-sense	GGGAATAATTTTGGAGTGGCTGT CCGAATGTCCCGGTACTCT	207
PA28β	Sense Anti-sense	GGTGCGCCTGAGTGGG AGGGAGTCCTCGTGCAAA	138
β-actin	Sense Anti-sense	CAACCGTGAGAAGATGACC ATCTCCTGCTCGAAGTCC	239

with guinea pig anti-WHs produced by immunization with serum-derived WHsAg which recognized all three envelope proteins, i.e., WHpreS1, WHpreS2 and WHmS (Michalak and Lin, 1994). WHV core antigen (WHcAg) was detected with rabbit anti-WHc antibodies produced by immunization with WHV core particles (Michalak *et al.*, 1989). The reactivity of guinea pig antibodies was identified with Cy5-conjugated goat anti-guinea pig IgGs and that of rabbit antisera with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgGs (both from Jackson ImmunoReasearch Laboratories Inc., West Grove, PA). Cells were examined using an Olympus FluoView™ FV300 confocal system (Olympus America Inc. Melville, NY) equipped with an Olympus BX50WI microscope. WCM-260 hepatocytes transfected with empty pcDNA3.1 vector and stained exactly as test cells, as well as WCM-260 transfected with p-WHV, plasmids encoding for individual WHV proteins or p-WHV mutants incubated with pre-immune sera were used as controls.

To evaluate presentation of class I MHC antigen on the transfected and control WCM-260 hepatocytes, the unfixed cells were incubated for 45 min on ice with mouse monoclonal antibody (mAb) against woodchuck class I MHC heavy chain (B1b.B9), previously prepared in this laboratory (Michalak *et al.*, 1995) or with phosphate-buffered saline, pH 7.6 (PBS), a negative control. After washing with PBS, cells were exposed to FITC-conjugated goat anti-mouse IgG (H+L) antibodies (Jackson ImmunoReasearch Laboratories Inc.) for 45 min, followed by washing with PBS. Subsequently, the cells were fixed with 4%

paraformaldehyde in PBS and analyzed by flow cytometry using a FACS Calibur flow cytometer (Becton-Dickinson, Palo Alto, Calif.). Flow cytometry data were interpreted with assistance of CellQuest software (Becton-Dickinson) or WinMDI software (The Scripps Research Institute, La Jolla, Calif.). Geometric mean fluorescence intensity (MFI), calculated automatically from the plots of flow cytometric histograms, was used to compare the level of class I MHC expression. The results from three independent experiments were used to calculate the means. WCM-260 cells transfected with empty pC-DNA3.1 vector served as a control and their MFI values was taken as 100%.

Moreover, WCM-260 hepatocytes transfected with p-WHV, plasmids encoding for individual WHV proteins or mutated p-WHV sequences with disabled expression of the selected viral proteins, as well as naive WCM-260 and those stably transfected with empty pC-DNA3.1 vector were examined for class I antigen display after treatment with exogenous rwIFNγ, as described above, and compared to the cells not treated with this cytokine.

5.3.11 Statistical analysis.

The statistical analysis was carried out using GraphPad software (GraphPad Software Inc., San Diego, Calif.) *P* values were determined by unpaired *t* test and $P < 0.05$ was considered significant.

5.3.12 Nucleotide sequences accession numbers.

The accession numbers for the woodchuck nucleotide sequences established in this study submitted to GenBank are as follows: LMP2, AY726002; LMP7, AY7260023; MECL-1, AY726004; PA28α, AY726005, and PA28β, AY726006.

5.4 Results

5.4.1 Validation of p-WHV expression in hepatocytes.

Expression of p-WHV, carrying more than the full-length WHV genome, was driven by the CMV promoter contained in the pC-DNA3.1 vector and most likely by WHV genome intrinsic promoter/enhancer sequences, as illustrated in Fig. 5.1. After transient transfection of WCM-260 hepatocytes with p-WHV, WHV RNA transcripts of the C, S and X genes were identified by RT-PCR using primer pairs specific for non-overlapping regions of the respective open reading frames (ORFs) (Fig. 5.3A). The specificity of the mRNA detections was ascertained by the absence of DNA signals when the RT step was omitted from the RT-PCR and by Southern blot hybridization analysis of the amplified products. In addition, total RNA isolated from the liver of a woodchuck with chronic WHV hepatitis yielded RT-PCR amplicons with molecular sizes identical to those detected in the transfected WCM-260 cells when amplified with the same primer pairs (Fig. 5.3A). WCM-260 cells stably transfected with p-WHV gave the same results (Fig. 5.3B).

The synthesis of WHsAg and WHcAg in the transfected cells was examined with specific antibodies by confocal microscopy. As shown in Fig. 5.3C, both antigens were co-localized in the cytoplasm of WCM-260 cells transiently transfected with p-WHV. The cells transfected with empty pC-DNA3.1 plasmid remained entirely negative (data not shown), confirming specificity of the antigen detections. The efficiency of the transfection, estimated based on the number of WHsAg or WHcAg-positive cells identifiable by immunostaining was between 10% and 20%. A comparable result was obtained when WCM-260 cells were transiently transfected with a plasmid coding green fluorescent protein (pEGFP; Clontech, Mountain View, CA) (data not shown).

Further, to determine whether WHV virions were produced by WCM-260 hepatocytes transfected with p-WHV, culture supernatants from these cells transiently transfected with this plasmid were concentrated by ultracentrifugation, as described elsewhere (Coffin *et al.*, 2004), and injected intravenously into a healthy WHV-naive woodchuck. The animal developed WHV infection characterized by the persistent presence of WHV DNA in serum, peripheral lymphoid cells and liver tissue when tested at week 57 post inoculation. The infection progressed in the absence of serological markers of WHV infection, i.e., WHsAg and anti-WHc, and its pattern was comparable to that of the primary

Figure 5.3. Expression of WHV genes in WCM-260 hepatocytes transfected with p-WHV. WCM-260 hepatocytes (A) transiently transfected (after 48-72 h) or (B) stably transfected (after 2-3 mo in the presence of G418) with p-WHV were analyzed for expression of individual virus gene mRNAs by RT-PCR using primer pairs specific for S, C or X gene. The amplified products were identified by Southern blot hybridization by probing with complete rWHV DNA. Total RNA isolated from the liver of a woodchuck with chronic WHV hepatitis was examined each time as a positive control. RNA samples not transcribed to cDNA (RT -) were analyzed in parallel with those subjected to RT reaction (RT +) to exclude possible contamination with DNA. The molecular sizes (bp) of the detected PCR products are marked on the right side of the panel. In panel C, WHV preS1/preS2 and core proteins were visualized by immunostaining in WCM-260 hepatocytes transiently transfected with p-WHV. Following exposure of the cells to anti-WHV preS and anti-WHc antibodies, secondary antibodies conjugated with Cy5 or FITC were applied to detect WHV preS (left) and WHcAg (center), respectively. The overlayed image (right) is shown to illustrate preS1/preS2 and WHcAg co-localization. Bars represent 20 μM.

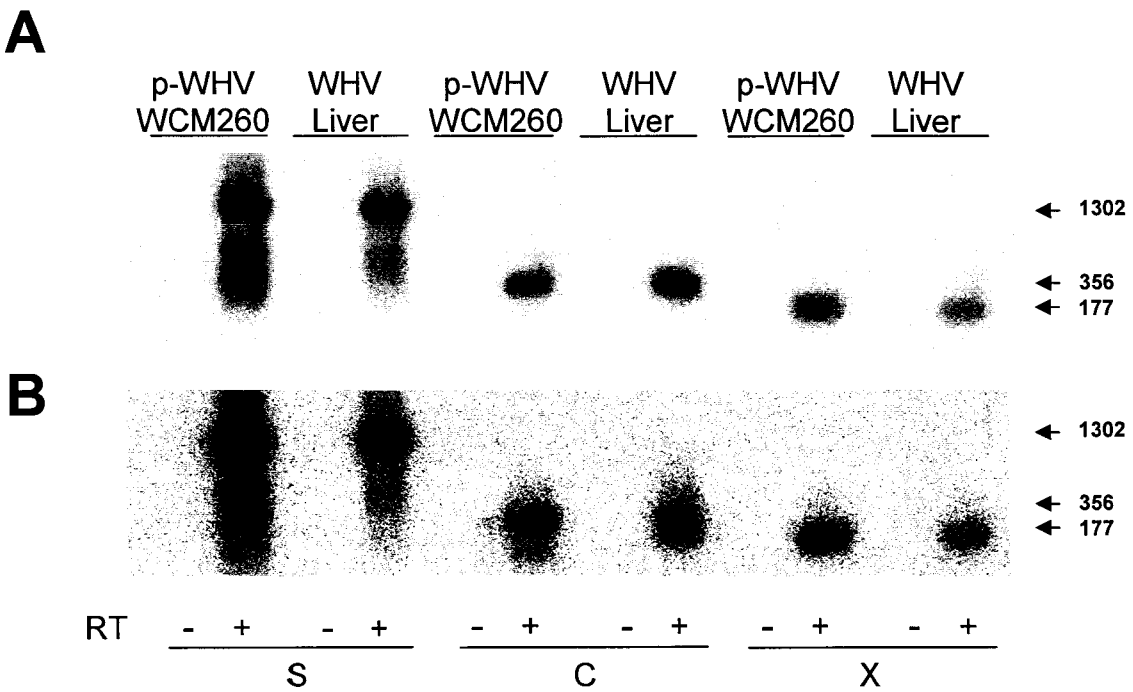

A
p-WHV WCM260
WHV Liver
p-WHV WCM260
WHV Liver
p-WHV WCM260
WHV Liver
1302
356
177
B
1302
356
177
RT - + - + - + - + - + - + - +
S
C
X

Figure 5.3 A&B

C

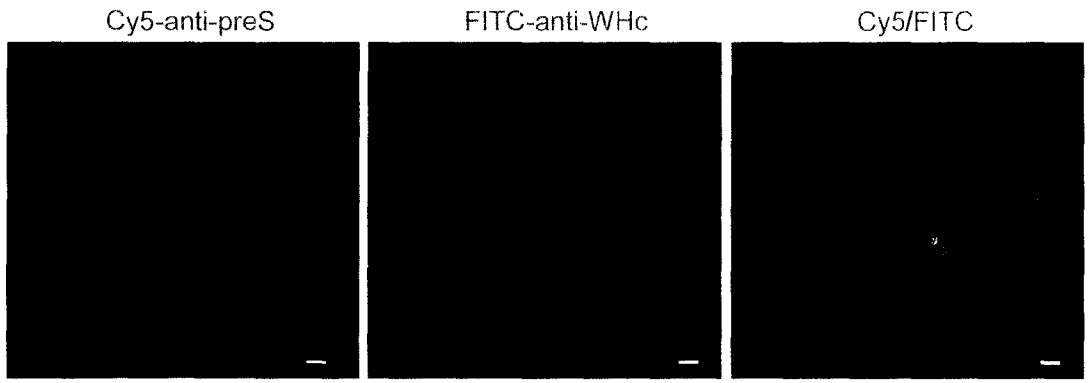


Figure 5.3C

occult infection which with time spreads to the liver, as described previously (Coffin and Michalak, 1999; Michalak *et al.*, 2004). Taken together, these findings showed that p-WHV was not only transcriptionally active in WCM-260 hepatocytes but also that infectious virions were produced. However, due to the relatively small number of hepatocytes displaying WHV antigens after transient transfection ($\leq$ 20% by immunofluorescent staining), it was concluded that the use of the stably transfected hepatocytes, which all *a priori* express viral genome, would be better to examine WHV influence on class I MHC expression.

5.4.2 Validation of plasmids encoding individual WHV proteins.

To assess which of the translation products of the WHV genome may modify class I MHC expression, WCM-260 hepatocytes were transfected with plasmids carrying WHV sequences encoding for WHpS1, WHpS2, WHmS, WHc or WHx protein. As illustrated in Figure 5.4A, when total RNA extracted from WCM-260 transiently transfected with the above plasmids was analyzed with appropriate specific primer pairs, the resulting RT-PCR amplicons showed the same molecular sizes as those obtained by amplifying cDNA derived from RNA extracted from the liver of a woodchuck with chronic WHV hepatitis (Fig. 5.4A). The signals were detected only in RNA samples subjected to RT but not in those in which the RT step was omitted, as confirmed by Southern blot hybridization analysis (Fig. 5.4A). These results showed that the genes encoding for individual WHV proteins were successfully transcribed in WCM-260

Figure 5.4. Validation of the expression of the plasmid constructs carrying WHV subgenomic sequences encoding individual virus proteins. WCM-260 hepatocytes (A) transiently transfected or (B) stably transfected with p-WHpS1, p-WHpS2, p-WHmS, p-WHc or p-WHx were analyzed for presence of relevant mRNA by RT-PCR using primer pairs specific for preS1 (S1), preS2 (S2) or mS region of S gene or for C or X gene. The amplicons were identified by Southern blot hybridization by probing with rWHV DNA. For other details see the legend to Figure 5.3.

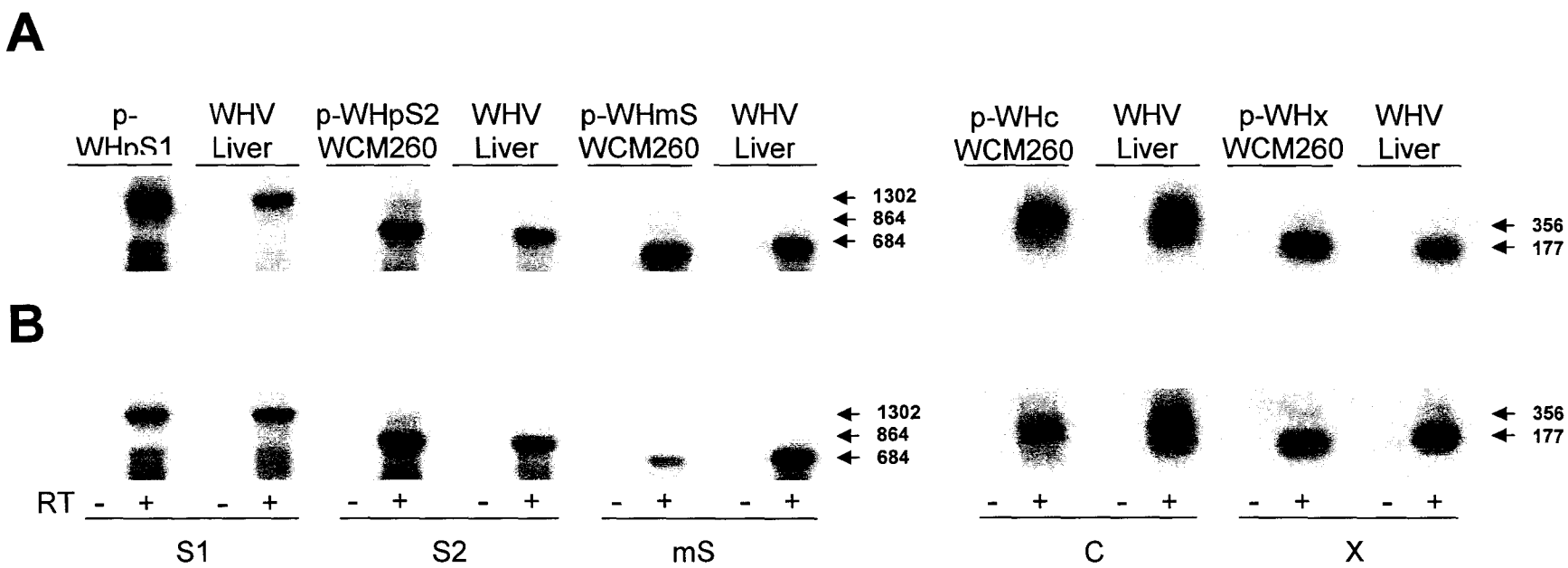

A
p-WHpS1
WHV Liver
p-WHpS2 WCM260
WHV Liver
p-WHmS WCM260
WHV Liver
p-WHc WCM260
WHV Liver
p-WHx WCM260
WHV Liver
1302
864
684
356
177
B
1302
864
684
356
177
RT - + - + - + - + - + - + - + - + - + - +
S1
S2
mS
C
X

Figure 5.4

hepatocytes. The same was found to be true for WCM-260 stably transfected with the above plasmids (Fig. 5.4B).

Similarly as it was found for the cells transfected with p-WHV (see Fig. 5.3C), WCM-260 transiently transfected with p-WHc displayed cytoplamic localization of WHcAg in 10% to 20% of the cells. WHsAg was detected in a similar proportion of the cells after transfection with either p-WHpS1, p-WHpS2 or p-WHmS (data not shown). The display of WHx protein was not examined due to the lack of an appropriate antibody. The WCM-260 cells stably transfected with the same plasmid constructs were seemingly WHcAg and WHsAg non-reactive by immunostaining, although they were positive when analyzed by Western blotting (data not shown). This suggested that the antigens were expressed but at the levels which were below the detection limit of the immunofluorescent method applied. In subsequent experiments, it was clearly established that these expression levels were sufficient to exert a consistent modifying effect on class I MHC antigen presentation.

5.4.3 Expression of the complete WHV genome and its subgenomic sequences encoding for preS1 and preS2 proteins inhibits hepatocyte class I MHC antigen display.

Stable transfection of WCM-260 cells with p-WHV resulted in a very significant (20-35%; $P = 0.004$) suppression of the surface class I MHC antigen display, as determined by flow cytometry and compared to the control WCM-260 cells transfected with empty pC-DNA3.1 vector (Fig. 5.5A) or to naive WCM-260

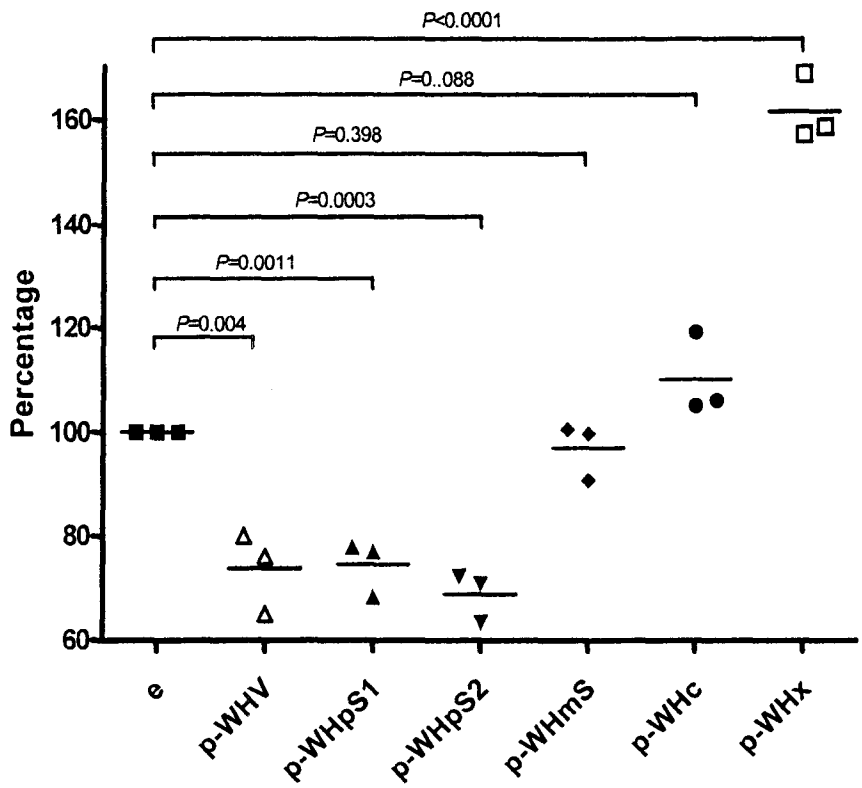
cells (data not shown). Similarly, WCM-260 transfected with WHV sequences encoding for WHpS1 or WHpS2 protein showed a meaningful inhibition of the surface display of class I antigen (22-36%; $P = 0.001$ and $P = 0.0003$, respectively). In contrast, WCM-260 cells transfected with p-WHmS or p-WHc did not show a noticeable change in the class I antigen expression level (Fig. 5.5A). Interestingly, transfection with p-WHx led to a dramatic upregulation (>50%; $P = 0.0001$) in the class I antigen presentation (Fig. 5.5A). Figure 5.5B illustrates the detected shifts in the class I MHC heavy chain fluorescence on WCM-260 cells transfected with p-WHV, p-WHpS1 p-WHpS2 or p-WHx, as well as after transfection with other plasmids when compared to the cells transfected with the empty vector. Overall, the data revealed that while the expression of whole WHV genome downregulated class I MHC antigen display on WCM-260 hepatocytes, expression of its individual proteins had a variable modifying effect from the inhibition exerted by WHpreS1 and WHpres2, through the lack of a noticeable effect in the case of WHmS and WHc, to the considerable augmentation in the class I antigen presentation when the cells were transfected with p-WHx.

5.4.4 Effects of WHV genome site-specific mutations on hepatocyte class I MHC antigen presentation.

To determine whether individual WHV translation products which were found to be capable of modifying hepatocyte class I MHC antigen display when expressed alone would yield a similar effect when expressed in the

Figure 5.5. Modulation of the class I MHC antigen display on WCM-260 hepatocytes stably transfected with p-WHV or with plasmid constructs encoding individual WHV proteins. (A) The class I antigen presentation on WCM-260 hepatocytes stably transfected with p-WHV, p-WHpS1, p-WHpS2, p-WHmS, p-WHc or p-WHx was assessed by flow cytometry by comparing the means (horizontal short lines) of MFI values obtained from three independent experiments. The data are presented as the percentages where the MFI values given by WCM-260 stably transfected with empty pC-DNA3.1 vector (e) were taken as 100%. *P* values were determined as indicated in Materials and Methods. (B) A representative example of flow cytometry histograms illustrating the display of the class I antigen on WCM-260 hepatocytes from one of three experiments shown in panel A. The levels of the class I antigen were determined on the hepatocytes prior to (IFNγ -) and after (IFNγ +) treatment with rwIFNγ, as described in Materials and Methods. The data are shown as overlayed histograms representing the cells transfected with plasmids carrying WHV sequences (open) and the cells transfected with empty vector (filled).

A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P=0.398
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P=0.0011
P=0.004
Percentage
160
140
120
100
80
60
e
p-WHV
p-WHpS1
p-WHpS2
p-WHmS
p-WHc
p-WHx

Figure 5.5A

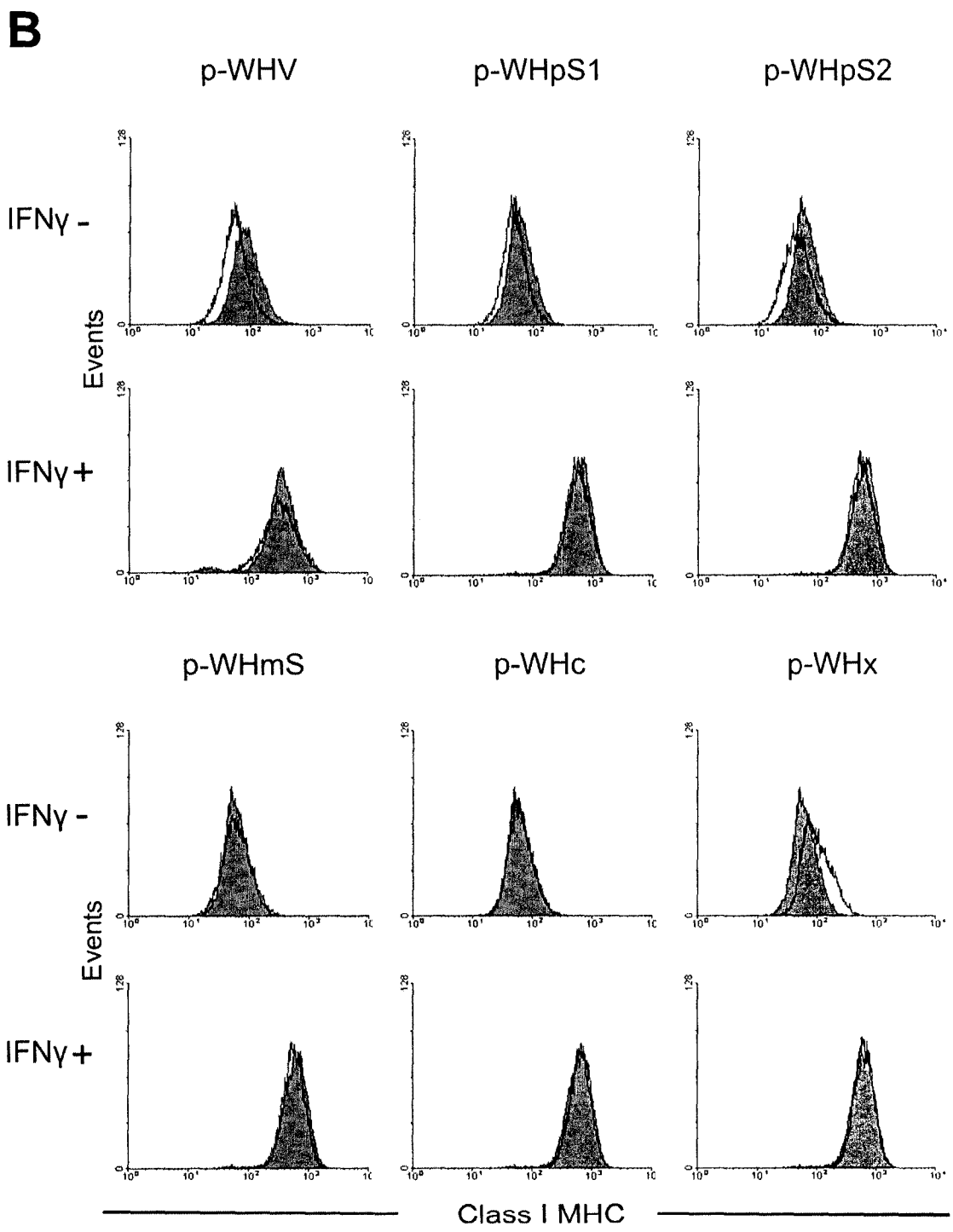

B
p-WHV
p-WHpS1
p-WHpS2
IFNγ -
IFNγ +
Events
p-WHmS
p-WHc
p-WHx
IFNγ -
IFNγ +
Events
Class I MHC

Figure 5.5B

context of the complete WHV genome, WCM-260 hepatocytes were transfected with p-WHV mutants in which transcription of selected genes was blocked by premature stop codons (see Fig. 5.2). As far as the availability of specific antibodies allowed, transcription of individual WHV proteins or their lack and the fact that the introduced mutations did not influence the expression of virus envelope and core proteins was confirmed by immunofluorescence staining (Fig. 5.6). Thus, WCM-260 cells transfected with p-ΔWHc and stained with anti-WHc were WHcAg negative, while those transfected with plasmids carrying mutated ORFs other than C ORF were WHcAg reactive (Fig. 5.6), as hepatocytes transfected with p-WHc shown in Fig. 5.3C. Similarly, preS1/preS2 antigenic reactivity was detected after staining with anti-preS antibodies in WCM-260 transfected with p-ΔWHc, p-ΔWHpS1, p-ΔWHx and p-ΔWHp, but not in those transfected with p-ΔWHmS (Fig. 5.6). It is important to emphasize that anti-preS antibodies recognize both WHpreS1 and WHVpre2 proteins, but not WHmS (see Materials and Methods). Therefore, WCM-260 transfected with p-ΔWHpS1 should be reactive, as they were, since expression of WHpreS2 was not affected. These findings not only verified that WHcAg and WHsAg synthesis was abrogated in WCM-260 cells transfected with p-ΔWHc and p-ΔWHmS , but also implied that the absence of a single protein in the context of expression of the whole WHV genome in our system was without a major effect on the synthesis of other virus proteins.

Figure 5.6. Determination of the competence of p-WHV mutants with the disabled expression of selected structural or nonstructural proteins for encoding of virus envelope and core proteins. WCM-260 hepatocytes transiently transfected with p-ΔWHpS1, p-ΔWHmS, p-ΔWHc, p-ΔWHx or p-ΔWHp were probed with anti-WHV preS or with anti-WHc by immunostaining, as described in Materials and Methods and in the legend to Figure 5.3. Bars represent 20 μM.

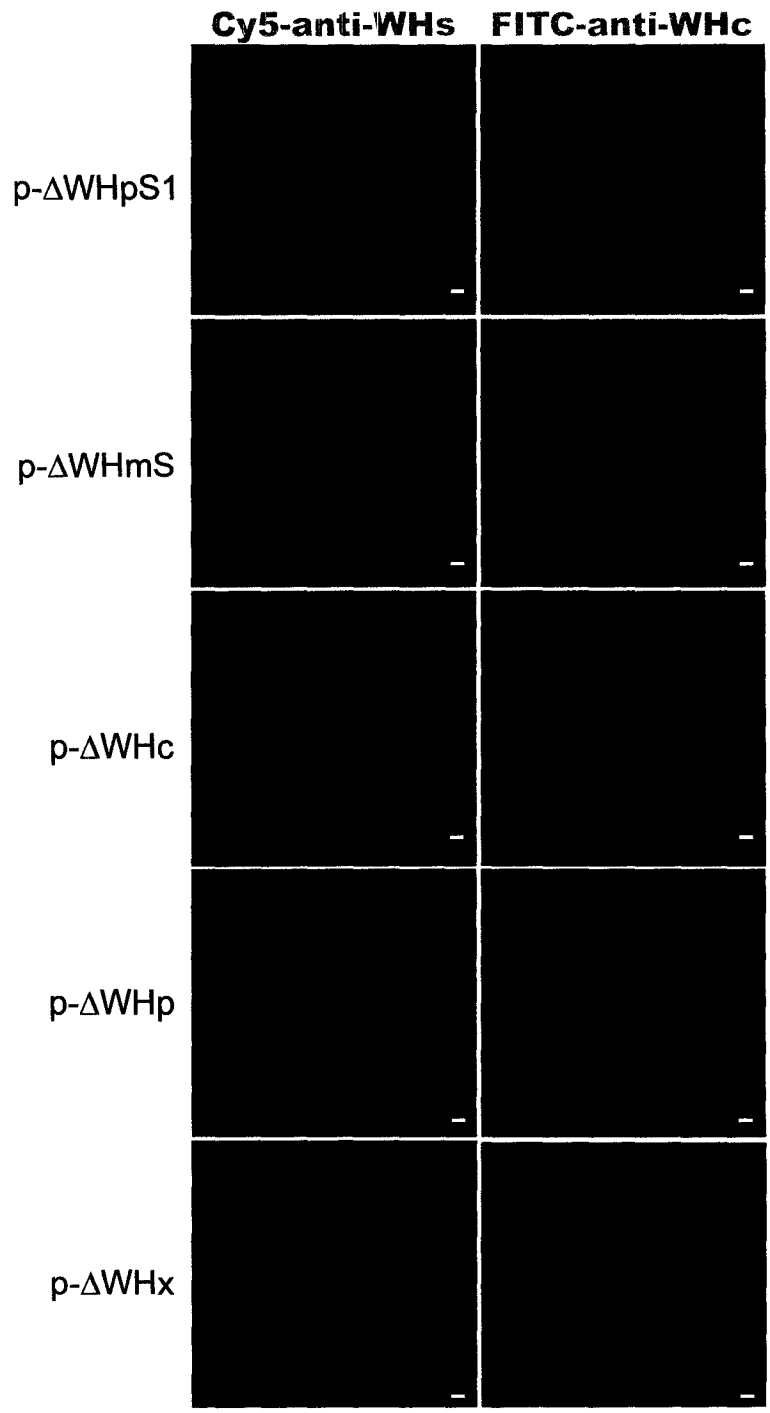
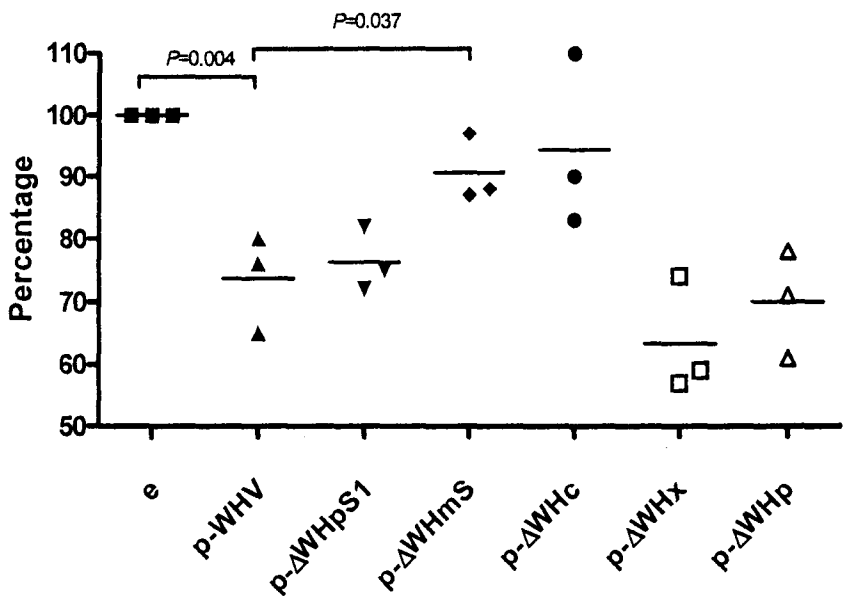


Figure 5.6

As shown in Figure 5.7A, the introduction of the premature stop codon in the WHmS-encoding region, which abrogated expression of not only WHmS but also WHpS1 and WHpS2 proteins (see Fig. 5.2), resulted in a significant enhancement ($P < 0.05$) in the class I MHC surface presentation over that on hepatocytes transfected with p-WHV. On the other hand, blocking of the expression of WHx led to a profound suppression in the class I antigen display when compared with the control cells transfected with the empty vector ($P = 0.0024$). Overall, the above results were in good agreement with the findings from WCM-260 hepatocytes transfected with plasmids encoding for WHpS1, WHpS2 or WHx (see Fig. 5.5A). Furthermore, when WCM-260 cells transfected with p-ΔWHpS1, which contained the stop codon disabling transcription of WHpreS1 but still carried functional intrinsic start codons for WHpreS2 and WHmS, were analyzed, it was found there was no change in the class I antigen display when compared to the cells transfected with p-WHV (Fig. 5.7A). This finding strengthened the notion that WHpreS2 protein was mainly, if not entirely, responsible for the observed inhibition of the class I antigen on WCM-260 transfected with p-WHV. Further, transfection with p-ΔWHp was without any statistically significant effect on the class I antigen staining. Finally, although the mean expression of class I antigen on WCM-260 transfected with p-ΔWHc was somehow enhanced compared to the cells transfected with p-WHV, the difference was not statistically significant and it was likely related to a variation in

Figure 5.7. The class I MHC antigen display on WCM-260 hepatocytes transfected with p-WHV mutants in which expression of selected viral proteins was disabled by introduction of appropriate premature stop codons. (A) Hepatocytes stably transfected with p-ΔWHpS1, p-ΔWHmS, p-ΔWHc, p-ΔWHx or p-ΔWHp or with p-WHV or empty vector (e), as controls, were assessed for class I antigen surface presentation by flow cytometry, as described in the legend to Figure 5.5 and in Materials and Methods. (B) A representative example of flow cytometry histograms demonstrating the class I antigen display on WCM-260 hepatocytes transfected with different p-WHV mutants from one of three experiments depicted in panel A prior to (IFNγ -) and after (IFNγ +) treatment with rwIFNγ. For further details see the legend to Figure 5.5 and Materials and Methods.

A


P=0.037
P=0.004
110
100
90
80
70
60
50
Percentage
e
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p-ΔWHmS
p-ΔWHc
p-ΔWHx
p-ΔWHp

Figure 5.7A

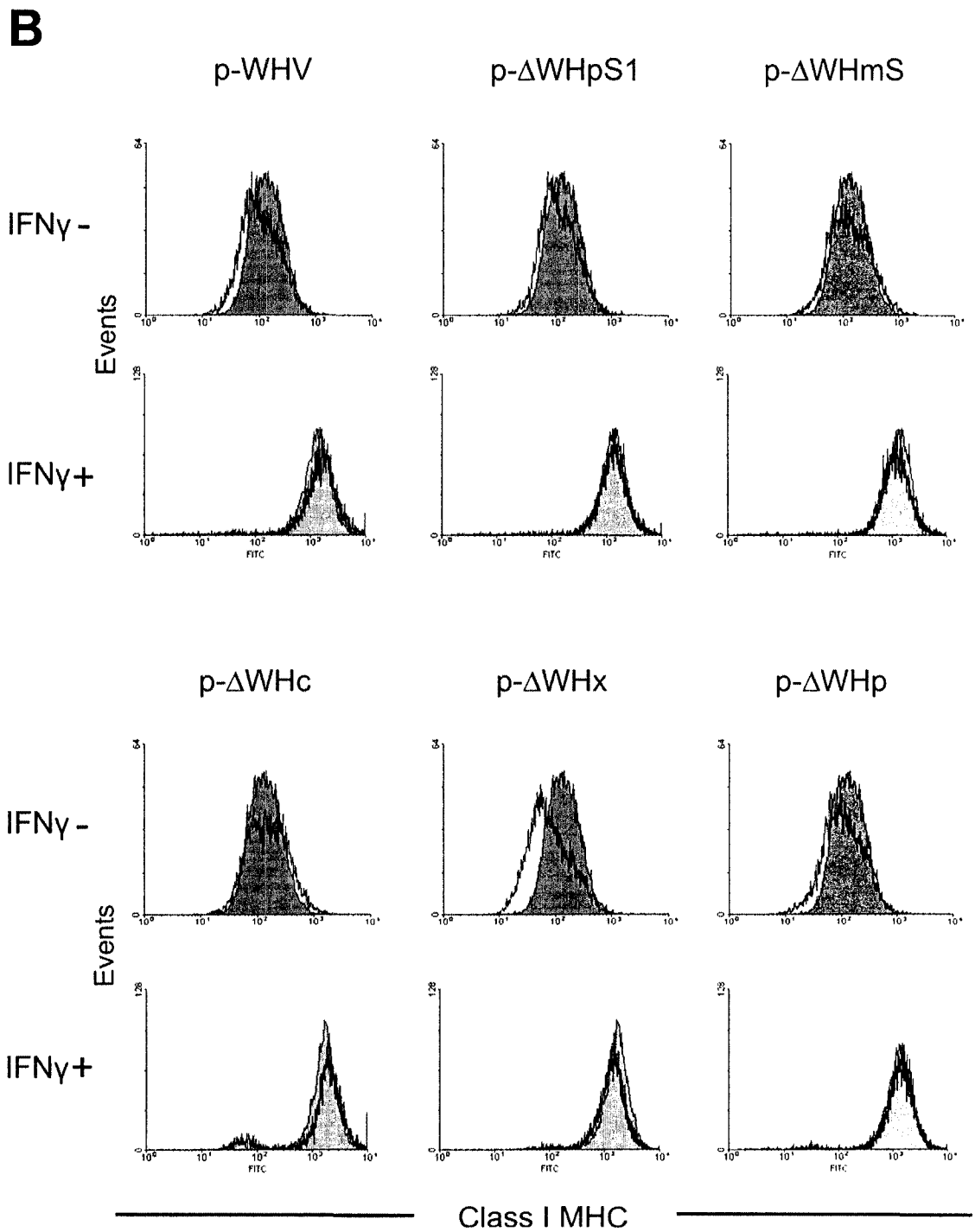

B
p-WHV
p-ΔWHpS1
p-ΔWHmS
IFNγ -
IFNγ+
Events
FITC
p-ΔWHc
p-ΔWHx
p-ΔWHp
Class I MHC

Figure 5.7B

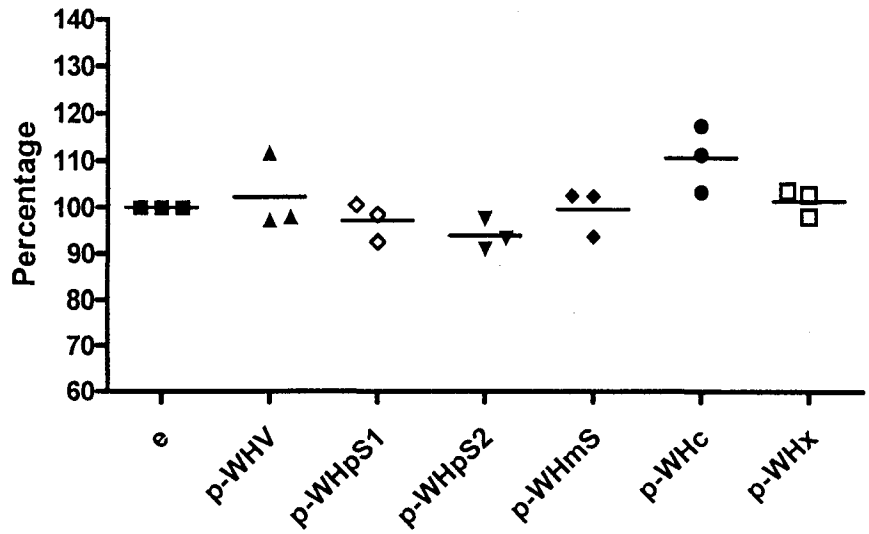
one of the experiments. Figure 5.7B shows examples of the changes in the class I antigen surface presentation detected on WCM-260 hepatocytes transfected with different p-WHV mutants.

5.4.5 Inhibition of class I MHC antigen presentation by WHV can be fully restored by treatment with IFN-γ.

Exposure of WCM-260 hepatocytes transfected with p-WHV or with either p-WHpS1 or p-WHpS2 to exogenous rwIFNγ led to upregulation in the class I MHC antigen to the level compatible with that detected on WCM-260 transfected with empty pC-DNA3.1 or on naive WCM-260 cells following their treatment with rwIFNγ (Figs 5.8A and 5.5C). It is of note that the observed augmentation in the class I antigen induced by rwIFNγ was significantly greater (11.7 $\pm$ 1.6 fold *vs.* 1.6 $\pm$ 0.06 fold; $P = 0.01$) than that mounted by transfection of hepatocytes with p-WHx. Also, treatment with rwIFNγ of WCM-260 hepatocytes expressing mutated p-WHV sequences, including p-ΔWHx, led to a comparable increase in the class I MHC antigen surface display (Figs 5.8B and 5.7C). Taken together, the above experiments clearly showed that the inhibition of class I MHC antigen on cultured hepatocytes expressing whole WHV genome or its fragments encoding particular proteins was not permanent but can be reversed by treatment with exogenous IFN-γ.

Figure 5.8. The display of class I MHC antigen on the surface of WCM-260 hepatocytes transfected with different WHV expression constructs following treatment of the cells with IFNγ. (A) Hepatocytes stably transfected with p-WHV, p-WHpS1, p-WHpS2, p-WHmS, p-WHc or p-WHx or (B) with p-ΔWHpS1, p-ΔWHmS, p-ΔWHc, p-ΔWHx or p-ΔWHp and control cells transfected with empty pC-DNA3.1 vector (e) or with p-WHV were treated with rwIFNγ prior to staining for class I antigen with B1b.B9 mAb, as outlined in Materials and Methods. It is of note that the treatment with rwIFNγ led to the unified augmentation in the class I antigen display to approximately the same level on hepatocytes transfected with different WHV DNA constructs and on the control cells. The data should be interpreted in the context of those shown in Figures 5.5 and 5.7.

A


Percentage
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130
120
110
100
90
80
70
60
e
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p-WHpS1
p-WHpS2
p-WHmS
p-WHc
p-WHx

B

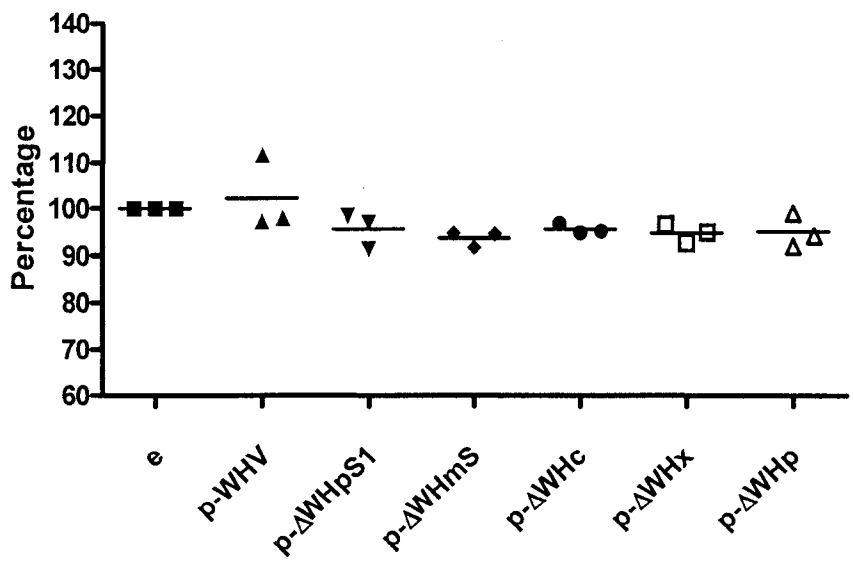
Percentage
140
130
120
110
100
90
80
70
60
e
p-WHV
p-ΔWHpS1
p-ΔWHmS
p-ΔWHc
p-ΔWHx
p-ΔWHp

Figure 5.8

5.4.6 WHV-induced inhibition of class I antigen presentation is not due to suppressed transcription of class I MHC- or proteasome-linked genes.

Quantitation of the mRNA levels of the class I MHC-related genes (*i.e.*, class I MHC heavy chain, β2m, TAP1 and TAP2) and the genes encoding subunits of immunoproteasome (*i.e.*, LMP2, LMP7, MECL1, PA28α and PA28β) revealed no meaningful differences between WCM-260 hepatocytes transfected with p-WHV or with sequences encoding for individual virus proteins of the virus and naive WCM-260 cells or those transfected with empty vector (Fig. 5.9). Similarly, there was no noticeable variation in the transcription level of these genes between WCM-260 cells transfected with p-WHV or p-WHV mutants and the control cells (data not shown). Taken together, the results revealed that the inhibited or augmented presentation of the class I MHC antigen on WCM-260 cells transfected with WHV sequences identified in this study as those capable of modifying the class I antigen display was not connected to the transcriptional activity of the genes examined. In a supplementary experiment, analysis of the class I MHC- and proteasome-linked gene mRNA levels in hepatocytes transfected with p-WHV, their mutants or WHV subgenomic fragments and in control hepatocytes following treatment with wrIFNγ showed the genes' transcriptional activity was upregulated to approximately the same extent in all hepatocytes tested (Fig. 5.9 and data not shown).

Figure 5.9. Quantitation of the class I MHC- and proteasome-linked genes expression in hepatocytes transfected with p-WHV and with constructs encoding for individual WHV proteins and subjected or not to treatment with IFNγ. The data were generated in three independent experiments by real time RT-PCR. They are presented as the mean gene copy numbers per reaction which were calculated using serial 10-fold dilutions of internal plasmid standards and normalized against house-keeping woodchuck β-actin cDNA level. The open bars represent hepatocytes not treated with rwIFNγ, while the stratified bars those treated with the cytokine.

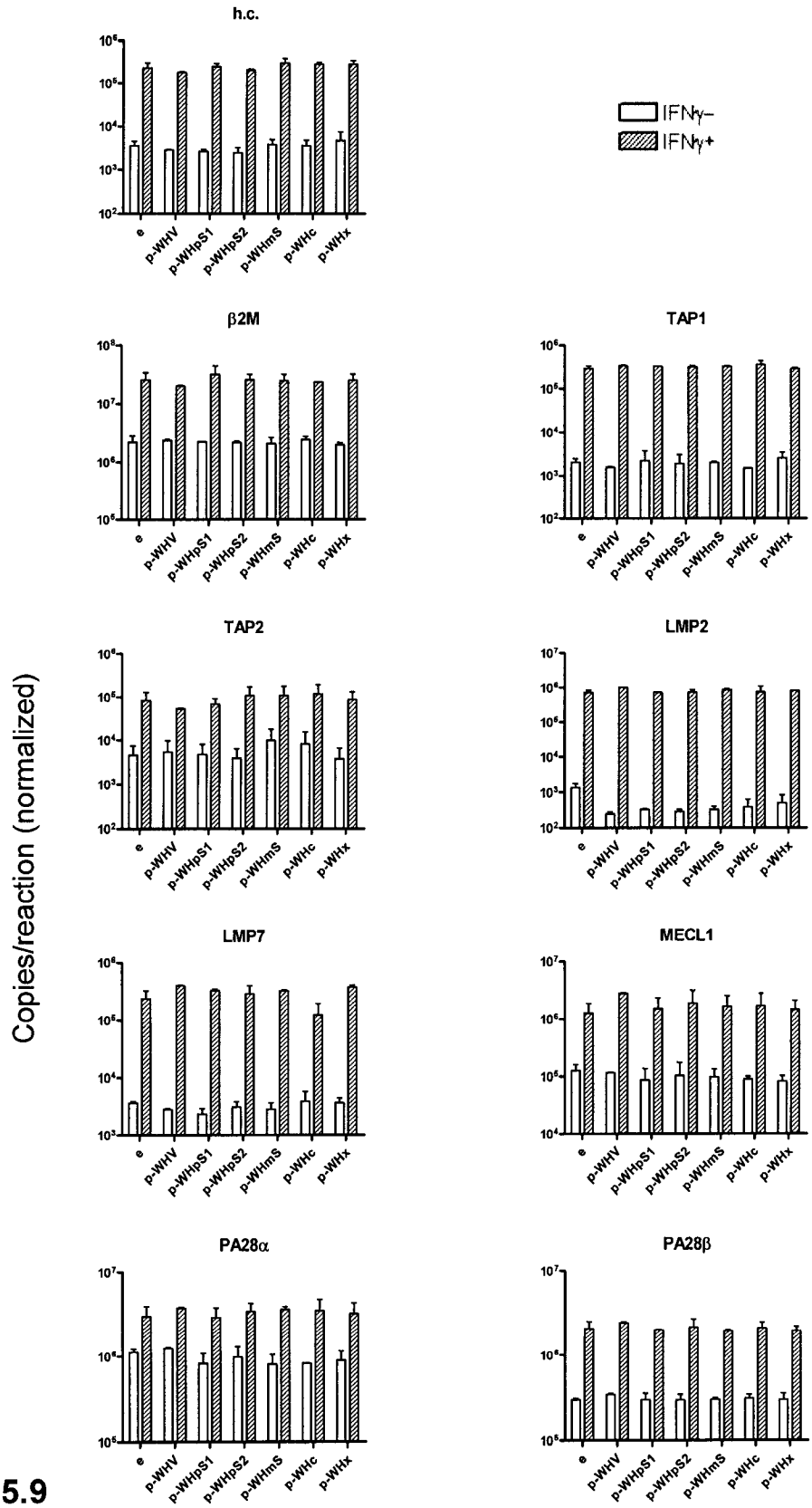
h.c.
IFNγ-
IFNγ+
β2M
TAP1
TAP2
LMP2
LMP7
MECL1
PA28α
PA28β
Copies/reaction (normalized)
e
p-WHV
p-WHpS1
p-WHpS2
p-WHmS
p-WHc
p-WHx

Figure 5.9

5.5 Discussion

To investigate the impact of WHV expression on the class I antigen presentation on the hepatocyte surface, we generated a panel of woodchuck hepatocyte lines transcribing complete WHV genome, subgenomic fragments coding individual viral proteins. In addition, we produced WHV genome mutants in which transcription of selected products in the context of expression of an otherwise complete genome was disabled by site-specific mutagenesis. The transcriptional activity of individual virus genes or their fragments was confirmed by determining the presence of relevant gene-specific mRNAs and, when the availability of specific antibodies allowed, expression of viral proteins. The data acquired showed that the stable transfection with complete WHV genome consistently resulted in a significant suppression of the class I MHC antigen on the hepatocyte surface, although no virus antigens could be visualized by immunofluorescence using available antibodies and relatively small amounts of virions were apparently assembled when hepatocyte supernatants were examined for infectivity by injecting into a naive woodchuck.

Importantly, the level of class I antigen inhibition on hepatocytes transfected with the whole WHV genome was comparable to that identified on the cells expressing alone either WHpreS1 or WHpreS2. In contrast, transfection with p-WHmS did not alter the antigen display when compared to the intact hepatocytes or those stably transfected with empty plasmid vector. Taking under consideration that all three envelope proteins are encoded within the single S

ORF, which has three in-frame translation initiation codons (see Fig 5.1), and that all of them share the same C-terminal portion while differing in the N-terminal amino acid sequences, the above finding implied that the sequence shared by both WHpreS1 and WHpreS2, hence most likely located within WHpreS2, was responsible for the inhibitory effect observed. The analysis of hepatocytes transfected with the complete WHV genome in which expression of WHpreS1 was selectively disabled, provided supporting evidence which helped to resolve this issue. Thus, transfection with p-ΔWHpS1, which was unable to translate WHpreS1 due to the stop codon introduced into the extreme 5'-terminal portion of the S gene (see Fig. 5.2), but was still able to translate WHpreS2 from the downstream initiation site, did not restore suppressed class I antigen display. This indicated again that the preS2 segment of the WHpreS1 protein plays a critical role in class I antigen suppression. Furthermore, the data from the transfection experiments with p-ΔWHmS, which was unable to initiate translation of WHmS, showed that the hepatocyte class I antigen display was restored almost to the same level as that displayed by hepatocytes transfected with empty vector. Because the C-terminal portion of both WHpreS1 and WHpreS2 proteins is constituted by WHmS, this suggests that the complete intact sequence of WHpreS2 protein is required to act as a class I antigen inhibitor. Taken together, this differential analysis provided cumulative evidence that suppression of the class I antigen on WCM-260 hepatocytes was mediated by WHpreS2 protein.

Remarkably, but not unexpectedly, transfection with p-WHx induced a dramatic increase in hepatocyte class I antigen display. X protein of

hepadnaviruses is known to be a promiscuous transactivator capable of promoting expression of a variety of viral and cellular genes, including those involved in control of cell growth, and can interact with cellular organelles, such as mitochondria and proteasomes, and modify their functions (Bouchard *et al.*, 2001; Diao *et al.*, 2001; Fischer *et al.*, 1995; Hu *et al.*, 1999; Zhang *et al.*, 2000). In regard to HBV X and class I MHC expression, it has been reported that transfection of the same cells with HBV X gene together with a reporter plasmid driven by class I MHC promoter led to augmented expression of luciferase reporter, suggesting that X protein was responsible for this event (Zhou *et al.*, 1990). This result seems to corroborate with the findings in our study, although significantly different experimental approaches were used. It has also been shown by employing a two-hybrid system that HBV X protein can interact with PSMA7 and PSMA1 of the proteasome complex (Fischer *et al.*, 1995; Hu *et al.*, 1999; Zhang *et al.*, 2000). It was postulated that this interaction may modify the function of both X protein and immunoproteasome. However, the effect of these expected modifications of the class I MHC antigen presentation was not characterized in these studies.

The observed diametrically opposed effects on class I MHC presentation following transfection with p-WHpS1 or p-WHpS2 or with p-WHx and the fact that the expression of the whole WHV genome strongly inhibited the antigen display suggest that a highly complex interplay between individual virus products is involved in suppression of the class I antigen. Thus, if we assume that, in the context of expression of the complete virus genome, both WHpreS2 and WHx

exerted their respective inhibitory or stimulatory action to the same degrees as those identified on hepatocytes transfected with the sequences encoding WHpreS2 or WHx alone, this may suggest that WHpreS2 is a very potent class I antigen inhibitor which is able not only to counteract the stimulating effect of WHx but also to suppress the antigen significantly below the level displayed on intact hepatocytes. This intriguing possibility will require further examination, for example by co-transfecting cells with both p-WHpS2 and p-WHx. It also remains to be established whether the same event occurs in naturally infected hepatocytes.

Exposure to rwIFNγ completely restored the class I antigen presentation on hepatocytes transfected with the whole WHV genome, with gene sequences encoding for WHpreS1 and WHpreS2 or with WHV DNA with an introduced stop codon disabling transcription of the preS1 region. These findings demonstrated that the inhibition of class I antigen was reversible in all hepatocyte lines in which defective class I antigen display was detected. This supports the notion that intrahepatic induction of IFNγ could not only suppress virus replication, as it has been shown for HBV in a transgenic mouse model (Guidotti *et al.*, 1996b), but also enhance class I MHC expression on chronically infected hepatocytes, promoting their recognition by virus-specific CTL. This is further supported by the results from the present study showing that treatment with IFNγ was highly effective in augmenting expression of the genes encoding different components of the endocytic antigen presentation pathway and proteasome subunits in

hepatocytes with suppressed class I antigen display. Therefore, treatment with this cytokine or with functionally equivalent agents may enable hepatocytes in chronic hepadnaviral infection to process and present viral peptides more efficiently in the context of class I antigen and, in consequence, increase their accessibility for recognition by specific CTL. Furthermore, as a result of the increased expression of IFNγ-inducible proteasome subunits, the assembly of immunopreoteasomes might also be enhanced, which has been shown to be required for the generation of certain HBV-specific CTL epitopes (Sijts *et al.*, 2000).

The precise molecular mechanism of WHpreS2-mediated suppression of the class I MHC antigen remains to be determined. However, as the quantitative analysis of the mRNA levels of several class I MHC- and proteasome-affiliated genes revealed, the class I antigen inhibition was not related to hepatocyte downregulated transcription of any of the genes examined. This suggests that the suppression has to be due to WHpreS2-dependent posttranscriptional interference. This conclusion is consistent with the findings from the previous study where, based on the analysis of hepatocytes naturally infected with WHV derived from woodchucks with chronic hepatitis, a virus-dependent posttranscriptional event was suspected as a reason behind the impaired hepatocyte presentation of class I molecules (Michalak *et al.*, 2000).

The present study provides experimental evidence that the envelope preS2 protein of WHV is functioning as a suppressor of class I MHC antigen in

hepatocytes actively transcribing virus genome. Identification of the translation product responsible for a defect in class I antigen presentation on hepatocytes, which are the main targets of hepadnavirus invasion and the fount of clinical symptoms, advances our understanding of how hepadnaviruses may subvert the host immune system, persist, and uphold protracted liver damage. Moreover, this study demonstrates that IFNγ can completely reverse the virus-induced defect in the class I antigen presentation. This provides an indication of how the host immune system, either intrinsically or due to exogenous stimulation, may overcome this deficiency in the course of chronic hepadnaviral infection.

CHAPTER 6. BICISTRONIC WOODCHUCK HEPATITIS VIRUS CORE AND GAMMA INTERFERON DNA VACCINE CAN PROTECT FROM SEROLOGICALLY EVIDENT INFECTION AND HEPATITIS BUT DOES NOT ELICIT STERILIZING ANTIVIRAL IMMUNITY

6.1 Summary

The immunity elicited against the nucleocapsid of hepatitis B virus (HBV) and closely related woodchuck hepatitis virus (WHV) has been shown to be important in resolution of hepatitis and protection from infection. Further, the activity of gamma interferon (IFN-γ), which may directly inhibit hepadnavirus replication, promotes antiviral defense and favors T helper cell type 1 (Th1) response, which is seemingly a prerequisite for HBV clearance. In this study, to enhance induction of protective immunity against hepadnavirus, healthy woodchucks were immunized with a bicistronic DNA vaccine carrying WHV core (WHc) and woodchuck IFN-γ (wIFNγ) gene sequences. Three groups, of 3 animals each, were injected once or twice with 0.5 mg, 0.9 mg or 1.5 mg per dose of this DNA vaccine. In addition, 4 animals received twice 0.6 mg or 1 mg WHc DNA alone. All animals were challenged with WHV. The results showed that 4 of 9 animals injected with the bicistronic vaccine and one of 4 immunized with WHc DNA became protected from serologically evident infection and hepatitis. This protection was not linked to induction of WHcAg-specific antibodies or a T cell proliferative response, and was not associated with enhanced transcription of Th1 cytokines or 2',5'-oligoadenylate synthetase.

Strikingly, all animals protected from serologically positive infection and hepatitis became reactive for WHV DNA and carried low levels of replicating virus in hepatic and lymphoid tissues after challenge with WHV. This study shows that the bicistronic DNA vaccine encoding for both hepadnavirus core antigen and IFN-γ was significantly more effective in preventing hepatitis than that encoding for virus core alone, but neither of them could mount sterile immunity against the virus.

6.2 Introduction

Hepatitis B virus (HBV) is one of the most common human pathogens that affects as a chronic, serum HBV surface antigen (HBsAg)-positive infection over 350 million people with an estimated 2 billion individuals exposed to the virus during their lifetime (Lee, 1997). The virus is the main causative factor of chronic hepatitis that frequently advances to cirrhosis and hepatocellular carcinoma (Chisari, 2000). It is now evident that HBV also elicits serologically silent, i.e., serum HBsAg-negative, infection which persists indefinitely after resolution of acute hepatitis B or due to primary asymptomatic exposure (Brechot *et al.*, 2001; Michalak *et al.*, 1994). This occult infection is associated with an increased risk of developing liver cancer (Pollicino *et al.*, 2004). It also became evident that HBV normally replicates in the lymphatic system, although less vigorously than in the liver (Lew and Michalak, 2001; Michalak, 2000; Michalak *et al.*, 1994). Studies of woodchucks (*Marmota monax*) infected with woodchuck hepatitis virus

(WHV), which represent the closest pathogenic animal model of hepatitis B, significantly contributed to recognition of these and other remarkable characteristics of hepadnaviral infection (reviewed in Tennant and Gerin, 2001). Among others, the woodchuck studies showed that WHV invariably invades the lymphatic system and persists for life independent of whether the infection is symptomatic or serologically undetectable and that exposure to low virus doses ($\leq 10^3$ virions) induces occult infection restricted to the lymphatic system which with time may engage the liver (Coffin and Michalak, 1999; Coffin *et al.*, 2004; Hodgson and Michalak, 2001; Lew and Michalak, 2001; Michalak *et al.*, 2004; Michalak *et al.*, 1999; Mulrooney and Michalak, 2003).

It is acknowledged that the immune responses play a pivotal role both in the pathogenesis and in the control of HBV infection (Chisari, 2000). Polyclonal cytotoxic T cell (CTL) and T helper type 1 (Th1) cell responses directed toward multiple antigenic epitopes of HBV are associated with resolution of acute hepatitis. This is preceded by intrahepatic upregulated expression of antiviral, Th1 cytokines, such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) (Guidotti *et al.*, 1996b; Guidotti *et al.*, 1999). In contrast, chronic hepatitis B is accompanied by a weak and narrowly focused HBV-specific T cell reactivity. It has been postulated, based on the data from other microbial infections (Boehm *et al.*, 1997), as well as chimpanzee and woodchuck models of hepatitis B (Guidotti *et al.*, 1999; Menne *et al.*, 2002b), that induction of a prevailing Th1 response and IFN-γ should be advantageous in the control of HBV infection. In this context, it has been shown that IFN-γ is highly effective in

steering T cell response toward Th1 type (Boehm *et al.*, 1997). In addition, IFN-γ strongly enhances class I and II major histocompatibility complex (MHC) expression, and hence augments antigen presentation by both endocytic and exocytic pathways (Boehm *et al.*, 1997; Fruh and Yang, 1999).

Purified HBV envelope particles or recombinant proteins carrying HBsAg reactivity have been applied as vaccines to prevent HBV infection, with overall efficacy of ~95% (Margolis *et al.*, 1995). However, their effectiveness is lower in elderly individuals, immunocompromised patients, and infants born to HBV-infected mothers (Sjogren, 2005), and breakthrough infections have been reported (McMahon *et al.*, 2005). Also, the HBsAg-based vaccines have a very limited or no beneficial effect in patients with chronic hepatitis B, who normally have poor anti-HBV humoral and cellular immune responses (Dienstag *et al.*, 1982; Jung *et al.*, 2002). Of note is that an attempt to augment an antibody response against WHV surface antigen (WHsAg) by inducing T helper type 2 cells in chronic WHV infection led to severe liver damage, particularly when high levels of antibodies to WHsAg (anti-WHs) were produced (Hervas-Stubbs *et al.*, 1997). This suggested that a similar approach to treatment of chronic hepatitis B would be futile.

The protective effect of immunization with HBV core antigen (HBcAg), an inner structural protein of the Dane particle, was first demonstrated in chimpanzees (Iwarson *et al.*, 1985; Murray *et al.*, 1984; Murray *et al.*, 1987). After injections with liver-derived or a recombinant HBcAg, antibodies against

HBcAg (anti-HBc) were induced and apparent complete protection from HBV was observed in some animals (Iwarson *et al.*, 1985; Murray *et al.*, 1984). A similar outcome has been seen in woodchucks immunized with native WHV core antigen (WHcAg) or recombinant core protein prior to WHV challenge (Roos *et al.*, 1989; Schodel *et al.*, 1993). The immunization prevented emergence of serologically evident infection in some woodchucks and the results suggested that priming of a specific Th1 type response, rather than antibodies to WHcAg (anti-WHc), could be important in establishing protective immunity.

Immunizations with DNA encoding for selected structural or nonstructural viral proteins have been shown to induce specific humoral and cellular immune responses (reviewed in Donnelly *et al.*, 2005). This is because the protein encoded by the delivered DNA is de novo synthesized and can enter both class I and class II MHC presentation pathways and prime CD8+ and CD4+ T cells simultaneously. The immune responses elicited by DNA vaccination have been shown to be protective against pathogens in a variety of experimental systems (Huygen *et al.*, 1996; Manickan *et al.*, 1995; Michel *et al.*, 1995; Sedegah *et al.*, 1994; Ulmer *et al.*, 1993; Wang *et al.*, 1993; Xiang *et al.*, 1994). Moreover, it was uncovered that increased efficacy of DNA vaccination and skewing toward a preferable T helper cell response can be achieved by co-delivery of plasmids encoding for molecules acting as adjuvants, such as cytokines, chemokines, and co-stimulatory factors (Calarota and Weiner, 2004; Chow *et al.*, 1998; Kwissa *et al.*, 2003; Lu *et al.*, 2005).

A number of DNA immunization protocols have been tested, with highly varying success, in animal models of hepadnaviral infection (Davis *et al.*, 1996; Lu *et al.*, 1999; Lu *et al.*, 2005; Michel *et al.*, 1995; Musacchio *et al.*, 2001; Pancholi *et al.*, 2001; Rollier *et al.*, 1999; Sallberg *et al.*, 1998; Thermet *et al.*, 2004; Triyatni *et al.*, 1998; Wild *et al.*, 1998). In woodchucks, to boost and to shift toward a preferable Th1 antiviral response, co-administration of plasmids encoding for WHcAg and IFN-γ (Siegel *et al.*, 2001) or interleukin-12 (IL-12) (Garcia-Navarro *et al.*, 2001), a cytokine promoting maturation of Th1 cells and production of IFN-γ, has been tested. As a result, it was shown that the protective immunity against WHV infection could be meaningfully enhanced over that induced by WHc DNA alone. However, protection was never achieved in all animals vaccinated and it remains unclear whether the protection was complete.

In the present study, in an attempt to enhance the effectiveness of the DNA vaccine encoding for WHcAg by inducing simultaneous production of IFN-γ to prime Th1 cell response and, at the same time, to fully control the co-delivery of the antigen- and the cytokine-encoding sequences, a bicistronic vector capable of concomitant expression of WHcAg and woodchuck IFN-γ (wIFNγ) was constructed. This expression vector was investigated in parallel with the plasmid encoding for WHcAg alone for the ability to mount complete (sterilizing) protection against the hepadnavirus. This was achieved by challenging the vaccinated woodchucks with a massive dose of WHV and by applying highly sensitive assays to detect WHV genomes and their replication. We also examined the ability of the bicistronic plasmid to elicit WHcAg-specific humoral

and T cell immune responses, and to upregulate expression of Th1 cytokines and IFN-α responsive 2',5'-oligoadenylate synthetase in peripheral lymphoid cells and hepatic tissue. We have found that immunization with the bicistrionic DNA was significantly more effective than that with WHc DNA alone in preventing WHV infection identifiable by serological assays. However, all animals who were free of serologically evident infection and hepatitis after vaccination had acquired low levels of replicating virus following challenge with WHV, indicating that the protection was not absolute. The reported findings raise a concern about the actual prophylactic potency of vaccination with DNA encoding for the internal protein of hepadnavirus. They also reveal that prevention against disease and protection from infection may not be synonymous in the case of hepadnaviral infection.

6.3 Material and Methods

6.3.1 Animals.

Fifteen healthy, adult woodchucks housed in the Woodchuck Hepatitis Research Facility at Memorial University, St. John's, Canada were used in this study. The possibility of prior exposure of the animals to WHV was excluded based on negative serological results for WHV infection markers, i.e., WHsAg and anti-WHc, and the absence of WHV DNA tested by a nested PCR/nucleic acid hybridization (PCR/NAH) assay (sensitivity, ≤10 virus genome equivalents [vge]/ml) using DNA from randomly selected serum, peripheral blood

mononuclear cell (PBMC), and liver biopsy samples, as described (Coffin *et al.*, 2004; Michalak *et al.*, 2004; Michalak *et al.*, 1999). Animal experimental protocols were approved by the Institutional Presidents' Committee on Animal Bioethics and Care.

6.3.2 Construction of pC-WHc and pWHc-wIFNγ expression vectors.

Full-length WHV core (WHc) gene sequence (567 bp in length; GenBank accession number J02442) (Galibert *et al.*, 1982) was amplified by PCR and inserted into the *EcoR*I site of the pCI-vector (Promega, Madison, WI). The integrity of the resulting plasmid, designated as pC-WHc, was confirmed by sequencing. To express simultaneously WHcAg and woodchuck IFN-γ (wIFNγ), a bicistronic vector containing both WHc gene and full-length wIFNγ cDNA was constructed. Briefly, wIFNγ cDNA was amplified with sense primer 5'-GCGC*GGATCC*ATGAAATACACAAGTTATTT and antisense primer 5'-GCGC*GGTACC*TTATTTGGATGCTCTCCGAC with incorporated *BamH*I and *Kpn*I sites (shown as underlined italics) using recombinant wIFNγ as template, which was previously generated in this laboratory (Wang and Michalak, 2005). Encephalomyocarditis virus internal ribosome entry site (IRES) sequence was amplified from pIRES2-EGFP vector (Clontech, Palo Alto, CA) with sense primer 5'-GGTGGGAGGTCTATATAAGCAGAGC and anti-sense primer 5'-GCG*AGATCT*GGTTGTGGCCATATTATCATCG with an inserted *Bgl*II site. The final construct, designated as pWHc-wIFNγ, was generated by sequentially and directionally inserting into pcDNA3.1 eukaryotic expression vector (Invitrogen,

Carlsbad, CA) full-length wIFNγ cDNA, then IRES, and finally WHc sequence released from pC-WHc by digestion with *EcoR*I restriction enzyme. The transcription of the WHc insert was driven by a cytomegalovirus immediate early promoter (PCMVIE) (see Fig. 6.1A). Large quantities of both pC-WHc and pWHc-wIFNγ plasmids were prepared using a giga-preps kit (Qiagen, Mississauga, Ontario) and re-suspended in phosphate-buffered saline, pH 7.4 (PBS) at 1 μg/μl for injections.

6.3.3 WHcAg and wIFNγ expression in transfected hepatocytes.

To assess competence of the constructed expression vectors, cultured woodchuck WCM-260 hepatocytes, previously derived and characterized in our laboratory (Diao *et al.*, 1998; Lew and Michalak, 2001), were transiently transfected with pC-WHc or pWHc-wIFNγ using Lipofectamine 2000 reagent (Invitrogen). Briefly, 1.5 x 10^5 WCM-260 cells were seeded on an 18-mm round glass cover slip in the well of a 12-well culture plate (Nunc, Rochester, NY). Next day, 1.6 μg of test plasmid and 4 μl of Lipofectamine 2000 reagent were first diluted separately in 100 μl of serum-free, antibiotics-free Dulbecco's modified Eagle medium (DMEM) (GIBCO BRL, Grand Island, NY) and then combined together. After incubation for 20 min at ambient temperature, the mixture was added to WCM-260 cell culture in which complete hepatocyte culture medium (Churchill and Michalak, 2004) had just been replaced with serum-free, antibiotics-free DMEM. After incubation in a humidified, 5% CO_2 atmosphere for

8 h at 37 °C, DMEM was substituted with complete hepatocyte culture medium and cells maintained for 48-72 h before evaluation.

WHcAg was detected in WCM-260 transfected with pC-WHc or pWHc-wIFNγ by immunofluorescence after staining with rabbit anti-WHc, produced as reported before (Michalak *et al.*, 1989). For this purpose, WCM-260 cells grown on the cover slip were fixed for 3 min with cold ethanol ether (Michalak *et al.*, 1989), exposed to rabbit anti-WHc for 30 min on ice, washed, and stained with goat anti-rabbit IgG conjugated with rhodamine (Jackson Immunoresearch Labs, Inc., West Grove, PA). Finally, the cover slip was mounted on a slide with 50% glycerol in PBS and examined with a Fluoview 300 confocal imaging system (Olympus America, Melville, NY).

Expression of wIFNγ was determined by assessing upregulation of the class I major MHC antigen in WCM-260 hepatocytes, which normally express very low levels of this antigen on their surface (Wang and Michalak, 2005). To simultaneously detect WHcAg and class I MHC antigen in WCM-260 hepatocytes transfected with the pWHc-wIFNγ construct, the cells, prepared and incubated with Rb-anti-WHc, as described above, were exposed for 30 min on ice to B1Bb9 monoclonal antibody (mAb) specific for the heavy chain of woodchuck class I MHC molecule. This mAb was previously prepared and characterized in this laboratory (Michalak *et al.*, 1995; Michalak *et al.*, 2000). Then, the cells were exposed to FITC-conjugated goat anti-mouse IgM/IgGs antibodies (Jackson Immunoresearch Labs, Inc.) for 30 min on ice and examined by confocal microscopy.

6.3.4 DNA immunization and WHV challenge.

In the first phase of the study, 4 healthy woodchucks were immunized twice with pC-WHc alone (Table 6.1; pC-WHc group). Two of the animals were injected with 0.6 mg and two with 1.0 mg of pC-WHc DNA per injection. As a control, one woodchuck was injected twice with 1 mg DNA of pCI empty vector. For each injection, half of the plasmid DNA was administered subcutaneously at 10-15 sites on the back and another half intramuscularly injected to the quadriceps of the hind leg at 3-5 sites under general anesthesia. Approximately two mo after the first immunization, a boost DNA injection with the same dose was given.

In a parallel experiment, 9 woodchucks were injected with the pWHc-wIFNγ DNA (Table 6.1; pWHc-wIFNγ group). The animals were divided into three subgroups each of which received 0.5 mg, 0.9 mg or 1.5 mg of the total plasmid DNA using the same mode of DNA administration as for the pC-WHc group. The pWHc-wIFNγ doses of 0.9 mg and 1.5 mg were calculated to contain DNA encoding for WHcAg at equivalent amounts to those contained in pC-WHc doses of 0.6 mg and 1.0 mg, respectively. Two months after primary immunization, a boost DNA injection with the same dose as the primary injection was given to selected woodchucks (see Table 6.1). After 2-3 mo, woodchucks belonging to pC-WHc and pWHc-wIFNγ groups were challenged by intravenous injection with WHV/tm2 inoculum at 1.1×10^{10} DNase-protected vge (GenBank accession number AY628096) (Coffin *et al.*, 2004). In addition, one woodchuck

received 1.3 mg DNA of wIFNγ plasmid (pC-wIFNγ) for each of the two injections prior to challenge.

6.3.5 Sample collection.

Sera were collected biweekly from the time of DNA immunization until 6 mo after challenge with WHV and then monthly. PBMC were obtained biweekly after DNA immunization and weekly following challenge with WHV for 2 months, then bi-weekly for 4 months, and monthly thereafter. The cells were isolated by density gradient centrifugation on Ficoll-Paque (Pharmacia Biotech, Baie d'Urfé, Quebec, Canada) as described elsewhere (Lew and Michalak, 2001). Liver biopsies were obtained by surgical laparotomy (Michalak *et al.*, 1999). The first biopsy was taken before DNA immunization, the second 6 or 7 weeks after challenge with WHV, and the third at autopsy. In some animals, the second biopsy was obtained 2 wks after the challenge, a third biopsy 6 wks later and a fourth at autopsy. At autopsy, in addition to liver tissue, samples of serum, PBMC and lymphatic organs were collected and preserved, following procedures described before (Hodgson and Michalak, 2001; Michalak *et al.*, 1999).

6.3.6 Serological and WHV DNA, cccDNA and mRNA detection assays.

Serum WHsAg, anti-WHc and anti-WHs were determined by specific enzyme-linked immunosorbent assays as described (Michalak *et al.*, 1999). For detection of WHV DNA in serum, PBMC, liver biopsies and in autopsy lymphatic and hepatic tissue samples, DNA was extracted by the proteinase K-phenol-

chloroform method (Michalak *et al.*, 1999). The isolated DNA was subjected to direct and, if required, nested PCR using WHV core gene-specific primers, conditions, and controls described previously (Michalak *et al.*, 1999). For detection of WHV cccDNA by specific PCR, mung bean nuclease treatment, primers and conditions previously established, were used (Lew and Michalak, 2001; Michalak *et al.*, 2004). The presence of WHV mRNA was examined using total RNA extracted with Trizol (Invitrogen). Samples were treated with DNase I following instructions of a DNA-free kit (Ambion, Austin, TX) before reverse transcription (RT) to cDNA (Coffin and Michalak, 1999; Michalak *et al.*, 1999). In all instances, DNA and RNA isolations were carried out in parallel with mock samples containing water instead of test nucleic acid samples. In addition, DNA from serum, or DNA or RNA from PBMC or liver tissue of a healthy animal and from a woodchuck with WHsAg-positive chronic hepatitis were included as negative and positive controls, respectively. In the case of RT-PCR, test RNA not transcribed in RT reaction was used to rule out potential carryover of WHV DNA (Hodgson and Michalak, 2001). Southern blot hybridization analysis of the amplified PCR products with ^{32}P-labeled complete recombinant WHV DNA was routinely used to verify the specificity and to enhance the sensitivity of the virus sequence detection, as described elsewhere (Lew and Michalak, 2001; Michalak *et al.*, 1999).

6.3.6 Real-time RT-PCR.

To quantify expression of wIFNγ and woodchuck TNF-α (wTNFα), IFN-α responsive 2',5'-oligoadenylate synthetase (wOAS), CD3ε (wCD3), β-actin and WHV RNA in the liver and PBMC, real-time RT-PCR assays were established using Light Cycler Faststart Master SYBR I kit (Roche Diagnostics, Laval, Quebec) and a LightCycler (Roche Diagnostics). Briefly, cDNA derived from approximately 25 ng of total RNA was used as template for each RT reaction. For PCR amplifications the following primer pairs were used: sense primer 5'-AGGAGCATGGACACCATCA and anti-sense primer 5'-CCGACCCCGAATCGAAG for wIFNγ, sense primer 5'-TGAGCACTGAAAGTATGATCC and anti-sense primer 5'-TGCTACAACATGGGCTACAG for wTNFα, sense primer 5'-TCAGGCAAAGGCACTACCC and anti-sense primer 5'-ACTTCTCTTTCGGACATGCT for wOAS, sense primer 5'-CTGGGACTCTGCCTCTTATC and anti-sense primer 5'-GCTGGCCTTTCCGGATGGGCTC for wCD3, and sense primer 5'-CAACCGTGAGAAGATGACC and anti-sense primer 5'-ATCTCCTGCTCGAAGTCC for woodchuck β-actin. WHV RNA was quantified using sense primer 5'-ATGCACCCATTCTCTCGAC and anti-sense primer 5'-TCAGGCAAAGGCACTACCC. Upon completion of PCR, a melting curve analysis was routinely carried out to determine the specificity of the amplicons. Ten-fold serial dilutions of respective recombinant gene cDNA fragments and

Light Cycler-quantification software (Roche Diagnostics) were used for enumeration of copy numbers or virus load in test samples. As controls, water instead of test nucleic acid and DNase-treated RNA samples not subjected to RT were included to rule out contamination or DNA carryover, respectively.

6.3.7 WHV-specific T cell proliferation assay.

WHV-specific T cell proliferation response was measured using isolated PBMC and [2-^{3}H]-adenine incorporation assay, as previously reported (Gujar and Michalak, 2005; Menne *et al.*, 1997a). As stimulators of WHV-specific T cell proliferation, recombinant WHV proteins produced in the pET41 *Escherichia coli* expression system (Novagen, Madison, WI), as previously described (Wang and Michalak, 2005), were used. Thus, recombinant WHV core protein (rWHcAg) was encoded by the sequence spanning nucleotides 2021 and 2584 of WHV genome, recombinant truncated WHcAg (1-149 amino acids) corresponding to the predicted partial sequence of WHV e antigen (rWHeAg) was encoded by nucleotides 2021 - 2467, and recombinant WHV X protein (rWHxAg) by nucleotides 1503 -1928 (numbers denote the positions of the nucleotides in WHV/tm3 genome according to Michalak et al. (GenBank accession number AY334075; Michalak *et al.*, 2004). Each protein was tagged with an octamer polyhistidine at the C-terminus to facilitate affinity purification (Wang and Michalak, 2005). In addition, a synthetic WHV core peptide, encompassing amino acids located in positions 97 to 100 of the virus nucleocapsid protein ($WHc_{97\text{-}110}$) and containing a WHV core T cell immunodominant epitope (Menne

et al., 1997b), was prepared (Synprep Corp., Dublin, CA). Also, native WHV envelope particles carrying WHsAg specificity, which were purified from plasma of a woodchuck with chronic hepatitis (Michalak *et al.*, 1990), were used as a T cell stimulator.

In brief, PBMC (1 x 10^5/well) were cultured in the presence of a recombinant WHV protein or WHsAg at 1 μg/ml and 2 μg/ml or with 2-fold serial dilutions of $WHc_{97\text{-}110}$ peptide ranging from 10 to 1.2 μg/ml. Five two-fold serial dilutions of Concanavalin A (ConA; Pharmacia Fine Chemicals, Uppsala, Sweden) ranging from 20 to 1.2 μg/ml, were included as positive controls and indicators of the natural T cell proliferation capacity. PBMC cultured in the absence of any stimulating antigen or ConA served as background controls. After 96 h culture, the cells were supplemented with 0.5 μCi of ^{3}H-adenine (Amersham Pharmacia Biotech, Piscataway, NJ) and incubated for an additional 12-18 h. Then, cells were harvested on a glass fibre filter membrane (Wallac Oy, Turku, Finland) using an automatic cell harvester (Harvester 96; Tomtec, Hamden, CT), and counts per minute (cpm) in each well were determined in a Topcount Scintillator (PerkinElmer, MA). All tests were performed in triplicates. The average cpm from readings of the triplicates, containing either stimulated or control cells, were calculated to define mean cpm values. The stimulation index (S.I.) was calculated by dividing mean cpm obtained after stimulation with test antigen or mitogen by mean cpm detected in the absence of a stimulant. An S.I. value equal to or above 3.1 was considered as positive (Gujar and Michalak, 2005).

6.3.8 Liver histology.

Histological examination of liver tissue samples was done after routine processing to paraffin, sectioning and staining (Michalak *et al.*, 1990). Morphological alterations encountered in hepatocellular, extrahepatocellular intralobular and portal compartments of hepatic parenchyma were graded on a numerical scale from 0-3, as described in previous works (Diao *et al.*, 2003; Hodgson and Michalak, 2001; Michalak *et al.*, 1990) and referred to herein as the histological degree of hepatitis.

6.4 Results

6.4.1 WHcAg and IFN-γ expression in cells transfected with pC-WHc or pWHc-wIFNγ.

The translational competence of pC-WHc and the bicistronic WHc-wIFNγ plasmids (Fig. 6.1A) were examined in transient transfection experiments using WCM-260 hepatocytes as targets. As shown in Figure 6.1B, WHcAg was detected in the cells transfected with either pC-WHc or pWHc-wIFNγ. On the other hand, synthesis of wIFNγ was monitored indirectly by detection of the enhanced expression of class I MHC antigen. The results showed that the display of class I antigen was significantly increased on hepatocytes transfected with pWHc-wIFNγ, but not on those transfected with pC-WHc alone. In addition, the cells transfected with a control pC-wIFNγ plasmid also showed upregulated

Figure 6.1 The pWHc-wIFNγ construct and the assessment of its expression competence in woodchuck WCM-260 hepatocytes. (A) Schematic presentation of the bicistronic plasmid pcDNA3.1 containing complete WHc gene, encephalomyocarditis-derived IRES sequence, and the whole wIFNγ cDNA sequence. Expression of WHc was driven by the cytomegalovirus immediate early promoter, PCMVIE. BGHpA represents bovine growth hormone polyadenylation signal. (B) Staining of WHcAg and class I MHC antigen in woodchuck WCM-260 hepatocytes transfected with pC-WHc (A and B), pWHc-wIFNγ (C and D) or with control pC-wIFNγ expression vector (E and F). After 72 h of transfection, cells were incubated with B1bB9 mAb against woodchuck class I MHC heavy chain followed by goat anti-mouse antibodies conjugated with FITC (B, D and F) and then with rabbit anti-WHc followed by goat anti-rabbit antibodies conjugated with rhodamine (A, C and E).

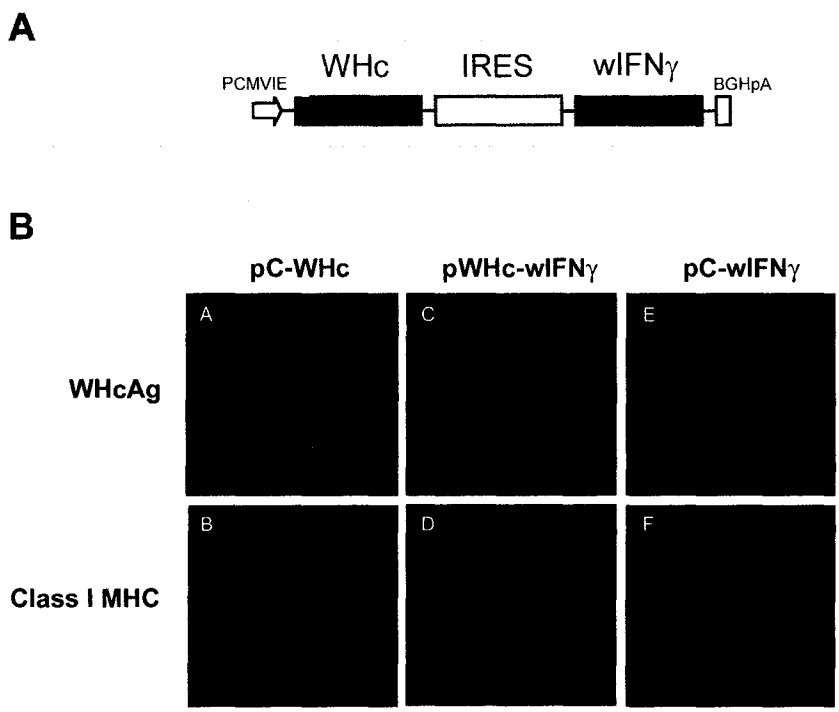
A
PCMVIE
WHc
IRES
wIFNγ
BGHpA
B
pC-WHc
pWHc-wIFNγ
pC-wIFNγ
WHcAg
Class I MHC
A
C
E
B
D
F

Figure 6.1

expression of class I MHC antigen, while they remained WHcAg non-reactive. These data implied that neither transfection with lipofectamine reagent nor expression of WHcAg enhanced class I MHC antigen display. This supported the contention that the observed increase in the class I MHC display was a specific consequence of synthesis of functionally active IFN-γ in cells transfected with pWHc-wIFNγ or pC-wIFNγ.

6.4.2 WHcAg-specific antibody and T cell responses after immunization with WHc DNA.

In the first phase of the study, 4 naive woodchucks were immunized with pC-WHc DNA (Table 6.1; group pC-WHc). Two of the animals (A/M and B/M) injected twice with 0.6 mg of pC-WHc did not produce anti-WHc (Fig. 6.2A). However, two others (C/M and D/M), which received two 1-mg doses of DNA, developed anti-WHc 4 to 5 weeks after the first injection with the plasmid (Fig. 6.2A). The level of anti-WHc elicited was on average ~25% higher in C/M than that in D/M. As expected, a control woodchuck injected with empty pCI vector was anti-WHc negative (not shown). WHV-specific lymphoproliferative response was not measured in these animals.

In the subsequent experiments, to test whether introduction of wIFNγ DNA into the WHc DNA construct would strengthen induction of immune responses towards WHcAg and augment protection against hepadnavirus, three groups of woodchucks, each of which contained 3 animals, were injected with 0.5, 0.9 or 1.5 mg per dose of pWHc-wIFNγ DNA (Table 6.1; group pWHc-wIFNγ). Among

Table 6.1. WHc DNA immunization protocols and features of WHV infection after challenge with WHV

Treatment group and animal No./Sex	Plasmid DNA mg/dose	Number of injections	Follow-up period (wk)[a]	After DNA injections[b]	After WHV challenge[c]						
										Degree of hepatitis[e]	
				Anti-WHc	Serum WHsAg	Duration of WHsAg positivity (wk)	Anti-WHc	Anti-WHs	Serum WHV DNA[d]	2-8 wk p.i.	Autopsy
pC-WHc group											
A/M	0.6	2	50	-	+	1	+	+	+	0.5	
B/M	0.6	2	48	-	+	5	+	+	+	0.5	1.0
C/M	1.0	2	51	+	-	0	+	+	+	0	0
D/M	1.0	2	41	+	+	39	+	n.a.	+	0.5	2.0
pWHc-wIFNγ group											
1/F	0.5	1	45	+	-	0	+	-	+	0	0
2/F	0.5	2	22	-	+	18	+	-	+	1.5	0.5
3/M	0.5	2	22	-	+	1	+	+	+	<0.5	<0.5
4/F	0.9	1	21	+	-	0	+	+	+	0	0
5/F	0.9	1	43	-	+	17	+	-	+	2.5	0.5
6/M	0.9	2	22	-	+	5	+	-	+	3	<0.5
7/M	1.5	1	27	-	-	0	+	-	+	0	0
8/M	1.5	2	22	+	-	0	+	-	+	0	0
9/M	1.5	2	15	-	+	1	+	-	+	0	0

Abbreviations: M, male; F, female; p.i., post infection; wk, week; n.a., not applicable.

[a] From time of challenge with WHV.

[b] Period from first DNA injection untill challenge with WHV.

[c] Period between challenge with WHV and the end of follow-up.

[d] Serum WHV DNA detected by direct or nested PCR/NAH with WHc gene-specific primers using DNA extracted from 100 μl of serum.

[e] Degree of hepatitis determined on scale from 0-3 based on severity of liver alterations according to the criteria described before (Hodgson and Michalak, 2001; Michalak *et al.*, 1999). 0 indicates normal liver histology and 3 most severe necroinflammatory lesions.

Figure 6.2. Serological markers of WHV infection, WHV DNA detection and liver histology in woodchucks immunized with pC-WHc DNA and challenged with WHV. Two groups, each consisting of two animals, were injected twice with either 0.6 mg/dose (A) or 1 mg/dose (B) of pC-WHc DNA at the time points indicated by solid vertical lines. Subsequently, the animals were challenged with 1.1×10^{10} vge of WHV at week 0. The appearance and duration of serum WHsAg, anti-WHc and WHV DNA are indicated as black bars. Liver tissue samples were collected at the time points shown by arrows and examined for WHV DNA presence (+ or -) and by histology. Morphological alterations encountered in liver samples are presented as the histological degree of hepatitis ranging from 0 to 3.

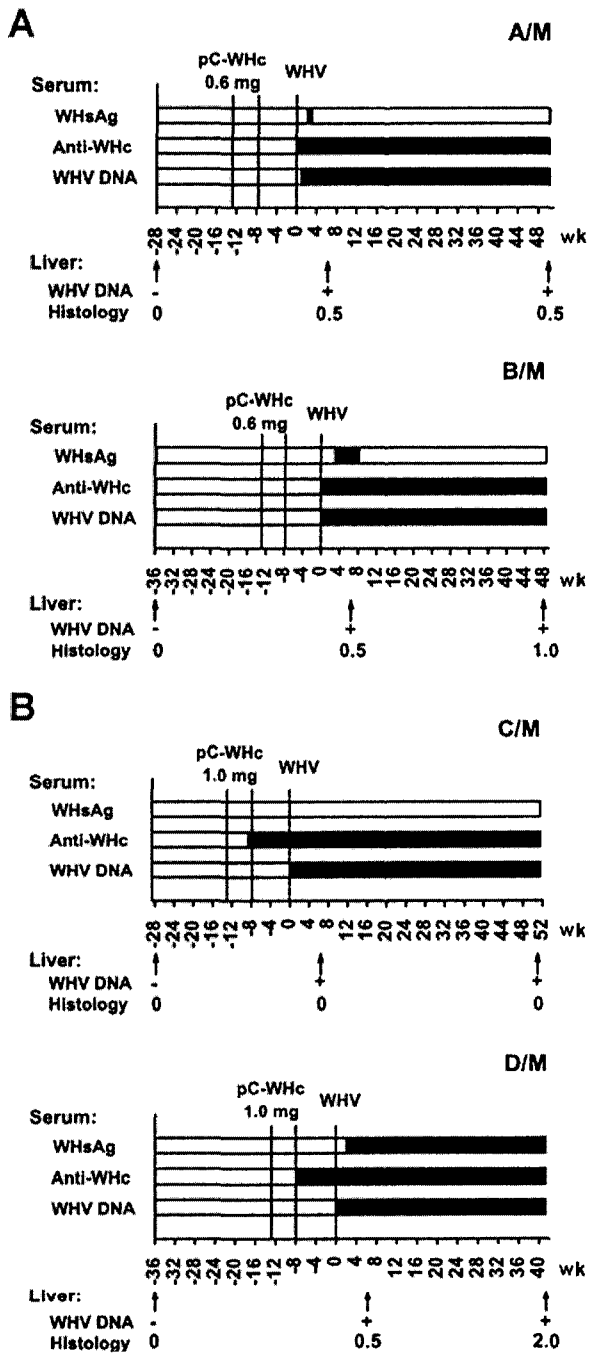
A
A/M
pC-WHc
0.6 mg
WHV
Serum:
WHsAg
Anti-WHc
WHV DNA
-28 -24 -20 -16 -12 -8 -4 0 4 8 12 16 20 24 28 32 36 40 44 48 wk
Liver:
WHV DNA - + +
Histology 0 0.5 0.5
B/M
pC-WHc
0.6 mg
WHV
Serum:
WHsAg
Anti-WHc
WHV DNA
-36 -32 -28 -24 -20 -16 -12 -8 -4 0 4 8 12 16 20 24 28 32 36 40 44 48 wk
Liver:
WHV DNA - + +
Histology 0 0.5 1.0
B
C/M
pC-WHc
1.0 mg
WHV
Serum:
WHsAg
Anti-WHc
WHV DNA
-28 -24 -20 -16 -12 -8 -4 0 4 8 12 16 20 24 28 32 36 40 44 48 52 wk
Liver:
WHV DNA - + +
Histology 0 0 0
D/M
pC-WHc
1.0 mg
WHV
Serum:
WHsAg
Anti-WHc
WHV DNA
-36 -32 -28 -24 -20 -16 -12 -8 -4 0 4 8 12 16 20 24 28 32 36 40 wk
Liver:
WHV DNA - + +
Histology 0 0.5 2.0

Figure 6.2

Figure 6.3. Serological markers of WHV infection, WHV DNA detection and liver histology in woodchucks immunized with pWHc-wIFNγ DNA. Three groups of woodchucks, each consisting of three animals, were immunized with 0.5 mg/dose (A), 0.9 mg/dose (B) or 1.5 mg/dose (C) of pWHc-wIFNγ DNA at the time points indicated by solid vertical lines. The animals then were challenged with 1.1×10^{10} vge of WHV at week 0. WHsAg, anti-WHc and WHV DNA and liver samples were evaluated and the results presented as described in Materials and Methods and in the legend to Fig. 6.2.

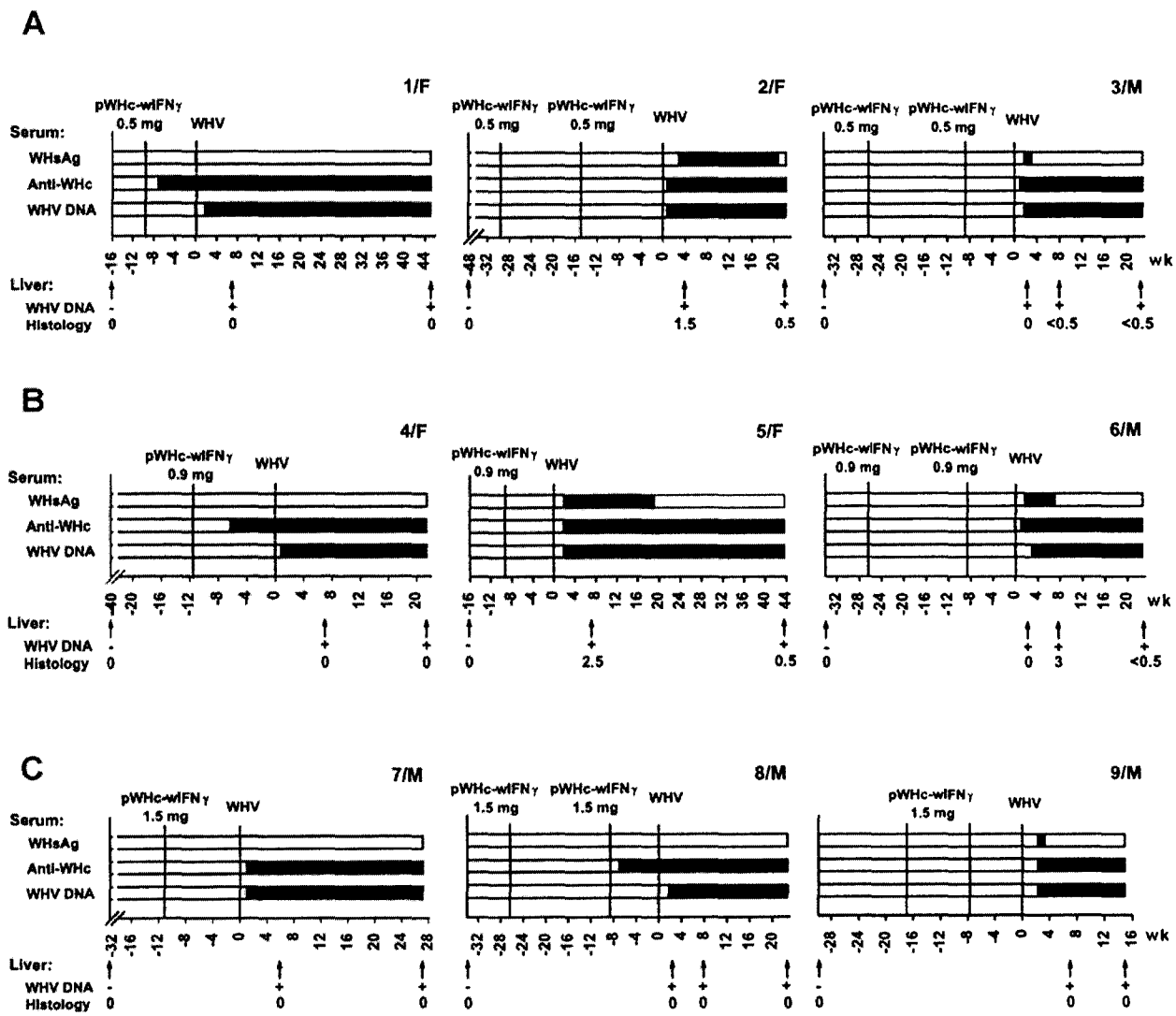
A
1/F
2/F
3/M
pWHc-wIFNγ
0.5 mg
WHV
Serum:
WHsAg
Anti-WHc
WHV DNA
wk
Liver:
WHV DNA
Histology
0 0 0
0 1.5 0.5
0 0 <0.5 <0.5
B
4/F
5/F
6/M
pWHc-wIFNγ
0.9 mg
WHV
Serum:
WHsAg
Anti-WHc
WHV DNA
wk
Liver:
WHV DNA
Histology
0 0 0
0 2.5 0.5
0 0 3 <0.5
C
7/M
8/M
9/M
pWHc-wIFNγ
1.5 mg
WHV
Serum:
WHsAg
Anti-WHc
WHV DNA
wk
Liver:
WHV DNA
Histology
0 0 0
0 0 0 0
0 0 0

Figure 6.3

these 9 animals, 3 responded by producing anti-WHc (Fig. 6.3). The antibodies appeared at 3 and 5 wks after first immunization in 1/F and 4/F, respectively, and in one week after booster injection in 8/M, independently of the dose of pWHc-wIFNγ given. Therefore, in contrast to the pC-WHc group, there was no relation between the amount of pWHc-wIFNγ given and induction of anti-WHc. A woodchuck injected with pC-wIFNγ was anti-WHc negative.

In 4 animals vaccinated with pWHc-wIFNγ, WHV-specific T cell proliferative response was measured prior to and after challenge with WHV. Two of the animals, 2/F immunized twice with 0.5 mg and 8/M injected twice with 1.5 mg of the plasmid, mounted specific T cell response, as shown in Figure 6.4. As expected, this response was directed against proteins encoded by WHV core gene, *i.e.*, rWHcAg and rWHeAg, and $WHc_{97\text{-}110}$ peptide, but not toward rWHxAg or WHsAg, confirming antigenic- restricted specificity of the T cell response induced by pWHc-wIFNγ. Also it is of note that a significantly higher magnitude of WHc-specific T cell response was detected in 2/F (maximum S.I. 13.7) than in 8/M (maximum S.I. 4.2). Overall, the results revealed that immunization with pWHc-wIFNγ DNA was able to elicit WHc-specific T cell response. However, this response was not induced uniformly among the animals immunized, suggesting that host factors played a decisive role in its development.

Figure 6.4

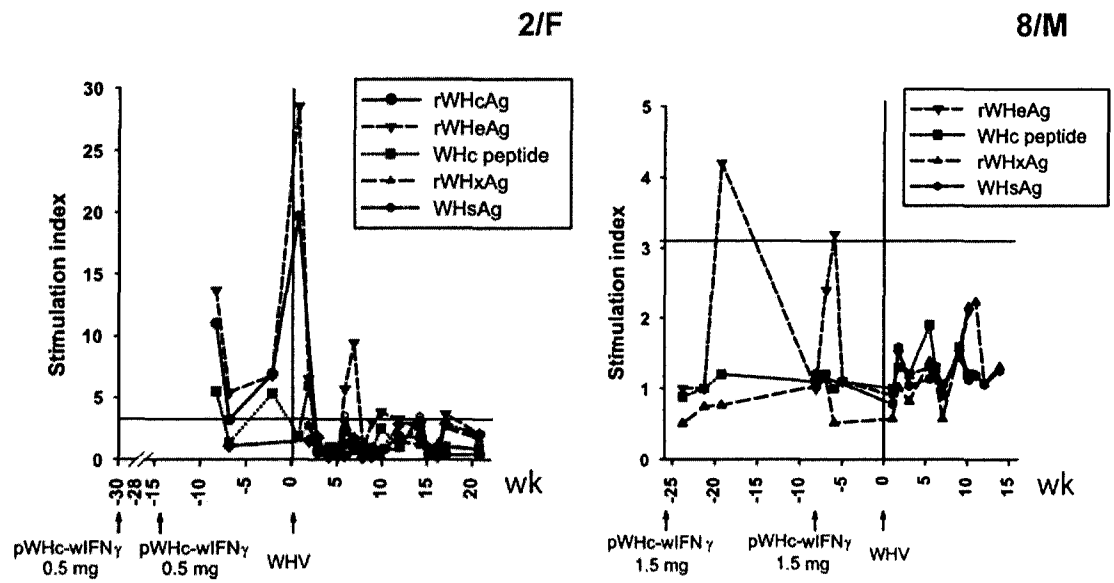

Figure 6.4. Profiles of WHV-specific cellular immune response in woodchucks immunized with pWHc-wIFNγ DNA. Animal 2/F was immunized twice with 0.5-mg doses and 8/M twice with 1.5-mg doses of pWHc-wIFNγ DNA at the time points indicated by arrows. Then the animals were challenged with 1.1 x 10^{10} vge of WHV at week 0. Proliferative T cell response to WHV antigens indicated in the inserts was measured by ^{3}H-adenine incorporation assay as described in Materials and Methods. The stimulation index was calculated by dividing mean cpm obtained after stimulation with test antigen by mean cpm detected in the absence of a stimulant. A stimulation index equal to or greater than 3.1, marked by the horizontal line, was considered as positive.

6.4.3 Immunization with WHc DNA can protect from serologically evident WHV infection and hepatitis.

Animals immunized with pC-WHc or pWHc-wIFNγ DNA were challenged with a massive dose of WHV to determine protective effectiveness of the vaccination strategy tested. Among 4 woodchucks which were injected with pC-WHc DNA, C/M developed anti-WHc and remained serum WHsAg non-reactive during the 12-mo follow-up after inoculation with virus (Fig. 6.2B). Liver biopsy obtained at 6 wks after challenge showed entirely normal histology, whereas that obtained 12 mo later displayed minimal nonspecific parenchymal alterations with sparse lymphocytic infiltrations in a few portal areas. In contrast, D/M which also developed anti-WHc following DNA vaccination established serum WHsAg-positive, histologically evident chronic hepatitis lasting until the end of the 12-mo observation period. Two remaining woodchucks, A/M and B/M, immunized with 0.6-mg doses of pC-WHc each developed transiently serum WHsAg-positive infection and self-limited acute hepatitis (Fig. 6.2A). Anti-WHc antibodies appeared in these two animals after WHV challenge and remained at high levels throughout the 12-mo observation period. Liver samples collected after acute phase of infection showed residual minimal inflammatory alterations persisting in both animals to the end of follow-up. These features were similar to those found in remote resolved hepatitis, as previously described (Coffin *et al.*, 2004; Hodgson and Michalak, 2001; Michalak *et al.*, 1999). A control animal

immunized twice with 1 mg of empty pCI vector and infected with WHV developed classical serum WHsAg-positive, self-limited hepatitis which was followed by persistent minimal inflammatory alterations in liver parenchyma.

As shown in Figure 6.3 and summarized in Table 6.1, 4 of 9 animals immunized with pWHc-wIFNγ remained serum WHsAg negative after challenge with WHV. Three of them (1/F, 4/F and 8/M) acquired anti-WHc due to DNA vaccination, while one (7/M) remained anti-WHc negative and produced the antibodies shortly after challenge with WHV. In addition, a very brief episode of WHs antigenemia, identifiable only in a single serum sample, was detected in 3/M and 9/M. The remaining 3 animals (2/F, 5/F and 6/M) developed serum WHsAg-positive infection lasting between 3 and 20 weeks (Fig. 6.3). All of these 5 animals became anti-WHc reactive in one to 4 wks after inoculation with WHV. Anti-WHs reactivity was detected in 2 of the woodchucks (Table 6.1).

Importantly, histological examination of 2 or 3 liver tissue samples collected from each animal during follow-up lasting for up to 45 weeks after inoculation with WHV showed consistently normal morphology in 5 of 9 woodchucks (Table 6.1). Among them were 4 animals (1/F, 4/F, 7/M and 8/M) which remained serum WHsAg nonreactive after inoculation with WHV and one (9/M) which had a one-week long episode of borderline WHs antigenemia (Fig. 6.3C). Three (7/M, 8/M and 9/M) of those 5 woodchucks were immunized once or twice with 1.5 mg of pWHc-wIFNγ (Table 6.1). Contrastingly, animals which established serum WHsAg-positive infection (2/F, 3/M, 5/F and 6/M) developed, as expected, acute hepatitis which subsided to minimal residual inflammatory

changes persisting to the end of the observation period. A control animal immunized twice with 1.3 mg of pC-wIFNγ DNA and then inoculated with WHV had classical serum WHsAg-positive self-limiting acute hepatitis (not shown).

It is of note that 2/F woodchuck, which developed a robust WHc-specific T cell proliferative response but no anti-WHc antibodies after DNA vaccination and displayed a swift and strong lymphoproliferative response to WHcAg and related antigens following challenge with WHV (maximum S.I. of 29) (Fig. 6.4), failed to be protected (Fig. 6.3A). In this animal, T cell proliferation response against multiple WHV antigens, including rWHxAg and WHsAg, was detected around 7 week post-infection, as well as in several other woodchucks infected with the same WHV inoculum but not immunized with WHc DNA which were investigated in parallel (Gujar and Michalak, manuscript in preparation). In contrast to 2/F, animal 8/M which developed a relatively weak WHc-specific T cell response and anti-WHc following vaccination, was free from WHs antigenemia and protected from hepatitis.

6.4.4 Transcription of IFN-γ, TNF-α, OAS in livers and lymphoid cells after vaccination with pWHc-wIFNγ.

To recognize whether induction of antiviral and Th1 cytokines may play a role in protection against WHV infection and hepatitis, the levels of wIFNγ, wTNFα and wOAS mRNAs were quantified by real-time RT-PCR in hepatic tissue and PBMC samples collected before DNA immunization and after challenge with WHV. As shown in Fig. 6.5A, intrahepatic transcription of wIFNγ

and wTNFα was not noticeably different prior to and after exposure to virus in any of the 4 animals protected from serologically evident infection and hepatitis, except 7/M which had an unexplained increase in wTNFα mRNA prior to WHV challenge. This was accompanied by relatively stable levels of wOAS and CD3 mRNA in individual animals, except 7/M (Fig. 6.5A). In contrast, an enhanced transcription of intrahepatic wIFNγ (2.5 to 5.7-fold increase) and wTNFα (4.2 to 6.3-fold increase) in comparison to the levels prior to WHV challenge was seen in woodchucks not protected from WHV infection (2/F, 5/F and 6/M) (Fig. 6.5B). These increases were observed in the acute phase of infection and were accompanied by upregulated expression of wOAS and CD3. In 3/M and 9/M which showed a brief episode of WHs antigenemia, the mRNA levels tested between 2 and 8 weeks post-infection were only moderately elevated or remained at the pre-challenge values. Analysis of the expression of the same genes in serial PBMC samples did not reveal measurable differences between the protected and non-protected animals or between the pre- and post-challenge phases (not shown). Overall, the data implied that protection from WHV infection and hepatitis induced by pWHc-wIFNγ was not correlated with measurable changes in the expression of Th1 cytokines or IFN-α in either peripheral lymphoid cells or hepatic tissue. However, this does not exclude a contribution of other anti-viral cytokines or immune mechanisms which were not tested in this study.

Figure 6.5. Profiles of relative expression of wIFNγ, wTNFα, wOAS, and CD3 genes in liver biopsy samples collected prior to and after challenge with WHV from woodchucks protected (A) and non-protected (B) from WHV infection after immunization with pWHc-wIFNγ DNA and challenge with WHV. The time of challenge with 1.1×10^{10} vge of WHV is indicated by arrow and corresponds to week 0. The mRNA levels of indicated woodchuck genes were quantified by specific real-time RT-PCR. The results are shown as the number of copies of a given gene per reaction after normalization against ~3×10^4 copies of woodchuck β-actin, which was quantified in parallel with each test sample.

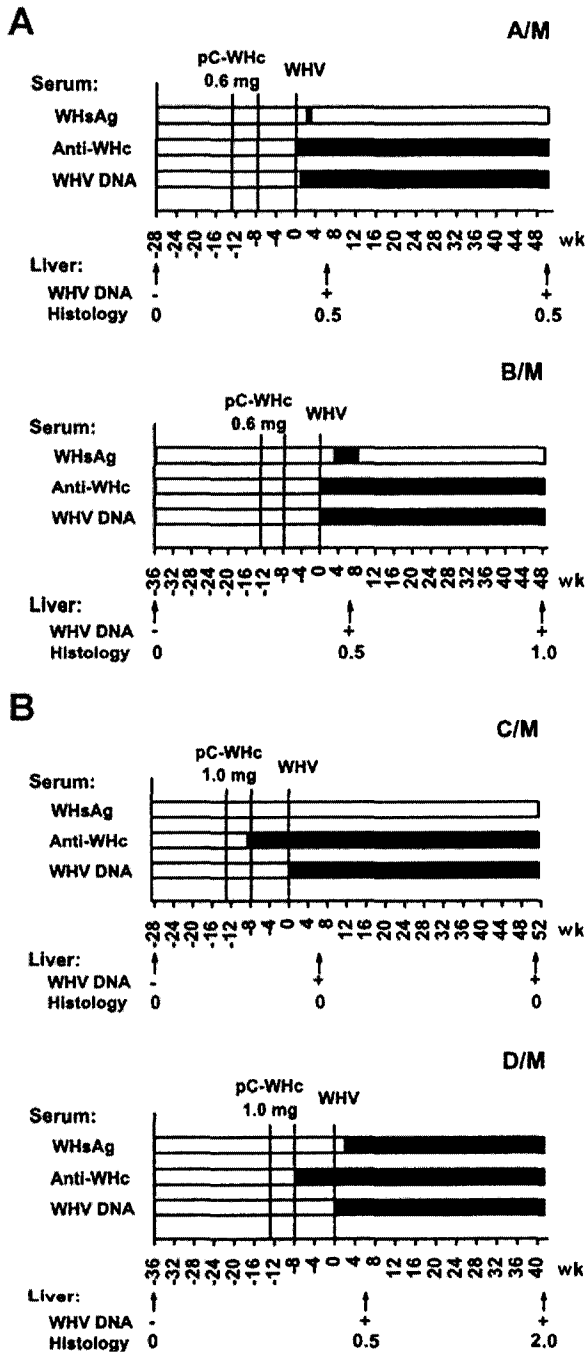
A
A/M
pC-WHc
0.6 mg
WHV
Serum:
WHsAg
Anti-WHc
WHV DNA
-28 -24 -20 -16 -12 -8 -4 0 4 8 12 16 20 24 28 32 36 40 44 48 wk
Liver:
WHV DNA - + +
Histology 0 0.5 0.5
B/M
pC-WHc
0.6 mg
WHV
Serum:
WHsAg
Anti-WHc
WHV DNA
-36 -32 -28 -24 -20 -16 -12 -8 -4 0 4 8 12 16 20 24 28 32 36 40 44 48 wk
Liver:
WHV DNA - + +
Histology 0 0.5 1.0
B
C/M
pC-WHc
1.0 mg
WHV
Serum:
WHsAg
Anti-WHc
WHV DNA
-28 -24 -20 -16 -12 -8 -4 0 4 8 12 16 20 24 28 32 36 40 44 48 52 wk
Liver:
WHV DNA - + +
Histology 0 0 0
D/M
pC-WHc
1.0 mg
WHV
Serum:
WHsAg
Anti-WHc
WHV DNA
-36 -32 -28 -24 -20 -16 -12 -8 -4 0 4 8 12 16 20 24 28 32 36 40 wk
Liver:
WHV DNA - + +
Histology 0 0.5 2.0

Figure 6.2

6.4.5 Woodchucks otherwise successfully immunized with pWHc-wIFNγ vaccine acquire traces of replicating WHV after challenge.

Although 5 of the 13 animals immunized with pC-WHc or pWHc-wIFNγ were protected from serologically apparent WHV infection and hepatitis, their serial serum sample collected after inoculation with WHV revealed low levels of WHV DNA when analyzed by sensitive PCR/NAH assays. This unexpected finding was investigated in detail using approaches previously established for identification of occult hepadnavirus persistence (Coffin and Michalak, 1999; Coffin *et al.*, 2004; Hodgson and Michalak, 2001; Lew and Michalak, 2001; Michalak *et al.*, 2004; Michalak *et al.*, 1999). Thus, the analysis of serial serum samples obtained after inoculation with WHV and at autopsy demonstrated WHV DNA at levels usually not exceeding 10^2 vge/ml. In all 5 animals apparently protected, WHV DNA was also detected in sequential PBMC samples collected after challenge and in lymphatic organs gathered at autopsy (not shown). The estimated levels of WHV genomes ranged between 0.005 and 0.5 vge/10^4 lymphoid cells, calculated as described previously (Michalak *et al.*, 1999).

In the animals protected from serologically evident infection, less than 2 x 10^3 WHV vge per μg of total DNA was detected in liver samples collected after challenge and at the time of autopsy, compared to an estimate of up to 2 x 10^7 vge per μg of total DNA detected in acute phase of hepatitis in the non-protected animals. At the end of follow-up of the non-protected woodchucks, WHV DNA in

the liver dropped to the loads comparable to those found in the protected animals, i.e., about 10^3 vge per μg of total DNA, with the exception of D/M which developed serum WHsAg-positive chronic hepatitis. Importantly, WHV cccDNA was also identified in autopsy liver tissue samples (not shown). Further, to determine the magnitude of WHV replication in the livers of the protected woodchucks, WHV pre-genomic RNA was quantified by real time RT-PCR in liver biopsies collected from 1/F, 4/F, 7/M and 8/M between 2 and 8 weeks after challenge and at autopsy. As shown in Figure 6.6A, less than 100 copies of WHV mRNA per 3 x 10^4 copies of β-actin mRNA was found in these liver samples. The WHV mRNA levels remained stable (in one $\log_{10}$ range) for up to 45 weeks after challenge. In woodchucks not protected from infection, i.e., 2/F, 3/M, 5/F, 6/M and 9/M, one to four log higher WHV RNA levels than those occurring in the livers of the protected animals were identified at 2 to 8 weeks post-infection, except 3/M. These hepatic loads progressively declined reaching less than 100 copies of WHV RNA per 3 x 10^4 copies of β-actin mRNA at the end of the observation period. Interestingly, 9/M animal, which showed unaltered liver histology after inoculation with WHV (Fig. 6.3C), carried WHV RNA at a high level at week 7 after challenge and this level declined to less than 100 copies at week 15 after challenge with WHV.

Figure 6.6

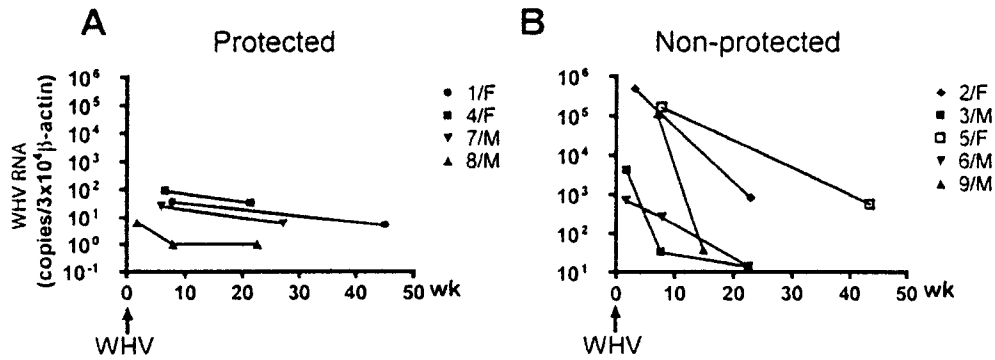

Figure 6.6. Detection of WHV RNA in liver tissue samples collected from woodchucks protected (A) and non-protected (B) from WHV infection after vaccination with pWHc-wIFNγ DNA. The time of challenge with WHV is indicated by arrow and corresponds to week 0. The WHV RNA levels were quantified by real-time RT-PCR and normalized against β-actin, as described in the legend to Figure 6.5. It is of note that WHV RNA was consistently detected, although at levels below 100 copies per ~3 x 10^4 copies of β-actin, in all liver samples acquired from animals which were otherwise protected from WHV infection and hepatitis after immunization with pWHc-wIFNγ DNA.

6.5 Discussion

In previous reports, it has been shown that immunizations of WHV-naive woodchucks with native or recombinant WHcAg or with plasmids encoding for this antigen were able to protect individual animals against experimental WHV infection, as determined by the absence of serum WHsAg and WHV DNA by PCR assays with detection limits of 5 x 10^2 to 10^3 vge/ml (Lu *et al.*, 1999; Lu *et al.*, 2005; Roos *et al.*, 1989; Schodel *et al.*, 1993). It was also demonstrated that co-administration of plasmids encoding for WHcAg and woodchuck IFN-γ or IL-12, enhanced the efficacy of the protection when compared to that elicited by WHc DNA alone (Garcia-Navarro *et al.*, 2001; Siegel *et al.*, 2001). In the present work, we confirmed that vaccination with WHc DNA can, although only occasionally, prevent animals from acquiring serologically detectable WHV infection and hepatitis. We also showed that the protective efficacy of this vaccine can be improved by delivery of DNA encoding for WHcAg and wIFNγ incorporated into a single plasmid vector. This result indicates that the bicistronic vector approach, in which the expression of the cytokine-encoding sequence is translated via IRES, represents a valid strategy for cytokine-facilitated enhancement of protective immunity against hepadnavirus. In other studies, the bicistronic plasmids encoding for HBV preS2 and IL-2 elicited a stronger Th1 response than HBV preS2 DNA alone when given to mice (Chow *et al.*, 1997). Similarly, immunization with HIV gp120/granulocyte-monocyte colony-stimulating

factor (GM-CSF) bicistronic DNA was able to enhance gp120-specific CD4+ T cell response in mice in comparison with co-application of plasmids encoding for each of these two molecules separately (Barouch *et al.*, 2002). An increased T cell response was also observed when a bicistronic plasmid encoding for HCV NS 3, 4 and 5 proteins and GM-CSF were injected to rats (Cho *et al.*, 1999). Therefore, the current data support the view that immunization with the DNA constructs encoding for viral antigen and an adjuvant acting cytokine can significantly enhance specific immune response to the antigen of interest.

In the present study, immunization with the bicistronic pWHc-wIFNγ DNA vaccine protected 4 of 9 (44.5%) animals from serologically detectable WHV infection. The protection was not related to the number of the DNA injections given, since a single dose was just as effective as two injections. However, among three woodchucks which received the greatest amount of the bicistronic DNA, two animals did not develop serologically evident infection and all three were free from hepatitis after inoculation with the virus (see Table 6.1 and Fig. 6.3C). Among the remaining 6 woodchucks injected with the intermediate or the low doses of pWHc-wIFNγ, two animals receiving a single injection with 0.9 mg or 0.5 mg of DNA became protected (see Table 6.1; pWHc-wIFNγ group). In contrast, among 4 animals immunized with pC-WHc alone, only C/M injected twice with 1 mg of the plasmid remained serologically negative and free from hepatitis after challenge (see Table 6.1; WHc group). These data showed that the protection induced by pWHc-wIFNγ was related to the amount of the DNA administered but not to the number of injections given, and that pWHc-wIFNγ

was clearly more potent than pC-WHc in preventing serologically evident infection.

Among woodchucks immunized with pWHc-wIFNγ who acquired protection, three animals produced anti-WHc prior to challenge (see Table 6.1). However, 7/M, which did not develop anti-WHc, was also protected from serologically positive infection. An even stronger indication that anti-WHc had little or no contribution to the protective immunity in our study, came from the experiment with woodchucks immunized with pC-WHc. Specifically, two animals injected twice each with 1-mg doses of pC-WHc produced anti-WHc. However, while C/M was protected from WHsAg-positive infection and liver injury, D/M established serum WHsAg-positive, histologically evident chronic hepatitis (Fig. 6.2B). This is a rare outcome of WHV infection in adult animals which is observed in no more than 15% of woodchucks infected during adulthood with a massive dose of the virus in our laboratory (Michalak, 1998b; Michalak, 2000). Overall, we did not observe an interdependence between the induction of anti-WHc and protection from experimental infection. This finding is consistent with the data from other studies in which a relation between humoral response to WHcAg and the outcome after challenge with WHV was investigated (Garcia-Navarro *et al.*, 2001; Lu *et al.*, 2005; Siegel *et al.*, 2001).

There was also no evident relation between WHcAg-specific T cell proliferative response induced by vaccination with pWHc-wIFNγ and protection from infection. Specifically, 2/F woodchuck which mounted a readily detectable T cell proliferation following stimulation with WHV nucleocapsid-related antigens

and a very strong specific T cell memory response within 1-2 wk of WHV inoculation (see Fig. 6.4A), developed WHs antigenemia and hepatitis (see Fig. 6.3A). In contrast, 8/M animal which showed borderline WHcAg-specific lymphoproliferation after vaccination and no memory response after challenge (see Fig. 6.4B), was protected from serologically positive infection and hepatitis (see Fig. 6.3C). These results differ from those reported by others for woodchucks immunized by co-administration of WHc and IFN-γ plasmids, where all three animals examined showed lymphoproliferative response to WHcAg and were protected from infection, as determined by assessing serum WHV DNA by PCR with the detection limit of ~10^3 vge/ml (Siegel *et al.*, 2001). However, in the same study, another animal, which had been injected with WHc DNA and not produced measurable T cell response to WHcAg, was also protected. Taken together, we tend to believe that immune responses other than those meaningfully involving specific T cells, for which activity is measured after ex vivo stimulation with WHV nucleocapsid-related antigens, play a prevailing role in the protection elicited by vaccination with WHV core encoding DNA in the presence of IFN-γ as a vaccine adjuvant. In this regard, a contribution of specific CTL or innate immunity primed by vaccination could be considered. However, this hypothesis can not be easily tested, since there are no assays measuring these types of immune responses in woodchucks available at the present time.

Vaccinated animals which mounted WHcAg-specific lymphoproliferative response or those which became protected from serologically evident infection and hepatitis did not show any measurable differences in expression of wIFNγ or

wTNFα, as well as wOAS, in serial samples of circulating lymphoid cells, when compared to those in the non-protected animals. Similarly, quantification of the mRNA levels of these cytokines in liver samples from the protected and the unprotected animals did not provide a lead to a possible mechanism underlying the elicited protection.

Liver tissue from the woodchucks protected from serologically positive WHV infection was free from inflammatory alterations, as evidenced by histological examinations. The lack of lymphocytic infiltrations and inflammation was confirmed by unaltered expression of intrahepatic CD3, IFN-γ and TNF-α (Hodgson and Michalak, 2001). Although the nature of the protection elicited by pWHc-wIFNγ vaccine remains uncertain, these findings convincingly showed that when this protective response was successfully induced it was sufficient to prevent entirely histological and molecular signs of hepatitis. In addition, the data from 9/M woodchuck, which developed a brief episode of WHsAg-positive infection but no hepatitis (see Table 6.1 and Fig. 6.3C), suggested that this response was robust, since no residual inflammatory changes were found in liver samples of this animal after challenge with WHV, although such alterations are frequently encountered even years after resolution of WHsAg-positive infection (Coffin *et al.*, 2004; Michalak *et al.*, 2004; Michalak *et al.*, 1999). In contrast, liver samples from woodchucks developing serum WHsAg-positive infection displayed typical histological features of hepatitis which subsided to minimal inflammatory changes persisting throughout the entire observation period, as reported before (Coffin *et al.*, 2004; Hodgson and Michalak, 2001; Michalak *et al.*, 1999). The

temporal upregulation in hepatic expression of CD3 and IFN-γ and, in some animals, TNF-α coincided with the acute phase of liver inflammation, as observed previously (Hodgson and Michalak, 2001; Nakamura *et al.*, 2001).

Despite the fact that vaccination with pWHc-wIFNγ was able to prevent hepatitis, all animals protected acquired low levels of WHV after challenge and trace virus replication persisted to the end of the experiment. This showed that the immunity elicited by vaccination with DNA encoding for hepadnavirus core antigen was unable to provide a sterile protection against the virus. Accordingly, our data argue for a possibility that the mechanisms governing protection from hepadnaviral infection and prevention of liver disease caused by this infection may not be identical.

The lack of sterile protection against hepadnavirus found in our study could be a consequence of at least two coinciding events. Firstly, since WHcAg is an inner structural antigen, immunity raised against this antigen may not be as effective as that directed towards virus envelope epitopes in preventing the initial attachment and entry of the virus to hepatocytes and lymphoid cells, which both are naturally targeted by the virus (Lew and Michalak, 2001; Michalak *et al.*, 2004; Michalak *et al.*, 1999). Therefore, virus replication can be established, at least at a low level, and virus can persistently propagate in the liver and lymphatic system, as shown by our study. On the other hand, the immunity raised against the core antigen, particularly in the presence of IFN-γ, can be highly effective in suppressing virus replication, possibly by a noncytopathic pathway (Guidotti *et al.*, 1996b), and in limiting virus spread to uninfected cells. This mechanism can

keep the virus under control, i.e., at the levels which are not pathogenic to the liver (see below) (Michalak, 2000; Michalak *et al.*, 1999). It has been documented in a series of previous studies that once WHV replication is established it can be suppressed but never completely eradicated (Coffin and Michalak, 1999; Hodgson and Michalak, 2001; Michalak *et al.*, 1999). The same appears to be true for humans who recovered from HBsAg-positive acute hepatitis B (Michalak *et al.*, 1994; Rehermann *et al.*, 1996a). The data from the present work are in full agreement with this previously established fact. Based on this, it can be conclusively stated that the protection against experimental WHV infection observed in our study was not complete.

Secondly, all animals vaccinated in our study were challenged with a massive dose of WHV normally causing hepatitis in naive animals and the woodchucks otherwise protected from infection and hepatitis carried low levels of virus in both lymphoid cells and their livers after challenge. We have previously uncovered that there is a significant difference in the threshold level of WHV required to infect lymphoid cells and that to infect hepatocytes (presumably 100- to 1000-fold) (Michalak *et al.*, 2004). We have found that WHV doses lower than or equal to 10^3 virions cause serologically concealed infection restricted to the lymphatic system, termed primary occult infection (POI). Contrastingly, WHV at higher doses induces serologically evident infection, involving both the lymphatic system and the liver, and causes hepatitis, which when resolved is followed by indefinitely long residual infection in the liver and lymphoid tissues, termed as secondary occult infection (SOI) (Coffin *et al.*, 2004; Michalak, 2000; Michalak *et*

al., 2004). Therefore, detection of low levels of WHV in the liver of the otherwise protected animals suggests that hepatocytes were initially exposed to a high dose of virus (greater than 10^3 virions) and infected, but the infection was most likely swiftly suppressed leaving behind residual WHV replication, which always escapes elimination by immunological and intracellular antiviral mechanisms. Hence, it is possible that the pWHc-IFNγ vaccine tested, as well as other vaccines based on immunization against hepadnavirus nucleocapsid antigens, can protect the liver, but unlikely the immune system, from infection with low virus doses.

In conclusion, our findings indicate that delivering simultaneously to the same location both WHcAg and IFN-γ encoding sequences has a potency to suppress virus replication and prevent development of hepatitis in woodchucks experimentally exposed to a massive virus dose. However, this vaccination strategy is unable to induce sterile protection against the virus. It is expected this approach might be of value in therapeutic treatment of hepatitis B, but unlikely in prophylaxis against HBV infection.

CHAPTER 7. GENERAL DISCUSSIONS

The aims of these studies were to investigate selected aspects of viral-host interactions which could be involved in the establishment of WHV persistence and to design experimental approaches to reverse the virus-caused cellular defects possibly supporting protracted virus infection in the liver, as well as to promote the host anti-viral immune responses. In particular, our studies focused on identifying how WHV can modify class I MHC antigen presentation on hepatocytes. Further, we attempted to discover whether an antiviral and immunoregulatory cytokine, such as IFNγ, could restore the inhibitory effect exerted by hepadnavirus on class I MHC antigen expression. Finally, we explored whether the DNA vaccine aiming at promoting anti-viral Th type 1 cell response by incorporating IFNγ DNA as the adjuvant could be used to prevent hepadnaviral infection in the woodchuck model of hepatitis B.

In order to address these issues, we first generated recombinant IFNγ and rwTNFα using both baculoviral and *E. coli* expression systems. The biological activities of the cytokines produced were confirmed not only by conventional virus protective or cytotoxic assays, but also by examining their immunoregulatory ability to enhance expression of class I MHC antigen on woodchuck hepatocytes. In subsequent studies, we have established an in vitro model to investigate whether WHV could interfere with the expression of class I MHC, a key molecule for recognition of infected cells by cytotoxic T cells. We have found that more than one of the WHV proteins is involved in modulating

hepatocyte class I MHC antigen display and that WHV envelope preS2 and X proteins act in diametrically opposing ways in this regard. The data revealed that the preS2 protein is a dominant class I MHC inhibitor when the hepatocyte transcribes the complete virus genome. Although the precise molecular mechanism of this event remains to be established, our data suggested the preS2 protein-induced posttranscriptional mechanism is responsible for this defect. Importantly, we have shown that rwIFNγ treatment of hepatocytes transcribing either whole WHV DNA or the preS2 encoding sequences negated the WHV-caused class I MHC antigen suppression and restored its presentation to the same level as that observed on control cells.

In another study, we designed a bicistronic plasmid containing DNA sequences coding for WHc and IFNγ and tested the efficacy of the construct as a vaccine in preventing WHV infection in woodchucks. We have found that a significant proportion of the vaccinated animals was protected from hepatitis, as revealed by the absence of serological markers of WHV infection and normal liver histology. The protection was accompanied by a dramatic decrease in liver WHV RNA, the intermediate of WHV replication, when compared to the non-protected animals. This inhibition of WHV replication is mediated by a mechanism which does not appear to involve intrahepatic activation of IFNγ, TNFα and 2'-5' OAS. However, in spite of the disease-free state, residual WHV infection was detected in all the animals, indicating that sterile immunity against hepadnavirus is unlikely to be achieved by this approach. Nevertheless, our findings do not exclude a possibility that the same approach might be effective as

a therapeutic vaccine against chronic hepatitis. It is now recognized that hepadnavirus replication once established never can be totally eliminated, even in cases in which apparently successful response to antiviral therapy with IFNα or nucleoside analogues is achieved. Therefore, the approach tested in our current study should be further investigated in animals chronically infected with WHV, both alone and in combination with the presently available antiviral agents, to determine its efficacy in suppressing liver disease in chronic infection.

The complexity of the hepadnavirus-host interactions, influenced by the host's individual variability in the range and strength of its antiviral immune responses and by variations in the intrahepatic cytokine milieu, makes it exceedingly difficult to inspect the viral influence on hepatocyte class I MHC expression in *in vivo* conditions. This is further complicated by a very low efficiency of HBV and WHV infections in cultured hepatocytes, which impedes the dissection of this issue *in vitro*. In the current studies, we have circumvented these difficulties by establishing a woodchuck hepatocyte line stably transfected with more than the full-length WHV genome to mimic the situation occurring in chronic infection. In addition, we created hepatocyte lines transcribing genes encoding individual WHV proteins and complete WHV DNA knock-out mutants generated by the site-specific mutagenesis. We have found that WHV suppresses class I MHC presentation on hepatocytes in our model, as we have earlier observed for naturally infected hepatocytes in the course of chronic WHV hepatitis (Michalak *et al.*, 2000). In addition to the significance of these data summarized and discussed in Sections 5.5 and 8, the results obtained suggest

that the WHV preS2 protein may act as a viral transactivator, in addition to WHV X protein (Murakami, 1999). In this regard, it has been reported that HBV preS1/2 protein may play a role in regulating host gene transcription (Hildt *et al.*, 2002; Hildt *et al.*, 1996). Moreover, such an interference with the host gene transcription has been suggested to result in the development of HCC, a common consequence of chronic HBV infection (Kekule *et al.*, 1990). In order to avoid recognition by host CD8+ CTL, one of the strategies shared by both malignant tumors and viral infections is suppression of cell surface class I MHC display to abrogate class I MHC-TCR CTL interaction. Our data indicate that hepadnavirus preS2 protein inhibits expression of class I MHC antigen, which may greatly enhance the chance of virus to persist in infected hepatocytes. These data offer an alternative explanation for the reported association between integration of the HBV sequence encoding preS1/preS2 proteins and HCC development.

The data from our *in vitro* hepatocyte-WHV system do not allow for comment on whether suppression of the class I MHC presentation could promote the activation of innate cellular responses, particularly of NK cells whose activity is triggered by the decreased class I MHC display on the surface of targeted cells (Karre, 2002). The answer may rely on several factors, among which *in vivo* accessibility of hepatocytes to NK cells, generally low levels of class I MHC expression on naïve hepatocytes, and massive amounts of virus envelope material circulating in chronic infection, which has been shown to compromise NK cell activity (de Martino *et al.*, 1985), need to be considered.

Our data also revealed that IFNγ could reverse WHV-caused inhibition of hepatocyte class I MHC expression. This suggests that the host's immune system can potentially overcome the virus-induced inhibitory effect. Analysis of the class I MHC-affiliated gene transcription also indicated that the endocytic presentation pathway was activated upon IFNγ stimulation, including cells actively transcribing WHV genome. The class I MHC-linked genes investigated in our study are all known to be involved in processing, transporting or loading of endogenously generated peptides to class I MHC molecules. The enhanced expression of these genes by IFNγ would lead to a more efficient presentation of viral peptides by class I MHC to virus-specific CTL. Therefore, treatment with IFNγ could make virally infected cells more recognizable by the immune system. Overall, our results support the notion that induction of intrahepatic IFNγ can contribute to the clearance of HBV not only by directly inhibiting virus replication, as suggested by others (Guidotti and Chisari, 2001), but also by promoting virus-specific immune responses. Understanding of how, when and to what extent such intrahepatic IFNγ should be induced would add another dimension to the possibility of successful management of chronic hepatitis B.

It has been well established that anti-viral T cell responses play a critical role in eliminating hepadnavirus infection. Therefore, induction of this response should be highly favorable to the host. One of the strategies is to use DNA immunization, which has been shown to have superior advantages by eliciting not only B cell but also T cell immune responses (Donnelly *et al.*, 2005). In our DNA vaccine design, introduction of DNA encoding for IFNγ, which is a Th type 1

polarizing cytokine, should skew the immune response toward the generation of viral-specific CD8+ CTL reactivity. Moreover, providing IFNγ should also enhance antigen processing and antigenic epitope presentation via stimulation of the endocytic presentation pathway, as our data confirmed. Taking this into consideration, there is a significant difference in the principle of operation between the currently available HBV vaccine and our DNA vaccine. Unlike the current HBsAg-based vaccine, which presumably blocks virus cell entry via induction of neutralizing anti-HBs and enhance virus clearance by the monocyte-macrophage system, the virus-specific CD8+ CTL induced by DNA immunization recognize target cells which have already been infected. In other words, virus-specific CD8+ CTL cannot block the entry of infectious virions and they can act only when the infection and synthesis of viral proteins have been established. Therefore, this type of vaccine should be of value in controlling already progressing infection, as it is in the case in chronic hepatitis B. On the other hand, as the data from our and other laboratories indicated, this type of vaccine can prevent hepatitis. Therefore, the question is how this can happen, especially since the DNA sequence coding for the internal virus antigen was used.

Our data revealed that the introduction of IFNγ DNA to the WHc DNA vaccine gave a significant advantage over administration of WHc DNA alone in protecting woodchucks from serologically evident infection and liver necro-inflammation. Although residual WHV DNA and RNA were detectable in the liver and the lymphatic system by sensitive PCR-based techniques, the response primed by the WHc-IFNγ DNA was able to keep the virus replication under tight

control. The detection of small quantities of WHV DNA was not completely surprising since persistence of viral genomes and their replicative intermediates, as we now know, universally continues years after apparent complete resolution of AH, which is normally accompanied by a strong, multivalent virus-specific CTL and T helper cell response (Penna *et al.*, 1996; Rehermann *et al.*, 1996a). Nevertheless, our findings provided conclusive data indicating that sterilizing immunity against hepadnavirus cannot be achieved using immunization with DNA encoding for the virus nucleocapsid antigen. This is entirely new information which contradicts the majority of the previous studies in which less sensitive techniques were applied for hepadnavirus detection.

Considering the fact that very close contact is required between virus-specific T cells and infected hepatocytes, it was rather surprising to find that none of the protected animals developed any sign of intrahepatic lymphomononuclear infiltrations, as judged by liver histology at the time corresponding to pre-acute phase (2 weeks p.i.) or acute infection (6-8 weeks p.i.). This suggests that the bicistronic WHc-IFNγ DNA vaccine was able to protect or induce almost complete virus clearance by a non-inflammatory pathway. This finding may suggest that controlling hepadnavirus replication can be achieved without damaging or compromising the liver function. In our study, there was also no relation between protection and the production of core antigen specific antibodies. Furthermore, the data obtained showed that there was also no association between protection and elevated intrahepatic expression of IFNγ, TNFα or 2'-

5'OAS, which has been suggested to contribute to HBV clearance from the liver in the HBV transgenic mouse model (Guidotti and Chisari, 2001). One potential explanation could be that enhanced IFNγ, TNFα and/or 2'-5'OAS expression occurred at a very early stage, *i.e.,* between 0 and 2 weeks after challenge with WHV. Alternatively, our data cannot rule out involvement of another not yet identified anti-viral molecule. If a similar anti-viral state can be elicited in chronic hepatitis B, the resolution of HBV infection would probably happen without severe liver inflammation, which is a side effect of the activation of antiviral immunity. Further investigation of the underlying mechanisms may contribute to the development of novel therapies against chronic HBV infection.

One of the issues which was not investigated in our study was to what extent the innate immunity could be involved in defending against infection following vaccination with WHc-IFNγ DNA. As one of the first lines of host defense, innate immunity has been shown to be very potent in elimination of a variety of pathogens (Kawai and Akira, 2006; Portnoy, 2005). Activation of NK and NK T cells, as well as administration or induction of IFNα has been reported to be able to limit HBV infection (Baron *et al.*, 2002; Kakimi *et al.*, 2000; Kakimi *et al.*, 2001). Recruitment of neutrophils to the liver was also found to participate in the immunopathogenesis of liver injury in chronic HBV infection (Sitia *et al.*, 2002). Therefore, limited activation of the innate immune responses in hepadnaviral infection may have a significant potential in limiting the virus spread, especially at the early stage of infection. We believe that in our model, the

observed protection from challenge with a massive virus dose was probably a consequence of the synergistic effect exerted by both the innate immunity and very early activation of T cell anti-viral response.

Hepadnavirus-host interaction is a highly dynamic and complex process. In this infection, the virus would replicate, produce an excess of envelope (surface) antigens, which most likely have immunomodulatory properties, and interfere on different levels with the host's immune defense machinery. On the other hand, the immune system, with its innate and adaptive branches of immunity, is set up to fight pathogens. The outcome of the infection is, therefore, determined by who is winning the battle, the virus or the host. In the case of chronic hepadnaviral infection, an effective therapy should act on both sides, *i.e.,* by inhibiting virus replication and by promoting host anti-viral immunity. At the present time, suppression of virus replication can be achieved by nucleoside analogs capable of inhibiting the activity of viral DNA polymerase. With the exception of the drug resistant infections, this kind of treatment can dramatically reduce the total virus load. Ideally, such a treatment can be combined with activation of both innate and virus-specific adaptive immunities. We believe that the sustained virological response can be eventually achieved in chronic HBV infection by such a combination of antiviral and immune-based therapy. As our data suggest, application of IFNγ or, more likely, functionally compatible synthetic analogs, could be a viable option to reverse HBV evasion. The effectiveness of this approach can be further enhanced by inducing or delivering the cytokine or

its functional equivalent to the site of infection to minimize the side effects seen in systemic administration of IFNγ. The ultimate goal would be to elicit virus-specific T cell and B cell responses. The former should combat the intracellular infection, while the latter should neutralize infectious virus outside the cell and limit spread of infection. However, one of the major challenges of this strategy will be to break the virus-specific immune tolerance which normally accompanies chronic hepatitis B. The introduction of IFNγ DNA into a therapeutic HBV DNA vaccine might be a valid approach.

CHAPTER 8. SUMMARY AND CONCLUSIONS

In the series of studies presented, we explored the woodchuck model of hepatitis B and the woodchuck hepatocyte culture system to investigate the nature of hepadnavirus-cell interactions potentially underlying the hepadnavirus ability to escape from immunological surveillance and the role of antiviral cytokines, predominantly IFNγ, in reversing a virus-caused defect and in modulating an anti-hepadnavirus immune response mounted by preventive DNA vaccine. For the purpose of our studies, several relevant woodchuck genes were identified and biologically active woodchuck cytokines, IFNγ and TNFα, were produced. In addition, an *in vitro* hepatocyte culture model was established to analyse WHV interference with the hepatocyte presentation of class I MHC molecules. Real-time RT-PCR quantification assays measuring transcriptional activity of WHV genes, as well as expression of woodchuck genes encoding components of the class I MHC presentation pathway and subunits of the proteasome were also developed. The results obtained can be summarized as follows:

1. The full-length woodchuck IFNγ and TNFα cDNAs have been successfully cloned and both cytokines were produced in the baculovirus-insect cell and the *E. coli* expression systems. The rwIFNγ derived from the baculoviral system was found to be biologically active, as determined by protection of woodchuck hepatocytes against ECMV-induced cytolysis,

inhibition of the cytolysis protection by specific antibodies raised against rwIFNγ, and by augmentation of the presentation of class I MHC antigen on woodchuck hepatocytes but not human HepG2 liver cells. In contrast, while rwIFNγ produced in *E. coli* was immunogenic, it was functionally less active.

The rwTNFα generated in the baculovirus system showed a classical ability to kill murine fibroblasts, which was specifically inhibited by cross-reactive antibodies to murine TNFα. The recombinant cytokine also upregulated class I MHC antigen display on woodchuck hepatocytes. The rwTNFα from *E. coli.* displayed substantially lower biologically activity. Overall, the study demonstrated that the expression of rwIFNγ and rwTNFα in the baculovirus system generated biologically active cytokines whose potency was considerably greater than those produced in *E. coli.*

2. To investigate the mechanism of WHV interference with the hepatocyte class I MHC antigen presentation, which has been identified as an unvarying feature of chronic WHV infection in the previous studies from this laboratory, we established a hepatocyte culture model stably transfected with the whole WHV genome, with sequences encoding for individual WHV proteins or with whole virus genomes in which transcription of selected proteins was disabled by site-specific mutagenesis. We discovered that the surface display of class I MHC

antigen was suppressed on hepatocytes transcribing the complete WHV genome or its subgenomic fragments encoding for the virus envelope preS2 protein or preS1 protein, which naturally encompasses preS2 amino acid sequence. Analysis of the mutated WHV genomes confirmed that the envelope preS2 protein was responsible for inhibition of the class I antigen display. Importantly, the class I antigen suppression was not associated with down-regulation of the genes encoding class I MHC heavy chain, β_2m, TAP1 and TAP2 or proteasome subunits. This suggested that it is unlikely that the interference occurred at the level of gene transcription. The results obtained imply that the defective presentation of class I MHC antigen on hepatocytes transcribing WHV is a consequence of intracellular posttranscriptional suppression exerted by the virus envelope preS2 protein.

3. In contrast to the inhibition of class I MHC presentation in hepatocytes expressing the complete WHV genome or subgenomic fragments encoding for the virus envelope preS2 and preS1 proteins, hepatocytes transcribing WHV X gene alone demonstrated a profound enhancement in class I antigen display. These remarkable contradictory findings indicate that different sequences of the hepadnavirus genome exert diametrically opposed effects on the class I MHC antigen presentation. This suggests that in the context of transcription of the complete WHV genome, a highly complex interplay between individual virus translation products is

responsible for inhibition of the hepatocyte class I MHC display and that the virus envelope preS2 protein acts as a dominant inhibitor of class I MHC expression.

4. WHV-induced class I MHC antigen suppression was completely reversed by exposure of cultured hepatocytes to IFNγ, which is known to be a potent stimulator of class I antigen expression. This may suggest that treatment with this cytokine or with functionally equivalent agents or promotion of the local (intrahepatic) production of the cytokine may enable hepatocytes in chronic hepadnaviral infection to overcome an inhibitory effect exerted by the virus preS2 protein on the class I antigen expression. This may allow for processing and effective presentation of viral peptides in the context of class I antigen, leading to their increased accessibility for recognition by specific CTL and, in consequence, enhance elimination of virally infected cells and clearance of the virus from the liver. Therefore, our study suggests that the induction of IFNγ, in addition to enhancing the host's innate immunity, might have a beneficial effect on the virus-specific immune response.

5. To examine the immunoregulatory function of IFNγ in promoting anti-viral immunity, we designed a bicistronic DNA vaccine encoding WHV core and woodchuck IFNγ within a single plasmid construct and investigated the

efficacy of this DNA vaccine in protecting woodchucks from experimental WHV challenge. We found that almost half (4 of 9) of the woodchucks immunized with the bicistronic vaccine became protected from serologically evident infection and hepatitis after challenge with WHV. In contrast, only one of four animals injected with WHc DNA alone was similarly protected. This protection was not linked to induction of WHcAg-specific antibodies or WHV-specific T cell proliferative response, and was not associated with enhanced intrahepatic transcription of Th1 cytokines or 2',5'-oligoadenylate synthetase, an indicator of IFNα activation.

Strikingly, all animals protected from serologically detectable infection and histologically evident hepatitis became reactive for WHV DNA and carried persistently low levels of replicating virus in hepatic and lymphoid tissues after challenge with a massive dose of WHV. Our data shows that the bicistronic DNA vaccine encoding for both hepadnavirus core antigen and IFNγ was significantly more effective in preventing hepatitis than that encoding for virus core alone, but neither of them could mount sterilizing immunity against the virus. Our findings indicate that immunization with DNA encoding for a hepadnavirus internal antigen is unlikely to be of value in prophylaxis against HBV infection, but they do not exclude that this approach might be applicable in therapeutic treatment of chronic hepatitis B.

CHAPTER 9. FUTURE DIRECTIONS:

The results obtained during the course of these studies have raised several important questions which are worthy of further investigation. Some of them are as follows:

1) Does inhibition of hepatocyte class I MHC expression by hepadnavirus compromise the host's immune defense against other intracellular pathogens infecting the liver? Since it is well known that the class I MHC-CTL interaction plays a critical role in clearing intracellular pathogens, the hepadnavirus-induced class I MHC suppression may not only promote persistence of HBV infection, but also prevent immune recognition of other viruses or parasites infecting hepatocytes. For example, when a liver is co-infected with HBV and HCV, the HBV-induced deficiency in the class I MHC antigen presentation may hinder the clearance of both viruses from the liver.

2) Would an induction of either intra or extrahepatic IFNγ promote the immune recognition of hepadnavirus in the liver in chronic hepatitis? Our *in vitro* data clearly showed that IFNγ can reverse the hepadnavirus-induced suppression of class I MHC antigen presentation on hepatocytes. It is a valid assumption that *in vivo* stimulation of the local (hepatic) or the extrahepatic production of IFNγ or providing the exogenous cytokine can

also restore the class I MHC inhibition on hepatocytes in chronically infected livers. Consequently, this could augment the recognition of infected cells by virus-specific CTL and, possibly, lead to the clearance of virus and termination or at least a decrease in the protracted liver injury which typically accompanies chronic hepadnaviral infection.

3) Since we have established the *in vitro* model of WHV infection in hepatocytes after their stable transfection with the complete WHV DNA, it would be feasible to examine whether IFNγ and/or TNFα directly suppress WHV replication, as it was reported for HBV in transgenic mice (Guidotti and Chisari, 2001). Our *in vitro* model offers a significant advantage by allowing for convenient, well-controlled manipulations and for a precise analysis of hepadnavirus replication status. In addition, woodchuck hepatocyte lines stably expressing IFNγ or TNFα and the availability of biologically active rwIFNγ and rwTNFα generated in the course of our study provide unique tools for these further investigations. Therefore, the system established can contribute to identification of the pathways by which the cytokines exert their postulated direct anti-viral action. This may subsequently aid in designing novel anti-viral agents.

4) In order to enhance the efficacy of DNA vaccination in preventing hepadnaviral infection, some further optimization procedures should be tested, particularly since we have found that immunization with DNA

encoding hepadnavirus core protein, even when supplemented by IFNγ DNA as an adjuvant, does not mount complete sterilizing immunity against the virus. For example, immunization *via ex vivo* manipulated DC can be tested. As professional antigen presenting cells, DCs express high levels of class I and class II MHC antigens as well as co-stimulatory molecules, and secrete numerous cytokines promoting cellular immune responses. Therefore, anti-hepadnaviral immunity could be greatly enhanced by using autologous DC transfected with DNA encoding the selected virus gene and transferring the cells back to the recipient. The availability of the woodchuck model of hepatitis B in this laboratory makes such testing highly feasible. Similar strategies have been successfully applied in eliciting anti-tumor immunity (Nestle *et al.*, 2005).

5) It will be highly valuable to establish a technique for evaluation of the WHV-specific CTL responses in the woodchuck model of hepatitis B. This is important because the aim of both the restoration of class I MHC antigen presentation in chronically infected livers and the anti-viral DNA vaccination is to ultimately induce effective CTL response against WHV epitopes. The currently available assays measure WHV antigen-specific T cell proliferative response, which detects CD4+ T helper but not CD8+ CTL reactivity. The development of CTL assays will help to better characterize the efficacy of the approaches enhancing the class I MHC expression on hepatocytes and DNA immunization protocols.

6) It would be important to understand how the immunization with WHV core DNA in fact protects the animals from WHV infection and hepatitis. Unlike conventional immunizations with HBsAg, which presumably block the infectivity by inducing specific antibodies inhibiting virus envelope-target cell interactions, T cell responses directed against hepadnavirus internal nucleocapsid epitopes are considered to be involved in the induction of protective immunity. However, since the nature of class I MHC presentation and class I MHC-CTL interactions is such that anti-viral T cell responses are induced after the virus invades the target cell and virus protein synthesis has been initiated, it is difficult to reconcile these opposing facts. Based on the above, we were rather surprised to see the lack of lymphocyte infiltrations in the livers of the protected woodchucks. Understanding the underlying mechanism will contribute to better recognition of the role of T cell response in the pathogenesis of hepatitis and may help in designing immunotherapy directed against chronic infection.

7) Although the DNA vaccination approach tested was successful in preventing experimental hepatitis only in some of the animals vaccinated and did not induce sterilizing protective immunity, its efficacy should be tested in chronic hepadnaviral infection to examine whether it can elicit curative virus-specific immune responses at least in some cases of

chronic hepatitis. The regimen may include pre-treatment of chronically infected patients or animals with a known anti-viral agent, such as lamivudine or adefovir, to reduce the virus load. This could be followed by DNA immunization to activate virus-specific T cell responses, which are generally weak and narrowly focused on singular virus epitopes in chronic infection. The addition of a Th1-type cytokine, such as IFNγ, may help to augment anti-viral T cell immune responses to a therapeutically meaningful level. The activated responses should keep the virus replication under tight control, but is unlikely to eliminate the virus completely, as the data from our and other studies indicate (Brechot *et al.*, 2001; Michalak *et al.*, 1999; Michalak *et al.*, 1994).

8) Why does the anti-viral immune response fail to completely eradicate hepadnavirus infection? Persistence of low levels of HBV and WHV replication after spontaneous or therapeutically induced recovery from hepatitis or, as the current study showed, after challenge of animals otherwise successfully protected by DNA vaccination, appears to be a common consequence of exposure to these viruses. The residual or secondary occult infection (SOI) is associated with a high risk of development of HCC in both humans (Pollicino *et al.*, 2004) and woodchucks (Michalak *et al.*, 1999). It might also be followed by the re-occurrence of symptomatic infection if the host's immune system became compromised by certain factors, for example, immunosuppressive therapy,

cancer, or co-infection with another virus. Therefore, the understanding of the mechanism of virus escape even from frequently very strong, polyvalent and epitope multispecific immune response, as is normally seen in self-limited acute hepatitis, would represent a significant advancement.

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APPENDIX A: FREQUENTLY USED MOLECULAR BIOLOGY BUFFERS AND REAGENTS

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

Binding buffer: 50 mM Sodium Phosphate (pH 8.0), 300 mM NaCl, 8 M Urea

Denaturing buffer: 50 mM Sodium Phosphate (pH 7.0), 300 mM NaCl, 8 M Urea

Elution buffer: 45 mM Sodium Phosphate (pH 7.0), 8 M Urea, 270 mM NaCl, 150 mM Imidazole

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

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

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

Isopropyl-beta-D-thiogalactopyranoside (IPTG): 100mM (100x stock)

Lysis buffer (DNA): 100 mM NaCl, 10 mM Tris-HCl buffer (pH 8.0), 25 mM EDTA, (pH 8.0), 0.5 % SDS and 0.1 mg/ml proteinase K (prepared fresh)

P1 solution: 50 mM Tris·Cl (pH 8.0), 10 mM EDTA, 100 µg/ml RNase A

P2 solution: 200 mM NaOH, 1% SDS (w/v)

P3 solution: 3.0 M potassium acetate (pH 5.5)

PBS-EDTA: 20 mM Phosphate buffer (pH 7.4), 150 mM NaCl, 5 mM EDTA

PBST: 20 mM Phosphate buffer (pH 7.4), 150 mM NaCl, 0.1% Tween 20

QBT buffer: 750 mM NaCl, 50 mM MOPS (pH 7.0),15% isopropanol (v/v), 0.15% Triton X-100 (v/v)

QC buffer: 1.0 M NaCl, 50 mM MOPS, pH 7.0;15% isopropanol (v/v)

QF buffer: 1.25 M NaCl, 50 mM Tris·Cl (pH 8.5), 15% isopropanol (v/v)

Semi-dry transfer buffer: 39 mM Glycine, 48 mM Tris, 0.03% w/v SDS, 20% Methanol

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

TBST: 10 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween 20.

Washing buffer: 50 mM Sodium Phosphate (pH 7.0), 300 mM NaCl, 8 M Urea, 7.5 mM Imidazole

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

All reagents were obtained from Sigma Chemical Co. unless otherwise noted.

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APPENDIX B: WOODCHUCK SEQUENCES CLONED DURING THE PRESENT STUDY.

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 respective GenBank accession number in a similar format at National Institute of Health GenBank, accessible at www.ncbi.nlm.nih.gov.

APPENDIX B.1

LOCUS AY726002 576 bp mRNA linear ROD 28-SEP-2004
DEFINITION Marmota monax low molecular mass protein 2 mRNA, partial cds.
ACCESSION AY726002
VERSION AY726002.1 GI:52547777
SOURCE Marmota monax (woodchuck)
FEATURES Location/Qualifiers
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/mol_type="mRNA"
/db_xref="taxon:9995"
CDS <1..>576
/note="LMP2; proteasome subunit"
/codon_start=1
/product="low molecular mass protein 2"
/protein_id="AAU81924.1"
/db_xref="GI:52547778"

/translation="TTIMAVEFDGGVVVGSDSRVSAGEAVVNRVFDKLSPLHHRIYCAL
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VAGWDQREGGQVYGTLGGMLTRQPFAIGGSGSTYIYGYVDAAYKPGMTPEEC
RRFTTDAITLAMNRDGSSGGVIYLVTVTAAGVDHRVILGNE"

ORIGIN
1 acaaccatca tggcagtgga gtttgatggg ggtgtcgtgg tgggttctga ttcccgggtg
61 tctgcaggag aggcagtggt caaccgggtg tttgacaagc tgtcccctct acaccatcgc
121 atctactgtg ccctctctgg ttcagccgct gatgcccaag ccgttgcgga catggccgcc
181 taccagctgg agttgcatgg gttggaactg gaagaacctc cactggttct ggctgctgca
241 aatgtgataa ggaatatcac ctacaaatat agggaagact tgtcggcaca cctcatggta
301 gctggctggg accaacgtga agggggccag gtatatggaa ccctgggagg aatgctgact
361 cgacagccct ttgccattgg tggatctggc agcacttaca tttacggtta tgtggatgca
421 gcatataaac caggcatgac ccctgaggag tgcaggcgct tcaccacaga tgctatcact
481 ctggccatga accgggatgg ctccagtgga ggtgtcatct acctggtcac tgtcaccgct
541 gctggtgtgg atcatcgagt aatcttgggg aatgag

APPENDIX B.2

LOCUS AY726003 471 bp mRNA linear ROD 28-SEP-2004
DEFINITION Marmota monax low molecular mass protein 7 mRNA, partial cds.
ACCESSION AY726003
VERSION AY726003.1 GI:52547779
SOURCE Marmota monax (woodchuck)
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/db_xref="GI:52547780"

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VHATHRD"

ORIGIN
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121 ctgtcagtac tgggagcgcc tgttggccaa agaatgcagg ctgtactact tgcggaatgg
181 ggagcggatc tcagtgtcag ctgcctccaa actgctctcc aacatgatgt gtcagtaccg
241 gggcatgggc ctctccatgg gcagtatgat ctgtggctgg gacaagaagg gtccaggact
301 ttactatgtg gatcacaatg ggactcggct ctcaggatgt atgttctcca ccggtagtgg
361 gaacagttat gcctacgggg ttatggacag tggctatctg cccaatctta gtcctgaaga
421 agcttatgac cttggtcgca gggctattgt tcatgccact cacagagaca a

APPENDIX B.3

LOCUS AY726004 746 bp mRNA linear ROD 28-SEP-2004
DEFINITION Marmota monax multicatalytic endopeptidase complex-like 1 mRNA, partial cds.
ACCESSION AY726004
VERSION AY726004.1 GI:52547781
SOURCE Marmota monax (woodchuck)
FEATURES Location/Qualifiers
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LLRTLSSPTEPIKRPDSYCFAPGTTAILTQTVKPLNLELLEET"

ORIGIN
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241 ctgcatccaa catggagcta cacgcaatgt ccactggacg cgagcctcgc gtggccacgg
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361 tcgtgggcgg agtagatctg accggaccgc aactctatgg cgtgcacccc cacggttcct
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601 ccgcatcagg tgcccagctg ctgcggacac tgagctcgcc cacagagcct ataaagaggc
661 ctgatagcta ctgctttgca cctggaacca cagccattct gacccagaca gtgaagccat
721 tgaacttgga gctcctagaa gaaact

APPENDIX B.4

LOCUS AY726005 595 bp mRNA linear ROD 28-SEP-2004
DEFINITION Marmota monax proteasome activator 28-alpha mRNA, partial cds.
ACCESSION AY726005
VERSION AY726005.1 GI:52547783
SOURCE Marmota monax (woodchuck)
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ORIGIN
1 gatttctgag ttggatgcat ttttaaagga accagctctc aatgaagtca acctgagcaa
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121 tcggaagaaa cagcaggaga aggaagataa ggatgagaag aagaaagggg aagatgaaga
181 caaaggtcct ccctgtggcc cagtgaactg caatgagaag attgtggtcc tcctgcaacg
241 tctgaaacct gaaatcaagg atgtcatcga gcaactcaac ctggtcacta catggttgca
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481 ttatcggcag ctggtccatg agttggatga agcagagtac cgggacattc ggctgatggt
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APPENDIX B.4

LOCUS AY726006 672 bp mRNA linear ROD 28-SEP-2004
DEFINITION Marmota monax proteasome activator 28-beta mRNA, partial cds.
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VERSION AY726006.1 GI:52547785
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TISKYFSERGDAVAKASKETHVMDYRALVHERDEAAYGELRAMVLDLRAFYAE
LYHIISSNLEKIVNP"

ORIGIN
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61 gaggctgagg aattcctcta cagattcttg ccacagaaaa tcatgtacct gagtcagctt
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241 gaagtaccca agtgtggctt tctccccggg aatgagaagg tcctggccct gcttgctcta
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