HEPADNAVIRAL LYMPHOTROPISM AND ITS ROLE IN VIRUS PERSISTENCE IN THE WOODCHUCK MODEL OF HEPATITIS B

CENTRE FOR NEWFOUNDLAND STUDIES

TOTAL OF 10 PAGES ONLY MAY BE XEROXED

(Without Author's Permission)

A

PATRICIA MARY MULROONEY-COUSINS







HEPADNAVIRAL LYMPHOTROPISM AND ITS ROLE IN VIRUS PERSISTENCE IN THE WOODCHUCK MODEL OF HEPATITIS B

by

© PATRICIA MARY MULROONEY-COUSINS

A thesis submitted to the School of Graduate Studies

in partial fulfilment of the requirements for

the degree of Doctor of Philosophy

Faculty of Medicine

Memorial University of Newfoundland

May, 2005

St. John's

Newfoundland



Library and Archives Canada Bibliothèque et Archives Canada

Published Heritage Branch

395 Wellington Street Ottawa ON K1A 0N4 Canada Direction du Patrimoine de l'édition

395, rue Wellington

Ottawa ON K1A 0N4

Canada

0-494-06686-5

NOTICE:

The author has granted a nonexclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or noncommercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis. Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

ABSTRACT

Hepatitis B virus (HBV) causes lifelong liver disease in up to 400 million persons worldwide. The true extent of HBV exposure is unknown, but could be as high as 2 billion people. This is mainly due to the existence of occult infection and the inadequate sensitivity of HBV-specific serological assays. Following our previous findings indicating that the lymphatic system is a site of hepadnavirus replication, the aims of this study were to determine virological factors underlying hepadnaviral lymphotropism as it pertains to virus persistence. Specifically, we focussed on the identification of how virus dose and the potential existence of variants may induce lymphatic system-restricted infection. We employed the woodchuck/woodchuck hepatitis virus (WHV) system, which represents the closest pathogenic model of human HBV infection and hepatitis B.

Our results revealed that a significant portion of circulating lymphoid cells are infected with WHV, whether or not infection is serologically evident or occult. This was documented by applying an *in situ* PCR combined with flow cytometry technique, established during this study, that enumerated WHV-infected cells without the necessity of nucleic acid extraction.

We identified that primary occult WHV infection, that is normally limited to the lymphatic system, is induced by exposure to low doses ($\leq 10^3$ vge) of wild-type virus and is unlikely due to infection with or the appearance of viral variants inclined to preferentially

invade lymphoid cells. We also showed that exposure to small amounts of WHV ($\leq 10^3$ vge)

did not induce protective anti-viral immunity in that the infected host remained susceptible to

i

infection with large WHV doses.

In a subsequent *in vitro* study, we documented that WHV could be serially passaged in both lymphoid cells and hepatocytes, which does not lead to the emergence of cell typespecific virus variants. The passaged WHV maintained its infectivity and pathogenicity when administered to virus-naive woodchucks. This proved that lymphotropism is a natural propensity of wild-type WHV in both *in vivo* and *in vitro* conditions.

By analysing animals intrahepatically transfected with recircularized, complete recombinant WHV DNA, we documented that recombinant WHV DNA initially establishes infection in lymphoid cells and, at this location, infectious virus is produced. The infection of the liver was always secondary.

Since dendritic cells (DC) are known to be a reservoir of virus replication in many persistent viral infections, we aimed to recognize whether this lymphoid cell subset is also the site of WHV persistent replication. We prepared monocytic derived DC from animals with serologically evident and occult chronic WHV infections and demonstrated that DC are indeed one of the cell types where the virus persistently propagates.

The new features of hepadnavirus infection uncovered in this study imply that when the host is exposed to a low hepadnavirus dose, the lymphatic system is a primary target of invading virus. Because of the similarities between WHV and HBV, it is reasonable to assume that infection of lymphoid cells also plays a major role in the initiation and long-term

ii

persistence of HBV in humans.

ACKNOWLEDGEMENTS

I would first like to express my gratitude to Dr. Thomas Michalak, who helped me in many ways over the course of my time as a graduate student. His emphasis on critical thinking, precise experimental procedures, and his example in designing and performing basic research was the basis of a mentorship beyond that normally provided by a supervisor. The opportunities he provided to showcase my work, and his constant encouragement to do so, instilled in me a self-confidence that will no doubt be invaluable in my career. His philosophy of belief in your own ideas, hard work, and his friendship will never be forgotten.

I also wish to thank Dr. Michael Grant and Dr. Gary Paterno, the members of my supervisory committee, for their help and advisement, and critique of my thesis. Thanks to the members of Dr. Michalak's research team, especially Norma Churchill and Colleen Trelegan, and some of his past and present students (you know who you are!), who helped discuss ideas, supported me, and were great friends. I also appreciate Canadian Blood Services for the Graduate Student Fellowship which provided financial support during the course of this study.

I dedicate this thesis to my family and husband; my sister, brothers and, especially, my parents (Pat and Shelia) who taught me to respect myself and to work hard. Their unconditional love and support was instrumental in the completion of this work. Finally, I

wish to thank Craig Cousins, for his love and help over the past few years. Words cannot

iii

begin to express my gratitude for you.

TABLE OF CONTENTS

ABSTRACT	i
ACKNOWLEDGEMENTS	iii
LIST OF TABLES	xi
LIST OF FIGURES	xii
ABBREVIATIONS	xv
LIST OF APPENDICES	xix
THESIS COAUTHORSHIP STATEMENT	xx
CHAPTER ONE: INTRODUCTION.	1
 1.1 HEPATITIS B VIRUS. 1.1.1 Molecular organization. 1.1.2 Ultrastructure. 1.1.3 Replication strategy. 	2 2 4 5
1.2 EPIDEMIOLOGY OF HBV INFECTION	5
 1.3 NATURAL PROGRESSION OF HBV INFECTION. 1.3.1 Symptomatic, serologically evident disease. 1.3.2 Asymptomatic, occult infection. 	7 7 9
 1.4 IMMUNOPATHOGENESIS OF HEPADNAVIRAL INFECTION. 1.4.1 Innate immunity. 1.4.2 Humoral immunity. 1.4.3 Cell-mediated immunity. 	12 13 16 19

THE WO	ODCHUCK MODEL OF HEPATITIS B	22
1.5.1	Woodchuck hepatitis virus (WHV)	22
1.5.2	Characteristics of WHV infection	24
	1.5.2.1 Symptomatic serologically evident hepatitis	24
	1.5.2.2 Primary occult WHV infection	27
	1.5.2.3 Silent residual WHV infection	29
	THE WC 1.5.1 1.5.2	 THE WOODCHUCK MODEL OF HEPATITIS B. 1.5.1 Woodchuck hepatitis virus (WHV). 1.5.2 Characteristics of WHV infection. 1.5.2.1 Symptomatic serologically evident hepatitis. 1.5.2.2 Primary occult WHV infection. 1.5.2.3 Silent residual WHV infection.

iv

1.6 GENERAL MECHANISMS OF VIRAL PERSISTENCE.	30
1.6.1 Latency of viral life cycle	30
1.6.2 Infection of immunologically privileged sites.	33
1.6.3 Induction of immunological tolerance.	34
1.6.4 Impairment of cytokine function.	35
1.6.5 Interference with MHC class I antigen processing and presentation	36
1.6.6 Interference with MHC class II antigen processing and presentation	38
1.6.7 Viral genomic variation	39
1.7 GENOME VARIATION IN HEPADNAVIRAL PATHOGENICITY	41
1.7.1 HBV genome mutations	41
1.7.1.1 Mutations found in fulminant hepatitis B	41
1.7.1.2 Mutations resulting in HBeAg-negative hepatitis B	43
1.7.1.3 Mutations arising after hepatitis B vaccination	44
1.7.1.4 Mutations arising during antiviral treatment	46
1.7.2 Woodchuck hepatitis virus variants	47
1.8 THE ROLE OF EXTRAHEPATIC REPLICATION IN HEPADNAVIR. PERSISTENCE.	AL 47
1.8.1 HBV infection of the lymphatic system	48
1.8.2 WHV lymphotropism.	50
1.9 PURPOSE OF THE STUDY	52
CHAPTER TWO: GENERAL MATERIALS AND METHODS	55
2.1 WOODCHUCKS	55
2.2 SAMPLE COLLECTION.	56
2.2.1 Blood collection.	56
2.2.2 Serum isolation.	56
2.2.3 Isolation and storage of peripheral blood mononuclear cells	57
2.2.4 Liver biopsies	57

V

2.4 DEPLETION OF VIRIONS AND VIRAL DNA FROM CELL SURFACE	62
 2.5 ISOLATION OF WHV DNA and RNA. 2.5.1 Isolation of cellular DNA. 2.5.2 RNA isolation. 2.5.3 Quantitation of nucleic acids. 	63 63 63 64
2.6 REVERSE TRANSCRIPTION REACTION	65
 2.7 POLYMERASE CHAIN REACTION (PCR). 2.7.1 Standard PCR conditions for WHV DNA detection. 2.7.2 Detection of WHV cccDNA. 2.7.2.1 Mung bean nuclease digestion. 2.7.2.2 Primers and cycling parameters. 2.7.3 PCR conditions for full-length WHV genome amplification. 	65 69 69 70 71
2.8 REAL-TIME PCR. 2.8.1 Detection of WHV core gene fragment.	73 73
 2.9 DETECTION OF WHV DNA. 2.9.1 Agarose gel electrophoresis. 2.9.2 Southern blot hybridization analysis. 2.9.2.1 DNA transfer to nylon membrane. 2.9.2.2 Random prime labeling and purification of recombinant DNA probes. 2.9.2.3 Southern blot hybridization. 2.9.2.4 Quantification of Southern bot hybridization signals by densitometry. 	74 74 75 75 75 76 77
 2.10 WHV DNA CLONING. 2.10.1 Purification of PCR products. 2.10.2 TA Cloning of DNA fragments. 2.10.3 Growth and storage of vector-transfected bacteria. 2.10.4 Mini-scale preparations of plasmid DNA. 2.10.5 Maxi-scale preparations of plasmid DNA 	78 78 79 79 80 80

2.11	WHV D	NA SEQUENCING	81
	2.11.1	Sequencing primer labeling with T4 kinase	81
	2.11.2	DNA fragment generation by PCR amplification	82
	2.11.3	Gel electrophoresis and analysis of amplified products	82

vi

2.12 HISTOLOGICAL EXAMINATION OF LIVER TISSUE	83
CHAPTER 3: QUANTITATIVE DETECTION OF HEPADNAVIRUS-INFECTED LYMPHOID CELLS BY IN SITU PCR COMBINED WITH FLOW	85
	05
3.0 SUMMARY	85
3.1 INTRODUCTION	86
3.2 MATERIALS AND METHODS. 3.2.1 Animals and lymphoid cell separation. 3.2.2 Optimization of assay conditions. 3.2.3 Cell surface DNase/trypsin/DNase treatment. 3.2.3 Cell surface DNase/trypsin/DNase treatment. 3.2.4 Cell fixation and permeabilization. 3.2.5 PCR primers and amplification conditions. 3.2.6 Flow cytometry analysis. 3.2.7 Hybridization analysis of amplified virus sequences in cells and in their PCR supernatants. 3.2.8 Statistical analysis. 3.2.8	88 92 94 94 95 96 99
 3.3 RESULTS	01 01 04 10
3.4 DISCUSSION	14

CHAPTER 4:

LOW DOSES OF HEPADNAVIRUS INDUCE INFECTION OF THE

LYMPHATIC SYSTEM THAT DOES NOT ENGAGE THE LIVER...... 123

4.0	SUMMARY	 123
1.2.2		_

4.1	INTRODUCTION	124	ł
-----	--------------	-----	---

vii

4.2.1 Woodchucks	126
4.2.2 WHV inocula from occult lymphatic system-restricted infection	127
4.2.3 Wild-type WHV inoculum	127
4.2.4 Sample collection and cell preparation	128
4.2.5 Serological and WHV DNA dot-blot assays	129
4.2.6 DNA extraction and PCR for WHV DNA and cccDNA	129
4.2.7 WHV DNA sequencing.	130
4.3 RESULTS	130
4.3.1 Transmission of primary occult WHV infection	130
4.3.2 Low-doses of wild-type WHV cause occult lymphatic system-restricted	120
4.2.2 Drimory accult W/UV infaction does not protect from	139
4.5.5 Frinary occur wriv intection does not protect from	147
supermicetion	14/
4.4 DISCUSSION	150
CHAPTER 5: MULTIPLE PASSAGE OF WILD-TYPE WHV IN LYMPHOID CELLS DOES NOT ALTER VIRUS GENOME SEQUENCE OR <i>IN VIVO</i>	
INFECTIVITY	154
5.0 SUMMARY	154
5.1 INTRODUCTION.	155
5.2 MATERIALS AND METHODS	157
5.2.1 Animals.	157
5.2.2 Cells	159
5.2.3 Preparation of splenocyte-derived WHV inocula	160
5.2.4 Multiple passage of splenocyte-derived WHV in lymphoid cells and	
hepatocytes	160
5.2.5 Detection of WHV DNA and cccDNA.	161
5.2.6 Preparation of culture supernatants from the final WHV passage in	

	5.2.7	lymphoid cells or hepatocytes. Inoculation of woodchucks with WHV recovered after multiple	162
		serial passage in lymphoid cells or hepatocytes	163
	5.2.8	WHV DNA sequencing.	163
5.3	RESUL	тѕ	164
	5.3.1	WHV-infected splenocytes secrete virus in culture.	164

viii

	5.3.2 Splenocyte-derived WHV is infect5.3.3 Multiple serial passage of lymphothepatocytes does not lead to the e	ious to naive lymphoid cells	5
	mutations		0
	5.3.4 <i>In vivo</i> infectivity of WHV after se hepatocytes	18	1
5.4	DISCUSSION		6
CH	APTER 6: OCCULT, LYMPHATIC SYSTEM-RE CAN BE INDUCED BY DIRECT LIVE COMPLETE rWHVDNA	STRICTED WHV INFECTION ER TRANSFECTION OF NAKED 192	2
6.0	SUMMARY		2
6.1	I INTRODUCTION		3
6.2	2 MATERIALS AND METHODS 6.2.1 Woodchucks		6

6.1	INTRO	DUCTION	193
6.2	MATER	LIALS AND METHODS	196
	6.2.1	Woodchucks	196
	6.2.2	Sample collection	196
	6.2.3	Full-length genome amplification and ligation of rWHV DNA	196
	6.2.4	Intrahepatic injection	203
	6.2.5	Sample collection.	203
	6.2.6	Preparation of WHV inocula from PBMC obtained during serologically	
		silent infection induced by rWHV DNA transfection	204
	6.2.7	Challenge with a liver pathogenic dose of wild-type WHV	205
	6.2.8	Preparation of monocyte-derived DC from animals injected with	
		rWHV DNA	205
	6.2.9	Detection of WHV DNA and cccDNA.	206
6.3	RESUL	тѕ	206
	6.3.1	Confirmation of recircularization of full-length monomeric rWHV DNA	
		constructs	206

6.3.2	Intrahepatic injection with rWHV DNA induces infection of lymphoid	
	cells.	207
6.3.3	Infectivity of WHV carried in lymphoid cells after animals were	
	transfected with rWHV DNA.	216

61	1 DISCUSSION	217
0.4	DISCUSSION	211

ix

CHAPTER 7: WHV PERSISTS IN DENDRITIC CELLS DURING LONG-TERM			
SYMPTOMATIC AND OCCULT INFECTIONS	225		
7.0 SUMMARY	225		
7.1 INTRODUCTION	226		
7.2 MATERIALS AND METHODS	229		
7.2.1 Woodchucks	229		
7.2.2 Derivation of immature and mature DC from monocytes	229		
7.2.3 DNA and RNA isolation.	232		
7.2.4 cDNA synthesis and PCR	232		
7.2.5 Cloning of woodchuck CD209.	232		
7.3 RESULTS	233		
7.3.1 Woodchuck DC are phenotypically comparable with their human			
counterparts	233		
7.3.2 DC from woodchucks with chronic hepatitis are a site of active			
replication	236		
7.3.3 DC from woodchucks with WHV persistently carry WHV	239		
7.4 DISCUSSION.	242		
CHADTED 9.			
CENERAL DISCUSSION	217		
ULINERAL DISCUSSION	241		
CHAPTER 9			
SUMMARY & CONCLUSIONS	261		
OLIADEED 10			
CHAPTER 10:			
FUTURE DIRECTIONS	203		
REFERENCES CITED			

APPENDIX A: COMPLETE WHV SEQUENCES CLONED DURING THE STUDY...... 300 APPENDIX B:

Х

LIST OF TABLES

C				
3				

Page

Table 3.1. Immunovirological characteristics of WHV infection at the time of analysis of WHV DNA in circulating lymphoid cells by <i>in situ</i> PCR combined with flow cytometry.	90
Table 5.1. Details on WHV passage experiments performed in the course of this study.	58
Table 5.2. Detection of WHV DNA in lymphoid cells after each of three sequential passages of splenocyte-derived WHV prepared from two woodchucks with chronic WHV hepatitis. 1	69
Table 5.3. Detection of WHV in cultured lymphoid cells and their supernatants after each passage of lymphotropic WHV derived from chronic WHV carrier WM.C	77
Table 5.4. Detection of WHV in cultured hepatocytes and their supernatants after each passage of lymphotropic WHV derived from WM.C chronic WHV carrier	78
Table 7.1. Immunovirological characteristics of chronic and SOI WHV infection in woodchucks studied and detection of molecular indicators of WHV infection in immature and/or mature DC. 2	30



LIST OF FIGURES

Page
Figure 1.1. Schematic diagram of WHV genome and encoded open reading frames
Figure 2.1. WHV-specific PCR oligonucleotide primers used in the study and sizes of resulting amplicons
Figure 3.1. Sensitivity and specificity of WHV DNA detection by PCR using the oligonucleotide primer pair homologous to the virus core gene sequence containing a fluorescein-conjugated sense primer.
Figure 3.2. Flow cytometry analysis of WHV-naive PBMC exposed to either WHV virions or cloned rWHV DNA and subjected or not to stepwise DNase/trypsin/DNase digestion and WHV DNA-specific PCR amplification
Figure 3.3 A and B. Detection of WHV DNA in lymphoid cells from WHV-infected woodchucks and a healthy control by <i>in situ</i> PCR coupled with flow cytometry or with hybridization analysis of cell supernatants obtained after PCR amplification
Figure 3.4. Representative plots from flow cytometry analysis of <i>in situ</i> amplified WHV DNA in PBMC samples obtained from two woodchucks with SOI persisting after termination of AH.
Figure 3.5. <i>In situ</i> PCR-flow cytometry analysis of PBMC from a woodchuck with POI who was born to a mother convalescent from acute WHV hepatitis
Figure 3.6. Detection of cccDNA in representative PBMC samples from woodchucks with silent persistent infection which were found WHV DNA reactive by <i>in situ</i> PCR/flow cytometry
Figure 4.1A and B. Serial passage of occult WHV infection restricted to the lymphatic system in virus-susceptible woodchucks

xii

Figure 4.4A and B. Serological and molecular profiles of WHV infection in woodchucks injected with increasing doses of a wild-type WHV and then challenged	
with a massive dose of the same inoculum	140
Figure 4.5. WHV DNA detection in serum, PBMC, and liver tissue samples collected from woodchuck 3/F.	143
Figure 4.6. WHV DNA detection in sequential liver biopsies obtained from animals 4/M and 5/F	145
Figure 4.7. Detection of WHV cccDNA in representative samples of PBMC obtained from woodchucks after inoculation with various doses of the WHV/tm3 inoculum but prior to challenge with a massive, liver-pathogenic dose of the same virus	148
Figure 5.1A and B. Detection of WHV DNA in supernatants collected at 72 h intervals during culture of WHV-infected splenocytes isolated from WM.C which were DNase treated or not.	166
Figure 5.2. Schematic outline of methodology used for serial passage of splenocyte- derived WHV in woodchuck lymphoid cell or hepatocyte cultures	168
Figure 5.3. Detection of WHV DNA in lymphoid cells and their supernatants collected after three serial passages of splenocyte-derived WHV from WM.A	171
Figure 5.4A and B. Detection of WHV DNA in lymphoid cells and WCM-260 hepatocytes and in their culture supernatants obtained after serial passage of WHV originating from WM.C splenocytes.	173
Figure 5.5. Detection of WHV cccDNA in representative samples of lymphoid cells and WCM-260 hepatocytes obtained after various passages of WHV derived from splenocytes of WM.C chronic WHV carrier.	179
Figure 5.6. WHV DNA (A) C and (B) preS sequence comparison of splenocyte- derived WHV inoculum, lymphoid cell and hepatocyte culture supernatants, and sera from WF 1 and WF 2	182

xiii

Figure 6.2. Southern blot hybridization analysis of the linearized plasmids and recircularized monomeric molecules of sp-rWHV DNA and ec-rWHV DNA	201
Figure 6.3A and B. Serological and WHV genome detection profiles and liver histology results in four woodchucks after intrahepatic transfection with either sp-rWHV DNA or ec-rWHV DNA.	208
Figure 6.4. Detection of WHV C gene sequence and cccDNA in representative PBMC samples obtained from woodchucks after liver transfection with either sp-rWHV DNA or ec-rWHV DNA prior to WHV DNA appearance in the liver	213
Figure 6.5. Profiles of serological and molecular markers of WHV infection in animals injected with inocula derived from PBMC obtained during the POI phase of infection induced by transfection with sp-rWHV DNA and ec-rWHV DNA	218
Figure 6.6. Detection of WHV C gene sequence and cccDNA in monocyte-derived DC samples obtained from WM2 and WM3 PBMC samples after liver transfection with either sp-rWHV DNA or ec-rWHV DNA, prior to WHV DNA appearance in the liver	er 220
Figure 7.1. Expression of CD209 in woodchuck and human DC	234
Figure 7.2. Detection of WHV DNA, cccDNA, and WHV RNA expression in immature and mature DC derived from two animals with serum WHsAg-positive, chronic WHV infection	e 237
Figure 7.3. Detection of WHV DNA and cccDNA in mature DC isolated from 2 animals with SOI persisting years after recovery from AH	.40
Figure 8.1. Schematic diagram of the involvement of the lymphatic system in the perpetuation of hepadnavirus infection induced by low WHV doses ($\leq 10^3$ vge) 2	.55



ABBREVIATIONS

AH	acute hepatitis
ALT	alanine aminotransaminase
anti-HBc	antibodies to hepatitis B virus core antigen
anti-HBe	antibodies to hepatitis B virus e 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
ASGPR	asialoglycoprotein receptor
AST	aspartate aminotransferase
AT	ambient temperature
bp	base pairs
С	core gene of HBV or WHV
cccDNA	covalently closed circular DNA
CD	cluster of differentiation
СН	chronic hepatitis
CMV	cytomegalovirus
cpm	counts per minute
CTL	cytotoxic T lymphocyte
DC	dendritic cells
DHBV	duck hepatitis B virus
DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide triphosphate
DR	direct repeat
EB	ethidium bromide

EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FasL	Fas ligand

XV

FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FRET	fluorescent resonance energy transfer
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
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HPV	human papilloma virus
HRPO	horseradish peroxidase
HSV	herpes simplex virus
IFNα	interferon alpha
IFNβ	interferon beta
IFNγ	interferon gamma
Ig	immunoglobulin
IL	interleukin
i.v.	intravenous
kb	kilobase pairs

LCMVlymphocytic choriomeningitis virusMCMVmurine cytomegalovirusMHCmajor histocompatibility complexminminuteMMLV-RTMoloney murine leukemia virus- reverse transcriptase

xvi

mo	months
NC×	negative control mean
NK	natural killer cell
nm	nanometres
ORF	open reading frame
Р	polymerase gene of HBV or WHV
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCR/NAH	nested PCR followed by Southern blot nucleic acid
	hybridization
p.i.	post-infection
POI	primary occult infection
preS1	large surface protein
preS2	middle surface protein
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
sec	seconds
SEM	standard error mean
SLAH	self-limited acute hepatitis
SOI	secondary occult infection
SSC	standard saline citrate
SS	single stranded
sssDNA	sonicated salmon sperm DNA

TCRT-cell receptorThhelper T cellTh1T helper type 1Th2T helper type 2TLRtoll-like receptor

xvii

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
X	X gene of HBV or WHV

xviii

LIST OF APPENDICES

APPENDIX A: STUDY	COMPLETE WHV SEQUENCES CLONED DURING THE	300
APPENDIX B: THE STUDY	WOODCHUCK CD209 GENE SEQUENCE CLONED DURING	328

Page



THESIS CO-AUTHORSHIP STATEMENT

This thesis is comprised of 10 chapters. Chapter 1 introduces the background and rationale for the thesis work. Chapter 8 gives a discussion that delineates the cohesiveness of the projects completed during this study, while Chapter 10 summarizes the major conclusions of the work. Chapters 3-7 contain the original data compiled from each of the projects completed for the thesis.

All of the work described in this thesis was performed by the author, whose primary role was in the collaboration of the design of the research methodology and in data collection and analysis. Chapter 3 was published as a principal-authored paper entitled "Quantitative detection of hepadnavirus-infected lymphoid cells by in situ PCR combined with flow cytometry: implications for the study of occult virus persistence" in the Journal of Virology, 2003, volume 77(2), pp. 970-979. The work comprising Chapter 4 was published as a co-authored paper entitled "Low doses of hepadnavirus induce infection of the lymphatic system that does not engage the liver" by T.I. Michalak, P.M. Mulrooney and C.S. Coffin in the Journal of Virology, 2004, volume 78(4), pp. 1730-1738. The thesis author significantly contributed to the content of this publication, specifically to the majority of the molecular determinations of WHV DNA and cccDNA presence, identification of the

complete nucleotide sequence of WHV/tm3 inoculum, and derivation of the sequence of

WHV amplicons. The work comprising Chapters 5-7 were completed solely by the thesis

author.

XX

The author has made other contributions to studies that parallelled the spectrum of this thesis. Data related to Chapter 4 describing the persistence of low levels of WHV DNA in the presence of antibodies to the virus core antigen were recently published as a paper entitled **"Persistence of isolated antibodies to woodchuck hepatitis virus core antigen is indicative of occult virus infection**" by C.S. Coffin, T.N.Q. Pham, P.M. Mulrooney, N.D. Churchill, and T.I. Michalak in *Hepatology*, 2004, volume 40(5), pp.1053-1061. The WHV cccDNA determinations and sequencing work for this paper were completed by the thesis author. In another related collaborative project, focussing on the occult persistence and lymphotropic nature of hepatitis C virus, the preliminary PCR data on HCV RNA detection in plasma and in mitogen-stimulated lymphoid cells were generated by the thesis author. This paper was entitled **"Hepatitis C"** by T.N.Q. Pham, S.A. MacParland, P.M. Mulrooney, H. Cooksley, N.V. Naoumov, and T.I. Michalak in the *Journal of Virology*, 2004, volume 78(11), pp.5867-5874.



CHAPTER ONE: INTRODUCTION

The term hepatitis is derived from the Greek words "hepar" (liver) and "itis" (inflammation). Although hepatitis can be attributed to many factors, the most common cause is viral. Many hepatitis viruses, designated as A, B, C, D, E, G, and TT, have been identified. These are able to infect the liver, causing hepatocellular necrosis, inflammatory cell infiltration, and subsequent hepatocyte regeneration.

The hepatitis B virus (HBV) causes significant morbidity and mortality, despite the availability of highly effective and safe vaccines. This is mainly due to the fact that established chronic HBV infection cannot be cured and that vaccination programs have not yet been implemented in many countries.

The identification of the surface antigen (Australia antigen), the envelope of the infectious virion, occurred in the 1960's when it was isolated from the serum of an infected Australian aboriginal man (Blumberg *et al.*, 1967). The ultrastructure of the infectious HBV virion was demonstrated in 1970 by Dane (Dane *et al.*, 1970) and, therefore, it is frequently called the Dane particle. It is generally accepted that HBV is essentially non-cytopathic (Guidotti and Chisari, 2001; Chisari and Ferrari, 1995; Curry and Koziel, 2000). However, in immunocompromised patients, *e.g.*, after re-infection of liver transplant, HBV can directly

destroy infected liver cells (Demetris et al., 1990). The symptoms of HBV infection are

mainly due to the host cytopathic immune responses directed against virus-infected

hepatocytes.

1.1 HEPATITIS B VIRUS

1.1.1 Molecular organization

HBV is the prototypic virus of the hepadnavirus family. This family is divided into two genera: Orthohepadnaviridae (mammalian viruses) and Avihepadnaviridae (avian viruses) (Marion et al., 1980). New viruses are being identified as members of these genera as more species are investigated. Well defined members of the mammalian subfamily include: HBV, woodchuck hepatitis virus (WHV), and ground squirrel hepatitis virus (GSHV). The best characterized member of the avian hepadnaviruses is the duck hepatitis B virus (DHBV), which has been extensively studied to determine hepadnavirus replication strategy and potential viral receptors (Mason et al., 1980).

All hepadnaviruses have similar molecular, structural, and antigenic properties. However, mammalian and avian hepadnaviruses also have some distinct characteristics. For example, avian hepadnaviruses lack an X gene, they synthesize two (not three) envelope proteins, and their envelope proteins do not exist as freely circulating filaments. As well, although chronic liver disease is induced by DHBV, hepatocellular carcinoma (HCC) does not appear to develop in infected animals (Cova *et al.*, 1994), unless alpha toxin is administered.

The HBV genome is a circular 3.2 kilobase (kb)-long, partially double-stranded DNA,

referred to as relaxed circular DNA (rcDNA). The circular structure of the genome is

maintained by a short cohesive overlap between the 5'-ends of plus and minus DNA strands.

The 5'-end of the DNA minus strand has a covalently linked protein, whereas the plus strand

has a 5'-RNA oligonucleotide primer attached, both of which are essential for viral replication.

The genome contains four overlapping open reading frames (ORF) encoding the four major translation products: the virus envelope or surface (S), core (C), polymerase (P) and X proteins. The three envelope proteins are encoded by the same ORF containing three inframe start codons, but they are derived from two different overlapping mRNA species (2.4 and 2.1 kb). These proteins have a common carboxy-terminus and differ at their aminotermini. They are referred to as: large (preS1), middle (preS2), and major or small (S) surface proteins (Gerlich *et al.*, 1992; Lau *et al.*, 1993).

The C ORF encodes the virus nucleocapsid protein, designated as the HBV core antigen (HBcAg), as well as another protein, which due to post-translational modifications, displays e antigen specificity (HBeAg) from the 3.5 kb pregenomic RNA (see Section 1.1.3). Although the amino acid sequences of the core and e proteins are nearly identical, except for an additional ten amino acid sequence encoded by the pre-core region and deletion of 34 residues encoded by the 3'-end of the C region, they induce the immune system to produce antibodies of distinct specificity (Ou *et al.*, 1986). The core protein is only found in infected cells and virions, whereas, HBeAg is detectable in the circulation and in hepatocytes, but does not exist as a part of the complete virion (Uy *et al.*, 1986; Salfeld *et al.*, 1989).

Transcription of the P ORF results from the 3.5 kb pregenomic RNA in formation of

1

a multidomain polypeptide with viral reverse transcriptase (RT), RNase, and DNA polymerase

activities. As well, this protein contains a packaging signal and primes reverse transcription

of the viral pregenome. This protein is essential for hepadnavirus replication (Bavand and Laub, 1988; Mack et al., 1988).

The X protein is synthesized by the smallest ORF and its derived mRNA of 0.7 kb. This protein has transcriptional trans-activating properties that may play a role in virus oncogenicity. This may be explained by the effect that the X protein has on cell death due to its interactions with anti-apoptotic cellular molecules, such as $Bcl-X_L$, thereby increasing cell survival and growth (Diao *et al.*, 2001a; Diao *et al.*, 2001b). The transactivating properties of the X-protein can affect other cellular proteins, including the expression of major histocompatibility complex (MHC) class I protein (Zhou *et al.*, 1990).

1.1.2 Ultrastructure

The infectious virion of HBV, or the Dane particle, exists as a 42 nanometer (nm)diameter sphere, consisting of a lipoprotein outer envelope made of the three virus surface proteins and host lipids (Gavilanes *et al.*, 1982). The envelope surrounds core (nucleocapsid) which houses the viral genome. Subviral particles carrying HBV surface antigen (HBsAg) reactivity are composed of envelope proteins and associated lipids and they form spheres (20nm diameter) and tubules (up to 230-nm long). These particles are not infectious, as they do not contain genetic material, but they can occur in the circulation in large quantities, as high as 50-300 μ g/mL, *i.e.*, 10¹² particles/mL (Peterson, 1981; Koff and Galambos, 1987; Dienstag

and Purcell, 1997). These subviral HBsAg particles purified from the blood of infected

individuals were used as the source material for preparation of HBV vaccines before the age

of recombinant protein synthesis technology (Hollinger et al., 1986).

1.1.3 Replication strategy

After a putative cell surface receptor(s) and virion interact to enable virus entry to the cytosol, it is presumed that the virus envelope is removed and the nucleocapsid migrates to the nucleus. There, HBV rcDNA is converted to covalently closed circular DNA (cccDNA) by host DNA polymerases and ligases. It is widely accepted that this event is the first step in viral replication and detection of HBV cccDNA is often used as the specific marker to monitor viral replication activity (Tuttleman et al., 1986). Four HBV mRNA transcripts (i.e., 3.5, 2.4, 2.1, and 0.7 kb) are transcribed from the cccDNA using host RNA polymerase and are exported from the nucleus. The 3.5 kb RNA is packed into core particles together with the viral polymerase in the nucleus of the infected cell. This pregenomic RNA is then reverse transcribed into minus strand DNA, which subsequently serves as a template for virus plus strand DNA synthesis (Summers and Mason, 1982). Once the plus stand is synthesized, new HBV rcDNA is formed. The mature nucleocapsid particles containing rcDNA are then either packaged into virions, which are exported from the cell, or recycled to the nucleus. Presently, hepadnaviruses are the only known DNA viruses that use reverse transcription in their replication cycle.

1.2 EPIDEMIOLOGY OF HBV INFECTION

While several other viruses are capable of inducing hepatitis (e.g., hepatitis A virus,

hepatitis C virus [HCV], hepatitis D virus, adenovirus, human cytomegalovirus [HCMV],

echoviruses, Epstein-Barr virus [EBV], rubella virus, and varicella zoster virus [VZV]), it is

well recognized that infection with HBV is one of the greatest worldwide health problems (Margolis *et al.*, 1991). This is primarily due to the fact that the virus is highly infectious (~50-times more than human immunodeficiency virus [HIV] and 4-5-times more than HCV) and frequently induces chronic hepatitis (CH), cirrhosis and HCC. This is taking place in the face of the availability of highly effective prophylactic vaccines. Recent estimates state that two billion people have been infected with the virus, with the global number of chronic HBV carriers (*i.e.*, serum HBsAg-positive individuals) approaching 400 million (World Health Organization, 2000). Of the people chronically infected, about 25% succumb to untreatable HCC (Beasley, 1988).

The geographic distribution of HBV infection varies greatly. Highly endemic areas include Southeast Asia and sub-Saharan Africa where up to 15% of the total population are chronically infected with HBV (*i.e.*, permanently serum HBsAg-positive), primarily due to vertical transmission from mother to child (Maynard *et al.*, 1989). In the developed world, including North America and Western Europe, infection rates are usually below 1%, and virus transmission occurs largely due to intravenous drug use and sexual contact. However, occupational exposure is still a concern for those not vaccinated (Gerberding, 1996). It is estimated that at least 270,000 Canadians are chronically infected with HBV, but the

prevalence of HBV infection varies considerably due to the heterogeneity of our population

(Canadian Consensus on the Management of Viral Hepatitis, 1999). For example, there is an

estimated chronic carrier rate of 6.9% among natives and 7.4% among immigrants from

Southeast Asia and Africa, whereas the rate is considered very low among non-immigrant

Canadians (Canadian Communicable Disease Report, 2001).

1.3 NATURAL PROGRESSION OF HBV INFECTION

1.3.1 Symptomatic, serologically evident disease

Clinical patterns of HBV infection can be classified into five distinct appearances: (1) asymptomatic 'subclinical' infection, (2) acute hepatitis (AH), (3) fulminant hepatitis, (4) CH, and (5) a serum HBsAg-positive healthy chronic carrier state. It is estimated that up to 70% of adults infected with HBV develop an asymptomatic infection (Chisari and Ferrari, 1996). This is usually identified by coincidental blood testing, although some of these individuals do demonstrate mild non-specific manifestations, such as fatigue and flu-like symptoms. The remaining 30% of the exposed individuals will develop serologically and clinically evident liver disease and experience flu-like symptoms, jaundice, abdominal pain, fatigue and anorexia, diagnosed as acute hepatitis B. Approximately 1% of people with acute HBV infection will develop a very severe form of liver damage, termed fulminant hepatitis (Saracco *et al.*, 1988; Hoofnagle *et al.*, 1995). This form of liver disease is associated with rapidly progressing massive hepatocyte necrosis coupled with the development of encephalopathy, and is usually fatal.

After the acute phase of disease, about 90% of adults spontaneously recover (selflimited acute hepatitis; SLAH) and enter the convalescent stage with apparent permanent immunity (Hoofnagle *et al.*, 1987). However, recent studies have demonstrated that recovery, which is characterized by the disappearance of clinical symptoms, the normalization of biochemical indicators of liver function (e.g., alanine aminotransaminase [ALT] and aspartate aminotransferase [AST]), the clearance of serum HBsAg, and the rise of antibodies to HBsAg (anti-HBs), does not reflect the complete elimination of the replicating virus (see Section 1.3.2) (Grob et al., 2000).

Patients with a continued presence of serological markers of active HBV infection (i.e., positive for HBsAg and antibodies to HBV core antigen [anti-HBc]) and with biochemical indicators of liver injury (e.g., ALT, AST) for longer than six months are deemed chronic HBV carriers. Of interest is the fact that the proportion of individuals who become chronic carriers is predominantly determined by the age of the patient when infected. For example, greater than 90% of infected neonates become symptomatic chronic carriers, as compared to 30-60% of children infected before the age of 4 years (Okada et al., 1976). However, a smaller proportion (5-10%) of individuals infected with HBV as adults progress to chronicity (Moyer and Mast, 1994). In these chronically infected patients, the pattern of CH can be variable. According to classical serological and histological diagnostic criteria, it includes chronic active (aggressive) hepatitis, chronic persistent (mild) hepatitis, and a HBsAg-positive healthy chronic carrier state without apparent morphological features of liver injury and with only occasional alterations in the levels of ALT and AST (Hoofnagle et al.,

1987).

Chronically infected individuals usually have progressive liver inflammation which

often leads to liver cirrhosis, frequently preceding the development of HCC. The risk of

developing HCC is almost 200-times greater in chronic HBV carriers than in uninfected

individuals (Beasley, 1988). Among factors potentially contributing to the development of HCC is the integration of HBV DNA into hepatocyte chromosomes, which potentially disrupts tumor suppressor gene functions or activates cellular genes (e.g., oncogenes and growth factors) leading to uncontrolled cell proliferation. Another potential factor is related to the perpetuating liver cell death and regeneration that increases the chance of mutations and subsequent tumour development during viral infection. Additionally, the HBV X protein has transactivating potential, which may play a role in HCC development (Murakami, 1999).

HBV can also induce extrahepatic disorders through the deposition of immune complexes of viral antigens and their specific antibodies. The pathogenic role of these immune complexes has been well documented in membraneous and membrane-proliferative glomerulonephritis, polyarteritis nodosa, and arthritis (Gocke, 1975; Michalak, 1978; Michalak and Krawczynski, 1981). There has also been reports of lymphoma relating to HBV infection (Ozaki et al., 1998).

1.3.2 Asymptomatic, occult infection

As mentioned (Section 1.3.1), the majority of individuals exposed to HBV develop asymptomatic infection and as many as 95% of patients with AH type B self-recover.

However, contrary to previously accepted opinions, recent studies demonstrated that these

individuals carry low levels of HBV for years after apparent complete spontaneous clinical

recovery (Blum et al., 1991; Michalak et al., 1994; Pardoe and Michalak, 1995; Rehermann

et al., 1995; Rehermann et al., 1996b; Conjeevaram and Lok, 2001).

Thus, HBV DNA sequences have been found in the serum, and HBV DNA and RNA in the liver and in lymphoid cells (i.e., peripheral blood mononuclear cells [PBMC]) years after convalescence from AH type B (Michalak et al., 1994). HBV DNA was detected even in the presence of otherwise protective antibodies (anti-HBs) when very sensitive polymerase chain reaction (PCR)-based assays were employed. Furthermore, the physicochemical characteristics of particles carrying HBV DNA were identical to that of DNase-resistant, complete virions. When less sensitive techniques like Southern blot hybridization were used, T cells, B cells, and monocytes were found to contain HBV replicative intermediates (Calmus et al. 1994). Continued activation of the host immune system after resolution of hepatitis B was also postulated since HBV-specific memory T cell responses could be detected in SLAH years after recovery (Rehermann et al., 1996a). The maintenance of this anti-viral state may be required to keep persisting HBV under control (Penna et al., 1996). This suggests that the low levels of virus genomes and viral proteins that remain after resolution of infection are capable of stimulating memory anti-viral T cell responses. In another study, it was shown that serum anti-HBc antibodies and HBV-specific cytotoxic T lymphocytes (CTL) in circulation persisted even 23 years after clinical recovery from HBV infection (Rehermann et al., 1996b), suggesting that the residual HBV persistence may trigger memory cellular responses to viral

antigens as well. The occult HBV persistence is not due to the virus adaptation to the host

through the generation of mutations that would enable the virus to evade immune recognition,

since genomic mutations were not detected even 30 years after resolution of AH type B

(Blackberg et al., 2001). Furthermore, this study also revealed that the HBV sequence in
liver samples collected decades after convalescence from AH were identical to that found at the time of initial infection. Importantly, it has also been shown that the occult persistence of HBV may not be detected when only serum is tested for HBV DNA presence. Thus, all (9/9) liver biopsies obtained an average of seven years after resolution of serologically evident hepatitis B carried HBV cccDNA, whereas only 2 of 9 serum samples showed the presence of S and X gene sequences (Yuki et al., 2003). Liver fibrosis and mild liver inflammation accompanied the persistent low level replication in 8 of the 9 patients studied. Notably, these findings are compatible with results from earlier studies in the woodchuck model of HBV infection (Michalak et al., 1999). Further, when sera from cases of CH of unknown etiology, as determined by histology, were examined for HBV DNA, 30% of the cases were found to harbour low levels of HBV DNA (less than 10⁴ copies/mL), suggesting the existence of occult, serologically undetectable infection. Almost 75% of the samples tested were anti-HBc positive, confirming a prior exposure to HBV (Chemin et al., 2001). Most recently, pathological relevance of occult HBV infection was documented by identification of HBV genome in 63.5% of HCC cases of unknown etiology, suggesting that the virus was a pathogenic factor in the development of liver cancer (Pollicino et al., 2004).

In contrast to occult HBV persistence continuing after apparent complete resolution

of hepatitis, a potential existence of primary asymptomatic occult hepadnavirus infection is

not yet recognized (see Section 4.1). It is known that 90% of children born to mothers with

chronic symptomatic HBV infection become chronic carriers of the virus. However, the

passage of occult HBV from mothers convalescent from AH type B to their children has not

been investigated. In the woodchuck experimental model of HBV infection, it has been shown that WHV can be passed from recovered mother to offspring as primary occult infection (POI), and that such acquired virus persists indefinitely and can be infectious to a healthy virus-naive animal (Coffin and Michalak, 1999). In terms of the parallel human situation, the ramifications of persistent carriage of low levels of hepadnaviral genomes, especially in cases where eradication of the virus is assumed, are currently unknown, although they could be epidemiologically (transfer of virus through blood transfusion and organ donation) and pathogenically (e.g., cryptic liver disease) important. Also, a possibility of induction of POI by very low virus doses, as observed in the woodchuck model of hepatitis B (see Section 1.5 and Section 4.1), and consequences of this form of silent HBV carriage have not yet been investigated in humans. However, the infection of the lymphatic system and the long-term HBV-specific T cell immune activity suggests that the establishment and maintenance of hepadnaviral persistence resisting immune elimination. In the human situation, the transplantation of liver from serologically HBV DNA negative, anti-HBc-positive individuals to virus-naive recipients led to re-infection of the recipient (Chazouilleres et al., 1994; Lowell et al., 1995; Hu et al., 2002). The fact that HBV infection of the recipient occurred points to the infectious competency of minute levels of persisting hepadnavirus.

1.4 IMMUNOPATHOGENESIS OF HEPADNAVIRAL INFECTION

The primary function of the immune system is the recognition and elimination of foreign molecules, including viral pathogens. The immune system of higher vertebrates

consists of natural (innate) and adaptive (specific) immunity. Upon initial viral entry to the host, it encounters the innate cellular immune system that, among others, includes monocytes, macrophages, natural killer (NK) cells, NKT cells and polymorphonuclear leukocytes. These cells recognize pathogenic molecules of foreign origin, causing the activation of cellular processes including phagocytosis, induction and synthesis of antimicrobial agents (e.g., nitric oxide, lysosomes), and cytokines (e.g., interferons [IFN] and tumor necrosis factor alpha [TNF\alpha]). If this primary line of defense does not neutralize and eliminate the virus, the adaptive (specific) immune responses become activated. These responses exhibit the classically defined properties of immunological memory and antigen specificity. In theory, the adaptive immune responses can be divided into humoral (antibody-mediated) and cellular arms. Pathogen-specific cellular immunity is mediated by two main groups of effector cells: cluster of differentiation marker (CD)4+ T helper (Th) cells, which provide help to B lymphocytes and to other immune effector cells, and CD8+ CTL, whose main function is to identify infected host cells through recognition of viral peptides presented by class I MHC. During infections with noncytopathic viruses, such as HBV, any overt clinical symptoms are generally a result of the host's immune responses against virus-infected cells, causing their

death and elimination (reviewed in Guidotti and Chisari, 1996).

1.4.1 Innate immunity

In general, the innate immune system, especially in the cases of viral infections, is controlled by NK and NKT cells, as well as monocytes and macrophages (Janeway and Medzhitov, 2002). These types of cells perform their actions by the secretion of antiviral cytokines and through cytolytic activity (Bendelac *et al.*, 1997; See *et al.*, 1997).

The peak of NK cell cytotoxic activity and proliferation normally occurs shortly after viral infection and is an important element of natural resistence to many viruses (Welsh *et al.*, 1979; Biron, 1997; Biron and Brossay, 2001). NK cells lyse infected targets in a non-MHC restricted pattern. NK cells are lymphoid-derived leukocytes that are able to lyse infected cells and secrete numerous effector cytokines after detection of reduced or aberrant expression of MHC class I molecules on a cell surface. Engagement of NK receptors with self-MHC prevents killing of the target cells by inhibitory signals. The three NK inhibitory receptor families are: (a) the CD94-NKG2 family (common to humans and rodents), (b) the Ly49 family (only in rodents), and (c) the KIR family (killer inhibitory receptor, occurring only in humans) (Lanier, 2003). Virus-infected cells with decreased surface expression of MHC class I are, theoretically, susceptible to NK-mediated lysis, predominately through perforin-dependent cytotoxicity (Kagi *et al.*, 1994; Sayers *et al.*, 1998). In the case of different viral pathogens, it has been shown that the ability of the host to mount a strong cytotoxic NK cell response very early in infection plays a decisive role in controlling virus

spread and limiting progression of the disease (Biron, 1997). It is also demonstrated that IFN-gamma (IFN γ), secreted by activated NK cells, may dramatically enhance defence against certain virus infections, for example during murine cytomegalovirus (MCMV) infection (Dokun *et al.*, 2001; Nguyen *et al.*, 2002). The NKT cell expresses markers of both T and NK cells. This cell type has a limited V α /V β T cell receptor (TCR) usage, and

contains one of the defined NK cell markers (*e.g.*, CD16 and Ly49). The activated NKT cell can produce high levels of cytokines, including interleukin (IL)-4 and IFN γ . The IFN γ production suggests that these cells have the potential to control viral infections, if they become activated. In addition to cytokine production, NKT cells also exhibit both Fas- and perforin-dependent cytolytic activity. Both human and mouse NKT cells recognize nonclassical MHC class I-like molecules (*i.e.*, CD1d) if associated with β 2-microglobulin (β 2-m) on professional antigen presenting cells (APC) via their TCR (Burdin and Kronenberg, 1999). It is of interest to note that NKT cells represent up to 50% of the mature T cells in the rodent liver (Watanabe *et al.*, 1995). However, the frequency of NKT cells in the human liver is much lower (Kenna *et al.*, 2003).

Currently, the role of NK and NKT cells in hepadnavirus infection is not well recognized. The murine liver is naturally abundant in NKT cells, where they may account for up to 30% of total intrahepatic lymphocytes (Bendelac *et al.*, 1999; Doherty *et al.*, 1999), and these cells can activate NK cells through cytokine secretion to control tumor growth and the spread of intracellular pathogens (Cui *et al.*, 1997; Bendelac *et al.*, 1999; Doherty *et al.*, 1999). It has been reported that AH type B is associated with enhanced NK cell cytotoxicity

and that HBsAg can inhibit the NK cell-mediated cytotoxicity *in vitro* (de Martino *et al.*, 1985), but further studies are required to fully understand the importance of the NK cellmediated response. There has been evidence that NKT and NK cells control hepadnavirus replication in HBV transgenic mice via IFN α/β and IFN γ release (Guidotti *et al.*, 1999; Cavanaugh *et al.*, 1998). As well, chimpanzee models of HBV infection support the idea that T cell-independent induction of hepatic cytokines, specifically IFN α/β , IFN γ , and TNF α can lead to a non-cytopathic inhibition of HBV replication (Kakimi *et al.*, 2000; Guidotti *et al.*, 1999). However, the precise role of these responses in natural hepadnaviral infections remains to be determined.

Dendritic cells (DC) play an important role in both innate and adaptive immunity. Not only are DC the only APC capable of inducing primary CTL and Th cellular responses *in vivo* (Bancherau and Steinman, 1998), they are able to interact with antigens through cell surface toll-like receptors. This interaction allows the DC to secrete IL-12, which in turn, stimulates NK and NKT cells to produce cytokines, including IL-4 and IFN γ , as discussed above (Bendelac *et al.*, 1997; See *et al.*, 1997). The release of these cytokines therefore link the innate and adaptive immune responses, since the recruitment and/or activation of specific B and T cells is enhanced in this milieu.

1.4.2 Humoral immunity

Specific humoral immunity is mediated by antibodies produced by plasma cells derived from antigen-specific B cells. Antibodies are essential in the early neutralization against viral

infections since they help, among other things, reduce the amount of freely circulating virus by preventing viral attachment and entry into host cells. They may also act as opsonins to enhance phagocytosis. Virus specific antibodies can also be involved in the elimination of infected cells that express viral antigens on their surface through complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity. Re-infection upon subsequent exposures with the same virus is usually prevented by the persistence of B cells productively secreting antibodies specific for the viral envelope. Stimulation of specific memory B cells which persist after the initial infection results in a rapid secondary immune response and production of large amounts of specific antibodies with increased affinity for the particular viral antigen (Abbas *et al.*, 2000).

The antibody response to HBV envelope glycoproteins (i.e., anti-HBs) is thought to be important in HBV neutralization and clearance. The loss of serum HBsAg and the subsequent seroconversion to anti-HBs is the classical marker of resolution of disease in patients with acute HBV infection, however in individuals with CH type B, anti-HBs is not detected, unless specific assays that detect traces of antibodies complexed to viral antigens are used (Maruyama et al., 1993). Neutralizing antibodies in HBV infection have been shown to be important since the successful use of HBsAg-based HBV vaccine (Beasley et al., 1988). Unfortunately, the antibody response to the envelope proteins could also be a potential contributor to liver injury. It has been reported that immune complex deposits containing HBsAg, immunoglobulin (Ig) and complement were detected in infected hepatic tissue from various stages of hepatitis B (Nowoslawski et al., 1972). Therefore, the humoral response to HBsAg remains operational in chronic infection, although it may cause pathological effects through deposition of immune complexes. In fact, it has been shown that about 10-20% of patients with HBV infection have extra-hepatic immunocomplex-associated disorders, including glomerulonephritis (Combes et al., 1971; Nowoslawski et al., 1975; Slusarczyk et al., 1980), polyarteritis nodosa (Gocke et al., 1970; Michalak, 1978), and arthritis (Csepregi

et al., 2000).

The biological roles of the antibody responses to HBcAg and HBeAg are less clear. Anti-HBc and antibodies to HBeAg (anti-HBe) are not neutralizing antibodies, as they coexist with the virus during both acute and chronic stages of hepatitis B. However, seroconversion to anti-HBe positive state has been associated with less severe hepatitis (Schmilovitz-Weiss et al., 1993). Additionally, it has been suggested that the decline of virus titer, which is usually observed after seroconversion from HBeAg to anti-HBe, might be the result of the elimination of infected cells via an antibody recognition of membrane bound HBe protein (Schlicht et al., 1991). The humoral response against HBeAg and HBcAg may also promote undesirable effects because the extrahepatic deposition of these immunocomplexes have been observed in glomerulonephritis (Slusarczyk et al., 1980; Ohba et al., 1997). The occurrence of anti-HBc antibodies in chronically infected patients might be because HBcAg can elicit a T cell-independent antibody response. This is in contrast to HBeAg which is exclusively a T cell-dependent antigen (Milich and McLachlan, 1986). Other studies have suggested that complement dependent cytotoxicity directed to hepatocytes expressing HBcAg, HBsAg or asialoglycoprotein receptor (ASGPR) may also contribute to the injury

of HBV-infected hepatocytes, particularly in patients with severe CH (Michalak et al., 1995).

The antibody response to the HBV P and X proteins are not monitored in clinical

situations, although they have been detected (Weimer et al., 1990). It has been reported that

antibody to carboxy terminus of virus polymerase may be an early marker of infection and reflects active HBV replication. Antibodies to HBxAg have been reported to show qualitative

and quantitative heterogeneity (Stemler *et al.*, 1990). They have been found at the highest levels and most frequently in patients with CH and usually at lower levels in acutely infected patients and asymptomatic carriers of HBV (Stemler *et al.*, 1990).

1.4.3 Cell-mediated immunity

The activation of CD4+ cells requires recognition by the polymorphic TCR of exogenous viral peptides presented by MHC class II molecules. These molecules are located on the surface of professional APC, which include DC, macrophages, and activated B lymphocytes. The 18-22 amino acid-long antigenic peptides presented by MHC class II are usually derived from extracellular antigens that are proteolytically processed in acidified endosomes or lysosomes after endocytosis by the APC (Abbas *et al.*, 2000).

There are two distinct subsets of CD4+ cells, each associated with a different arm of the immune system. The T-helper type 1 (Th1) subset, which produce cytokines such as IL-2 and IFN γ , are known to be involved in cell-mediated functions, such as clonal proliferation of CTL. In contrast, T-helper type 2 (Th2) cells, which secrete IL-4, IL-5 and IL-10, are involved in the maturation and differentiation of B cells (Mosmann and Sad, 1996). From a

murine model of leishmaniasis and other infection model systems, it has been proven that Th1 responses are particularly effective against intracellular pathogens, whereas protection against extracellular microbes require Th2 activity (reviewed by Paul and Seder, 1994). It is important to note that because viral encoded proteins are synthesized intracellularly they are mainly processed by the class I MHC presentation pathway. Therefore, the principle

mechanism of specific immunity against established non-cytopathic viral infections is a virusspecific MHC class I-restricted, CD8+ CTL-mediated response (Abbas *et al.*, 2000).

Once activated, CTL can kill a targeted cell by two contact mediated processes. These cytotoxic pathways operate via Fas (CD95)/Fas ligand (FasL) interaction or by perforin-granzyme release. In the Fas/FasL pathway, ligation and trimerization of Fas receptors on target cells by effector cells expressing FasL causes apoptosis of the infected cell (Shresta *et al.*, 1998). In the perforin-dependent pathway, CTL secrete perforin, a pore forming protein, that acts on the target cell membrane providing access for granzymes, which interfere with specific substrates involved in the ultimate death of the cell and/or they are transported to the nucleus where they may activate death substrates (Ando *et al.*, 1993). In addition, activated CTL secrete antiviral cytokines (such as TNF α and IFN γ) which can directly kill the target cell (Kagi and Hengartner, 1996; Kagi *et al.*, 1996).

In terms of the T cell responses to hepadnavirus, it is generally acknowledged that virus-specific CTL responses are responsible for hepatocellular damage that typically accompanies hepatitis (Chisari, 2000). As virally infected hepatocytes are recognized by the CTL effectors, the vigorous anti-viral immune response mounted against the infected organ is the causative factor of liver damage in HBV infection. Due to the restricted host range of HBV and the lack of cell cultures that efficiently support viral replication, studies on T cell involvement in the immunopathogenesis of HBV-induced hepatocellular injury have focused on the *in vitro* analysis of CTL and Th cells derived from patients with clinically evident hepatitis. In general, the HBV-specific T cell specific response is vigorous, polyclonal, and

multispecific in patients with AH, but weak and epitope-restricted in patients with chronic infection (reviewed by Chisari, 2000). Recently developed techniques involving the use of a fluorochrome-labeled peptide-MHC class I tetrameric complex that directly binds to the TCR of peptide-specific CTL (Ogg and McMichael, 1998) have found that the frequency of virus-specific CTL in HBV infection is 30 to 45-fold greater than that estimated by conventional limiting dilution analysis (Maini *et al.*, 1999). As well, besides the cytolytic acts CD8+ T cells perform, they also secrete anti-viral cytokines that may directly inhibit viral replication without cellular damage (Chisari, 2000).

The CD4+ T cells may contribute to long term control of trace levels of HBV replication persisting after recovery from hepatitis, as demonstrated by the detection of HBcAg-specific proliferative T-cell responses years after resolution of AH (Rehermann *et al.*, 1995; Penna *et al.*, 1996; Rehermann *et al.*, 1996b). As mentioned above (see Section 1.3.2), identification of HBV DNA by sensitive PCR/Southern blot hybridization assays in the sera and in PBMC many years after recovery from AH suggests that the HBV-specific CD4+ T-cell response is maintained indefinitely by minute amounts of persistently replicating virus (Michalak *et al.*, 1994; Penna *et al.*, 1996; Rehermann *et al.*, 1996b). The same could be true

with respect to continued maintenance of a strong anti-HBc antibody response that is

normally detectable throughout life after recovery from hepatitis B (see Section 1.3.2).

1.5 THE WOODCHUCK MODEL OF HEPATITIS B

1.5.1 Woodchuck hepatitis virus (WHV)

WHV was discovered in a colony of eastern North American woodchucks in the Philadelphia Zoological Garden, where a high rate of CH and HCC was observed (Summers et al., 1978). The WHV model is currently widely accepted as the most natural and suitable model for the study of HBV, since the viruses share a similar genomic organization, ultrastructure, antigenic cross-reactivity, range of targeted organs, and they induce comparable course of infection and pathological features of hepatitis, including development of HCC (reviewed in Michalak, 1998).

The virion of WHV has a diameter of 45 nm. The 3.3 kb WHV genome (Figure 1.1) is slightly longer than HBV DNA and shares overall about 70% nucleotide sequence homology to the human virus. The WHV envelope proteins demonstrate antigenic crossreactivity with those of HBV, to the extent that commercial assays for HBsAg and anti-HBs have been used to identify corresponding WHV antigens. As well, core antigens of both viruses have common antigenic determinants (reviewed in Marion et al., 1991). Currently, the outbred nature of the animals and the lack of reagents recognizing markers specific for

individual woodchuck lymphoid cell types are the major constraints in the use of this animal

system. However, the woodchuck model of hepatitis B is more accessible and substantially

less expensive than chimpanzees.

One of the major differences between the HBV and WHV lies in the oncogenic potential of WHV. HCC develops in almost all animals with serum WHV surface antigen Figure 1.1 Schematic diagram of the WHV genome and encoded open reading frames



Legend:

Double stranded DNA
Single stranded DNA
Encoded protein
Virus nick region

(WHsAg)-positive CH within 18 to 36 months after infection with the virus (Popper *et al.*, 1981; Korba *et al.*, 1989). It is believed that the activation of cellular oncogenes, particularly c-*myc* and N-*myc*, through the integration of viral promoter sequences near these genes or through rearrangements of these genes, is responsible for the higher rates of HCC in woodchucks. In contrast, HBV integrates in a random fashion into the human hepatocyte genome. Also, hepatitis B progression tends to be more frequently associated with the development of liver cirrhosis, while this outcome is not observed in woodchucks infected with WHV.

1.5.2 Characteristics of WHV infection

1.5.2.1 Symptomatic serologically evident hepatitis

The transmission of WHV through blood and body fluids, as well as from mother to offspring, parallels that of HBV. Similar to infection with HBV, woodchucks persistently infected with WHV develop chronic liver inflammation with different degrees of hepatocellular injury and lymphomononuclear cell infiltrations (Michalak, 1998). As in HBV infection, most infected neonates or those born to mothers with serum WHsAg-positive

chronic infection develop serologically evident CH that almost invariably progresses to HCC.

The fact that animal age, virus strain, and its dose may influence the rate of chronicity in

experimental WHV infection has been documented (Cote et al., 2000a). In adult animals,

WHV infection usually leads to SLAH and clearance of virus serological markers (Korba et al., 1989), but molecular indicators of residual WHV infection remain (Michalak et al., 1999).

Approximately 10-15% of animals infected with WHV progress to serum WHsAg-positive CH. This rate of progression to CH is similar to that in adults infected with HBV (5-10%). However, suppression of the immune response by cyclosporin A in experimentally infected adult woodchucks dramatically increases the rate of CH outcome, depending on the age of the animal at the time of drug administration (Cote *et al.*, 1992). This suggests that the maturity of the immune system is critical in the pathogenesis of hepadnaviral infection and its persistence. Furthermore, the existence of autoantibodies against ASGPR has been associated with the development of CH in the woodchuck-WHV model (Diao and Michalak, 1997; Diao *et al.*, 2003).

Hepadnavirus clearance without massive immune-mediated destruction of infected hepatocytes occurs in WHV infection. In one study, hepatocytes were labeled during the peak of acute infection, when nearly all liver cells are infected (Kajino *et al.*, 1994). After elimination of WHV from the liver, many labeled hepatocytes were still present, suggesting that non-cytopathic viral clearance occurs in woodchucks infected with WHV. More recently, studies investigating the role of anti-viral cytokines (*i.e.*, IFN γ and TNF α) as determinants of progression of AH to SLAH or CH have suggested that the host hepatic cytokine milieu during early AH is important in determining the outcome of hepatitis in both neonatally acquired and adult infection (Cote *et al.*, 2000b; Hodgson and Michalak, 2001). Elevated hepatic IFN γ , TNF α , and CD3 expression, coupled with a lower hepatic viral load and increase in liver inflammation preceded recovery from AH, while a reverse status of these parameters was associated with progression of AH to CH (Hodgson and Michalak, 2001). The establishment of assays measuring the proliferative T-cell response in woodchucks has further demonstrated that WHV-specific CD4+ T cells may play an important role in preventing development of chronic infection. The antiviral effect of CD4+ T cells appears to be primarily through their ability to help initiate and sustain the virus-specific CTL response, possibly mediated by the secretion of proliferative cytokines (*e.g.*, IL-2) and antiviral cytokines (*e.g.*, IFN γ), as in HBV infection (Penna *et al.*, 1997). Similar to HBcAg, WHV core antigen (WHcAg) and some of its synthetic peptides effectively stimulate proliferation of T cells derived from acutely infected woodchucks, but not those obtained from animals with CH (Menne *et al.*, 1997).

It has been documented that chronic WHV infection, but not acute WHV hepatitis, is associated with the incorporation of large quantities of virus envelope proteins in hepatocyte plasma membranes (Michalak and Churchill, 1988; Michalak and Lin, 1994). It was suggested that this fact, together with the abundant amounts of these proteins normally occurring in the circulation in chronic infection might constitute an immunological barrier at the hepatocyte surface preventing efficient immune elimination of WHV-infected cells.

WHcAg and WHV e antigen (WHeAg) were also found to be associated with the outer

membranes of infected hepatocytes (Michalak and Churchill, 1988; Michalak *et al.*, 1990; Michalak and Lin, 1994). Interestingly, while antibodies to WHV core antigen (anti-WHc) were readily detectable on hepatocyte plasma membranes, antibodies to WHV e antigen (anti-WHe) could only be detected on membranes from animals that had recovered from AH (Michalak *et al.*, 1990). This may suggest that, similar to HBV infection in humans, a humoral response against e antigen may contribute to the resolution of acute WHV infection, presumably by the elimination of infected hepatocytes through antibody-mediated cytolysis, as was suggested for chimpanzees vaccinated with HBeAg (Schlicht *et al.*, 1991). As discussed later (Section 1.6.5), the decreased cell surface expression of MHC class I on hepatocytes in chronic WHV hepatitis may potentially impact the effectiveness of CD8+ T cell-mediated antiviral immunity (Michalak *et al.*, 2000).

WHV infection commonly triggers the production of both organ non-specific and liver specific ASGPR autoantibodies (Dzwonkowski and Michalak, 1990; Diao and Michalak, 1997). Antibodies against ASGPR (anti-ASGPR), in particular, could be capable of inducing hepatocyte cytolysis in the presence of complement (Michalak *et al.*, 1995, B; Diao *et al.*, 1998). Recently, it has been shown that hepatocytes in chronic WHV hepatitis could be prone to anti-ASGPR-mediated cytopathic effects due to the deposition of ASGPR-anti-ASGPR immune complexes on their plasma membranes (Diao *et al.*, 2003). This autoreactivity may contribute to the pathogenesis and prolonged recovery from liver injury in viral hepatitis (Diao and Michalak, 1996; Diao *et al.*, 1998; Diao *et al.*, 2003).

1.5.2.2 Primary occult WHV infection

In the woodchuck model, it has been shown that maternal transmission of WHV to offspring occurs not only when the mother has serologically evident chronic WHV infection, but also when it is convalescent from AH (Coffin and Michalak, 1999). All offspring from these recovered dams carried very low levels of WHV genomes and WHV mRNA in the lymphatic system, but the liver was infected in only about half of the offspring studied. Interestingly, no serological markers of infection, such as WHsAg, anti-WHc or antibodies against WHV surface antigen (anti-WHs) were detected in the offspring. WHV cccDNA was detectable in the lymphatic organs and in WHV DNA-positive livers. In addition, WHV DNA reactive particles occurring in serum of these offspring displayed features of enveloped complete virions. Thus, they migrated with comparable velocity and had the buoyant density of intact WHV virions. The significance of this form of silent infection is most obvious in the observation that the inocula prepared from serum and lymphoid cells of these offspring, irrespective of whether infection was lymphatic system-restricted or engaged the liver, was able to induce WHV infection in naive animals. Moreover, these offspring were not protected from infection when challenged with a large dose of WHV (*i.e.*, 1.1 x 10¹⁰ virus genome equivalents [vge]) (Coffin and Michalak, 1999).

The consequences of infection in infants born to mothers with resolved HBV have never been studied. However, the similarities of WHV to HBV suggest that the persistence of small amounts of the virus, especially in the extrahepatic reservoir, could have a significant

impact, in terms of transmission of infection, as well as induction of disorders which are not

yet considered to be related to persistent hepadnavirus infection (Coffin and Michalak, 1999;

Michalak et al., 1999). POI initiated by minute amounts of the virus carried across the

placenta in blood, or by circulating lymphoid cells, may induce this occult infection, similar

to the chronic infection induced in neonates born to mothers with symptomatic CH (Shimizu

et al., 1991). A similar situation is seen in other virus infections. For example, mice infected

at birth with high levels of lymphocytic choriomeningitis virus (LCMV) become life-long carriers and fail to develop virus-specific responses (Jamieson *et al.*, 1991).

1.5.2.3 Silent residual WHV infection

Remnant asymptomatic hepadnavirus infection, *i.e.*, secondary occult infection (SOI), continuing after resolution of HBV and WHV hepatitis have been documented in both humans and woodchucks (Michalak, 2000). The anti-viral immunity established after an encounter with a dose of either WHV or HBV causing hepatitis does not completely eradicate the virus and virus genomes and trace virus replication persists in both hepatocytes and cells of the lymphatic system in the host.

In the woodchuck model, WHV DNA and RNA persistence after recovery from AH is lifelong when highly sensitive PCR assays coupled with Southern blot nucleic acid hybridization (PCR/NAH) of the amplified products are utilized (Michalak *et al.*, 1999). This life-long carriage is not restricted to the PBMC and lymphatic organs, but also involves the liver. Animals that have serologically cleared WHV often show transient minimal to moderate

inflammatory alterations in the liver up to the end of their lifespan (Michalak et al., 1999), as

has also been reported subsequently for humans with SLAH (Yuki et al., 2003). Moreover,

up to 20% of the recovered animals finally develop HCC (Korba et al., 1989; Michalak et al., 1999).

In other studies, it was demonstrated that the harboured WHV was capable of replication, as evidenced by the detection of viral cccDNA in both the liver and cells of the

lymphatic system (Lew and Michalak, 2001). The SOI is always accompanied by anti-WHc and frequently by anti-WHs (Michalak *et al.*, 1999). This may reflect the continuous restimulation of the immune system by a progressing and low-level production of viral proteins. The silently persisting virus appears to retain its oncogenic potential, with approximately 20% of animals finally developing HCC (Korba *et al.*, 1989; Michalak *et al.*, 1999; Coffin *et al.*, 2004).

1.6 GENERAL MECHANISMS OF VIRAL PERSISTENCE

Viruses that persist in a host need to possess two fundamental characteristics. They must be able to maintain their genome in host cells for a prolonged period of time and be able to avoid recognition and elimination by the host's immune response (Oldstone, 1989; Oldstone, 1998). Viruses have evolved multiple mechanisms to avoid detection by immune effector cells and antibody immune responses. Most viruses that persist have established a complex virus-host relationship and may use many mechanisms to avoid the antiviral immune response and subsequent elimination. In the following sections, the best recognized

mechanisms contributing to virus evasion in general, and likely pertaining to hepadnavirus

persistence, will be briefly summarized.

1.6.1 Latency of viral life cycle

The ability of a virus to establish a lifelong latent infection in the host is one of the most intellectually challenging aspects of virology. An effective mechanism of evading host

defences is for a virus to withdraw into a latent state in which the viral DNA is present at a low copy number, few viral proteins are expressed, and the cellular defences are not mobilized. At a later time, perhaps when conditions are more conducive to virus replication, the virus can reenter the lytic, vegetative cycle. Herpesviruses often employ this strategy. A striking example is the latency involving VZV, a herpesvirus that causes chicken pox, but commonly sets up a latent infection in the neurons in the dorsal root ganglia (Hyman *et al.*, 1983; Croen *et al.*, 1988). When immunity has subsided decades after the initial infection and acute disease, the virus can re-initiate lytic replication, resulting in new lesions (shingles) in the body segment innervated by the affected dorsal root ganglion (Hope-Simpson, 1965). This reactivated virus can even be transmitted to a new host. In general, the mechanisms underlying establishment and release from latency are not well understood.

Many other well-studied viruses have been shown to use this silent presence to establish persistent infections. Perhaps the best-studied example of limited gene and protein expression is that of herpes simplex virus (HSV). During latent infection, the only viral transcripts that are detected are the latency-associated transcripts and no proteins are

expressed (Stevens et al., 1987). The virus enters nerve endings and is transported to the

nucleus of sensory nerves innervating the mucosal epithelium. In latently infected neurons,

viral genomes acquire the characteristics of endless or circular DNA and no replicating virus

can be detected in the sensory ganglia innervating the site of inoculation (Spivack and Fraser,

1988). In a fraction of neurons harboring latent HSV, the virus is periodically reactivated,

usually upon injury to the initially infected area. Infectious virus is carried to peripheral

tissues by axonal transport, usually to cells at or near the site of initial infection. The latent life cycle ensures that the host's immune system cannot identify the virus as foreign, as proteins are rarely generated.

Similarly, latent EBV infection is associated only with expression of one protein, EBV nuclear antigen-1 (EBNA-1) (Wu *et al.*, 2000). A wide range of B cells, from pro-B to antigen-experienced memory cells (but not plasma cells), appear susceptible to EBV infection and transformation *in vitro*, reflecting expression within the B-cell lineage of the principal EBV receptor, CR2 (CD21) (Jondal *et al.*, 1976). Upon infection of B cells the virus can either progress to a lytic or latent infection (Petti *et al.*, 1990; Swaminathan, 2003). Latent EBV persistence has also been shown to be dependent on the proliferation of daughter cells with low copy episomes that attach to mitotic spindles through EBNA-1 (Kapoor and Frappier, 2003).

Another virus that causes latent infection that escapes immune recognition is cytomegalovirus (CMV). CMV infects myeloid-lineage hematopoietic cells (including progenitors that give rise to granulocytes, macrophages, dendritic cells and possibly endothelial cells), which eventually become important targets for lifelong latency. The study

of the impact of latent CMV infection is hampered, since only 0.01% of PBMC are infected,

even when highly sensitive PCR-driven in situ hybridization techniques are used

(Soderberg-Naucler et al., 1997).

1.6.2 Infection of immunologically privileged sites

Another viral immune escape mechanism is the infection of tissues and cells that are not readily accessible to the immune system. A site of persistence employed by many viruses, including HSV, LCMV, VZV, and measles, is the central nervous system (Oldstone et al., 1993). Central nervous system cells allow viral evasion since the blood-brain barrier impedes lymphocyte extravasation. As well, the lack of MHC class I expression on neurons ensures that viruses (or their processed peptides) cannot be recognized by virus-specific CTL (Joly et al., 1991).

To escape immune recognition, many viruses infect lymphocytes, the very cells which are required to eliminate virus and enable viral clearance (Oldstone et al., 1989). The disruption of function of these cells, therefore, can result in immunosuppression, which may be either virus-specific or generalized. Measles virus is a well-characterized example of a lymphotropic virus that causes generalized immunosuppression (Niewiesk et al., 2000). It has been postulated that measles virus impairs both the primary and secondary immune responses by inhibiting T cell proliferation. However, the other functions of these cells, such

as cytokine secretion, does not seem to be compromised.

HIV is another example of a lymphotropic virus that infects CD4+ cells, the majority of which are Th cells and macrophages. This infection eventually destroys these types of cells, with the overall effect of generalized immunosuppression, enabling opportunistic

infections which cannot be eradicated, frequently causing death of an infected individual (Embretson et al., 1993; Cohen and Fauci, 2001).

Infection of DC is another mechanism whereby viruses can remain hidden from immune responses. In various viral infections, such as vaccinia (Engelmayer *et al.*, 1999), measles (Servet-Delprat *et al.*, 2000), and CH type C (Auffermann-Gretzinger *et al.*, 2001; Bain *et al.*, 2001), the invasion of DC is associated with impaired DC differentiation and function. Overall, the downregulation of costimulatory molecules and the impairment in expression of maturation markers on DC can be responsible for a decrease in immunostimulatory activity. As mentioned in Section 1.4.1, the decreased function of DC may not only affects adaptive antigen specific responses but also overall innate immune mechanisms.

1.6.3 Induction of immunological tolerance

The effectiveness of the host immune response to any particular pathogen also depends upon the maturity of the immune system at the initial time of exposure. Theoretically, antigens that are encountered in early stages of development are not likely to initiate an immune response and will not be identified as foreign, whereas the same antigens seen by a developed immune system will be immunogenic (Ahmed, 1992). In this regard,

vertically transmitted LCMV infection in mice is one of the best studied models of tolerance.

Mice infected at birth become life-long carriers and fail to develop a virus-specific response

(Jamieson et al., 1991). Similarily, in chronic HBV infection of children infected via vertical

transmission, a potential mechanism of impairment of virus clearance may be due to the

presence of viral antigens during immunological development, whereby viral antigens are not

identified as foreign, thereby inducing tolerance to viral products.

In adult viral infection, viral antigens may initially induce a strong antigen-specific effector T cell response. However, in later phases of the infection, the virus can overwhelm the CD8+ CTL virus-specific response, causing their clonal deletion from the lymphocyte population (Ahmed and Gray, 1996). This has been suggested to occur during LCMV infection in adult mice who fail to eradicate the virus leading to its persistence (Oldstone *et al.*, 1993). A similar situation may exist in HBV infection, whereby individuals with chronically high levels of the virus remain in a fairly healthy, asymptomatic state.

1.6.4 Impairment of cytokine function

Cytokines are an integral component of antiviral immunity. They behave in a complex network to inhibit viral replication, inhibit proliferation and destroy infected cells, control the inflammatory response, and contribute to other immune effector mechanisms. Studies have demonstrated that proteins from several viruses can interfere with these cytokine functions. Three adenovirus early proteins (E3-14.7K, E3-10.4K/14.5K, and E1B-19K) can protect mouse cells which are sensitive to TNF α -induced apoptosis. The mechanism by which these

adenovirus proteins counteract TNF α appears to be through inhibition of cytosolic phospholipase A2 (Krajcsi *et al.*, 1996).

A different mechanism used by EBV is to impede antigen presentation. EBV expresses the protein BCRF1 which is a homologue for human IL-10. Studies have shown that BCRF1 inhibits the expression of TAP-1 (transporters associated with antigen processing protein-1), as would IL-10 (Zeidler *et al.*, 1997). This cytokine mimicry would hamper the transport of processed peptide antigens into the endoplasmic reticulum (ER), which would prevent MHC class I molecules from being loaded with EBV specific peptides.

Viral peptides that mimic host cytokine receptors or inhibit activation of cytokines have also been described. For example, vaccinia and myxoma viruses encode proteins capable of binding IFN α and TNF α (reviewed by Alcami and Koszinowski, 2000). Additionally, the mousepox virus produces a secretable binding protein for IL-18 and, therefore, prevents the induction of IFN γ production.

It has also been demonstrated that measles virus is capable of inhibiting macrophage IL-12 production, which is required for antiviral responses in infected cells, through the binding of hemagglutinin protein to CD46 (Atabani *et al.*, 2001).

1.6.5 Interference with MHC class I antigen processing and presentation

One of the major mechanisms of viral persistence is to prevent the presentation of viral peptides by MHC class I molecules on the surface of infected cells. Because the stable cell surface expression of MHC class I molecules requires association between the heavy chain

and β 2-m, as well as an endogenously produced synthesized viral peptide, any interference

with antigen processing or presentation will interfere with CTL surveillance. For example,

many viral proteins have been reported to interact with TAP and other components of MHC

class I antigen presentation. In particular, it has been shown that the C-terminal domain of

the HIV Tat protein, a transactivator of HIV transcription, represses transcription of MHC

class I genes. This impediment arises due to the interaction of Tat with a component of the

transcription factor TAFII250 (Weissman et al., 1998) that binds to the MHC class I heavy chain promoter. The repression of MHC class I transcription is one of the mechanisms that HIV utilizes to avoid immune recognition (Weissman et al., 1998). HSV infection also reduces cell surface levels of MHC class I molecules (Jennings et al., 1985). The decrease in this expression is due to peptide transport interference by a viral protein product of the immediate early gene, US12, also known as infected cell protein 47 (ICP47) (York et al., 1994). This cytosolic protein associates with the peptide binding domain of TAP, inhibiting peptide translocation to the ER. This results in the production of empty, unstable MHC class I molecules. Other viruses prevent peptide loaded, intact MHC class I molecules from being transported to the membrane. Adenoviruses express the protein E3/19K, the early region 3 transmembrane glycoprotein, which is a prime inhibitor of class I MHC surface expression. This protein binds to the alpha 1 and alpha 2 helices of the MHC class I heavy chain (Flomenberg et al., 1994), preventing its transport to the cell surface. Even in uninfected lymphoid cells which have been transfected with E3/19K, this decreased surface MHC class I expression has been shown (reviewed in Burgert et al., 2002).

In the case of WHV, it has been shown that MHC class I expression on the hepatocyte

cell surface in animals with serumWHsAg-positive CH decreases nearly to the levels present

on non-infected normal hepatocytes (Michalak et al., 2000). The corresponding data showing

that CD3 and IFNy expression in the liver are also at near normal levels in chronic infection

suggests that alteration in MHC class I expression in the presence of moderate to high viral

loads may compromise immune recognition. The precise mechanism of this interference is

not yet established. However, since the levels of MHC class I-associated transcripts in hepatocytes from animals with chronic WHV hepatitis are essentially identical to those in healthy animals, it has been proposed that the process is post-transcriptional (Michalak and Hodgson, 2001).

Other studies have shown that accessory molecules, such as lymphocyte function associated antigen-3 (LFA-3) and intracellular adhesion molecule-1 (ICAM-1), are involved in viral escape from CTL recognition. Specifically, a reduced level of LFA-3 and ICAM-1 on EBV-positive Burkitt's lymphoma cells allows escape from virus-specific CTL lysis (Gregory *et al.*, 1988). However, the mechanism of suppression of these adhesion molecules is not well understood.

1.6.6 Interference with MHC class II antigen processing and presentation

Overall, there is less known about the interference of viral proteins with MHC class II than with MHC class I expression (see Section 1.6.5). In terms of viruses which invade humans, HCMV, HIV and human papilloma virus (HPV) have been shown to impede MHC class II expression. Most of the viruses inhibit the IFNγ-signal transduction cascade required

to induce upregulation of MHC class II transcription. This mechanism has been reported for

infection with HPV, where a protein termed E5 inhibits acidification of endosomes, destroying

functional MHC class II molecules (Straight et al., 1995). With respect to HCMV, inhibition

of MHC class II expression results from disruption of the IFN γ -stimulated Jak/STAT signal transduction pathway (Miller *et al.*, 1999). Because the induced expression of MHC class II

by IFN γ is likely to play a key role in antigen presentation, interference with this step could prevent the generation of an effective immune response against the virus. In terms of HIV infection, it has been demonstrated that the nef protein interferes with MHC class II processing (Kanazawa and Matija-Perterlin, 2001).

1.6.7 Viral genomic variation

Nucleotide substitutions in viral genomes can have several effects, including evasion of natural or vaccine-induced immunity, drug resistance, changes in pathogenicity, and alterations in tissue or species tropism, leading to viral persistence (Domingo et al., 1993). This usually occurs in viruses which replicate through reverse transcription due to the errorprone nature of the reverse transcriptase, since it lacks 5'-3' exonuclease activity (Wain-Hobson, 1996). This replicative mechanism can lead to significant genomic changes in the virus, thereby creating protein modifications which may evade virus-specific B and T-cell mediated immune responses. As mentioned before (Section 1.1.3) hepadnavirus is the only DNA virus that has this feature.

The mutation of viral proteins at sequences that serve as epitopes for antibody

recognition provides effective escape of the virus from the host humoral immunity. Mutations

in hypervariable region 1 of the HCV E2 glycoprotein, for example, may generate viral

species able to escape recognition by specific antibodies (van Doorn et al., 1995). Similarly,

in the HIV sequence, a single amino acid substitution in the conserved region of gp120, the envelope glycoprotein, can lead to a loss of recognition by antibodies (Watkins et al., 1993).

In an analogous manner, mutations in epitopes involved in the binding of viral peptides to MHC molecules or in TCR recognition of MHC/viral peptide complexes can enable the virus to evade detection by the immune system. The presence of CTL escape mutants was initially demonstrated in LCMV infection. Transgenic mice carrying a TCR specific for one LCMV epitope enabled a study of responses to epitopes with single amino acid substitutions (Pircher *et al.*, 1990). This study proved that for LCMV, a single substitution in the peptide that constitutes the TCR contact site resulted in suppression of the CTL response and virus persistence. The conclusion from this study is limited by the single TCR specificity used in the model, so that in the situation of a polyclonal and multispecific CTL repertoire, a similar viral advantage may not exist. However, it has been shown that CTL escape variants occur during natural HIV infection (Borrow *et al.*, 1997) and HBV infection (Bertoletti *et al.*, 1994).

A similar mechanism by which viral antigenic variation can enable viral escape of the host immune recognition is TCR antagonism. The interaction of the mutated viral peptides with the TCR causes the T-cell to be unresponsive to the engagement. Influenza virus and

HIV have been postulated to use this antagonistic form of immune evasion. The exact

mechanism of this event is not yet understood, however, it provides a highly effective means

of evading the virus specific CTL response. The efficacy of this strategy is evident in that the

variants could block CTL-mediated lysis of cells that are co-infected with wild-type virus.

This would allow the survival of the wild type virus in the presence of an ongoing CTL

response.

1.7 GENOME VARIATION IN HEPADNAVIRAL PATHOGENICITY

1.7.1 HBV genome mutations

Due to the structural and pathogenic properties of HBV, the implications of viral genome mutations can be extensive in terms of alterations in the viral replication cycle, immune recognition, and disease development. Virus "mutations" and "variants" are terms often used interchangeably, however, only mutations are relatively permanent changes in the viral genome, leading to a subspecies, whereas variants are considered to be only slightly different from wild type virus sequences (<10% nucleotide sequence variation in comparison to wild type). Even though virus mutations have been identified in different forms of HBV infection, the contribution of these mutations to virus infectivity, escape from immune recognition, and modulation of disease progression and severity is not clear. The unique strategy of HBV replication, involving reverse transcription, is associated with a high mutation rate of $1.75 \times 10^{-5} - 7.62 \times 10^{-5}$ misincorporations/site/year (Roberts *et al.*, 1988; Orito *et al.*, 1989). However, the compact genome structure of hepadnavirus, with four overlapping reading frames, may frequently led to nonproductive mutations since they can

affect more than one gene product.

1.7.1.1 Mutations found in fulminant hepatitis B

As stated previously, fulminant hepatitis is a rare but deadly form of hepatitis type B (Section 1.3.1). Only about 1% of those acutely infected with HBV will develop this disease, of which almost all will die unless they receive a liver transplant. The HBV genomic sequence

carried in this form of hepatitis has been extensively studied. Although cases of fulminant hepatitis have been linked to specific mutations (see below), this disease can also exist without viral sequence variation. On the other hand, presence of the same mutations is not always associated with fulminant disease, suggesting the importance of host-specific mechanisms (Karayiannis *et al.*, 1995; Protzer *et al.*, 1996; Naumann *et al.*, 1997).

HBV genome sequence analysis in source and index patients have shown that the majority of cases in outbreaks of fulminant hepatitis were derived from the same virus, with greater than 99% homology between sequences taken from different patients (Asahina *et al.*, 1996). Overall, three epidemics of fulminant hepatitis have been reported, and all of them arose due to infection with of HBeAg-negative strains originating from chronically infected HBeAg-negative patients (reviewed in Gunther *et al.*, 1999).

The specific HBV mutation associated with the fulminant disease is from G to A at position 1896 (G1896A). This point mutation disables the secretion of HBeAg by introducing a stop codon that only permits translation of a short pre-C peptide. Reports from different countries showed that the G1896A point mutation commonly occurs in patients with

fulminant hepatitis B (Carman et al., 1991; reviewed in Carman, 1996). Presently, the HBV

pre-core defective mutant is the only known hepadnavirus mutation associated with progression and/or outcome of hepatitis B.

The mechanism by which the HBeAg defective mutant mediates the induction of fulminant hepatitis was suggested based on experiments in HBV transgenic mice. It was observed that the change of a single residue in the HBV core/e-specific T cell epitopes led to

the loss of HBeAg tolerence and, in consequence, altered the type of Th cell responses (Milich *et al.*, 1998). The dysregulation of the Th1-Th2 balance may result in an increased and rapid inflammatory response, causing destruction of the liver. A potential role of antiviral cytokines, such as IFN γ and TNF α , in the initiation of fulminant hepatitis has also been suggested in a transgenic mouse model of HBV infection (Ando *et al.*, 1993).

1.7.1.2 Mutations resulting in HBeAg-negative hepatitis B

Hepatitis B accompanied by the absence of HBeAg reactivity arises from mutations either in the HBV pre-core region creating a stop codon or due to frame-shift mutations. As previously stated, the G1896A hypermutation, which prevents the synthesis of pre-core protein, may account for the severe liver disease in patients with serum HBeAg-negative hepatitis (Brunetto *et al.*, 1989; Carman *et al.*, 1989). Many longitudinal studies have shown that seroconversion to anti-HBe is commonly associated with the disappearance of biochemical markers of hepatitis and a decrease in viremia (Okamoto *et al.*, 1990; Gunther *et al.*, 1992; Maruyama *et al.*, 1998; Milich and Liang, 2003), as compared to chronic carriers without anti-HBe. Therefore, it can be suggested that the absence of HBeAg due to genomic

mutation would be associated with increased severity of liver disease and a greater HBV viremia during infection, and that anti-HBe may contribute to viral clearance. A common occurrence of pre-core defective HBV is seen in patients with end-stage liver disease. After liver transplantation in these patients, variant HBV, which is not capable of producing HBeAg, is thought to reinfect the donor liver (Angus *et al.*, 1995; Naumann *et al.*, 1997).

Other studies carried out in HBeAg transgenic mice demonstrated the tolerogenic potential of HBeAg. This may explain both the induction of fulminant hepatitis and the *in utero* tolerence to HBV antigens induced in the fetus of HBeAg-positive mothers (Milich *et al.*, 1990). These studies also provide insight into the pattern of induced HBV disease in the absence of HBeAg. Further, provided that HBeAg can cross the placenta, its interaction with the immune system prior to perinatal infection may down-regulate the HBcAg/HBeAgspecific Th cell response, which is assumed to play a role in HBV clearance (Ferrari *et al.*, 1990). In mice, HBeAg preferentially elicits a Th2 cell response which produce cytokines that are anti-inflammatory (Milich *et al.*, 1997). Also, HBeAg has the potential to deplete HBeAg-specific and core-specific Th1 cells, probably by apoptosis (Milich *et al.*, 1998). Thus, the suppressive effect of HBeAg on the immune system's response to HBV may partially explain fulminant hepatitis and newborn tolerance to HBV. The dysregulation of this tolerence caused by the aberrant HBV lacking the ability to express HBeAg may affect the pattern of induced liver disease.

1.7.1.3 Mutations arising after hepatitis B vaccination

Over the past 10 years, despite effective vaccination protocols against HBV yielding protective anti-HBs, outbreaks of HBV infection in individuals thought to be protected have occurred. Up to 99% of antibodies induced after HBV vaccination are directed toward the "a" antigenic determinant of HBsAg, which is conserved across all HBV subtypes. Normally, these antibodies are protective. However, "a" antigenic determinant with mutations between

amino acid residues 137 and 149 have been found in the HBV sequence in patients with breakthrough infections (Howard et al., 1995). One of these mutations lies at position G145R (Carman and Howard, 1992; Okamoto et al., 1992). This mutation has shown to be responsible for break-through infections, which occur in 5-19% of children vaccinated in endemic countries (Whittle et al., 1991; Fortuin et al., 1994). Thus, approximately 8% of children from Gambia have evidence of such HBV infection (Fortuin et al., 1994), and many of them also harbour another mutation, K141E (Karthigesu et al., 1994). It is well known that there are three epitopes in the "a" determinant and that vaccine-induced antibodies may differ in reactivity profiles compared to those developed over the course of natural HBV infection followed by recovery (Thanavala et al., 1986). This suggests that a single amino acid change in the virus envelope protein may render neutralizing antibodies generated by vaccination useless (Karthigesu et al., 1999). A similar situation exists when mothers vertically transmit HBV infection, and their newborns are vaccinated. These children generally develop HBV with mutations in the "a" determinant (Lee et al., 1997; Ngui et al., 1997) and become asymptomatic HBeAg-positive carriers, with no wild-type sequence

reversion over time (Carman et al., 1990; Hsu et al., 1997).

The increasing selection of the aforementioned variants due to vaccination, their potential to establish a chronic carrier state, and their possible transmission may lead to a spread of "a" determinant variants. This may cause potential problems for successful global vaccination strategies. Surveillance of the epidemiology of these variants is required. However, follow-up studies of children infected with "a" determinant variants completed so

far do not indicate a significant spread of these mutants (Oon et al., 1996; Hsu et al., 1997).

1.7.1.4 Mutations arising during antiviral treatment

Antiviral drugs currently used to treat CH type B, such as lamivudine, often select mutations in the HBV P gene (Colacino and Staschke, 1998; Melegari *et al.*, 1998). Resistance to therapy and break-through infection are observed with the emergence of these mutations. Lamivudine therapy induces amino acid changes in the YMDD motif, namely M to V or I (Ling *et al.*, 1996; Niesters *et al.*, 1998). After one year of treatment, up to 39% of immunocompetent patients develop mutations in this motif (M550V or M550I) (Honkoop *et al.*, 1997). Besides the active site of the RT, the B domain of the polymerase, upstream from the YMDD domain, is also an area where mutations can develop which overcome antiviral therapy with lamivudine. Famciclovir therapy often shows amino acid changes emerging from the B domain of the polymerase. These changes can often compensate for the deleterious effect of the mutations in the YMDD motif (Fu and Cheng, 1998). Even though these mutations re-establish P protein function, the *in vitro* activity of the transfected mutant

P gene was only about 10% of that compared to the wild-type controls (Melegari et al.,

1998). This may explain why viremia levels for breakthrough infections during treatment only

reach 10% of the pretreatment levels (wild-type sequence) (Neisters et al., 1998), and why

after treatment ends, wild-type HBV quickly re-emerges (Buti et al., 1998).
1.7.2 Woodchuck hepatitis virus variants

Considering that WHV is the closest natural model of HBV, some of the aforementioned mutations which were found in HBV-infected people were also identified in WHV. For example, the G1896A mutation in the pre-core region of WHV parallels that occurring in HBV. This mutant has only been found in chronic WHV infection (Li *et al.*, 1996).

X protein deletion variants have been found in WHV. They contain deletions and duplications/insertions near the direct repeat (DR) sequence within the X gene (Sugata *et al.*, 1994). In HBV infection, these mutations tend to appear in chronic infection in children with post-transfusion hepatitis, and in patients infected with HBV after renal dialysis (Feitelson *et al.*, 1995). Similarly the X protein deletion variants have only been found in chronic WHV (Li *et al.*, 1996).

In studies on antiviral agents, such as lamivudine, performed using the woodchuck model, P gene mutations identical to those described above for HBV have been identified. Notably, the A566T mutation in the FLLA motif of the polymerase B domain and the M589V

mutation in the RT domain of the P gene also gives rise to a viral genome capable of re-

establishing infection (Tatti et al., 2002).

1.8 THE ROLE OF EXTRAHEPATIC REPLICATION IN HEPADNAVIRAL PERSISTENCE

Since the mid-1980's, it has been shown that although hepatic HBV infection and

induced liver pathology is the leading source of clinical manifestations, the virus also productively infects cells of the lymphatic system, at least in chronic symptomatic hepatitis B. This fact holds true for all hepadnaviruses, in both avian and mammalian models of HBV infection (Pontisso *et al.*, 1984; Yoffe *et al.*, 1986; Blum *et al.*, 1991; Michalak *et al.*, 1994; Rehermann *et al.*, 1995; Penna *et al.*, 1996; Michalak *et al.*, 1999). Ultimately, the consequences of the potential existence of lymphotropic hepadnavirus variants, if they exist, and a role of lymphoid cell infection in the course of hepadnaviral hepatitis remain to be elucidated.

1.8.1 HBV infection of the lymphatic system

In chronic, serum HBsAg-positive HBV infection, the presence of HBV DNA was detected using Southern blot hybridization analysis of PBMC DNA. Replicative intermediates, cccDNA and viral RNA, were also detected in lymphoid cells of these patients (Pontisso *et al.*, 1984; Laskus *et al.*, 1999). However, usually these studies did not employ methods to differentiate between intracellular and potentially cell surface adhered virions or

free viral DNA. Moreover, HBV antigens have been found in PBMC isolated from some chronically infected individuals (Chemin *et al.*, 1992).

The development of sensitive techniques able to detect genome quantities below 100 vge/mL by applying PCR amplification of viral DNA, demonstrated the presence of HBV in serum and PBMC in patients recovered from AH type B (Michalak *et al.*, 1994; Penna *et al.*, 1996; Rehermann *et al.*, 1996b), as well as in liver biopsy samples from such patients (Yuki

et al., 2003). This persistence of the virus proves that the appearance of otherwise protective anti-HBs does not indicate total virus eradication (Michalak et al., 1994).

The detection of HBV genome and its replicative intermediates observed in the lymphatic system of patients with occult HBV suggests ongoing HBV replication. Transiently increased levels of HBV replication may occur at times due to many factors. Stimulation of the immune system due to common infections or more serious factors, *i.e.*, co-infection with HIV or HCV, and administration of immunosuppressive agents during transplantation or chemotherapy, may upregulate virus production. An example of how significant low levels of lymphoid cell-derived HBV can be is exemplified in a report describing a patient who was HBV seronegative with no detectable HBV DNA that had undergone hematopoietic cell transplant from a donor that was serum HBsAg-negative but anti-HBs and anti-HBc-positive (Carpenter et al., 2002), indicating convalescence from AH type B. Serum HBV DNA was apparently negative in the donor described in this study, as tested by real time PCR (sensitivity: 25 vge/mL). However, upon administration of the bone marrow, the recipient became HBsAg-positive at 7 months post-transplantation. The most probable explanation of the source of HBV infection in this patient was from the donor's cells. The recipient was treated with immunosuppressive drugs, which may have allowed increased virus replication. Studies employing enzymatic treatment of PBMC isolated from patients convalescent

from AH to remove any traces of viral DNA and potentially attached virions proved that HBV

expression was intracellular (Michalak et al., 1994). However, due to ethical and practical

considerations in sample collection, investigations of liver biopsies from such recovered

patients have not been extensive. Nevertheless, recent studies have shown that there are longterm histologic and virologic consequences after clearance of silent infection continuing after termination of AH (Yuki *et al.*, 2003). Thus, liver tissue samples obtained at a median of 7.2 years after recovery harboured HBV DNA S and X gene sequences, as well as HBV cccDNA in all individuals examined. Furthermore, mild inflammatory infiltrations and fibrous expansion of portal areas were observed upon histological examination. Further studies are required to determine the clinical relevance of these observations. However, these findings are consistent with earlier detailed studies of liver alterations in woodchucks years after recovery from experimental WHV hepatitis (Michalak *et al.*, 1999).

Importantly, not only low levels of HBV DNA persist in PBMC during occult infection but also virus-specific T cell responses and anti-HBc antibodies, supporting the notion that low level HBV replication progresses in these apparently healthy individuals (Rehermann *et al.*, 1996b). Importantly, HBV DNA sequences encountered in these individuals did not differ from wild-type HBV even 30 years after recovery from AH type B (Bläckberg and Kidd-Ljunggren, 2001).

1.8.2 WHV lymphotropism

Many of the findings mentioned above have been extensively studied in the woodchuck model of HBV infection. Initially, extrahepatic replication in lymphoid tissues had been shown by detection of WHV DNA in the PBMC of animals with serum WHsAgpositive CH (Korba *et al.*, 1987; Ogston *et al.*, 1989). It was later shown that the expression

of WHV replication intermediates could be upregulated in lymphoid cells from these woodchucks in the presence of non-specific mitogens (Korba *et al.*, 1988). Further, it was demonstrated that PBMC from animals with CH could also display WHsAg and WHcAg, in addition to WHV DNA and RNA (Chemin *et al.*, 1993).

The potential pathogenic relevance of the lymphotropic nature of WHV became evident in studies of WHV occult infection. Examination of circulating and organ lymphoid cells during the life-time follow-up of the animals recovered from acute WHV hepatitis showed the presence of WHV DNA and RNA (Michalak *et al.*, 1999). Importantly, as described in Section 1.5.2.3, HCC has developed years after recovery in about 20% of these animals, indicating that the harboured virus retained its oncogenic potential. Other *in vitro* studies from our laboratory showed that virus derived from naturally infected lymphoid cells could infect cultured virus-naive lymphoid cells and hepatocytes (Lew and Michalak, 2001). The supernatants from the lymphoid cell cultures injected intravenously (i.v.) into virus-naive woodchucks produced a serologically silent WHV infection (see below).

It was documented that woodchuck dams chronically infected with WHV transmit infection to their offspring and that up to 90% of the offspring develop serologically evident CH (Gerin, 1990). It has also been shown that dams that apparently completely recovered from WHV infection and were anti-WHs positive pass the virus to their babies (Coffin and Michalak, 1999). This infection was asymptomatic and progressed in the absence of WHsAg, anti-WHs and anti-WHc antibodies. WHV genomes were detected at levels of 10-100 vge/mL in serum for up to almost 4 years after birth (Coffin and Michalak, 1999). Moreover, WHV DNA in some animals persisted in the lymphoid organs and circulating lymphoid cells, but not in the liver. Importantly, although this virus was not evidently troubling to the offspring (*e.g.*, no biochemically or histologically evidence of liver pathology), it was infectious when injected to virus-naive adult woodchucks.

The nature of WHV lymphotropism and the lymphatic system-restricted infection are not recognized. Also, the pathological consequences of the persistence of small amounts of biologically competent virus at this extrahepatic site requires further and more detailed investigations.

1.9 PURPOSE OF THE STUDY

Due to recent identification of the existence of persistent occult hepadnaviral infection continuing after recovery from SLAH and demonstration of predisposition of hepadnavirus to infect the host's lymphatic system, many areas of research concerning the virus lymphotropism and its contribution to the long-term persistence of hepadnavirus need to be elucidated. Also, pathobiological properties of the virus replicating within lymphoid cells are not fully determined. The possible existence of lymphotropic viral variants, which might be

a causative factor of the lymphatic system infection, also is not recognized. The potential, but currently not fully recognized, epidemiological and pathological consequences of occult hepadnavirus persistence makes investigations in this area clinically relevant. The difficulties with such studies in humans are mainly due to practical and ethical problems with collection of appropriate materials, in particular liver biopsies, and with determination of the onset of HBV invasion. This gives way to the use of the natural experimental model of HBV infection, the woodchuck-WHV system. The aims of the present studies were as follows:

1. To determine the extent of WHV infection in lymphoid cells in different stages of hepadnaviral infection, including chronic WHV hepatitis, primary occult infection (POI) and secondary occult infection (SOI), continuing after recovery from WHV hepatitis. In this regard, to establish a method allowing direct detection of hepadnavirus genome in intact lymphoid cells and to quantify numbers of WHV-infected lymphoid cells during both serologically evident and silent virus persistence.

2. To elucidate if the induction of occult WHV infection might be related to the dose of the invading virus and, if so, to identify an amount of the virus that is required to cause this form of infection in the woodchuck model of hepatitis B.

3. To identify whether the existence of a particular virus genomic variant could be

responsible for the lymphotropic nature of WHV. For this purpose, to design experimental

conditions facilitating enrichment of a lymphotropic virus. Subsequently, to analyze nucleotide sequences of the WHV obtained after multiple serial passages of wild-type virus in cultured woodchuck lymphoid cells and hepatocytes. To determine if this approach may generate changes in the virus genome which would be specific for a particular cell type and which may modify the virus' *in vivo* infectivity.

4. To assess the engagement of the lymphatic system in WHV infection established by the intrahepatic transfection with complete recombinant WHV genome (rWHV DNA). To assess if the virus in this form of artificially induced infection invades and replicates in DC.

5. To examine if DC are a reservoir of actively replicating virus in animals with serologically evident and occult long-term WHV persistence.



CHAPTER TWO: GENERAL MATERIALS AND METHODS 2.1 WOODCHUCKS

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

Woodchucks infected with WHV were kept separately from healthy animals. Healthy woodchucks had no serological markers of past WHV infection, *i.e.*, they were negative for serum WHsAg, anti-WHc and anti-WHs antibodies (Section 2.3). In addition, DNA extracted from sera, PBMC, and liver biopsies from these animals were WHV DNA non-reactive by WHV-specific PCR/NAH of the resulting PCR products (sensitivity <10 vge/mL) (Section 2.7).

Initiation of WHV infection was considered when WHV DNA became first detectable

in the circulation (i.e., serum and/or PBMC). The beginning of the acute phase of WHV

infection (i.e., AH) was diagnosed when serum WHsAg was detected for the first time. The

existence of AH was routinely confirmed by histological examination of liver biopsy obtained

by laparotomy 4-6 weeks after inoculation with WHV. Recovery from AH was diagnosed

when serum WHsAg was permanently cleared. At this stage, anti-WHs were usually, but not

always, detectable.

Chronic WHV infection was defined when an animal was continuously positive for serum WHsAg, anti-WHc, and WHV DNA for at least six months (mo). Diagnosis of CH was confirmed by histological examination of liver biopsy (Section 2.12).

2.2 SAMPLE COLLECTION

2.2.1 Blood collection

Blood was obtained aseptically from the digitalis vein of woodchucks under isofluorane-induced anaesthesia (CDMV Inc., St. Hyacinthe, Quebec). Blood was collected into tubes with no additives (red top Vacutainer; Becton Dickinson, Rutherford, New Jersey) for serum isolation (Section 2.2.2) or into tubes containing sodium ethylenediamine tetraacetic acid (EDTA; lavender top Vacutainer; Becton Dickinson) for plasma and PBMC isolation (Section 2.2.3).

2.2.2 Serum isolation

Untreated blood was allowed to clot at ambient temperature (AT) for approximately

one hour (h) before serum isolation and then kept at 4°C for 18 h to collect any remaining

serum. The tube was then centrifuged at $720 \ge g$ for 10 minutes (min). The isolated serum

was aseptically aliquoted to small volumes and stored at -20°C for future use.

2.2.3 Isolation and storage of PBMC

Approximately 5 mL of EDTA-treated blood was overlaid on 3 mL of Ficoll-Paque (Pharmacia Biotech, Baie d'Urfé, Quebec) and centrifuged at 330 x g for 30 min. The plasma layer was collected and stored at -20°C. The interface between the two layers which contains PBMC was removed and diluted with 10 mL of sterile phosphate buffered saline (PBS), pH 7.4 with EDTA (PBS-EDTA), and centrifuged at 330 x g for 10 min. Red blood cells remaining in the cell pellet were lysed with 3 mL of buffered ammonium chloride solution for 10 min, then washed with PBS-EDTA under the same conditions as indicated above.

Viable cells were counted with a hematocytometer using trypan blue exclusion. Approximately 10⁷ cells were pelleted by centrifugation using the conditions described above and suspended in fetal calf serum (FCS) containing 10% dimethyl sulfoxide (DMSO) at a concentration of no more than 10⁷ cells/mL in cryogenic vials. The vials were stored in liquid nitrogen after a slow cooling process. Upon thawing on ice, these cells could be used for culture or serve as a source for nucleic acid isolation.

2.2.4 Liver biopsies

Biopsy liver tissue was obtained by surgical laparotomy under aseptic conditions. Each animal was sedated by an intramuscular injection of ketamine (23 mg/kg; Ketaset; CDMV Inc.) and xylazine (10 mg/kg; Lloyd Laboratories, Shenandoah, Iowa) and then anaesthetized using 2-4% isofluorane (CDMV Inc.). Each biopsy was divided aseptically into several fragments. Liver pieces (1-2 mm³ each) were immediately frozen in liquid nitrogen

for future nucleic acid analyses. Other tissue fragments (about 5-mm³ each) were fixed in 10% buffered formalin (Fisher Scientific, Nepean, Ontario) for histological examination or embedded in HistoPrep (Fisher Scientific) and then frozen in isopentane pre-cooled in liquid nitrogen for future immunofluorescent (IFL) examination.

2.2.5 Collection of specimens at autopsy

Prior to autopsy, animals were injected with an overdose of ketamine-xylazine. Blood was collected by cardiac puncture and used for isolation of serum (Section 2.2.2), PBMC, and plasma (Section 2.2.3). The liver, spleen, lymph nodes, bone marrow, kidneys, pancreas, and fragments of skeletal muscle were removed aseptically. Tissue samples were preserved for further investigations, as described above (Section 2.2.4). In some cases, splenocytes were isolated by gently pressing the splenic tissue through a fine wire mesh (Jin et al., 1996). The resultant cells were resuspended in 10 mL PBS-EDTA and then processed following the protocol for PBMC (Section 2.2.3).

2.3 ASSAYS FOR SEROLOGICAL MARKERS OF WHV INFECTION

2.3.1 Detection of woodchuck hepatitis surface antigen (WHsAg)

WHsAg was detected using a WHsAg-specific enzyme-linked immunosorbent assay (ELISA) developed in this laboratory. First, a 96-well flat bottom plate (Linbro/Titertek; ICN Biomedicals, Aurora, OH) was coated with anti-WHs antibodies at 1 µg protein in 50 µL PBS per well, incubated at 4°C overnight, and washed three times. Non-specific binding was

blocked by adding 300 μ L of 0.25% Tween-20 (Sigma Chemical Co., St. Louis, MO) in PBS (blocking buffer) and incubating AT for 1 h. After washing, 50 μ L of test serum, or the appropriate controls, was added to the wells and incubated for 1 h at AT. Then, 50 μ L of the secondary antibody, mouse anti-WHpre-S2, was added (diluted 1:1000 in blocking buffer) and incubated for 1 h AT. For detection, an alkaline phosphatase (AP)-conjugated goat antimouse antibody was added (diluted 1:1000 in blocking buffer) and incubated for 1 h AT. For detection, an alkaline phosphatase (AP)-conjugated goat antimouse antibody was added (diluted 1:1000 in blocking buffer) and incubated for 1 h AT. After washing, the assay was developed by adding 50 μ L soluble alkaline phosphate substrate (*p*-nitrophenyl phosphate; Sigma Chemical Co.) The reactions were terminated after 30 min by addition of 50 μ L of 0.1 M EDTA. Absorbance was read at 400 nm (A₄₀₀) using a microplate reader (BioRad Laboratories, Hercules, CA). Sera from healthy animals were used as negative controls. Positive controls included sera from WHsAg-positive woodchucks chronically infected with WHV. Specimens that gave a value equal to or above 2.1 (determined by dividing A₄₀₀ sample by the negative control mean [NC \bar{x}]) were considered WHsAg reactive.

Alternatively, we used a cross-reactive AUSRIA-II kit for detection of HBsAg (Abbott Laboratories, N. Chicago, IL). Serum or plasma samples were tested directly for

WHsAg following the manufacturer's recommended procedure. Briefly, 200 μ L of each sample test and control sample were incubated for 16 h at AT with beads coated with anti-HBs. After washing, the beads were incubated for 1 h at 45°C with 200 μ L ¹²⁵I-labeled anti-HBs. Bound radioactivity was counted in a gamma counter. Specimens giving counts per minute (cpm) equal to or greater than the cutoff value (determined by multiplying the NC \times

count by a factor of 2.1) were considered WHsAg reactive. Based on detection of purified WHsAg, the assay sensitivity was estimated to be 3.25 ng protein/mL (Michalak *et al.*, 1989).

2.3.2 Detection of antibodies to WHsAg (anti-WHs)

Anti-WHs antibodies were measured using a cross-reactive ELISA (AUSAB EIA, Abbott Laboratories) detecting anti-HBs. The cross reactivity of this assay allowing detection of anti-WHs was established previously (Michalak *et al.*, 1989 and 1990). Briefly, polystyrene beads coated with HBsAg were incubated overnight with 200 μ L of either test woodchuck serum or plasma, or samples of the appropriate positive and negative control sera supplied by the manufacturer, as well as anti-WHs positive and negative woodchuck sera. Unbound material was removed by washing with water and the beads were incubated with 200 μ L of a mixture of HBsAg tagged with biotin and then with rabbit anti-biotin conjugated with horseradish peroxidase (HRPO) for 2 h at 40°C. Next, the beads were washed to remove unbound conjugates, transferred to fresh tubes, and incubated with 300 μ L of freshly prepared *o*-phenylenediamine solution containing hydrogen peroxide. After incubation at AT

for 30 min, the beads were transferred to fresh tubes and the enzyme reaction was stopped

by addition of 1 mL of 1N H_2SO_4 . The colour intensity was measured at 492 nm using a Quantum II dual-wavelength analyser (Abbott Laboratories). The presence or absence of anti-WHs reactivity was calculated automatically by comparing the absorbance values of the sample tested to the cutoff value (NC \times +0.05). Samples with absorbance values greater than or equal to the cutoff value were considered positive.

2.3.3 Detection of antibodies to WHcAg (anti-WHc)

Anti-WHc was detected using a specific competition ELISA developed previously in this laboratory (Michalak et al, 1999). This assay is based on a principle that any anti-WHc present in the test sample competes with HRPO-labeled anti-WHc for binding to immobilized WHcAg. To this end, a 96-well, flat-bottom plate (Linbro/Titertek; ICN Biomedicals) was coated with woodchuck anti-WHc antibodies at 1 µg protein in 50 µL of PBS per well, incubated at 4°C overnight, and washed three times. Nonspecific binding was blocked as above (Section 2.3.1) by adding blocking buffer and incubating at AT for 2 h. After washing, the plate was directly used or stored at -20°C. Before the assay, the plate was thawed and the wells washed briefly with PBS. To each well, 0.5 µg of WHcAg in 50 µL of blocking buffer was added and the plate was incubated at AT for 2 h in a humid chamber. The plate was then washed 4 times with PBS, blotted dry, and 20 µL of blocking buffer, 5 µL of the test serum sample, or the appropriate controls, and 25 µL of anti-WHc labeled with HRPO (diluted 1:2,500 in blocking buffer) was added to each well. After a 2-h incubation, the wells were washed 3 times with PBS, and 50 µL of freshly prepared 3,3',5,5'-tetramethylbenzidine

(TMB) substrate (BioRad Laboratories) was added to each well. The reaction was stopped

after 30 min by addition of 50 µL of 1N H₂SO₄. The A₄₅₀ was determined using a microplate

reader (BioRad Laboratories). Sera from healthy animals were used as negative controls.

Positive controls included sera from WHsAg-positive woodchucks chronically infected with

WHV. The degree to which the test sample inhibited the binding of HRPO-labeled anti-WHc was calculated as follows: percent inhibition = $100 - (\text{test sample optical density (OD)} \div$

negative control OD \times 100). The assay results were accepted when the positive controls inhibited \geq 95% of the HRPO-anti-WHc binding to WHcAg and the negative controls gave no inhibition. Samples that produced \geq 50% inhibition were considered positive for anti-WHc.

2.4 DEPLETION OF VIRIONS AND VIRAL DNA FROM CELL SURFACE

In order to ensure that analysed WHV DNA originated exclusively from cells but not from virions or free DNA fragments potentially attached to the surface, cells were subjected to limited enzymatic treatment and extensive washing. Under standard conditions, cells, either freshly isolated or recovered from liquid nitrogen by thawing on ice, were washed twice with PBS-EDTA by centrifugation at 330 x g for 5 min. The resulting cell pellet was resuspended in 900 μ L of sterile PBS. Then, the cells were supplemented with 100 μ L of 10 X DNase buffer (500 mM MgCl₂ in 100 mM Tris-HCl buffer, pH 8.0) and 10 µL of 1 mg/mL DNase I (type IV from bovine pancreas; activity, 2 U/µg; Sigma Chemical Co., St. Louis, MO) and incubated for 30 min at 37°C. After DNase digestion, 10 µL of 0.1 M CaCl₂ and 10 µL of 10 mg/mL trypsin (type IX from bovine pancreas; activity, 7.3 U/µg; Sigma Chemical Co.) were added and cells incubated on ice for 30 min. Subsequently, 20 µL of 10 mg/mL trypsin inhibitor (type II-O from chicken egg white; Sigma Chemical Co.) was added and cells briefly mixed at room temperature. In the next step, DNase digestion was repeated by adding 10 µL of 1 mg/mL DNase and using conditions described above. The treated cells were washed twice with PBS-EDTA and counted after trypan blue staining. After treatment,

the cells were used directly for nucleic acid isolation or aliquoted as outlined in Section 2.2.3 in 10% DMSO in FCS and frozen in liquid nitrogen for long-term storage.

2.5 ISOLATION OF WHV DNA AND RNA

2.5.1 Isolation of cellular DNA

For DNA isolation, 100 mg tissue fragments were homogenized in 100 μ L of Hank's balanced salt solution (HBSS) or 100 μ L of serum, plasma, or culture supernatants was combined with 200 μ L of DNA lysis buffer (10 mM Tris-HCl, pH 8.0, with 10 mM NaCl, 1 mM EDTA, and 0.5% sodium dodecyl sulfate [SDS] containing 200 μ g proteinase K (Invitrogen, Carlsberg, CA). The samples were incubated overnight in a 42°C shaking incubator, then extracted two times with equal volumes of Tris-HCl-buffered phenol followed by chloroform/isoamyl alcohol (24:1) following a standard procedure (Strauss, 1997). DNA was precipitated from the aqueous phase with one-tenth volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol (Sigma Chemical Co.) for 12 h at -20°C. After centrifugation, the resultant pellet was washed with 70% ethanol. The DNA was resuspended

in sterile water to an approximate final concentration of 0.5 $\mu g/\mu L$ or 5-10 μL serum

equivalent/µL.

2.5.2 RNA isolation

Total RNA was isolated from tissue and cells using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Briefly, tissues or cells were pulverized in liquid

nitrogen, homogenized in 1 mL of TRIzol reagent and mixed for 5-30 min. After the addition of 200 μ L chloroform, the tubes were shaken vigorously for 15 seconds (sec) and then centrifuged at 12,000 x g for 15 min at 4°C. RNA was precipitated from the aqueous upper phase with 500 μ L of isopropanol (Sigma Chemical Co.) for 10 min at AT and collected by centrifugation at 12,000 x g for 10 min at 4°C. The RNA pellet was washed in 1 mL of RNase-free 75% ethanol. The final RNA pellet was briefly air-dried and resuspended in RNase-free water. RNA was quantitated (Section 2.5.3) and used immediately for reverse transcription reaction (Section 2.6) or stored at -70°C.

2.5.3 Quantitation of nucleic acids

DNA, RNA and synthetic oligonucleotide primers or probes used in this study were quantitated based on the ultraviolet (UV) absorbance at 260 nm using a DU 530 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). DNA and oligonucleotides were read after suspension in sterile water or TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer, respectively, whereas RNA was diluted in 0.1% diethyl pyrocarbonate (DEPC) in water to minimize degradation by RNases. The concentration of RNA was calculated

assuming an OD of 1 corresponds to 40 mg RNA/mL (Strauss, 1997), giving the formula:

RNA (mg/mL) = (OD260 nm - OD320 nm) x dilution factor x 40 mg/mL. DNA

concentrations were determined as above except that an OD of 1 corresponds to 50 mg

DNA/mL (Strauss, 1989). The concentration of oligonucleotides was determined using the specific mass per OD obtained in the certificate of analysis from the manufacturer (Invitrogen

or IDT Technologies, Coralville, IA). The purity of the nucleic acid preparations were determined using the 260:280 nm absorbance ratio. Only RNA with a 260:280 nm ratio of greater than 1.8 and DNA greater than 1.6 were used.

2.6 REVERSE TRANSCRIPTION REACTION

The reverse transcription (RT) reaction was used to convert RNA to single stranded The reaction reagents and their concentrations were as follows: 2-8 µg of total cDNA. RNA, 20 U RNasin (Promega Biosciences Inc., Madison, WI), 1X reaction buffer (50 mM Tris-HCl buffer, pH 8.3, with 75 mM KCl and 3 mM MgCl₂), 10 mM dithiothreitol, 1 mM of each deoxynucleotide triphosphate (dNTP, Invitrogen), 200 units (U) of Moloney murine leukemia virus reverse transcriptase (MMLV-RT; Invitrogen), and 100 ng of random primers in a total reaction volume of 40 µL. The reaction was incubated at 37°C for 1 h. After this incubation, the sample was heated to 95°C for 5 min to inactivate remaining enzymes and then chilled on ice before PCR amplification (Section 2.7).

2.7 POLYMERASE CHAIN REACTION (PCR)

2.7.1 Standard PCR conditions for WHV DNA detection

Routinely, three sets of primers (Figure 2.1) specific for non-overlapping regions of

WHV genome, i.e., C, S and X genes, were used for the detection of virus DNA by direct

PCR. For the first round of PCR amplification, the direct primer pairs were used. To enhance sensitivity of DNA detection, three other primers pairs, internal to the those **Figure 2.1 WHV gene-specific PCR oligonucleotide primers used in the study and the sizes of resulting amplicons.** Primers location and direction (sense, +; antisense, -) are shown. The positions of the primers and the size of the amplicons generated are indicated in the table. All primer positions refer to the WHV sequence published by Kodoma *et al.* (1985) (GeneBank accession number M11082), except for those indicated by an asterisk, which are numbered according to Galibert *et al.* (1993) (GenBank accession number J02442).





Figure 2.1 WHV gene-specific PCR oligonucleotide primers used in the study and the sizes of resulting amplicons

Primor pairs and

size of target					
direct PCR	PCNV (1983-2007) COR (2589-2605) 623 bp	PSW (2949-2968) SUW (894-917) 1277 bp	XPC (1891-1907) PXO (1522-1547) 386 bp	PGAP1 (1298-1322)* MCOR (2429-2453)* 1156 bp	FGP2 (1908-1925) FGM2 (1892-1907) 3308 bp
	FWHC (2089-2107) RWHC (2430-2446) 358 bp				
nested PCR	PPCC (2033-2049) CCOV (2439-2460) 428 bp	PSW (2949-2968) HWVN (391-407) 767 bp	PXX (1568-1584) XXC (1742-1760) 192 bp	CCCV (2303-2284)* XINT (1630-1653)* 674 bp	
		NSW (303-322) SSW (781-803) 500 bp			

mentioned above, were applied for nested PCR. The sequences of these oligonucleotide primers and their location in the WHV genome have been previously reported (Coffin and Michalak, 1999; Michalak *et al.*, 1999).

In general, direct PCR detection of WHV utilized 1-4 µg of total genomic DNA as template. The PCR reaction conditions consisted of 1 X reaction buffer comprising 1.5 mM MgCl₂ with 50 mM KCl in 20 mM Tris-HCl buffer (pH 8.4) and 2.5 U of *Taq* DNA polymerase, 200 µM of each dNTP (all from Invitrogen), 300 ng of each oligonucleotide primer in a 100 µL total volume. The reaction mixture was overlaid with 100 µL of mineral oil (Sigma Chemical Co.) to prevent 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 15 min. For each thermocycling reaction, rWHV DNA, containing the complete virus genome sequence, and DNA from liver, serum, or PBMC from a WHsAg-positive chronically infected animal were used as positive controls (Coffin and Michalak, 1999; Lew and Michalak, 2001). In addition, both a water and a mock sample, containing all reagents used during the DNA

extraction and PCR were included as negative controls. For nested PCR, 10 µL of the direct

PCR mixture was re-amplified under the same conditions as the direct reaction. PCR

amplifications were performed under conditions limiting the possibility of contamination, as

outlined in detail in previous studies (Coffin and Michalak, 1999; Michalak et al., 1999; Lew and Michalak, 2001).

2.7.2 Detection of WHV cccDNA

As outlined in Section 1.1.3, the hepadnavirus replication strategy is unique among DNA viruses. The genome is composed of a partially double-stranded DNA molecule, rcDNA. Before replication can be initiated, the rcDNA must be repaired by host DNA polymerases and ligases to form cccDNA. Therefore, the detection of cccDNA, since it indicates the first step in viral replication, is used as the specific marker to monitor virus replication activity (Tuttleman et al., 1996).

2.7.2.1 Mung bean nuclease digestion

To exclude the possibility of detecting the partially double-stranded WHV rcDNA when testing for the presence of WHV cccDNA, digestion of test DNA with a single-strand specific enzyme, mung bean nuclease, which cleaves single-stranded DNA at the 3'-end of adenine and thymidine (Kowalski et al., 1976), was performed. Employment of this enzymatic treatment differentiates between rcWHV DNA and cccDNA, by eliminating single stranded regions of rcDNA leaving the cccDNA intact.

Principles of this technique have been described for HBV cccDNA by others (Tuttleman et al., 1986; Wu et al., 1990) and adapted for WHV cccDNA in our laboratory (Lew and Michalak, 2001). Approximately 2-10 µg of extracted DNA or 200 pg of rWHV DNA, that was either denatured by boiling (as a negative control) or was left intact (as a positive control), was mixed with 2 µL of 10 X mung bean digestion buffer (New England

Biolabs Inc., Beverly, MA), 1 U/ μ g of mung bean nuclease (New England Biolabs Inc.), and supplemented with sterile water to a total reaction volume of 20 μ L. This reaction proceeded at 30°C for 30 min and was terminated by precipitation of the nucleic acid with 50 μ L 100% ethanol in the presence of 5 μ L 3 M sodium acetate at -20°C for 12 h. The DNA was recovered by centrifugation, resuspended in 20 μ L, and used for PCR amplification with WHV cccDNA specific primers.

2.7.2.2 Primers and cycling parameters

All PCR primer and cycling parameters specific for WHV cccDNA (*i.e.*, spanning the nick region of the WHV genome) were similar to those used in our other studies (*e.g.*, Lew and Michalak, 2001). Two pairs of primers that spanned the nick region of the genome were used for direct and nested amplification rounds. For the direct amplification, primers PGAP1 (TGGTGTGTGTGTGTGTGTGTGTGTGTGTGTGGACGC; position: 1298-1322) and MCOR (CCGGAAGAGTCGAGAGAATGGGTGC; position: 2453-2429) were used (Figure 2.1). If cccDNA was not detected after initial direct amplification, a 10 μ L aliquot of the direct PCR product was amplified in a nested reaction using the primers XINT

(CTTCGCTTCGCCCTGAGACGAGT; position: 1630-1653) and CCCV (GTCCCCAGGTGTCAGTGACA; position: 2303-2284). All numbers denote nucleotide positions in the WHV genome sequence based on Galibert *et al.*, 1993 (GenBank accession number J02442).

The mung bean nuclease-digested DNA (see Section 2.7.2.1) or a 10 µL aliquot of

the direct PCR product was added to a 100- μ L total reaction volume that was composed of 1.5 mM MgCl₂, 200 μ mol of each dNTP, 300 ng of each primer, 50 mM KCl in 20 mM Tris-HCl buffer (pH 8.4) and 2.5 U *Taq* DNA polymerase (all from Invitrogen). The cycling parameters were as follows: 1 cycle of 95°C for 5 min, 52°C for 2 min, 72°C for 3 min, followed by 40 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 1 min, followed by a final extension of the generated PCR products by incubation at 72°C for 15 min. Mock and water as templates in the reaction, in addition to the controls mentioned above, were routinely included as negative controls, as outlined in Section 2.7.1.

2.7.3 PCR conditions for full-length WHV genome amplification

In order to construct WHV replicons containing whole virus sequences, the complete WHV genome was amplified in one intact piece. For this purpose, primers were designed to contain the *Sap*I restriction enzyme site. Introduction of this site to the WHV-specific primers was extremely useful since it does not exist in the WHV genome and has non-palindromic sticky ends (Gunther *et al.*, 1995). Consequently, this allowed for cloning of the amplified WHV sequence, providing at the same time, for sequence excision from a vector.

Subsequent re-ligation of the introduced SapI sticky ends should facilitate the production of WHV replicons (see Chapter 6).

For the complete WHV DNA amplification, a back-to-back pair of primers with incorporated the restriction site for *Sap*I were designed using primer design software (OMIGA). Primers FGM2 (gctcttcATTTATGCCTACAGCCTCC; position: 1907-1892) and

FGP2 (gctcttcTAAATGCATGCGACTTCCG; position: 1908-1925), with the *SapI* site sequences, indicated in lower case, were synthesized (Figure 2.1). All numbers denote nucleotide positions in the WHV genome sequence reported by Kodoma *et al.*, 1985 (GenBank accession no. M11082).

Since the WHV genome is over 3.3 kb, extended PCR conditions were employed to ensure full genome amplification. Since this program was very long (over 6 h), an increased amount of *Taq* DNA polymerase was also required. Thus, 2-4 µg of total DNA, or DNA from a 100-µL serum equivalent was added to a reaction volume composed of 1.5 mM MgCl₂, 200 µmol of each dNTP, 300 ng of each primer, 50 mM KCl in 20 mM Tris-HCl buffer (pH 8.4) and 5 U *Taq* DNA polymerase (all from Invitrogen). The initial incubation consisted of 95°C for 5 min, 52°C for 2 min, 72°C for 3 min, followed by 10 cycles of 95°C for 40 sec, 52°C for 1 min 30 sec, 72°C for 3 min. Then, 10 cycles, each at 95°C for 40 sec, 52°C for 1 min 30 sec, and 72°C for 5 min, were performed. An additional 10 cycles, each performed at 95°C for 15 min. Mock and water instead of DNA were routinely included as negative controls, as outlined in Section 2.7.1. If the results of first-round PCR were

negative, sub-genomic fragments were amplified from the direct PCR amplified products

using standard C, S, and X gene-specific primers (see Section 2.7.1).

2.8 REAL-TIME PCR

2.8.1 Detection of WHV C gene fragment

Real time PCR using the Roche Light-Cycler (Roche Diagnostics, Mannheim, Germany) was employed to quantify WHV genome presence in selected woodchuck samples. For this purpose, primers specific for the C gene sequence named FWHc (CTTTCCTGATCTTAATGCT: position 2089-2107) and RWHc (AGTCGAGAGAATGGGTG: position 2430-2446) were designed (Figure 2.1). Hybridization probes labelled with fluorescein isothiocyanate (FITC) or LC 640 Red were used to detect amplified products through fluorescent resonance energy transfer (FRET). The WHc-FITC conjugated probe (TGGACACTGCTACTGCCTTG: position 2112-2131) catalyzed the fluorescent emission of WHc-LC 640 Red conjugated probe (TGAAGAAAAGCTAACAGGTAGGGAAC: position 2133-2160) when annealed to the amplified WHV core 358-base pair (bp) sequence amplified with FWHc and RWHc primers. Primers and probes were produced by Synthegen, LLC (Houston, TX) and used at a concentration of 0.5 μ M and 0.2 μ M, respectively. A reaction mixture contained 4 mM MgCl₂ and components of the FastStart DNA Master Hybridization Probes Kit (Roche Diagnostics).

The program parameters used for this amplification were initiated by Taq DNA polymerase activation at 95°C for 10 min. This was followed by 55 cycles performed at 95°C for 10 sec, 49°C for 10 sec, and 72°C for 13 sec. The melting curve of hybridization probes was generated by incubation at 35°C for 10 sec, and then slow heating to 95°C by increasing

the temperature by 0.2°C/sec. The sensitivity of this assay was 10 vge/mL, as determined by the amplification of 10-fold dilutions of complete rWHV DNA amplified in parallel with the test samples. After the reaction was completed, samples were collected and analysed by ethidium bromide (EB)-agarose gel electrophoresis (Section 2.9.1) to ensure validity of controls and to confirm the size of the amplified product. Sequence specificity of the products was ascertained by Southern blot hybridization analysis (Section 2.9.2).

2.9 DETECTION OF WHV DNA

2.9.1 Agarose gel electrophoresis

To detect PCR products, an 18-µL reaction aliquot (Section 2.7) was supplemented with 2 µL of DNA loading dye (10% Ficoll-400, 0.25% bromophenol blue, 0.25% xylene cyanol FF, 50 mM EDTA, 10 mM Tris-HCl buffer; (all from Sigma Chemical Co.). The mixture was pipetted into the wells of a 1% agarose gel (Invitrogen). The gel was made with 1 X TAE buffer (40 mM Tris-HCl buffer, pH 8.0, 1 mM EDTA,) containing 0.5 ng/mL of EB. Molecular markers (100 bp ladder or 1 kb ladder; Invitrogen) were run in parallel with test samples to determine size of amplified products. After electrophoresis at 100 V for 50

min, the DNA bands were visualized under a UV light and the image was recorded using a

low-light imaging system (ChemiGenius 2, Syngene; Frederick, MD).

2.9.2 Southern blot hybridization analysis

2.9.2.1 DNA transfer to nylon membrane

To confirm the specificity of DNA products, validity of negative and positive controls, and plasmid preparations, or to semi-quantify PCR amplified DNA, Southern blot hybridization, using ³²P-labeled cloned rWHV DNA or synthetic oligonucleotide probes, was performed. Thus, the agarose gel containing the PCR or restriction enzyme digestion products were denatured with 1.5 M NaCl with 0.5 M NaOH for 30 min. The DNA was then neutralized with two changes of 1.5 M NaCl in 1 M Tris-HCl buffer (pH 8.0) for 30 min each. Blotting of DNA from the gel to the nylon membrane (Amersham Biosciences, Uppsala, Sweden) was performed using downward capillary transfer. After completion of transfer (12-16 h), the membrane was baked at 80°C in a vacuum oven for 2 h.

2.9.2.2 Random prime labeling and purification of recombinant DNA probes

Complete rWHV DNA used in this study as a probe was excised from the plasmid vector using digestion with the restriction enzyme *Eco*RI. For this purpose, 10 µg of the recombinant plasmid DNA was incubated with 100 U of *Eco*RI, 1 X REACT 3 buffer (both

from Invitrogen), and 50 U DNase-free-RNase (Boehringer Mannheim, Quebec, Canada) for 2 h at 37°C. After digestion, the DNA insert was separated from the plasmid by electrophoresis at 50 V in a 1% low-melting point agarose gel (Invitrogen) made with 1 X TAE containing EB. The band containing the DNA of interest was excised from the gel and purified using the Wizard PCR Prep DNA Purification System (Promega Biosciences). To prepare the WHV DNA probe, a random primed DNA labeling system (Rediprime; Amersham Biosciences) using ³²P-dCTP (Amersham Biosciences) was employed. Briefly, 25 ng of the recombinant WHV DNA in 45 μ L of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) buffer was boiled for 5 min and then chilled on ice for 2 min. The denatured DNA and 5 μ L of ³²P-dCTP was added to a reaction tube containing lyophilized dATP, dGTP, dTTP, Klenow enzyme, and 9-mer random primers.

The ³²P-labeled recombinant DNA and oligonucleotide probes were separated from unincorporated ³²P-dCTP by fractionation on Sephadex G-50 NICK columns (Pharmacia Biotech), as per the manufacturer's instruction. Briefly, the column was initially washed with TE buffer, the probe mixture was applied onto the column and then eluted with 400 μ L of TE buffer. The first eluate was discarded and 450 μ L of new TE buffer was applied to the column. The recovered fraction containing the labeled probe was stored at 4°C. The radioactive incorporation of ³²P-dCTP in the probe (cpm/ μ L) was determined using a scintillation counter to determine the volume of probe required for 6 x 10⁶ cpm per hybridization reaction (Section 2.9.2.3).

2.9.2.3 Southern blot hybridization

Membrane DNA blots to be hybridized with radiolabeled rWHV DNA or

oligonucleotide probe were placed in a sealed glass tube with 6 mL of hybridization buffer

containing 100 µg/mL sonicated salmon sperm (sss) DNA (Sigma Chemical Co.), 5 mL 6 X

SSC (standard saline citrate), 0.5 mL 50 X Dendhardt's solution, and 0.5 mL 10% SDS. The

membrane was prehybridized at 65°C in a rotary hybridization oven for 1 h. After prehybridization, 6 x 10⁶ cpm of heat-denatured radiolabeled WHV DNA or oligonucleotide probe was added and the blot was hybridized overnight at 65°C in a rotary hybridization oven.

For hybridization with oligonucleotide probes, the nylon membranes were prehybridized at 42°C in a hybridization oven for 1 h with 6 mL of hybridization buffer (as above), but without sssDNA. The blot was then incubated for 16 h at 42°C with 6 x 10⁶ cpm of a ³²P-end-labeled oligonucleotide probe (Section 2.10.3).

After hybridization, blots were washed twice in 2 X SSC with 0.1% SDS for 10 min at AT followed by two washes with 0.2 X SSC with 0.1% SDS for 10 min each at the proper hybridization temperature, depending on the probe used. The membranes were then covered in plastic wrap and exposed to a multipurpose Phosphor screen or exposed to X-ray film (MRP-1; Eastman Kodak Co., Rochester, NY) at -70°C in autoradiography cassettes with intensifying screens.

2.9.2.4 Quantification of Southern blot hybridization signals by densitometry

In order to determine the relative amount of WHV DNA in test samples, hybridized

Southern blots that were exposed to Phosphor screens were analyzed using the Cyclone system (Canberra Packard Ltd., Montreal, Quebec). The relative intensity of signals derived from serial 10-fold rWHV DNA dilutions were used to generate a standard curve. These dilutions were amplified in parallel with test samples and analysed by Southern blotting, from which the quantity of WHV DNA was estimated through densitometric analysis of hybridization signals.

Alternatively, in some cases, the film image of the radioactive signal emitted by the hybridized probe was quantified using the Gene Tools software on the ChemiGenius-2 Bioimaging system (both from Syngene). As above, the density of signals from WHV DNA standard dilutions was used as a reference to approximate the content of WHV DNA.

2.10 WHV DNA CLONING

2.10.1 Purification of PCR products

PCR products destined for cloning were either used directly or after purification using the Wizard PCR preps DNA purification system (Promega Biosciences). Thus, 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 Eppendorf tube and melted at 72°C. Purification resin was added to the tube, mixed briefly, and filtered under vacuum through a Wizard minicolumn, as per the manufacturer's instructions. The column was washed twice with 80% isopropanol, dried under vacuum for 30 sec, and then centrifuged at 12,000 x g for 2 min. Any residual isopropanol collected by centrifugation was discarded. TE buffer (50 μ L) was

heated to 65°C and applied onto to the column. After incubation for 5 min, the column was

centrifuged at 12,000 x g for 20 sec. The eluate contained the purified DNA. The DNA

concentration was determined by spectrophotometric analysis as described above (Section

2.5.3).

2.10.2 TA cloning of DNA fragments

Small DNA fragments (less than 1.5 kb) amplified by PCR were cloned using the TOPO TA Cloning Kit (Invitrogen), while larger DNA fragments were cloned using the TOPO XL Cloning Kit (Invitrogen). The final cloning mixture contained 25 ng of PCR product, 1 X ligation buffer, 50 ng of linearized plasmid (pCRII in TOPO TA kit or pCRXL in TOPO XL kit) and 4 U T4 DNA ligase in a total volume of 10 µL. The reaction proceeded at AT for 5 min. After incubation, 2 µL of the reaction mixture was added to 50 µL of TOP-10 bacterial cells (Invitrogen). The mixture was chilled for 30 min on ice, heat shocked for 30 sec at 42°C, and placed on ice for 2 min. Subsequently, 250 µL of SOC medium, containing 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, and 20 mM glucose (Invitrogen) was added and the bacteria was incubated at 37°C for 1 h in a rotary shaker at 250 revolutions per minute (rpm).

2.10.3 Growth and storage of vector-transfected bacteria

A 100-µL aliquot of each transformation reaction mixture was spread onto 1.5% Bacto-Agar (Difco Laboratories, Detroit, MI) coated with 40 µL of X-Gal (Sigma Chemical

Co.) in 10-cm Petri dishes. The dishes were incubated at 37°C for 16 h. Single bacterial colonies were collected and inoculated into 5 mL of Luria-Bertani (LB) medium (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, pH 7.0) supplemented with 50 µg/mL kanamycin (Sigma Chemical Co.). Bacteria were grown overnight at 37°C rotating at 250 rpm. Once the colonies were shown to contain the insert of interest, 50% glycerol stocks of bacteria

culture were cryopreserved at -80°C for long-term storage.

2.10.4 Mini-scale preparations of plasmid DNA

For preparation of small amounts of plasmid DNA, a 1.5-mL aliquot of transformed bacterial cells that were grown overnight to saturation were pelleted at 20,000 x g for 20 sec. The cells were resuspended in 100 μ L of glucose in Tris-EDTA (GTE: 50 mM glucose, 25 mM Tris-HCl buffer, pH 8.0, and 10 mM EDTA) and left for 5 min at room temperature. The bacteria were lysed with 200 μ L of freshly prepared 0.2 N NaOH/1% SDS solution and ice chilled for 5 min. To neutralize the mixture and to remove chromosomal DNA and proteins, 250 μ L of 3 M potassium acetate (pH 5.5) was added to the tube, vortexed for 2 sec, and the mixture ice chilled for 5 min. The preparation was centrifuged at 20,000 x g for 3 min to pellet cell debris and chromosomal DNA. After centrifugation, the supernatant was transferred to an Eppendorf tube and the plasmid DNA was precipitated with 1 mL of 100% ethanol for 2 min at AT. 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 μ L of TE buffer. Mini-scale preparations were analyzed for the presence of the proper DNA inserts by

electrophoresis in EB-agarose gel, after appropriate restriction enzyme digestion of the plasmid DNA.

2.10.5 Maxi-scale preparations of plasmid DNA

A 50 µL bacterial aliquot containing the proper plasmid insert, as judged by analysis

of minipreps (Section 2.10.4), was grown to saturation in 250 mL of LB medium supplemented with 50 µg/mL of the appropriate antibiotic (kanamycin or ampicillin). The plasmid DNA was isolated using the components of the Hi-Speed Plasmid Purification Kit (Qiagen Inc., Mississaugua, Ontario), as per the manufacturer's instruction. Briefly, the bacteria was collected by centrifugation at 6,000 x g for 15 min at 4°C. The pellet was resuspended in 10 mL of Buffer P1. Bacteria were lysed by addition of 10 mL of Buffer P2 and by gentle mixing of the solution gently to avoid shearing of DNA. The viscous mixture was supplemented with 10 mL of Buffer P3 and mixed immediately. Then, the mixture was passed through a QIA filter cartridge that was equilibriated with Buffer QBT prior to use. The cleared lysate was allowed to pass through the resin, and was washed. Nucleic acid was eluted by the addition of 15 mL of Buffer QF and, after washing, the eluate was collected. Isopropanol was used to precipitate and recover the DNA, which was then collected by the QIA precipitator, as per the manufacturer's instruction. The final elution of DNA was carried out by incubation with 0.5 mL of TE buffer. The fragment of interest was analyzed by electrophoresis in EB-agarose gel.

2.11 WHV DNA SEQUENCING

2.11.1 Sequencing primer labeling with T4 kinase

Plus-strand WHV DNA genome primers (Figure 2.1) selected for use in sequencing reactions were radiolabeled using $[\gamma^{-32}P]$ -labeled ATP, using the *Fmol* DNA sequencing system (Promega Biosciences) following the manufacturer's instructions. For the labeling,

10 pmol of primer was resuspended in a 10- μ L volume containing 3.0 μ L of 3000 Ci/mmol [γ -³²P] ATP (Amersham Biosciences), 1 μ L of T4 polynucleotide kinase 10 X buffer, and 5 U T4 polynucleotide kinase. After incubation for 10 min at 37°C, it was inactivated by heating to 95°C for 2 min. The labelled primers were used immediately in the sequencing reaction (Section 2.11.2) or stored at -20 °C until required.

2.11.2 DNA fragment generation by PCR amplification

Nucleotide sequence analysis was performed using the *Fmol* DNA cycle sequencing system (Promega Biosciences.). Briefly, a cocktail containing approximately 40 fmol of PCR product or recombinant plasmid DNA, 1.5 pmol of sequencing primer end-labeled with ³²P-ATP (Amersham Biosciences) (Section 2.11.1), 2 mM of MgCl₂ in 50 mM Tris-HCl buffer, pH 9.0 (supplied as a 5 X buffer), and 5 U sequencing grade *Taq* DNA polymerase (Promega Corp.) was prepared. The cocktail was divided equally into of 4 tubes, each containing 2 μ L of either the G, A, T or C terminating dideoxy nucleotide mixture. Cycle sequencing was performed 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 μ L of formamide stop

solution was added to each tube and the tube was kept on ice until electrophoresis in a

sequencing polyacrylamide gel (PAGE), as per manufacturer's instruction.

2.11.3 Gel electrophoresis and analysis of amplified products

Before electrophoresis, each sample was heat-denatured for 2 min at 70°C, then
chilled on ice. The DNA samples were separated at 50 V on an 8% denaturing polyacrylamide gel containing 7 M urea (Invitrogen). After electrophoresis at 50°C (as monitored with a thermal probe) in TBE (90 mM Tris 90 mM borate 2 mM EDTA, pH 8.0) buffer, the gel was fixed in a 7.5% methanol, 7.5% 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) or to X-ray film (Kodak) overnight.

Alternatively, the complete sequences of WHV DNA were obtained by fluorescencebased automated DNA sequence analysis (LI-COR; LiCor Inc., Lincoln, NB). This service was provided by the Department of Genetics, Hospital for Sick Children or the York . University Core Facility (both in Toronto, Ontario).

To search for sequence variation or to confirm the identity of nucleotide sequences, 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 (Basic Local Alignment Research Tool) search software (<u>http://www.ncbi.nlm.nih.gov/BLAST</u>) was performed.

2.12 HISTOLOGICAL EXAMINATION OF LIVER TISSUE

Paraffin embedded liver fragments were cut to 5-µm sections. The sections were stained with hematoxylin and eosin, Mason-trichrome, periodic acid shiff or impregnated with silver. Sections were examined to assess and numerically score histological lesions.

Alterations in three liver parenchymal compartments were evaluated, *i.e.* hepatocellular, intralobular/extrahepatocellular and portal, as previously described in detail (Michalak *et al.*, 1990; Michalak and Lin, 1994; Hodgson and Michalak, 2000; Hodgson and Michalak, 2001). An overall histological grade of disease severity (0-3) was assigned for each liver specimen examined. All histological evaluations were performed on coded samples by Dr. T. I. Michalak.



CHAPTER 3: Quantitative Detection of Hepadnavirus-Infected Lymphoid Cells by *In Situ* PCR Combined with Flow Cytometry

This study has been published in the Journal of Virology in Jan. 2003, volume 77(2), pp. 970-979.

3.0 SUMMARY

The detection of small amounts of viral pathogens in infected cells by classical PCR is hampered by a partial loss of virus nucleic acid due to nucleic acid extraction and by difficulties in discrimination between truly intracellular virus genome material and that possibly adhered to the cell surface. These impediments limit reliable identification of virus traces within infected cells, which are a typical encounter in latent and persistent occult infections. Due to potential significant pathogenic and epidemiological implications of occult hepadnavirus persistence, the ability to diagnose the presence of small amounts of virus genome is imperative to ensure safety of blood used for transfusion and organ/cell transplantation. In this study, hepadnavirus-specific *in situ* PCR combined with the enzymatic elimination of extracellular virus and flow cytometry permitted detection of viral genomes in lymphoid cells without nucleic acid isolation and allowed quantification of infected cells

during the course of persistent infection with WHV. The validity of the procedure was

confirmed by nucleic acid hybridization analysis of the in situ amplified viral sequences. The

results showed that hepadnavirus genome can be directly detected within lymphoid cells not

only in serologically accountable infection, but also years after recovery from viral hepatitis

and in the course of a primary occult virus carriage. Percentages of infected peripheral

lymphoid cells in symptomatic WHV hepatitis fluctuate between 3.4 and 20.4% (mean 9.6%

 \pm standard error mean [SEM] 1.7), whereas those in persistent, serologically mute WHV infection range from 1.1 to 14.6% (mean 4.8% \pm SEM 0.8)(P = 0.005). The obtained data are consistent with our previous findings that WHV replication continues indefinitely in the lymphatic system independent of whether infection is symptomatic or serologically concealed. They also document that hepadnavirus can be detected in a significant proportion of circulating lymphoid cells both in serologically apparent as well as in occult persistent infection.

3.1 INTRODUCTION

The PCR has proven to be one of the greatest advances in molecular diagnostics that is now widely applied to detect pathogens and to identify clinical conditions. In its classical form, this technique requires the extraction of DNA or RNA that has to be reverse transcribed to cDNA prior to amplification. The isolation step is inevitably associated with a partial loss of nucleic acid and, therefore, it may pose a serious impediment when identification of traces of genomic material of pathogens occurring at minute quantities is required. This is particularly important in the diagnosis of latent or persistent occult viral infections where very

low virus loads are normally encountered within infected cells. In these infections, even a

relatively negligible decrease during virus nucleic acid recovery or its minor degradation may

predetermine whether a pathogen is detected or not.

HBV inflicts lifelong, serologically detectable (*i.e.*, serum HBsAg-positive) infection in an estimated 400 million people worldwide (Margolis *et al.*, 1991; World Health Organization, 2000). However, small quantities of this noncytopathic DNA virus, which are not identifiable by currently used and otherwise sensitive serological immunoassays, also commonly occur in the absence of clinical symptoms and biochemical evidence of liver injury. It was documented that patients who resolved AH type B continue to carry small amounts of HBV genomes in serum, circulating lymphoid cells and in the liver for decades after recovery, despite production of protective antiviral responses (Michalak et al., 1994; Rehermann et al., 1995; Penna et al., 1996; Yotsuyanagi et al., 1998; Cabrerizo et al., 2000; Blackberg and Kidd-Ljunggren, 2001). The investigations in the woodchuck model of hepatitis B confirmed these observations and revealed that minute amounts of infectious WHV consistently persist for life after resolution of AH or acquisition of a primary serologically occult infection (Michalak et al., 1999; Coffin and Michalak, 1999). This occult virus is transmissible from mothers to offspring as a primary, serologically silent infection (Coffin and Michalak, 1999). Regardless of whether the hepadnaviral infection is serologically evident or silent, lymphoid cells invariably support replication of pathogenic virus (Korba et al., 1987; Lew and Michalak, 2001) and are a reliable source of genomic material for detection of persisting virus (Michalak et al., 1999; Michalak et al., 2000; Lew and Michalak, 2001).

The protocols currently applied for identification of low copy numbers of hepadnavirus genomes require isolation of DNA and a multi-step and contamination prone procedure that involves nested PCR followed by hybridization analysis of the amplified virus sequences. This approach, although of superior sensitivity, is time-consuming, does not permit for determination of the number of infected cells, nor does it allow for reliable discrimination between the intracellular virus sequences, which might be indicative of virus replication, and those nonspecifically attached to the cell surface. To circumvent these drawbacks and to establish a simplified method facilitating further studies on the role of the lymphatic system in hepadnavirus infection, we developed a direct *in situ* PCR procedure employing intact lymphoid cells which, in conjunction with enzymatic elimination of extracellular virus, detects viral amplicons within the cells by flow cytometry. Although *in situ* PCR coupled with flow cytometry has been shown to be applicable for identification of HIV and HCV in PBMC (Re *et al.*, 1994; Muratori *et al.*, 1996), our approach overcomes an ambiguity posed by a possible identification of contaminating extracellular viral sequences and permits for specific detection of small quantities of viral genomes in both freshly isolated and archival, cryopreserved PBMC samples. In the present study, we employed WHV-infected lymphoid cells to develop the procedure and to assess its applicability for identification of lymphotropic virus during chronic infection accompanied by either abundant or minuscule loads of circulating virus.

3.2 MATERIALS AND METHODS

3.2.1 Animals and lymphoid cell separation

Samples of peripheral lymphoid cells examined in this study were derived from 4

group of animals. Group 1 included PBMC isolated from 4 randomly selected WHV carriers

with CH confirmed by histological examination of liver biopsy (see Section 2.12). All the

animals had naturally acquired chronic infection and were serum WHsAg-positive and anti-

WHc-reactive for at least 6 months prior to PBMC collection. Multiple PBMC samples were available for analysis from one of the animals (1/F) post infection (p.i.) (Table 3.1). In all woodchucks, serum WHV loads were in the range of 10¹¹ vge/mL, as determined by a dotblot hybridization assay (sensitivity ~10⁶ vge/mL) (Table 3.1).

Group 2 included PBMC obtained from 9 animals with a past history of a self-limiting episode of WHV-induced AH (Table 3.1). These animals were initially WHV-naive and they developed transient AH when infected with WHV as adults (Michalak, 2000; Hodgson and Michalak, 2001). The woodchucks in this study group were serum WHsAg-negative and anti-WHc-reactive, and some of them demonstrated anti-WHs antibodies at the time of or prior to PBMC collection (Table 3.1). After recovery from AH, all animals carried trace amounts of WHV DNA in serum, lymphoid cells and in the liver for life. The levels of WHV DNA in sera did not exceed 10³ vge/mL, although they were usually around 10 vge/mL, as determined by WHV-specific nested PCR/NAH (Lew and Michalak, 2001). PBMC were collected for up to 5.5 years after clearance of serum WHsAg, at the time when liver histology was normal or occasionally demonstrated features of minimal, intermittent inflammation. The molecular and pathological features of this residual infection were described in detail in

previous works from this laboratory (Michalak et al., 1999; Michalak, 2000; Hodgson and Michalak, 2001).

Group 3 consisted of PBMC obtained from 4 woodchucks born to mothers who resolved WHV hepatitis (Table 3.1). These offspring were consistently WHsAg, anti-WHs and anti-WHc negative, but they carried low levels of WHV in serum, circulating lymphoid

Category of infection and animal	Time of PBMC acquisition (mo) ^a	Serology			Serum	WHV DNA
		WHsAg	anti-WHs	anti-WHc	(vge/mL) ^b	(% positive) ^c
GROUP 1 Serologically evident,						
chronic infection						
1/F	1	+	_	+	4.7×10^{11}	20.4
	2	+	-	+	4.7×10^{11}	9.3
	4	+	-	+	4.1×10^{11}	6.6
	6	+		+	3.4×10^{11}	10.6
	8	+	_	+	1.7×10^{11}	63
	11	+	_	+	4.8 x 10 ¹¹	66
	15	+		+	1.8×10^{11}	10.1
	19	+		+	2.7×10^{11}	15.1
	17				2.7 X 10	10.5
2/M	9	+	-	+	5.5 x 10 ¹¹	3.4
3/F	13	+	_	+	3.5×10^{11}	7.1
4/F	33	+	-	+	5.2×10^{11}	5.4
	35	+		+	4.8×10^{11}	4.1
					Mean (± SEM):	9.6 ± 1.7
GROUP 2						
Serologically silent, residual infection ^d						
5/F	5.5		+	+	1×10^{3}	3.8
	17.5		+	+	~10	3.2
	21	1.2.2.2.2.2.2.2.2.2.2.2.2.2.2.2.2.2.2.2	+	+	~10	21
					10	2.1
6/F	8e	-	-	+	$1 \ge 10^3$	8.9
7/17	0.6					
//٢	8	-	-	+	~10	3.6
	20	-	-	+	~10	4.7
8/F	16	_	-	+	~10	5.6
	30	-	-	+	1×10^{3}	2.1
9/M	20 ^e		1	+	~10	11.2
	31 ^f	_	-	+	1×10^{3}	12.9

Table 3.1 Immunovirological characteristics of WHV infection at the time of analysis of WHV DNA in circulating lymphoid cells by in situ PCR combined with flow cytometry

90

10/F	26°	-	+	+	~10
	66 ^r	-	-	+	~10
11/M	31	-	+	+	~10
	43		+	+	1×10^{3}
	44	-	+	+	~10
	51 ^f		-	+	~10
12/M	58	-	-	+	~10
13/M	42		_	+	1×10^{3}
	60 ^f		-	+	~10
					Mean (± SEM):
ROUP 3					
Serologically silent					
primary occult					
infection ^g					
meetion	6	and the second second		1. A	~10
14/F	18				~10
14/1	26				~10
	20		-		~10
	30	-	-	-	~10
	10				10
15/15	12	-	-		~10
13/F	17				10
16/15	17				~10
10/F	15				1 - 103
17/5	45 50 h	-	-	-	1 x 10 ⁵
1 //F	52"	-	-	-	~10
					Mean $(\pm SEM)$:

^a From inoculation with WHV, or from birth to a mother convalescent from acute hepatitis, or from time of arrival to colony for animals with serologically evident, chronic WHV infection

^b WHV DNA detected by dot blot hybridization (sensitivity ~10⁶ vge/mL) and, when negative, by nested PCR with WHV core gene-specific primers and Southern blot hybridization of the amplified virus sequences (sensitivity ~10 vge/mL)

^c Positive by *in situ* PCR combined with flow cytometry

(

^d Adult animals who recovered after an acute episode of WHV hepatitis and carried for life trace amounts of WHV

°PBMC samples tested for WHV cccDNA and found positive

^fPBMC samples collected at the end of the natural lifespan of the animals examined

^g Animals born to mothers who resolved acute WHV hepatitis and carried for life trace amounts of WHV

^h PBMC samples tested for WHV cccDNA and found negative

0.0
8.0
2 (
2.6
1.9
0.0
8.0
3.7
1.2
12
0.9
0.7
12
1.4
15+08
4.5 ± 0.0
1.2
1.3
1.0
1.2
0.6
2.6
1.0
4.8
2.4
2.1
14.6
11.0
57
8.0
5.1 ± 1.6

91

cells, lymphatic organs and frequently, but not always, in the liver, as described previously (Coffin and Michalak, 1999). In the current study, circulating lymphoid cells acquired from 3 offspring (14/F, 16/F and 17/F) with WHV DNA detection in the liver and one offspring (15/F) without hepatic expression were examined. Multiple PBMC samples were available for analysis from 14/F woodchuck (Table 3.1). Only PBMC found reactive for WHV DNA by classical nested PCR/NAH were examined in the current study.

Group 4 comprised PBMC derived from healthy, WHV-naive animals. These cells were used to optimize the assay conditions and as negative controls.

Tests applied for identification of serological markers of WHV infection (see Section 2.3) and dot-blot hybridization and PCR/NAH used for determination of WHV DNA loads were described previously (Section 2.7.1).

PBMC were isolated from blood treated with sodium EDTA drawn using Vacutainer (see Section 2.2.3). Cells were suspended at 1 x 10⁷ cells in 1-mL aliquots of heat-inactivated FCS with 10% DMSO and stored in liquid nitrogen for up to 5.5 years prior to use.

3.2.2 Optimization of assay conditions

In order to detect solely intracellular virus sequences and, at the same time, to preserve integrity of cells through extended PCR cycling, various enzyme treatment and cell fixation, permeabilization, and washing protocols were examined. This included evaluations of different schemes of limited cell surface digestion with DNase and trypsin to remove WHV virions and virus DNA fragments potentially attached to the cell surface (see below), the use

of different fixatives to stabilize structural integrity of the cells, and testing of different washing protocols. In addition, since detection of virus DNA was intended in the cells stored frozen for a prolonged period of time, procedures of handling of these cells, which are more fragile than those freshly isolated, were examined. The steps outlined below are the results of these preceding investigations.

In a series of preliminary experiments, the conditions for effective removal of extracellular WHV particles and WHV DNA sequences by DNase/trypsin/DNase digestion were tested. In these experiments, PBMC from healthy animals were exposed to WHV virions which were semi-purified from the serum of a chronic WHV carrier by ultracentrifugation over a 60% sucrose cushion, as described previously (Coffin and Michalak, 1999), or spiked with full-length rWHV DNA (Pardoe and Michalak, 1995). For this purpose, 1 x 10⁶ cells per sample were incubated for 30 min at AT with approximately 300 DNase-protected WHV particles or with 300 vge of rWHV DNA per cell. In both situations, the cells were washed with 0.25% Tween-20 in PBS and then treated by applying different DNase/trypsin/DNase protocols.

In another optimization trial, it was established that fixation of PBMC in cold acetone

for 3 min was as efficient as that with 1% or 4% paraformaldehyde for 2 h or 30 min,

respectively. However, acetone fixation gave evidently lower background signals than

paraformaldehyde when PBMC were examined by in situ PCR/flow cytometry.

Furthermore, examination by light microscopy of PBMC before and after permeabilization with proteinase K and the enzyme inactivation revealed that the morphology

of cells remained intact, although their overall number decreased by up to 20%. This was without any effect on the final results since only 10⁴ cells after *in situ* PCR were required for flow cytometric analysis. It was also found that extensive washing of fixed or unfixed PBMC with 0.25% Tween-20 in PBS did not disturb the cell's morphological integrity and that omission of this washing increased background binding of the FITC-labeled primer.

3.2.3 Cell surface DNase/trypsin/DNase treatment

Under standard conditions, cells were removed from liquid nitrogen, thawed on ice and washed twice with 0.25% Tween-20 in PBS by centrifugation at 660 x g for 5 min to remove FCS and DMSO. The resulting cell pellet was resuspended in 900 μ L of sterile PBS and DNase/trypsin/DNase treated, as described in Section 2.4. The treated cells were washed twice with 0.25% Tween-20 in PBS and counted. They were aliquoted at 1 x 10⁶ of viable cells per a 0.6-mL, thick-wall PCR tube (Fisher Scientific) in which they were kept throughout all subsequent steps.

3.2.4 Cell fixation and permeabilization

Samples containing 1 x 10⁶ of DNase/trypsin/DNase-treated cells were fixed on ice in acetone (molecular grade; Sigma Chemical Co.), which was precooled at -20°C for 3 min and then washed twice with 0.25% Tween-20 in PBS by centrifugation at 660 x g for 5 min at AT. In the following step, the cells were permeabilized by incubation with 200 μ L of 10 μ g/mL proteinase K (activity > 20 U/mg; Invitrogen) in PBS at 37°C for 5 min. The enzyme was inactivated by boiling for 2 min to prevent excessive digestion, as in *in situ* hybridization protocols described by others (Bagsara *et al.*,1995; Nuovo, 1995). Then, the cells were washed in two changes of 0.25% Tween-20 in PBS.

3.2.5 PCR primers and amplification conditions

Cells obtained after this procedure were suspended in a 100 µL volume of PCR buffer as described in Section 2.7.1. The PCR mixture was covered with 80 µL of molecular grade mineral oil to prevent evaporation. In this study, oligonucleotide primers homologous to WHV C gene were employed for the detection of virus specific DNA. Thus, PPCC primer conjugated at the 5'-end with FITC with sequence 5'-TAGGAGGCTGTAGGCAT (2033-2049) was used as the sense primer (FITC-PPCC), whereas CCOV primer with sequence 5'-TCTCAATCGCCGCGTCGCAGA (2439-2460) was used as the antisense primer. In some instances, unlabeled PPCC and CCOV primers were employed in control reactions.

To amplify viral DNA, a PCR cycling program was used as outlined in Section 2.7.1. In all PCR runs, at least three samples of the same cell preparation, each containing 1×10^6 cells, were processed. One underwent PCR amplification as test sample and two others

served as controls. In one control reaction, both Taq DNA polymerase and the FITC-labeled

primer were omitted to assess background cell autofluorescence. In the second sample, Taq

DNA polymerase was omitted to determine whether the FITC-labeled primer may directly

hybridize to intracellular WHV DNA under the thermocycling conditions used. In preliminary

experiments, it was established that the 5'-end FITC label on PPCC primer did not affect

efficiency of WHV DNA sequence amplification, as proven in parallel reactions with the primer pair comprised by unlabeled PPCC and CCOV and rWHV DNA (Figure 3.1). All amplifications were performed in a TwinBlock thermal cycler (Ericomp Inc.).

In selected PBMC samples examined in this study, the presence of WHV cccDNA, indicative of replicating virus, was also assessed. This was accomplished following a standard PCR-based procedure previously established in this laboratory (Lew and Michalak, 2001) and described in Section 2.7.2.

3.2.6 Flow cytometry analysis

Following PCR, cells were spun down at 660 x g for 5 min, and the supernatant collected and saved for molecular hybridization analysis. The resulting cell pellet was gently suspended in 0.5 mL of 0.25% Tween-20 in PBS and washed by centrifugation under the same conditions as above. The final pellet was suspended in 200 μ L of PBS, transferred to a sterile 5-mL polypropylene tube, covered with Parafilm, and kept on ice until analysed in a fluorescence activated cell sorter (FACS) Star-Plus flow cytometer (Becton Dickinson). This examination was done within 12 h after completion of PCR.

Cells were gated by a forward and side scatter to exclude cellular debris. The data

generated from a flow of 1 x 10⁴ events were analysed with the help of Cell Quest software

(Becton Dickinson). Trace signals occasionally given by control cell samples subjected to

thermocycling in the absence of a FITC-labeled primer and *Taq* DNA polymerase were

subtracted from the signals given by test cells. These signals never exceeded the mean value

Figure 3.1. Sensitivity and specificity of WHV DNA detection by PCR using the oligonucleotide primer pair homologous to the virus core gene sequence containing a fluorescein-conjugated sense primer. Serial 10-fold dilutions of full-length rWHV DNA were amplified with either (A) PPCC-CCOV or (B) FITC#PPCC-CCOV primer pairs under the same PCR conditions. In parallel, supernatants from WHV-naive PBMC or PBMC derived from a chronic WHV carrier obtained after *in situ* PCR with FITC#PPCC-CCOV or, as controls, FITC#-PPCC in the absence of *Taq* DNA polymerase or in the absence of both FITC#PPCC and *Taq* DNA polymerase were analysed (B). The amplified WHV DNA sequences were identified by Southern blot hybridization to [³²P]-labeled rWHV DNA as a probe. The results show that either standard PCR procedure employing labeled or unlabeled PPCC primer or *in situ* PCR method using FITC#PPCC primer generate DNA fragments of identical molecular size (428 bp) and specificity, and that the use of FITC#PPCC primer does

not modify the sensitivity of WHV C gene detection.



of 0.7% (SEM 0.08%) (see below) of the total counted events. In some experiments, supernatants from cells after *in situ* PCR and cells not utilized for cytometric analysis were examined by dot-blot hybridization, as described below.

3.2.7 Hybridization analysis of amplified virus sequences in cells and in their PCR supernatants

To confirm validity of the flow cytometry results, a dot-blot hybridization assay was adopted to examine presence of virus amplicons in both PBMC and their PCR supernatants. For this purpose, after *in situ* PCR, the cells were spun down, counted and adjusted to the same cell concentration ($3-5 \times 10^5$ cells/dot). Then, they were lysed by three cycles of freezing and thawing with vortexing, which eliminated almost all ~99% cells, as evaluated by phase-contrast microscopy. The resulting suspension was blotted onto a nylon membrane (Hybond-N; Amersham Biosciences) using a dot-blot microfiltration apparatus (BioRad Laboratories) and then washed twice with 200 µL of sterile PBS.

When cell PCR supernatants were examined, 90 µL of each supernatant recovered after PCR was blotted onto a nylon membrane following the same procedure as above.

Subsequently, the nucleic acid was denatured, neutralized and the membrane baked and then

hybridized overnight at 65 °C with a [32P]-labeled rWHV DNA as a probe (Michalak et al.,

1999). After washing in 0.2 X SSC containing 0.1% SDS, the blots were exposed for

autoradiography to Kodak X-Omat film, as performed in Southern hybridization procedures

(see Section 2.9.2.3). In all instances, 2-fold serial dilutions of rWHV DNA were blotted as quantitative standards. In preliminary experiments, PBMC supernatants obtained after *in situ* PCR were subjected to electrophoresis on 1% agarose gels and analysis by Southern blot hybridization (Michalak *et al.*, 1999). These experiments showed that the amplified PCR products were of the expected molecular size and had WHV DNA specificity, as shown in Figure 3.1.

3.2.8 Statistical Analysis

Based on the mean and SEM of background signals detected in WHV-naive cells (mean $0.7\% \pm \text{SEM } 0.08$; n= 16), readings equal to or less than 1% were accepted as negative. Statistical comparison of background signals from WHV-negative cell samples to the low positive samples (1.0 - 2.1%) in samples obtained from serologically silent residual or serologically silent primary occult WHV infection gave a highly significant difference (*P*=0.0001), indicating that values above 1% were in fact positive. The significance of differences between percentages of WHV DNA-negative and weakly WHV DNA-positive PBMC and between WHV DNA-positive PBMC detected in the groups studied (see Table

3.1) was determined by Mann-Whitney nonparametric, unpaired test. Two tailed P values \leq

0.05 were considered significant.

3.3 RESULTS

3.3.1 Validation of enzymatic elimination of the cell surface adhered viral DNA

To validate the effectiveness of DNase/trypsin/DNase treatment on the removal of extracellular attached WHV virions and/or WHV DNA and, at the same time, to optimize conditions for sole detection of intracellular virus sequences, PBMC from WHV-naive animals were incubated with an excess of either WHV virions or rWHV DNA, and then subjected to the stepwise enzyme treatment described. As illustrated in Figure 3.2 (upper panel), when cells were exposed to approximately the same amounts of virus genomes contained in either virions or protein-free recombinant WHV DNA preparation, they showed WHV DNA signals as detected by *in situ* PCR/flow cytometry. However, the percentage of WHV DNA-positive PBMC was much greater after incubation with free virus DNA (83.1%) than with virions (6.8%). These signals were removed following DNase/trypsin/DNase treatment, indicating that the treatment was effective and ensured that only intracellular viral DNA was subsequently detected. Comparable results were obtained when either freshly isolated or previously frozen WHV-naive PBMC were exposed to rWHV DNA and when the cells obtained when either freshly isolated or previously frozen WHV-naive PBMC were

exposed to rWHV DNA and when the cells were incubated with rWHV DNA suspended in

PBS or normal woodchuck serum (data not shown). In general, the data demonstrated that

WHV virions or viral DNA fragments may in fact attach to the lymphoid cell surface and,

therefore, compromise specificity of PCR aimed at exclusive detection of intracellular virus sequences. They also showed that washing cells alone is not sufficient to dissociate the

Figure 3.2. Flow cytometry analysis of WHV-naive PBMC exposed to either WHV virions or cloned rWHV DNA and subjected or not to stepwise DNase/trypsin/DNase digestion and WHV DNA-specific PCR amplification. PBMC isolated from a healthy woodchuck were incubated with either 3 x 10⁸ WHV virions (approximately 300 virions/cell) (upper panel) or 1 ng of rWHV DNA (approximately 300 vge/cell) (lower panel), washed and treated or not with DNase/trypsin/DNase before PCR and flow cytometric examination. Adsorption of virions or protein-free WHV DNA to PBMC is evident in the absence of enzyme treatment, whereas following treatment, no positive cell signal was observed, indicating successful elimination of the attached WHV particles or nucleic acid sequences.







surface bound viral DNA and that the enzymatic digestion applied in this study was effective at its elimination.

3.3.2 In situ PCR coupled with flow cytometry detects WHV in PBMC of animals with either high or minuscule levels of circulating virus

PBMC isolated from serum WHsAg-reactive, chronically infected woodchucks with serum loads of WHV around 10¹¹ vge/mL (Group 1; Table 3.1), showed readily detectable intracellular WHV DNA when examined by in situ PCR-flow cytometry. In this disease situation, percentages of WHV genome containing cells ranged between 3.4 and 20.4% (mean $9.6\% \pm \text{SEM 1.7}$; n = 12) (Table 3.1) or, in other words, there was 3.4×10^4 to 20.4×10^4 infected cells per each 1 x 10⁶ of PBMC analysed.

Considering individual samples obtained during follow-up of animals with CH, the number of WHV DNA-positive PBMC did not fittingly correlate with the load of circulating virus. This fact is well illustrated for 1/F woodchuck from which 8 PBMC and parallel serum samples obtained during a 19-mo observation period were available for analysis (Table 3.1). Thus, although the level of circulating WHV did not fluctuate meaningfully (1.7-4.8 x 10¹¹

vge/mL) in this animal, the percentage of infected PBMC ranged between 6.3 and 20.4% during the same time period.

The proportion of WHV DNA reactive PBMC in woodchucks with a serologically

concealed infection that continued after recovery from AH (Group 2) or with a primary occult

WHV infection (Group 3), where the serum levels of WHV were usually around 10 vge/mL,

ranged between 1.1 and 14.6% (mean 4.8% \pm SEM 0.8; n = 27). When the percentage values of WHV-positive PBMC determined for woodchucks with residual or occult WHV infection (Groups 2 and 3) were compared to those detected in animals with WHsAg-positive CH (Group 1), the difference was highly significant (P = 0.005). This indicates that although some animals with very low levels of circulating virus and serologically silent infection may carry numbers of WHV-positive PBMC comparable to those seen in woodchucks with high WHV loads and serologically demonstrable infection, the population of WHV DNA-positive PBMC is overall significantly greater in serologically evident than serologically silent infection. PBMC samples derived from WHV-naive animals showed minimal background signals, of which the mean was $0.7\% \pm$ SEM 0.08 (n = 16).

Figure 3.3A shows representative plots from flow cytometry analyses of PBMC isolated from animals with actually progressing, WHsAg-positive CH or with a past episode of SLAH. In addition, this figure illustrates the results obtained from control reactions. As expected, PBMC derived from a healthy animal were negative for WHV DNA. In contrast, populations of WHV DNA-positive cells were readily detectable in PBMC taken from

woodchucks with either active or residual WHV infection. As it is also shown in Figure 3.3A

(middle row) PBMC treated under the thermocycling conditions in the absence of both FITC-

labeled, virus-specific primer and Taq DNA polymerase were WHV DNA non-reactive.

Occasionally, these control reactions gave a trace background autofluorescence which did not

exceed 1% of the total events counted. These signals, if occurring, were subtracted from the

values given by relevant test samples.

Figure 3.3. Detection of WHV DNA in lymphoid cells from WHV-infected woodchucks and a healthy control by *in situ* PCR coupled with flow cytometry or with hybridization analysis of cell supernatants obtained after PCR amplification. (A) Representative flow cytometry results showing WHV DNA-positive populations of PBMC from an animal with progressive WHsAg-positive CH (evident infection) and from a woodchuck with silent WHV infection continuing for 12 months after resolution of AH (silent infection) and from a control, WHV-naive woodchuck (top row). The same cell samples treated under identical cycling conditions but in the absence of FITC#PPCC primer and *Taq* DNA polymerase (pol.) showing almost nonexisting background autofluorescence (middle row) or after omitting *Taq* DNA polymerase illustrating signals obtained due to hybridization of FITC# PPCC primer with WHV DNA in PBMC from chronic and residual WHV infections but not from a healthy animal (bottom row). (B) Dot-blot hybridization analysis of supernatants collected after

PBMC underwent in situ PCR, confirming the validity of positive flow cytometry signals and

negative controls shown in panel A.



A

Evident infection



B

rWHV DNA (ng/dot)

1 0.5 0.25 0.12 0.06 0.03



PBMC supernatant



Interestingly, when infected PBMC were subjected to the standard PCR conditions in the presence of FITC-labeled, WHV-specific primer but without Taq DNA polymerase, WHV genome positive cells could also be detected, as illustrated in Figure 3.3A, bottom row. Overall, the percentages of WHV DNA reactive cells detected by this method were on average 5% lower than those identified by the standard in situ PCR/flow cytometry protocol. Therefore, this approach did not allow for detection of infected cells where low numbers (< 5%) of WHV-positive PBMC were identified by in situ PCR/flow cytometry. It became evident that the signals generated in the absence of Taq DNA polymerase were due to a specific hybridization of FITC-PPCC primer with intracellular WHV DNA. This conclusion was based on the facts that neither PBMC from WHV-naive animals treated under the same thermocycling conditions in the presence of FITC-PPCC nor WHV-infected PBMC treated with FITC-labeled HCV-specific primer produced positive signals (data not shown). It is conceivable that the in situ hybridization method, utilizing a fluorochrome-labeled oligonucleotide as a probe, might constitute a base for future development of even a more simplified flow cytometry technique applicable for specific detection of pathogens when they

are present at moderate to high levels within infected cells, as observed in this study.

In order to validate the results obtained by the in situ PCR-flow cytometry and, at the

same time, to test whether hybridization of the amplified sequences with a radiolabeled probe

may increase sensitivity of WHV DNA detection, PBMC supernatants collected after *in situ*

PCR were probed by dot-blot hybridization with [32P]-labeled, cloned WHV DNA. This

approach was based on our observation that enzymatic permeabilization of cells leads to the

escape of some amplicons during the PCR procedure and, therefore, may allow for assessment of the cell positivity by analysing reaction mixtures obtained after PCR and removal of PBMC. As illustrated in Figure 3.3B, the intracellular amplified WHV DNA was detectable by this dot-blot hybridization method, validating the positive and negative signals obtained for PBMC presented in Figure 3.3A. However, although this approach gave results closely comparable to those generated by *in situ* PCR-flow cytometry in regard to positivity or negativity of individual PBMC samples tested, it did not improve the overall sensitivity of WHV DNA detection. Similarly, in another set of experiments, when either infected PBMC alone or together with their supernatants were analysed by dot-blot hybridization after standard *in situ* PCR, there was no meaningful enhancement in the sensitivity of WHV genome detection over the standard *in situ* PCR-flow cytometry procedure (data not shown). Nevertheless, the data revealed that the *in situ* amplified sequences can be alternatively detected by a DNA hybridization method in situations where a flow cytometer might not be available.

110

3.3.3 Frequency of virus genome-containing PBMC in WHV POI and SOI

Two groups of animals known from our previous studies to persistently carry low levels of WHV in the lymphatic system, as determined by classical PCR amplification techniques (Coffin and Michalak, 1999; Hodgson and Michalak, 2001; Michalak *et al.*, 1999), were examined by *in situ* PCR-flow cytometry. Group 2, composed of PBMC from animals who spontaneously overcame an acute episode of WHV hepatitis (*i.e.*, SOI) and continued to have lifelong residual WHV infection, showed percentages of infected PBMC ranging from 1.1 to 12.9% (mean 4.5% \pm SEM 0.8; n = 19). Five of the PBMC samples tested were collected at the end of the natural lifespan of the recovered animals (*i.e.*, approximately 3 to 5.5 years after clearance of WHsAg from serum) (Table 3.1). The percentages of positive cells in these particular samples ranged between 1.1 and 12.9%, indicating that the population of infected PBMC may significantly vary even in the very late period after resolving acute infection.

Figure 3.4 illustrates representative plots of WHV DNA-positive lymphoid cells from two woodchucks (5/F and 11/M; Table 3.1) with apparent complete resolution of experimentally induced AH. Animal 5/F had finally developed HCC at 36.5 months after clearance of WHsAg and seroconversion to anti-WHs, whereas 11/M has been euthanised due to crippling senility at 51 months after seroconversion. As shown, approximately 15% of PBMC collected during AH from 5/F were WHV DNA reactive, whereas 3.8% and 3.2% of cells were infected at 5.5 and 17.5 months after WHsAg clearance, respectively. For 11/M, 5.9% of PBMC demonstrated detectable levels of virus DNA during the late phase of AH,

and 3.7% and 1.2% cells were infected at approximately 3.5 and 4 years after disappearance

of serum WHsAg, respectively. As expected, PBMC obtained prior to inoculation with WHV

were WHV DNA non-reactive.

In Group 3, PBMC collected from animals with POI acquired by vertical transmission

of WHV from mothers who resolved AH were included (Table 3.1). The percentages of

WHV infected cells detected in these animals by in situ PCR-flow cytometry ranged between

Figure 3.4. Representative plots from flow cytometry analysis of *in situ* amplified WHV DNA in PBMC samples obtained from two woodchucks with SOI persisting after termination of AH. PBMC obtained during acute phase of WHV hepatitis and at the indicated time points after clearance of serum WHsAg and seroconversion to anti-WHs were subjected to DNase/trypsin/DNase treatment, PCR amplification and flow cytometry analysis. The percentage of WHV DNA reactive PBMC is indicated in each panel. PBMC collected prior to inoculation with WHV from the same animals are shown as negative controls.



WHV-naive

Acute hepatitis





11/M





0

100

101

Resolved acute hepatitis



1.2 and 14.6% (mean 5.1% \pm SEM 1.6; n = 8). Three representative plots of PBMC obtained at approximately 6 months, and at 2 and 3 years after birth from the same offspring (14/F) are shown in Figure 3.5.

No significant difference in the percentages of WHV-infected PBMC was observed between animals with SOI and those with a POI. Statistically significant differences were found between animals with serum WHsAg-positive chronic infection and those with SOI (P= 0.008) and POI (P = 0.04).

As an indicator of active hepadnavirus replication, the presence of WHV cccDNA was assessed by the nick region-specific PCR in selected PBMC samples which were found WHV DNA reactive by *in situ* PCR/flow cytometry. WHV cccDNA was detected in 4 of 5 PBMC samples tested, as confirmed by Southern blot hybridization analysis. The results are shown in Table 3.1 and illustrated in Figure 3.6.

3.4 DISCUSSION

This study shows that hepadnavirus genome can be readily detected in lymphoid cells by using a direct PCR method combined with flow cytometry. This one-step PCR procedure

detects hepadnavirus DNA with sensitivity (~10² vge/mL) comparable to that previously

achieved only by nested PCR and it allows for reliable unveiling of the virus genomes in the

lymphatic system during persistent, serologically concealed infection. The PCR-flow

cytometry technique established in this study offers several important advantages over the

classical PCR protocols utilized for identification of hepadnaviruses in lymphoid cells. It

Figure 3.5. *In situ* **PCR-flow cytometry analysis of PBMC from a woodchuck with POI who was born to a mother convalescent from acute WHV hepatitis**. PBMC obtained at the indicated time points after birth were examined following the standard protocol. The percentages of WHV DNA reactive cells detected in individual samples are indicated in each panel.





6 mo











Figure 3.6. Detection of cccDNA in representative PBMC DNA samples which were obtained from woodchucks with serologically silent, persistent infection found WHV DNA reactive by *in situ* PCR/flow cytometry. DNA isolated from PBMC was subjected to mung bean nuclease digestion and PCR amplification. PCR products were analysed by Southern blot hybridization to confirm molecular size (674 bp) and specificity. For immunovirological characteristics of animals from which the PBMC samples shown originated, see Table 3.1. Contamination controls included water added instead of DNA and amplified by a direct (D) or a nested (N) reaction and mock (M) treated as test DNA. The positive control consisted of DNA derived from PBMC of a woodchuck with WHsAgpositive, chronic WHV infection.



 Negative
 Negative

 0/M - 20 mo
 9/M - 20 mo

 17/F - 8 mo
 17/F - 52 mo

 10/F - 26 mo
 10/F - 26 mo

Positive Control

←674 bp


abrogates the need for extraction of nucleic acid and, by minimizing manipulations, maximizes preservation of virus genomes within structurally intact cells decreasing, at the same time, the risk of accidental contamination. By including limited enzyme treatment of cells with DNase/trypsin/DNase prior to PCR, detection of intracellular viral DNA, but not that potentially nonspecifically attached to the cell surface from plasma or other body fluids, is secured. Furthermore, for the first time, quantification of infected lymphoid cells in the course of hepadnaviral infection becomes feasible. This method might be further adopted to multiparametric analysis of infected lymphoid cells, their isolation, and identification of cell subsets in which hepadnavirus preferentially replicates, as well as to detection of replicative forms of hepadnavirus genomes.

The results obtained in this study validate previous findings demonstrating that silent WHV persistence continuing after recovery from hepatitis or progressing POI is associated with the sustained infection of the host's lymphatic system (Michalak, 2000). Comparing, on a case by case basis, the levels of serum WHV DNA determined by the classical PCR protocol with the percentages of infected PBMC identified by the *in situ* PCR-flow cytometry (Table 3.1), it is evident that a barely detectable level of virus in serum can be accompanied by a

relatively large population of infected PBMC. This situation has been encountered both in

the residual infection continuing after resolution of SLAH and in POI. In these animals, the

percentages of WHV-positive PBMC were occasionally as high as those detected in WHsAg-

reactive CH. This indicates that serologically mute infection does not necessarily infer a low

number of infected lymphoid cells. Although, it is likely that the amount of virus per

individual infected cell is considerably greater in serologically evident than in serologically silent infection. Furthermore, as the present study suggests, the proportion of infected PBMC may significantly fluctuate, even in a short period of time, during the course of both silent and serologically detectable infections. This is consistent with another observation from our study, indicating that a high proportion of WHV-infected PBMC may appear even years after apparent complete resolution of hepatitis and in the late stage of lifelong POI. Nevertheless, taken together, the percentages of infected PBMC were found to be significantly greater (P = 0.005) in the serologically evident than in the silent virus infections (*i.e.*, POI and SOI).

In addition, the presence of WHV cccDNA in PBMC samples found positive by *in situ* PCR/flow cytometry supports the conclusion that virus replication occurs in these cells. This is consistent with other previous findings where expression of WHV DNA and WHV cccDNA in the lymphatic system, and infectivity of lymphoid cell-derived WHV were documented (Coffin and Michalak, 1999; Michalak *et al.*, 1999; Lew and Michalak, 2000). Therefore, it is not surprising that variable WHV genome levels are expressed in the lymphatic system of animals after SLAH or in offspring born to convalescent dams. This current study describes a new approach by which intracellular WHV DNA in lymphoid cells can be detected.

The percentages of WHV-positive lymphoid cells identified in our study appear to be

similar to those delineated for other infections with lymphotropic viruses, when the in situ

PCR-flow cytometry method was applied for detection of infected PBMC. In one reported

work, HCV-specific sequences were detected in 0.2 to 8.1% of PBMC, often in the absence

of overt clinical symptoms (Muratori *et al.*, 1996). In another work, the percentages of viruspositive PBMC identified in HIV-infected patients did not exceed 20%, with a range of positivity from 0.6 to 20% (Re *et al.*, 1994). This study also showed that when the data were viewed on a case by case basis, highly variable populations of HIV-positive cells occurred regardless of the CD4+ T cell count and viral load estimated by measuring HIV-1 p24 antigenemia. This observation may corroborate our findings in WHV infection. Considering the data from the *in situ* PCR-flow cytometry analyses mentioned above, it can be perceived that the WHV lymphotropic potential, seen as virus ability to establish infection in lymphoid cells, is comparable to that displayed by other known lymphotropic viruses.

The present study reinforces the notion that serologically silent hepadnavirus carriage should be monitored not only by evaluation of virus genome in serum, but also by examining its expression in peripheral lymphoid cells. It is evident that testing of circulating lymphoid cells may offer an important diagnostic advantage and identify virus in apparently negative cases. Other findings from this laboratory have shown that the systematic analysis of serial sera, PBMC and liver tissue samples collected during the natural lifespan of woodchucks with SOI allows for detection of WHV DNA with a higher frequency in PBMC (78%) than in sera

(68%), although the liver remains the most reliable source for the identification of silently persisting virus (Michalak *et al.*, 1999; Michalak, 2000). The same could be true for serologically silent HBV infection. Cumulative data from a growing number of studies documenting the persistence of HBV traces following spontaneous or therapeutically induced resolution of hepatitis B and the appearance of HBV infection in recipients of organs from

apparently HBV negative donors (Michalak, 2000; Brechot *et al.*, 2001) strengthen the epidemiological and pathogenic significance of the cryptic form of HBV infection (see Section 1.3.2 and Section 1.10).

In the course of this study, a relatively simple approach to the specific detection of intracellular hepadnavirus has been established. This procedure should provide a valuable tool for further investigations on hepadnaviral lymphotropism and the role of the lymphatic system in the natural course of hepadnaviral infection. This new method could also be applied to monitor the progression of symptomatic or concealed infection and to assess the effectiveness of antiviral agents against hepadnavirus propagating within lymphoid cells.



CHAPTER 4: Low Doses of Hepadnavirus Induce Infection of the Lymphatic System That Does Not Engage the Liver

This study has been published in the Journal of Virology, Feb. 2004, volume 78(4), pp. 1730-1738.

4.0 SUMMARY

As it was previously documented in this laboratory, WHV invades the host's lymphatic system and persists for life in lymphoid cells independently of whether infection is symptomatic and serologically evident or concealed (occult). In this study, we show, using the woodchuck model of hepatitis B, that hepadnavirus can establish an infection that engages only the lymphatic system, but not the liver, and persists in the absence of virus serological markers, including anti-viral antibodies. This primary occult hepadnaviral infection (*i.e.*,POI) is caused by wild type virus invading the host at a quantity usually not greater than 10³ virions and is characterized by trace virus replication progressing in lymphatic organs and PBMC that, with time, may also spread to the liver. The infection is transmissible to virus-naive hosts as an asymptomatic, indefinitely long, occult carriage of small amounts of biologically competent virus. In contrast to residual silent WHV persistence which normally endures after the

resolution of viral hepatitis and induces the liver (i.e., SOI), POI restricted to the lymphatic

system does not protect against reinfection with a large, liver-pathogenic dose; however, the

occult infection is associated with a swift recovery from hepatitis caused by the

superinfection. This study clearly documents that the lymphatic system is a primary target of

WHV infection when small quantities of virions invade the susceptible host.

4.1 INTRODUCTION

Replication and retention of virus in cells of the immune system characterize many persistent viral infections and are a major hindrance to sterilizing antiviral therapy. HBV and WHV are noncytopathic hepadnaviruses which cause a similar course and outcome of liver disease (Summers et al., 1978; Tennant and Gerin, 1994; Michalak, 1998; Menne and Tennant, 1999; Chisari, 2000). Besides the estimated 350-400 million people worldwide who are chronically infected carriers of serum-HBsAg reactive HBV, there are many more individuals that harbour virus at quantities only detectable by highly sensitive molecular assays (Liang et al., 1990; Liang et al., 1994; Michalak et al., 1994; Penna et al., 1996; Rehermann et al., 1996b; Yotsuyanagi et al., 1998; Marusawa et al., 2000; Yuki et al., 2003). The epidemiologic and pathogenic importance of this serologically silent occult HBV carriage are increasingly evident, particularly in regard to: (1) transmission of virus traces through seemingly HBV-negative blood transfusion, hemodialysis or organ transplantation (Hoofnagle et al., 1978; Chazouilleres et al., 1994; Prieto et al., 2001); (2) reactivation of infection due to cytotoxic or immunosuppressive therapy (Lok et al., 1991; Hu, 2002); (3) contribution to the pathogenesis of liver diseases currently considered to be cryptogenic, e.g. HCC (Liang

et al., 1991; Cacciola et al., 1999; Yotsuyanagi et al., 2000; Chemin et al., 2001). In the

woodchuck-WHV model, the lifelong silent virus persistence is an invariable consequence of

resolved viral hepatitis (Michalak et al., 1999; Michalak, 2000; Hodgson and Michalak, 2001)

and is accompanied by development of HCC in about 20% of the recovered animals (Korba

et al., 1989; Michalak et al., 1999).

Analysis of HBV genome expression years after recovery from an episode of AH showed that virus traces persist in the presence of virus-specific immune responses (Michalak *et al.*, 1994; Penna *et al.*, 1996; Rehermann *et al.*, 1996b; Yotsuanagi *et al.*, 1998; Cabrerizo *et al.*, 2000; Chisari, 2000; Yuki *et al.*, 2003). As previously discussed (Section 1.5.2.2 and 1.8.2), WHV normally invades the host's lymphatic system, and lymphoid cells are a reservoir of replicating virus independent of whether the primary infection is serologically evident or occult (Coffin and Michalak, 1999; Michalak *et al.*, 1999; Michalak, 2000; Mulrooney and Michalak, 2003). It has also been shown that WHV produced by lymphoid cells can establish productive infection in cultured hepatocytes and lymphoid cells, and viral hepatitis and HCC in susceptible animals (Michalak *et al.*, 1999; Lew and Michalak, 2001). Hence, the data accumulated indicate that although hepatocytes are the site of the most vigorous replication of HBV and WHV in serologically diagnosed infection, both viruses also are lymphotropic and can propagate in the lymphatic system.

Our recent findings in the woodchuck model of hepatitis B revealed yet another form of occult hepadnavirus persistence, where under certain natural or experimental conditions *i.e.*, in offspring born to dams recovered from WHV hepatitis (Coffin and Michalak, 1999)

and in adult animals inoculated with lymphoid cells obtained long after resolution of AH (Michalak *et al.*, 1999) or with very small WHV doses (Lew and Michalak, 2001), hepadnavirus can elicit POI confined to the lymphatic system, which is characterized by low levels of circulating virus and by minimal virus replication solely in lymphoid cells (Coffin and Michalak, 1999; Michalak *et al.*, 1999; Lew and Michalak, 2001; Mulrooney and Michalak,

2003). The nature of a viral prerequisite leading to establishment of this extrahepatic form of occult hepadnavirus persistence was unknown. We hypothesized that the dose of invading virus and/or the existence of a lymphotropic virus variant might be the underlying reason. To test these possibilities and to delineate features of this newly identified form of hepadnaviral carriage, we have applied two complementary investigative approaches. The first aimed at elucidation of whether occult, naturally acquired neonatal infection limited to the lymphatic system is transmissible to virus-naive, immunocompetent hosts and, if so, whether this infection retains its molecular and immunovirological properties through the passage. The second aimed at generation of a reproducible model of occult, lymphatic system-restricted infection using a wild-type, serum-derived virus and to test how this infection affects the host's susceptibility to infection after challenge with a massive virus dose known to cause hepatitis. Our findings imply that the lymphatic system-restricted POI is caused by wild type virus and that the quantity of invading virions predetermines whether the infection is silent and confined to the lymphatic system or serologically evident and liver pathogenic.

4.2 MATERIALS AND METHODS

4.2.1 Woodchucks

Infection experiments were carried out in 2-3 year old healthy woodchucks housed

in the Woodchuck Research and Breeding Facility at Memorial University. Animals were

housed and screened for prior exposure to WHV as described in Section 2.1.

4.2.2 WHV inocula from occult lymphatic system-restricted infection

Woodchuck 3B/M, born to dam B, which resolved AH and was positive for anti-WHs, carried WHV DNA in serum and the lymphatic tissues, but not in the liver, during 15 mo follow-up after birth (Coffin and Michalak, 1999). WHV derived from serum of offspring 3B/M was i.v. injected at ~1 x 10⁴ vge into adult 260/M. The 260/M woodchuck acquired POI with serum WHV DNA levels not exceeding 10² vge/mL, as reported (Coffin and Michalak, 1999). For the current study, inocula were prepared from plasma of 3B/M and from plasma and splenocytes of 260/M (Figure 4.1A). Thus, plasma collected at autopsy from 3B/M (20 mL) and 260/M (10 mL) were ultracentrifuged at 200,000 x g for 18 h in a SW 50.1 rotor and the concentrates containing $\sim 1 \times 10^3$ and $\sim 5 \times 10^2$ WHV vge were i.v. injected into 2115/M and 2117/M, respectively. Sera and PBMC were collected weekly until week 10 p.i., then biweekly. Liver biopsies were obtained before and at week 6 and 22 p.i. At week 23 p.i., the animals were challenged with WHV/tm3 inoculum at 1.1 x 10¹⁰ DNaseprotected WHV/dose (see below). Liver samples were collected at week 6, 29 and 46 thereafter. 2120/M was i.v. injected with 6.2 x 10⁶ splenocytes (80% viability) isolated from 260/M, and was then euthanised at week 18 p.i.

4.2.3 Wild-type WHV inoculum

WHV/tm3 inoculum was prepared from a pool of sera of a single chronic WHV carrier. This inoculum has proven to be highly liver pathogenic and at 1.1×10^{10} vge/dose induced serum WHsAg-positive AH in ~85% of adult woodchucks (Michalak *et al.*, 1999;

Michalak, 2000). It contained wild-type WHV DNA sequence identical with that in the liver and the spleen of the donor, as revealed by sequencing of the cloned complete WHV genomes (GenBank accession numbers: AY334075 for WHV/tm3 inoculum, AY334076 for liverderived WHV and AY334077 for spleen-derived WHV; see Appendices A.1-3). To determine whether a wild-type virus can establish POI, 9 WHV-naive animals were injected with increasing doses of WHV/tm3 ranging from ~10 to 1 x 10⁷ DNase-protected virions (Michalak *et al.*, 1999). Thus, 1/F, 2/F and 3/F were inoculated with ~10 vge, 4/M and 5/F with 1 x 10³ vge, 6/M and 7/F with 1 x 10⁵ vge, 8/F with 1 x 10⁶ vge, and 9/F with 1 x 10⁷ vge. The animals were bled weekly until week 6 p.i. and then biweekly. Liver biopsies were collected before inoculation and at week 5 and 12 p.i. At week 18 p.i., each animal, except 1/F and 8/F, was challenged with 1.1 x 10¹⁰ vge of WHV/tm3 inoculum. Sera and PBMC were collected for up to month 12 and liver samples at week 6, 36 and 48 after challenge. 1/F was euthanised at week 18 p.i. to assess WHV expression in lymphoid organs, while 8/F had CH and was followed until HCC developed at month 25 p.i.

4.2.4 Sample collection and cell preparation

PBMC were harvested from sodium EDTA-treated blood on Ficoll-Paque, as described in Section 2.2.3. Liver biopsies were obtained by surgical laparotomy as outlined in Section 2.2.4. At autopsy, serum, PBMC, liver, spleen, bone marrow, lymph nodes, and other organs were also collected (see Section 2.2.5). Liver sections were stained and hepatic lesions assessed as previously described (see Section 2.12). In some instances, PBMC and

splenocytes were cultured with phytohemagglutinin (PHA) (5 µg/mL) for 72 h to enhance expression of WHV DNA and cccDNA, as previously demonstrated (Coffin and Michalak, 1999; Michalak *et al.*, 1999). DNase/trypsin/DNase treatment preceded DNA extraction to eliminate potential carryover of virions and virus DNA fragments on the cell surface (see Section 2.4; Mulrooney and Michalak, 2003).

4.2.5 Serological and WHV DNA dot-blot assays

WHsAg, anti-WHs, and anti-WHc were assayed as described in Section 2.3. The serum WHV DNA was evaluated by dot-blot hybridization using [³²P]-labeled complete, cloned WHV DNA (Coffin and Michalak, 1999; Lew and Michalak, 2001). Hybridization signals were quantified by a phosphorimage densitometry using 2-fold serial dilutions of rWHV DNA as a standard, as outlined in Section 3.2.6. When negative, WHV DNA was assayed by PCR/NAH as described in Sections 2.7 and 2.9.

4.2.6 DNA extraction and PCR for WHV DNA and cccDNA

Total genomic DNA was isolated as outlined in Section 2.5. WHV DNA was

amplified by PCR using primers and conditions described in Section 2.7.1. For PCR detecting

WHV cccDNA, mung bean nuclease treatment, primers and conditions outlined in Section

2.7.2 were applied. The amplicons' specificity and the validity of controls were routinely

confirmed by Southern blotting (see Section 2.9.2).

In selected cases, WHV DNA was quantified by real-time PCR detecting WHV C gene amplicons through FRET (sensitivity of 10² vge/mL), as described in Section 2.8.1.

4.2.7 WHV DNA sequencing

DNA from selected serum, lymphoid cell and tissue samples was subjected to PCR with back-to-back primers amplifying the complete WHV genome, as described in Section 2.7.3. When required, fragments of the full length WHV DNA was further amplified with WHV gene specific primers (reported in Section 2.7.1). The PCR products were either directly sequenced or cloned using the TA-cloning system as summarized in Section 2.10 and sequenced (see Section 2.11).

4.3 RESULTS

4.3.1 Transmission of primary occult WHV infection

To determine the infectivity and pathogenic competence of WHV in POI, inocula derived from woodchuck 3B/M, that was born to a mother convalescent from WHV hepatitis and had serologically undetectable infection with small amounts of WHV DNA in serum,

PBMC and lymphatic organs but not in the liver (Coffin and Michalak, 1999), were i.v. injected into virus-naive adults 260/M and 2115/M (Figure 4.1A). Subsequently, plasma and splenocytes from 260/M, which developed POI (Coffin and Michalak, 1999), were i.v. administered into 2117/M and 2120/M, respectively (Figure 4.1A). Animals 2115/M, 2117/M and 2120/M, similarly to 3B/M and 260/M (Coffin and Michalak, 1999), developed

Figure 4.1 Serial passage of occult WHV infection restricted to the lymphatic system in virus-susceptible woodchucks. (A) Outline of experiment showing animals and features of WHV infection at the time of acquisition of virus inocula. (B) Serological and WHV genome expression profiles and results of liver histology after inoculation of virus-naive adult woodchucks with WHV derived from animal 3B/M, with neonatally acquired POI confined to the lymphatic system, and from woodchuck 260/M with the same type of infection acquired after of 3B/M serum. The graphs display the times of the primary inoculation (week 0) and the challenge with WHV (week 23), the appearance and the duration of serum WHsAg (and anti-WHc (\Box), and the expression of WHV DNA in serum, PBMC, and liver biopsy samples. Anti-WHs antibodies were not detected (not shown). WHV DNA in serum, PBMC, and liver samples was detected by direct or nested PCR, followed by Southern blot identification of the amplified sequences. The serum WHV DNA content did not exceed 10 vge/mL during follow-up and is represented by open bars. The levels of WHV genomes in PBMC are shown as follows: open bars, 0.005 to 0.5 vge/10⁴ cells; light-grey bars, 0.5 to 5

vge/10⁴ cells; dark-grey bars, 5 to 50 vge/10⁴ cells; and black bars, > 50 vge/10⁴ cells. The quantities of WHV genomes in liver samples obtained at the time points marked by arrowheads were assessed as described above and are expressed as estimated numbers of WHV vge per10⁴ cells. Liver alterations are presented as the histological degree of hepatitis ranging from 0 to 3, assessed according to previous work (Hodgson and Michalak, 2001).



B



infection negative for WHsAg, anti-WHc and anti-WHs, and for WHV DNA by dot-blot hybridization (sensitivity: 2 x 10⁶ vge/mL). Nevertheless, WHV DNA was detected in serum and PBMC from day 7 or 14 p.i. in all animals by nested PCR/NAH (Figure 4.1B).

Liver biopsies gathered before and at week 6 p.i. from woodchuck 2115/M (injected with \sim 5 x 10² WHV vge derived from 3B/M plasma) and from woodchuck 2117/M (injected with \sim 1 x 10³ vge obtained from 260/M plasma) were entirely non-reactive for WHV DNA and had normal histologies (Figures 4.1B and 4.2). However, liver tissue obtained 4 mo later displayed WHV DNA at 0.2-2 vge/per 10⁴ cells in both animals (Figures 4.1B and 4.2). These virus signals occurred in the absence of liver alterations in 2115/M, while minimal lymphomononuclear infiltrations and a moderate proliferation of bile ducts were seen in woodchuck 2117/M.

Liver samples obtained prior to and at week 6 and 18 p.i. from animal 2120/M, injected with $\sim 3 \times 10^2$ vge contained within splenocytes of 260/M (Figure 4.1A), had normal histologies and were virus DNA non-reactive (Figure 4.3). Despite this, autopsy tissues collected at week 18 p.i. unveiled WHV confined to the lymphatic system (Figure 4.1A), as it was observed in animals 3B/M and 260/M (Coffin and Michalak, 1999). Thus, only the

spleen, lymph nodes and bone marrow of 2120/M were WHV DNA reactive at ~5 vge/10⁴ cells (Figures 4.1B and 4.3), while PBMC carried WHV at ~0.05-0.5 vge/10⁴ cells between weeks 3 and 16 p.i. (Figure 4.1B). Comparable results were obtained by real-time PCR. WHV cccDNA was detected in the spleen and bone marrow of 2120/M. WHV preS, S, C and X gene sequences from 2120/M spleen and bone marrow did not show alterations

Figure 4.2. WHV DNA expression in serum, in PBMC, and in liver biopsy samples collected from woodchuck 2117/M. This animal was inoculated with WHV derived from an animal with neonatally acquired, lymphatic system-restricted POI and was then challenged with a massive, liver-pathogenic dose of WHV. Total DNA isolated from liver biopsy samples and from parallel PBMC and serum samples obtained prior to inoculation (week -2), after inoculation with ~5 x 10² WHV vge from 3 B/M plasma (weeks 6 and 22 p.i.), and after challenge at week 23 p.i. with the WHV/tm3 inoculum at a dose of 1.1 x 10¹⁰ WHV vge (weeks 6, 29, and 46 after challenge) was tested for WHV DNA by direct and, if negative, by nested PCR with WHV C gene-specific primers. The amplicons were detected by Southern blot hybridization to rWHV DNA. For contamination controls, water was added instead of DNA and the sample was amplified by a direct (D) or nested (N) reaction, as was a mock (M) treated sample. The positive controls consisted of DNA isolated from the serum, PBMC, or liver from a woodchuck with chronic WHV infection that was positive for serum

WHsAg. Positive signals showed the expected 623 bp (direct PCR) or 428 bp (nested-PCR)

nucleotide fragments.





Figure 4.3. Analysis of WHV DNA expression in liver and lymphoid tissues collected from woodchuck 2120/M. This animals was inoculated with splenocytes isolated from animal 260/M, which had acquired POI after injection with the serum of offspring 3B/M. Five-microgram samples of total DNA from liver biopsy specimens collected before (week -2) and after (weeks 6 and 18 p.i.) inoculation with splenocytes carrying an estimated 3 x 10² WHV vge and 1 µg of DNA from the spleen, bone marrow, or lymph nodes collected at autopsy (week 18 p.i.) were tested for WHV DNA by nested PCR with C, S, and X genespecific primers and by Southern blot analysis of the products derived. Contamination controls included samples with water added instead of DNA (N) and a mock (M) that had been extracted and treated under conditions identical to those as test DNA samples. Positive samples showed the expected molecular sizes of the amplified virus C (428 bp), S (500 bp),

and X (192 bp) gene fragments.





compared with wild-type WHV present in 3B/M spleen and in two liver biopsies obtained from its mother (dam B) before and after parturition (Coffin and Michalak, 1999).

4.3.2 Low-doses of wild-type WHV cause occult lymphatic system-restricted infection

To recognize the viral prerequisite initiating POI, normal adult woodchucks were injected with increasing doses of a serum-derived inoculum that carried homogenous, wildtype WHV genome (*i.e.*, WHV/tm3 inoculum) (Figure 4.4). This experiment revealed that WHV doses equal or below 1 x 10³ virions caused POI with WHV DNA in serum, PBMC and lymphoid tissues, but not in the liver (Figure 4.4), even when 5 μ g of total liver DNA was used for direct PCR amplification. Conversely, all animals infected with doses >1 x 10³ virions developed AH with WHV DNA readily detectable in the liver and lymphoid cells. Thus, woodchucks 1/F, 2/F and 3/F (injected with ~10 virions) became serum WHV DNA positive from day 7 p.i. at 10-10² vge/mL and the animals carried virus in PBMC from weeks 2 to 3 p.i. onwards at 0.05-0.5 vge/10⁴ cells (Figure 4.4B). These loads were confirmed by real-time PCR, in situations when WHV DNA signals could be detected by this technique. WHsAg and anti-WHc in serial sera (Figure 4.4B) and WHV DNA in liver samples collected

at week 6 and 12 p.i. from these animals were non-reactive (Figures 4.4B and 4.5). WHV

cccDNA was detected in PBMC from 2/F and 3/F at week 8 or 10 p.i. (Figure 4.6) and WHV

sequence identical to that in WHV/tm3 inoculum was identified in these cells. Animal 1/F,

euthanised at week 18 p.i. (Figure 4.4), had POI and carried WHV DNA at 0.05-0.5 vge/10⁴

cells in bone marrow and in PHA-stimulated splenocytes. WHV cccDNA was detectable at

Figure 4.4. Serological and molecular profiles of WHV infection in woodchucks injected with increasing doses of a wild-type WHV and then challenged with a massive dose of the same inoculum. Healthy, initially WHV naive woodchucks were injected (week 0) with the indicated amounts (vge) of the serum-derived WHV/tm3 inoculum. At week 18 p.i., the animals (except 1/F) were challenged with the same WHV/tm3 inoculum at a dose of 1.1×10^{10} vge, known to normally induce AH in naive animals. (A) Detection of serum WHsAg (**1**) and anti-WHc (**1**). Anti-WHs were transiently identified in 5/F, 6/M, and 7/F after the clearance of WHsAg from serum following primary WHV inoculation and in 2/F, 3/F, and 4/M after challenge with a dose of 1.1×10^{10} vge (not shown). (B) Detection of WHV DNA in sequential serum, PBMC, and liver samples. Estimated WHV vge levels detected in serum are depicted as follows: open bars, 1 to 10 vge/mL; light-grey bars, 10 to 10^2 vge/mL; dark-grey bars, 10^2 to 10^3 vge/mL; and black bars, $>10^3$ vge/mL. WHV DNA quantities identified in PBMC are shown as follows: open bars, 0.005 to 0.5 vge/10⁴ cells;

light-grey bars, 0.5 to 5 vge/10⁴ cells; dark-grey bars, 5 to 50 vge/10⁴ cells; and black bars,

>50 vge/10⁴ cells. Likewise, the levels of WHV DNA detected in liver biopsy or autopsy

samples at the time points indicated by arrowheads were shown as the estimated number of

WHV vge/10⁴ cells.





Figure 4.5. WHV DNA detection in serum, PBMC, and liver tissue samples collected from woodchuck 3/F. This animal was injected with a dose of 10 WHV vge of the WHV/tm3 inoculum, and subsequently challenged with a massive dose of the same inoculum. DNA extracted from parallel serum, PBMC, and liver biopsy samples obtained prior to inoculation (week -12), after inoculation with ~10 vge of WHV (weeks 6 and 12 p.i.), and after challenge at week 18 p.i. with 1010 vge of the WHV/tm3 inoculum (weeks 6, 36, and 48 after challenge) were assayed for WHV DNA by direct or nested PCR by using the C genespecific primers and Southern blotting for the detection of amplified sequences. Relevant DNA samples from a WHsAg-positive chronic carrier were included as positive control, and samples with water added instead of DNA (D and N) and a mock-treated sample (M) extracted in parallel with test samples were used as negative controls. Hybridization signals

showed the expected 623- or 428-bp band.





Figure 4.6. WHV DNA detection in sequential liver biopsies obtained from animals 4/M and 5/F. These animals were infected with 10³ WHV vge per dose prior to challenge with a liver pathogenic dose of WHV/tm3 inoculum. DNA extracted from liver samples taken before and after inoculation with WHV (weeks 6 p.i. and 12 p.i.) were assayed for WHV DNA by nested PCR/NAT using C and S gene-specific primers. As contamination controls, water was added instead of DNA and the sample was amplified by a direct PCR and, if required, a nested (N) reaction, as was a mock (M) prepared and treated under conditions identical to those for test DNA samples. The positive controls consisted of DNA isolated from the liver of a woodchuck with serum WHsAg-positive chronic WHV infection and show the expected amplicon sizes for WHV C (432 bp) and S (500 bp) gene fragments.







4/M



the same locations. The preS, S, C and X sequences of WHV occurring in splenocytes and plasma of this animal were identical with those in WHV/tm3 inoculum.

Interestingly, 4/M and 5/F (injected with 1×10^3 vge) produced contrasting profiles of WHV infection. While animal 4/M established POI, woodchuck 5/F developed serum WHsAg-positive AH with WHV DNA detectable in serum, PBMC and liver (Figure 4.4). Interestingly, the development of AH, and protection from subsequent WHV infection (see Section 4.3.3), seemed to be related to the prolonged presence of WHV DNA in the liver before the time of challenge. WHV DNA was detectable at week 6 p.i. in animal 5/F, but was not evident in animal 4/M until 12 weeks p.i. (Figure 4.6). Nevertheless, WHV cccDNA gave comparable density signals in PBMC samples from both animals obtained at week 12 p.i. (Figure 4.7) and WHV sequences in these cells were identical. In the remaining four animals inoculated with doses $>1 \times 10^3$ vge, serologically evident AH developed (Figure 4.4A). The disease was self-limiting in three animals, while 8/F established serum WHsAg-positive CH superseded by HCC. In recovered woodchucks 5/F, 6/M, 7/F and 9/F, low levels of WHV persisted in their sera, PBMC and livers to the end of follow-up, (Figure 4.4B), as expected (Michalak et al., 1999; Michalak, 2000; Hodgson and Michalak, 2001).

4.3.3 Primary occult WHV infection does not protect from superinfection

Woodchucks 2115/M, 2117/M, 2/F, 3/F and 4/M with POI and animals 5/F, 6/M, 7/F

and 9/F which recovered from AH were challenged with WHV/tm3 inoculum at 1.1 x 1010 DNase-protected vge. After challenge, all 5 animals with POI developed serologically evident be same locations. The preS, S, C and X sequences of WHV occurring index of this animal were identical with those in WHV/tm3 inoculum.

Figure 4.7. Detection of WHV cccDNA in representative samples of PBMC obtained from woodchucks after inoculation with various doses of the WHV/tm3 inoculum but prior to challenge with a massive, liver-pathogenic dose of the same virus. Total DNA isolated from PBMC was digested with mung bean nuclease to eliminate WHV DNAinterrupted molecules and to amplify the DNA with PCR primers spanning the nick region of the WHV genome. Nested PCR products were analysed by Southern blot hybridization to confirm the molecular size (674 bp) and WHV specificity. Contamination controls included a sample with water added instead of DNA (N) and a mock-treated sample (M). Mung bean nuclease-treated DNA from the PBMC of a healthy woodchuck (H) was used as a negative control.

Michalak et al., 1999; Michalak, 2000; Hodgson and Michalak, 2001



2/F 3/F 4/M 5/F 9/F WHV vge/animal ~10 10³ 10³ Negative 10⁷ Positive ~10 controls control weeks after inoculation ΝΜΗ 8 9 12 12 5.5





AH (Figures 4.1B and 4.4). Noticeably, the disease was transient in all cases and was followed, as expected, by a residual WHV persistence in both the liver and lymphoid cells. Not surprisingly, animals 5/F, 6/M, 7/F and 9/F were not susceptible to WHV challenge with a liver-pathogenic virus dose (Figure 4.4), as previously observed (Michalak *et al.*, 1999).

4.4 **DISCUSSION**

In this report, we show for the first time that the lymphatic system, not the liver, is the primary target of hepadnavirus when small quantities of virions invade the host. This tropism appears to be an intrinsic property of a wild-type virus. The infection produced is undetectable by immunovirological assays, is persistent, is transmissible to virus-naive hosts, does not induce immunoprotection, and, although initially confined to the lymphatic system, may with time spread to the liver. We found that the quantity of the invading virus is critical in determining whether the primary infection is occult, restricted to the lymphatic system or serologically apparent, involving the liver and causing hepatitis. In the WHV model, amounts below or near 10³ virions induce POI independently of whether the virus is transmitted from a host with established occult, lymphatic system-confined infection or from an animal with

classical, serum WHsAg-positive CH. The present study provides new insights to the natural

history of hepadnaviral infection and consolidates previous observations implying that an

asymptomatic, serologically silent infection might be procured from an exposure to a low

virus dose (Liang et al., 1990; Chu and Lok, 2002; Leblebicioglu et al., 2002; Shiao et al.,

2002).

Previous reports have implied that lymphoid cells are an invariable site of propagation of biologically competent WHV, even when the liver remains unaffected (Coffin and Michalak, 1999; Lew and Michalak, 2001). This study directly proves this concept. It also delineates molecular and immunovirological properties of POI. These characteristics are significantly distinct from those of SOI that endures in humans and woodchucks after recovery from hepadnaviral hepatitis (Michalak *et al.*, 1994; Rehermann *et al.*, 1996b; Michalak *et al.*, 1999; Michalak, 2000; Yuki *et al.*, 2003). Thus, contrary to SOI, where protracted WHV replication occurs both in the liver and in the lymphatic system and where circulating anti-WHc and anti-WHs, but not WHsAg, are detectable (Michalak *et al.*, 1999; Michalak, 2000), POI is limited to the lymphatic system and is not accompanied by immunovirological indicators of WHV exposure. However, in both POI and SOI, WHV persists at comparable levels in serum (≤ 100 vge/mL) and lymphoid cells ($<10^3$ vge/µg total DNA).

The absence of WHV in hepatic tissue of the animals infected with WHV at doses lower than or equal to 10^3 vge was confirmed by repeated nested PCR/NAH analysis of multiple DNA preparations obtained from these livers, including testing as much as 5 µg of

total liver DNA in the direct run of the PCR. Although the potential existence of minuscule amounts of WHV DNA in intrahepatic lymphoid cells and/or residual blood within the tissue samples examined cannot be completely excluded, these virus traces, if occurring, were not detectable by highly sensitive assays applied in this study. To provide a definite answer whether intrahepatic lymphoid cells carry virus during POI, isolation of these cells from the appropriate livers, their expansion *in vitro*, and analysis for expression of WHV DNA and virus genome replication intermediates will be required.

Animals with POI were not protected from WHV challenge and developed a transient episode of AH. This is consistent with our previous observations in offspring born to dams convalescent from WHV hepatitis (Coffin and Michalak, 1999) and in adult animals inoculated with low WHV doses (Lew and Michalak, 2001). On the other hand, this contrasts with the current (Figure 4.4) and previous (Michalak *et al.*, 1999) findings in woodchucks with SOI which were unresponsive to challenge with the identical dose of the same WHV pool. This apparent discrepancy between the minuscule persistent replication of virus, susceptibility of the animals to reinfection, and their consistent ability to effectively terminate hepatitis induced by superinfection might be explained by the possibility that infection with small virus amounts induces a specific immune response capable of forming memory. This response, although too weak to protect against challenge with a large virus dose, may have, due to continuous virus encounters, adequate recollection to mount a strong response capable of limiting liver disease produced by the reinfection.

The prerequisite virus characteristics governing establishment of the POI in the

woodchuck have been clearly defined in this study. The quantity of virus usually not exceeding 10^3 vge was found to be decisive. However, while doses below 10^3 virions consistently induced POI, doses of ~ 10^3 vge caused either POI (*i.e.*, animal 4/M) or AH (*i.e.*, animal 5/F). Also, amounts slightly above 10^3 vge (*e.g.*, ~ 1×10^4 vge in 260/M) occasionally produced POI. This suggests that the outcome of infection with borderline WHV quantities

(*i.e.*, 10^3 - 10^4 virions) is influenced not only by virus dose but also by the host milieu.

The present study reveals a significant difference (presumably 100 to 1000-fold) in the threshold of virus required to infect lymphoid cells or that required to infect hepatocytes. It is evident that WHV, at least at low doses, is predisposed to infect the host's lymphatic system, but the nature of this tropism is not yet fully explained (Jin *et al.*, 1996; Michalak, 2000). It is also unknown whether the same hierarchy in tissue tropism exists in infection with a large, liver pathogenic dose ($\geq 10^4$ virions). This can be tested by determining the initial site of WHV invasion and replication using lymphoid cells and liver biopsies serially collected after inoculation with such a dose but prior to appearance of hepatitis. This approach may also elucidate the fate of virus during the long incubation period that typically precedes hepatitis.

The pathogenic and epidemiological impacts of POI need to be determined. However, since this state is associated with trace propagation of infectious virus and viral loads similar to those in the silent infection persisting after resolution of hepatitis (*i.e.*, SOI), the expected consequences might also be similar. Since, virus can spread from the lymphatic system to the liver with time, it might also potentially be the cause of cryptogenic hepatic pathology.

Because HBV and WHV are highly compatible in their pathobiological properties, this data

likely represents the blueprint for a human situation when low HBV doses invade a

susceptible individual.

CHAPTER 5: Multiple Passage of Wild-Type WHV in Lymphoid Cells Does Not Alter Virus Genome Sequence or *In Vivo* Infectivity

SUMMARY

As our previous study showed, although a dose of invading virus appears to be a major factor in determining whether WHV infection is restricted to the lymphatic system or also engages the liver, the nature of WHV lymphotropism remains unclear and a role for a specific lymphotropic variant cannot be excluded. The availability of woodchuck lymphocyte and hepatocyte cultures susceptible to WHV infection allow investigation of this issue *in vitro*. In this study, we hypothesized that a repeated passage of wild-type WHV in lymphoid cells should lead to enrichment of a lymphotropic virus variant, if in fact it exists. For this purpose, WHV inoculum carrying a wild-type, homogenous WHV sequence was generated by culturing *in vivo* infected splenocytes, while PBMC from a single healthy woodchuck and a normal woodchuck WCM-260 hepatocyte line were used as WHV infection targets. The repeated serial passage of the splenocyte-derived virus for up to 13 consecutive times in both cell types did not lead to the emergence of WHV cell-type specific variants. Moreover, the

passaged virus remained infectious when injected into healthy woodchucks. The results

demonstrated that WHV lymphotropism is unlikely a consequence of the existence of specific

lymphotropic viral variant, but is a natural propensity of a wild-type hepadnavirus.
5.1 INTRODUCTION

It is now accepted that hepadnavirus is capable of replication not only in hepatocytes but also in cells of the immune system and that infection of lymphoid cells persists for life. In terms of symptomatic chronic HBV infection, which is characterized by continuous presence of HBsAg in serum and chronic liver necroinflammation, lifelong persistence of virus has been shown in both the liver and cells of the lymphatic system. However, the liver and lymphoid cells have also been found to be the sites of HBV replication in serum HBsAgnegative patients who recovered from AH when sensitive PCR-based assays were applied to detect the virus genome (Michalak et al., 1994; Rehermann et al., 1995; Penna et al., 1996; Yotsuyanagi et al., 1998; Cabrerizo et al., 2000; Blackberg and Kidd-Ljunggren, 2001). This occult form of HBV carriage might be a source of infectious virus available for transmission to healthy individuals through blood transfusion or organ donation. In this regard, recent retrospective studies of individuals with HCC of unknown etiology showed that low levels of HBV genome occurred in 63.5% of liver tissue samples tested and, in some cases, virus DNA integration into the hosts's genome was detected (Pollicino et al., 2004). This shows that traces of HBV, only detectable by highly sensitive methods, retain its pathogenic and

oncogenic potential.

Most recent studies employing the woodchuck-WHV model have shown the existence

of two distinct forms of occult hepadnaviral infection, POI and SOI (Michalak et al., 2004).

The occult virus persistence in the SOI form associated with development of HCC in about

20% of the animals affected (Michalak et al., 1999). It remains to be determined whether

persistent POI could also be accompanied by the development of HCC. We have also shown that transmission of minute amounts of virus from dams with SOI continuing after SLAH to their offspring led to the establishment of POI (Coffin and Michalak, 1999). POI is also developed when an animal is inoculated intravenously with less than 10³ virions (Chapter 4; Michalak *et al.*, 2004). In the above situations, lymphoid cells were infected, but the liver was not engaged. The results of our studies suggested that this extrahepatic form of WHV infection was not due to the invasion with organ-specific viral variants, but rather a consequence of infection with a low dose of virus.

However, the reason behind the development of lymphoid cell-restricted WHV infection is not fully explained. In order to account for the possibility that a relatively minor subpopulation of WHV might be responsible for preferential infection of the lymphatic system, we serially passaged splenocyte-derived virus in virus-naive lymphoid cells in an attempt to enrich any potentially existing cell-specific virus variants. For this purpose, we developed a reproducible *in vitro* system whereby WHV could be transmitted to normal lymphoid cells and hepatocytes. The results showed that the infectivity of hepadnavirus towards cells of the lymphatic system is an inherent property of wild-type virus and it is not

due to the existence or emergence of a viral variant in the core or S genes. As well, WHV

obtained after multiple serial passage in either lymphoid cells or hepatocytes remained

infectious to healthy animals. The serological and molecular profiles the infection induced

correlated with the dose of virus administered, but not with the cell type from which the virus

originated.

5.2 MATERIALS AND METHODS

5.2.1 Animals

Three animals chronically infected with WHV with comparable loads of WHV in serum (~10¹⁰ vge/mL) were used to obtain virus-infected splenocytes. Thus, WM.A chronic WHV carrier was infected in the wild and had sustained serum WHsAg-reactive infection during the 30-mo period before autopsy. The splenocyte culture supernatant from this animal was used for preliminary passage experiments 1 and 3 (Table 5.1). Two other animals, WF.B and WM.C, were i.v. infected with WHV in our colony. Both animals developed classical, serum WHsAg-reactive CH, which was confirmed by histological examination of liver biopsies. Thus, animal WF.B was infected with ~10⁶ DNase-protected WHV vge using WHV/tm3 inoculum (see Section 4.2). This animal became serum WHsAg-positive at 1 mo p.i. and the antigen persisted for its lifetime. The splenocytes from WF.B were used for the preliminary passage experiments 2 and 4 (Table 5.1). Animal WM.C developed chronic WHV infection after inoculation with WHV/tm2 inoculum (see Appendices A.4-5) containing ~10¹⁰ DNase-protected WHV vge. The full WHV genome sequence obtained from the liver, spleen and serum of WM.C, whose serum subsequently served as WHV/tm3 inoculum, was

determined to be wild-type and the same at all three locations tested (see Section 4.2).

WM.C splenocyte culture supernatant was used for the WHV serial passage experiments 5 and 6 (Table 5.1).

In addition, a healthy animal, L10, provided PBMC for *in vitro* WHV infection experiments described in this study. Also, two healthy animals, WF.1 and WF.2, were used

Experiment	Animal/Source of WHV Inoculum	WHV Cell Target	Number of WHV passage		
1	WM.A	lymphoid cells	3		
2	WF.B	lymphoid cells	3		
3	WM.A	lymphoid cells	3		
4	WF.B	lymphoid cells	3		
5A	WM.C	lymphoid cells	13		
5B	WM.C	hepatocytes	12		
6A	WM.C	lymphoid cells	13		
6B	WM.C	hepatocytes	12		

 Table 5.1 WHV passage experiments performed in the course of this study



to examine the *in vivo* infectivity of WHV recovered after multiple passages in either lymphoid cells or hepatocytes, *i.e.*, following experiments 5A and 6B, respectively (Table 5.1). Randomly selected samples of sera, PBMC and liver biopsies collected prior to the inoculation of these animals were negative for serological markers of WHV infection, *i.e.*, WHsAg, anti-WHc and anti-WHs, and for WHV DNA, as described in Section 2.2.1.

5.2.2 Cells

Splenocytes, composed of mainly lymphocytes, were isolated from WM.A, WF.B and WM.C using a metal 50-mesh cell dissociation sieve. After separation through a Ficoll-Paque gradient (Pharmacia Biotech) (Jin *et al.*, 1996; Michalak *et al.*, 1994), splenocytes were DNase/trypsin/DNase-treated, as described in Section 2.4. PBMC were isolated from L10 animal on Ficoll-Paque density gradient, as outlined in Section 2.2.3. These PBMC were used as infection targets in the passage experiments.

Normal woodchuck hepatocytes, WCM-260, were used as control WHV infection targets for experiments 5B and 6B. This cell line was derived from hepatocytes isolated from a liver biopsy of a healthy adult animal by two-step collagenase microperfusion (Diao *et al.*,

1998). WCM-260 were propagated in gelatin-coated flasks in hepatocyte medium consisting of 80% Hepato-STIM medium with 10 μ M dexamethasone (Becton Dickinson), epidermal growth factor (10 ng/mL; Becton Dickinson), L-glutamine (2 mM), penicillin (50 μ g/mL), and streptomycin (50 μ g/mL), supplemented with 20% (vol/vol) HepG2 cell culture supernatant (Diao *et al.*, 1998; Churchill and Michalak, 2004).

5.2.3 Preparation of splenocyte-derived WHV inocula

Splenocytes from WM.A, WF.B and WM.C treated with DNase/trypsin/DNasetreated (Section 2.4) were washed three times in HBSS and spun down at $320 \times g$. Then, they were supplemented with 5 mL of hepatocyte culture medium at $\sim 3 \times 10^6$ cells/mL and cultured in a 25-cm² tissue culture flask (Corning Costar Corp., Cambridge, Mass). Supernatants obtained after 72-h culture of splenocytes from WM.A and WF.B were used as inocula for preliminary passage experiments 1-4. The splenocyte-derived WHV from WM.C animal was prepared by culturing the cells for 12 days, with culture medium collected every 72 h. The supernatants were pooled and used as inoculum in experiments 5 and 6. One-mL samples of all splenocytes supernatants were preserved for WHV DNA analysis. Supernatants predestined for *in vitro* infection experiments were stored at 4°C for no longer than 7 days. In some cases, splenocyte-derived supernatants were subjected to digestion with DNase to examine the presence of enveloped-protected WHV virions, as described before (Michalak *et al.*, 1994).

5.2.4 Multiple passage of splenocyte-derived WHV in naive lymphoid cells and

hepatocytes

Naive woodchuck PBMC (~1 x 10⁷) or WCM-260 hepatocytes (~9 x 10⁵) were

seeded 24 h prior to infection in 25-cm² tissue culture flasks in 5 mL hepatocyte culture

medium. Then, the medium was removed and replaced with 4 mL of splenocyte culture supernatant (*i.e.*, lymphoid cell-derived WHV; $\sim 3 \times 10^5$ vge) supplemented with 1 mL of fresh

hepatocyte culture medium. The cells were incubated for 18 h at 37°C in a humidified 5% The inoculum was removed and the cells were treated with CO_2 atmosphere. DNase/trypsin/DNase to remove any cell-surface attached virions or WHV DNA (Section 2.4), washed, resuspended in 5 mL of fresh hepatocyte culture medium, cultured for 72 h, and harvested. Both adherent and non-adherent lymphoid cells were collected. Hepatocytes were removed by brief treatment with trypsin-EDTA (Invitrogen). Four-mL samples of supernatants from lymphoid cell or hepatocyte cultures were used for inoculation of naive cells in the next passage, while 1 mL was preserved for analysis. Cells were DNase/trypsin/DNase-treated and washed three times in HBSS before storage. Final cell washes were saved for WHV DNA analysis to ensure complete removal of extracellular virus material. In preliminary experiments 1-4 (Table 5.1), using splenocyte culture supernatants prepared from animals WM.A and WF.B, three consecutive passages of the virus (twice for each of the two inocula) were carried out. For multiple passage experiments (i.e., 12-13 passages), splenocyte-derived inoculum from WM.C animal was used (Experiment 5 and 6; Table 5.1). For each of the experiments, culture supernatants obtained from infected cells from the previous passage were used to infect naive lymphoid cells or WCM-260 hepatocytes

in the next passage.

5.2.5 Detection of WHV DNA and cccDNA

WHV DNA content in lymphoid cells, hepatocytes, cell supernatants, and cell washes

was determined using direct and, if required, nested PCR followed by Southern blot

hybridization analysis of amplified products, as previously described (Sections 2.7 and 2.9). In brief, DNA was extracted from lymphoid cells, hepatocytes or from 200 μ L of cell culture supernatants by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation (Section 2.5). For standard PCR detection of WHV DNA, total DNA from 200 μ L of cell culture supernatant or 1 μ g of DNA from lymphoid cells or hepatocytes was amplified using primers specific for WHV C, S, or X gene sequences, as outlined in Section 2.7 and Figure 2.1. Amplified WHV DNA was analysed by agarose gel electrophoresis and hybridized to complete rWHV DNA as a probe (Section 2.9).

In some cases, real-time PCR using the Roche Light-Cycler (Roche Diagnostics) was employed to quantify the amount of WHV DNA in culture supernatants (Section 2.8).

WHV cccDNA in cultured lymphoid cells and hepatocytes was detected as outlined in Section 2.7.2.

5.2.6 Preparation of culture supernatants from the final WHV passage in lymphoid cels or hepatocytes

In order to collect the entire amount of WHV produced after the final passage of virus in either lymphoid cells or WCM-260 cells from experiments 5A and 6B, respectively, culture

supernatants were ultracentrifuged at 200,000 x g for 24 h at 4°C in a Beckman SW50.1

rotor. The pellet was resuspended in 500 µL of PBS and a 100 µL-sample was retained for

DNA extraction and WHV DNA quantitation.

5.2.7 Inoculation of woodchucks with WHV recovered after multiple serial passage in lymphoid cells or hepatocytes

Animals WF.1 and WF.2 were i.v. injected with concentrated WHV derived from the final passages obtained after experiments 5A and 6B. The WHV DNA content in these inocula was determined by real-time PCR (Section 2.8). Subsequently, $\sim 1.5 \times 10^4$ vge from WHV-infected lymphoid cell culture supernatant (experiment 5A) and $\sim 8.8 \times 10^2$ vge from that of infected hepatocytes (experiment 6B) were i.v. injected into WF.1 and WF.2, respectively. Serum and PBMC samples were collected prior to the inoculation, weekly for up to 10 weeks p.i. and, then, biweekly. Liver biopsies were obtained prior to infection and at 6 weeks p.i., as described in Section 2.2.4. Animals were monitored for up to 7 mo p.i. by testing of serological markers of WHV infection and for WHV DNA content in the serum, PBMC, and liver biopsies. WHV DNA presence was also determined in the lymphatic organs and the liver collected at autopsy.

5.2.8 WHV DNA sequencing

WHV C and S gene fragments generated by PCR were directly sequenced using

WHV-specific primers and the Fmol sequencing kit, as outlined in Section 2.11. All

sequences were aligned with the published WHV sequences reported in GenBank, including

those identified in our studies in the serum (WHV/tm3 inoculum), spleen, and liver of WM.C

(see Section 4.2; Michalak et al., 2004).

5.3 RESULTS

5.3.1 WHV-infected splenocytes secrete virus in culture

Splenocytes isolated at autopsy from WM.A, WF.B and WM.C chronically infected with WHV, which had comparable WHV loads in serum, were treated with DNase/trypsin/DNase to eliminate potentially cell surface attached virions or virus DNA fragments. To ensure comparable conditions for *in vitro* WHV infection of lymphoid cells and WCM-260 hepatocytes, both cell types were cultured in hepatocyte culture medium containing 10 µM dexamethasone. This hormone has been previously shown to upregulate WHV DNA displayed in infected lymphoid cells when compared to cells maintained in its absence (Lew & Michalak, 2001). We also found that naturally infected woodchuck splenocytes can be cultured for at least 12 days without significant alterations in their viability, extending earlier findings showing no loss in the cell viability for up to 6 days (Lew & Michalak, 2001).

It was determined by real-time PCR that each of the splenocyte supernatants collected after 72-h culture contained WHV DNA at comparable levels \sim 3 x 10⁵ vge/mL.

In experiments 5 and 6, splenocytes isolated from WM.C animal were used as a source

of lymphotropic WHV. The virus occurring in this animal was well-characterized in our previous studies (Section 4.2) and was proven to be infective and oncogenic to woodchucks. As previously mentioned, the full WHV genomic sequences from the serum, liver, and spleen

of this woodchuck were analysed and confirmed to be wild-type and identical to each other.

As indicated in Section 5.2.3, to generate a pool of splenocyte-derived WHV from WM.C,

supernatants from the splenocyte culture were collected every 72 h for 12 days. As shown in Figure 5.1A, the amount of virus released increased over the course of the 12-day culture, except in the second stimulation interval (*i.e.*, 6-day culture). Using real-time PCR, WHV load detected in the pool of supernatants collected during 12-day culture was $\sim 3 \times 10^5$ vge/mL. Importantly, the amount of virus remained essentially the same after DNase digestion, indicating that WHV DNA detected was encapsidated in virions (Figure 5.1B).

5.3.2 Splenocyte-derived WHV is infectious to naive lymphoid cells

Figure 5.2 outlines the overall scheme used to serially transmit splenocyte-derived WHV in either virus-naive lymphoid cells or WCM-260 hepatocytes. As previously mentioned, we assumed that a lymphoid cell type-specific variant, if it exists, should be enriched after multiple passage in lymphoid cells, allowing its detection more readily. WCM-260 hepatocytes served as a control, since it was established before that they are susceptible to WHV infection *in vitro* (Lew and Michalak, 2001).

Preliminary experiments 1-4 (Table 5.2) were conducted to establish the conditions for lymphoid cell infection using culture supernatants from splenocytes of WM.A and WF.B

as inocula and freshly isolated virus-naive lymphoid cells as targets. In these preliminary

experiments, three sequential passages of WHV were performed. Table 5.2 illustrates WHV

DNA detection in both infected lymphoid cells and their supernatants. As shown, almost all

cells and supernatants examined were WHV DNA reactive, indicating that the virus released

to the culture supernatant was capable of transmitting WHV infection from one lymphoid cell

Figure 5.1. (A) Detection of WHV DNA in supernatants collected at 72-h intervals during culture of WHV-infected splenocytes isolated from WM.C. 1.7 x 10⁷ splenocytes derived from WM.C chronic WHV carrier were treated with DNase/trypsin/DNase, cultured for 12 days, with supernatants collected every 72 h, as described in Materials and Methods. DNA was extracted from 200-µL supernatant samples and assayed for WHV DNA by direct PCR with WHV C gene-specific primers. Serial 10-fold dilutions of complete rWHV DNA were used as quantification standards. Numbers under the panel represent the relative densitometric units (DU) given by the hybridization signals. (B) DNase-treatment of cell supernatants. 100-mL sample of pooled supernatants from WM.C splenocyte culture were either DNase-treated (T) or not (NT) prior to DNA extraction to determine the presence of envelope-protected WHV virions. As a control for DNase digestion, 1 ng of rWHV DNA was resuspended in healthy woodchuck serum and was either digested or not with DNase before DNA extraction. As the positive control, serum from a WHsAg-positive animal chronically infected with WHV (CH) was used. Contamination controls consisted of water

(DW) added to the direct PCR instead of DNA and a mock extracted sample (M). All samples were amplified by direct PCR using WHV C gene-specific primers. Specificity and

molecular size (623 bp) of amplicons were determined by Southern blot hybridization.



B

A

Negative controls	W spler super	M.C nocyte matant	rWHV	DNA	CH s	erum	Posi tive control
DW M	NT	Т	NT	Т	NT	Т	СН











 Table 5.2 Detection of WHV DNA in lymphoid cells after each of three sequential passages of splenocyte-derived WHV
 prepared from two woodchucks with chronic WHV hepatitis

		Passage Number											
Experiment	Animal/ Source of		1		2	3							
	WHV Inoculum	Cells ^a	Supernatant ^b	Cells ^a	Supernatant ^b	Cells ^a	Supernatant ^b						
1	WM.A	~5 x 10 ⁴	~1 x 10 ³	n.d.°	n.d.	~5 x 10 ²	~1 x 10 ²						
2	WF.B	$\sim 5 \times 10^4$	$\sim 1 \times 10^{3}$	$\sim 5 \times 10^2$	n.d.	n.d.	n.d.						
3	WM.A	$\sim 5 \times 10^4$	$\sim 1 \times 10^{3}$	n.d.	$\sim 1 \times 10^{2}$	$\sim 5 \times 10^3$	n.d.						
4	WF.B	$\sim 5 \times 10^4$	$\sim 1 \times 10^{3}$	$\sim 5 \times 10^2$	$\sim 1 \times 10^{2}$	$\sim 5 \times 10^2$	n.d.						

^a Presented as WHV vge detected in ~5 x 10⁶ lymphoid cells
^b Presented as WHV vge detected in total volume of supernatant

° n.d., not detected

169

culture to the next. However, the virus quantities produced during these short sequential passage experiments were relatively low (*i.e.*, in total $\sim 10^4$ vge per passage experiment), and at certain passages, *i.e.*, passage 2, WHV DNA levels were near the detection limit of the nested PCR/NAH assay, as shown for experiment 1 in Figure 5.3 (Table 5.2). Nucleotide sequence analysis of the WHV C and S gene fragments amplified from the initial inoculum, and the lymphoid cells and their supernatants obtained after the third passage from experiments 1 and 3 showed identical sequences, implying that no cell type-specific WHV variant(s) had arisen.

5.3.3 Multiple serial passage of lymphotropic WHV in lymphoid cells or hepatocytes does not lead to the emergence of cell type-specific virus mutations

Since three consecutive passages of splenocyte-derived WHV in lymphoid cells did not lead to the appearance of cell-specific variants, we aimed to continue WHV passage in both lymphoid cells and in control hepatocytes for a longer period of time. For these longterm experiments, we used WHV derived from splenocytes of WM.C chronic carrier (Table 5.1). In these experiments, $\sim 3 \times 10^5$ WHV vge was used for the direct (first round) infection.

WHV passaged 12 or 13 times in lymphoid cells (experiments 6A and 5A; Table 5.3) or in hepatocytes (experiments 6B and 5B; Table 5.4) continued to maintain its infectivity in both cell types until the end of the experiment. Shown in Figure 5.4 is the detection of WHV sequences amplified after selected virus passages in either lymphoid cells (Figure 5.4A), or hepatocytes (Figure 5.4B), and in corresponding culture supernatants, using PCR and

Figure 5.3. Detection of WHV DNA in lymphoid cells and their supernatants collected after 3 serial passages of splenocyte-derived WHV from woodchuck WM.A. 2 x 10⁷ splenocytes (SPL) isolated from WM.A were treated with DNase/trypsin/DNase and cultured for 72 h as described in Materials and Methods. The splenocyte culture supernatant (sSPL) was then passaged to virus-naïve freshly isolated PBMC. Exposed cells and their culture supernatants were collected after 72-h culture. DNA was extracted from 1 x 10⁶ cells or 200mL supernatant samples and assayed for WHV DNA by direct and nested PCR with WHV C gene-specific primers. Serial 10-fold dilutions of complete rWHV DNA were used as quantification standards. Water added to direct PCR (DW) and nested PCR (NW) instead of test DNA and a mock (M) prepared and treated as test DNA were used as contamination controls. Amplicons of 428 bp were detected by Southern blot hybridization with complete rWHV DNA probe. Relative DU values given by the hybridization signals are marked under







Figure 5.4. Detection of WHV DNA in cultured lymphoid cells and WCM-260 hepatocytes and in their culture supernatants obtained after serial passage of WHV originating from WM.C splenocytes. DNA extracted from cells (C) and supernatants (S) were amplified with primers specific for either WHV C, S, or X gene by direct PCR and, if required, nested PCR followed by NAH. WHV passaged in (A) lymphoid cells and (B) WCM-260 hepatocytes and recovered after passages 1, 3, 5, 7, and 12 are shown. Contamination controls consisted of water added to direct PCR (DW) and nested PCR (NW) instead of test DNA, and a mock (M) prepared and treated as test samples. Serum from a WHsAg-positive chronic WHV carrier served as a positive control. Arrows indicate the molecular sizes for WHV C (direct, 623 bp; nested, 428 bp), S (direct, 1277; nested, 500 bp), or X (direct 386; nested, 192 bp) gene fragments.









U Negative controls DW NW M

C

S

×

primers specific for the virus C, S, and X genes. There was no significant increase in the overall titre of WHV produced in each of the passages, regardless of the cell type used. Southern blot analysis showed that, periodically, the level of virus secreted was below the detection limit of the nested PCR/NAH assays used. However, it is also noteworthy that, although supernatants from passages (6-10) had undetectable WHV DNA when DNA extracted from 200- μ L samples was analysed, a pellet from a pool of these supernatants recovered after ultracentrifugation was WHV DNA reactive (Tables 5.3 and 5.4). This result indicates that the virus was in fact present in the culture supernatants but at very low levels (≤ 10 copies/mL). Nevertheless, this amount was sufficient to transmit infection to either virus-naive hepatocytes or lymphoid cells, as shown for subsequent passages in Figures 5.5A and 5.5B, and Tables 5.3 and 5.4.

WHV cccDNA analysis of selected WHV DNA-reactive lymphoid cells and hepatocyte samples confirmed that the transmitted virus was capable of active replication in both cell types (Figure 5.5).

When the sequences of the WHV C and S gene amplicons from supernatants collected after the third and the final 12 or 13 passage from either lymphoid cells (experiment 5; Table

5.3) or hepatocytes (experiment 6; Tables 5.4) were compared to the initial inoculum, no

sequence variation was detected. In the first instance, we analysed the core and S regions of

WHV amplified from supernatants after passage 3, as we did in the preliminary experiments

1-4, and we again found that these sequences were identical with that detected in the inoculum. We then assumed that a cell type-specific variation may require longer time to arise

Table 5.3 Detection of WHV in cultured lymphoid cells and their supernatants after each passage of WHV derived from WM.C chronic carrier

Expt.5A	Passage	Passage Number:														
	1	2	3	4	5	6	7	8	9	10	11	12	13	recovered		
cells*	~105	5x10 ⁴	5x10 ⁴	n.d.°	2x10 ²	n.d.	5x10 ⁴	5x10 ⁴	5x10 ⁴	5x10 ⁴	n.d.	5x10 ⁴	n.d.	4.5 x 10 ⁵		
supernatant ^b	~103	2x10 ²	2x10 ^{2d}	2x10 ²	2x10 ²	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2x10 ²	2x10 ^{2d,e}	2.2 x 10 ³		
						Onu	continugut	. · · · ·	10 111	vvgc						
Expt.6A	Pas	ssage Nur	nber:					on 2 A		v vgc				Cotol WHV		
Expt.6A	Pas	ssage Nur	nber: 2 3	3	4	5	6	7	8	9 1	0	11	T 12	Total WHV recovered		
Expt.6A cells ^a	Pas	ssage Nur 1 10 ⁵ 5:	nber: 2 3 x10 ⁴ 5x	3 10 ⁴ 5x	4 10 ⁴ 52	5 <10 ⁴	6 n.d. ^c 5x	7 :10 ⁴ 52	8 x10 ⁴ r	9 1 n.d. n.	0 d. :	11 n.d.	T 12 n.d.	Total WHV recovered 4.0 x 10 ⁵		

^a Presented as WHV vge detected in $\sim 5 \times 10^6$ lymphoid cells

- ^b Presented as WHV vge detected in total volume of supernatant
- ^c n.d., not detected WHV C and S gene sequenced
- ^e Supernatant ultracentrifuged and used as inoculum for WF.1
- ^f Total amount of WHV vge recovered over the course of the whole passage experiment
- ^g Indicated supernatant samples (200 µL each) were pooled and ultracentrifuged, as outlined in Section 5.2.6

Grand Total^f: ~4.0 x 10⁵

Table 5.4 Detection of WHV in cultured hepatocytes and their supernatants after each passage of WHV derived from WM.C chronic carrier

Expt.5B	Passage	Passage Number:														
	1	2	3	4	5	6	7	8	9	10	11	12	13	recover		
cells ^a	~104	5x10 ³	5x10 ³	n.d.°	5x10 ³	n.d.	5x10 ³	5x10 ³	5x10 ³	5x10 ³	5x10 ³	n.d.	n.d.	4.5 x 1		
upernatant ^b	~103	2x10 ²	2x10 ^{2d}	2x10 ²	2x10 ²	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.8 x 1		
						Ultra	acentrifuga	$ion^{g} \le 2 x$	10 ² WHV	/vge			Grand T	otal ^f : ~4.5 x		
Expt.6B	Pas	sage Nun	nber:			Ultra	acentrifuga	ion^{g} : < 2 x	10 ² WHV	/vge		_	Grand T	otal ^f : ~4.5 x		
Expt.6B	Pas	sage Nun	nber:	3	4	Ultra 5	acentrifuga 6	ion ^g : < 2 x	10 ² WHV	/vge 9 1	0	11	Grand T	otal ^f : ~4.5 x Total WHV recovered		
Expt.6B cells*	Pas ~1	sage Nun	nber: 2 :10 ³ 5:	3 x10 ³ 5	4 x10 ³	Ultra 5 5x10 ³	acentrifuga 6 n.d. ^c	ion ^g : < 2 x 7 x10 ³ n	10 ² WHV 8 .d. n	/vge 9 1 .d. n.	0 d. n	11 1.d.	Grand T 12 5x10 ³	otal ^f : ~4.5 x Total WHV recovered 4.0 x 10 ⁴		

^a Presented as WHV vge detected in $\sim 5 \times 10^6$ lymphoid cells

^b Presented as WHV vge detected in total volume of supernatant

^c n.d., not detected

^d WHV C and S gene sequenced

^e Supernatant ultracentrifuged and used as inoculum for WF.2 ^f Total amount of WHV vge recovered over the course of the whole passage experiment

^g Indicated supernatant samples (200 µL each) were pooled and ultracentrifuged, as outlined in Section 5.2.6

Grand Total^f: ~4.0 x 10⁴

178

Figure 5.5. Detection of WHV cccDNA in representative samples of lymphoid cells and WCM-260 hepatocytes obtained after various passages of WHV derived from splenocytes of WM.C chronic WHV carrier. Total DNA isolated from indicated cells was digested with a single-strand-specific mung bean nuclease to eliminate WHV DNA relaxed circular molecules and then amplified with PCR primers spanning the nick region of the WHV genome. Nested PCR products were analysed by Southern blot hybridization to confirm specificity and molecular sizes of the amplicons detected (674 bp). As contamination controls, water added to the direct (DW) or nested (NW) PCR and a mock extracted sample (M) were used.







and we would detect such variation later in the long-term serial passage experiment. However, when the supernatants from the final passages of experiment 5A (passage 13) and 6B (passage 12) were analysed, regions of the core gene from nucleotides 2049 to 2255 and the preS gene from positions 2990 to 290 showed no nucleotide changes when compared to the inoculum (Figure 5.6).

5.3.4 In vivo infectivity of WHV recovered after serial passage in lymphoid cells or hepatocytes

Since we found that the splenocyte-derived virus was infectious in vitro to both lymphocytes and hepatocytes and that the WHV gene sequences detected after multiple serial passage in these cells appeared to be the same as that of wild-type virus in the C and preS regions analysed (Figure 5.6), we wanted to determine if the passaged virus also retained its in vivo infectious potential. For this purpose, the final supernatants from lymphoid cells (experiment 5A) and hepatocyte (experiment 6B) cultures were concentrated by ultracentrifugation and the recovered virus injected into healthy WF.1 and WF.2 animals. Thus, WF.1 was inoculated i.v. with $\sim 1.5 \times 10^4$ vge obtained in the lymphoid cells and WF.2

with $\sim 8.8 \times 10^2$ vge after passages in hepatocyte cultures.

Figure 5.7 illustrates the profiles of serological markers of WHV infection and WHV

DNA detection in the serum, PBMC, and liver biopsies in WF.1 and WF.2. Regardless of the

source of WHV inoculum and the subsequent serum WHsAg and anti-WHc profiles, WHV

DNA appeared within 2 weeks after inoculation and persisted throughout the entire follow-up

However, when the supermatants from the final passages of experiment 5A (passage 13) and 6B (passage 12) were analysed, regions of the core gene from nucleotides 2049 to 2255 and the preS gene from positions 2990 to 290 showed no nucleotide changes when compared to the inoculum (Figure 5.6).

Figure 5.6. Core and preS sequence comparison. The core and preS sequence comparison of WHV DNA detected in splenocyte-derived WHV inoculum used for experiments 5 and 6, in the final culture supernatants obtained after serial passage of WHV in lymphoid cells (Experiment 5A) and WCM-260 (Experiment 6B) hepatocytes, and sera obtained at autopsy from WF.1 and WF.2 woodchucks, infected witht the final culture supernatant obtained after experiments 5A and 6B, respectively. Dots indicate sequence homology and dashes mark regions of unknown sequence. Nucleotide positions are indicated based on the sequence of WHV/tm3 (GenBank accession number AY334075).

182

with ~8.8 x 10" vge after passages in hepatocyte cultures.

Figure 5.7 illustrates the profiles of serological markers of WHV infection and WHT

NA detection in the serum, PBMC, and fiver biopsies in WF.1 and WF.2. Regardless of

· source of WHV inoculum and the subsequent serum WHsAg and anti-WHo profiles, WHV

DNA appeared within 2 weeks after inoculation and persisted throughout the entire follow-up

core

WM.C splenocyte super.	2041 agaattt ggt tcatcttatc agtt gtt gaa tt ttcttcct ttggacttct ttcct gatct taatgcttt g gt ggacact g ctact gcttt gt atgaaga
Lymphoid cell final super.	
WF.1 serum	
Hepatocyte final super.	,
WF.2 serum	
WM.C splenocyte super.	ctattagaca agetttagta tecteggate aattaactaa attgataget tegatgagtt etaacataac
Lymphoid cell final super.	
WF.1 serum	
Hepatocyte final super.	
WF.2 serum	

preS

WM.C splenocyte super. Lymphoid cell final super. WF.1 serum Hepatocyte final super. WF.2 serum	2990 acaacttt ga ct tttaaagg taaaccatat tett gggaac acagacaget agt geaacat aat gggeaac aacataaaag teacetteaa teeagacaaa atageagegt ggt ggeet ge agt gggeact
WM.C splenocyte super. Lymphoid cell final super. WF.1 serum Hepatocyte final super. WF.2 serum	tattacacaa ccacttaccc tcagaaccag tcagt gittic aaccagg gat ttatcaaaca acateett gg taaateecaa aacteaacaa gaact ggaet etgitet cat aaacagatae taaacagatag
WM.C splenocyte super. Lymphoid cell final super. WF.1 serum Hepatocyte final super. WF.2 serum	att ggaacac ttggcaagga tttccgt gg atcaaaaact accatt ggtc aacagggatc ctcccccaaa accagctcaa actttcgaaa tcaaacctgg gccaataata gt tcct gg aattcgg gac
WM.C splenocyte super. Lymphoid cell final super. WF.1 serum Hepatocyte final super. WF.2 serum	ataccacgt g gt ttagttee geeteaaate ceaacaaate gagateaagg gagaaageet acteeteeaa eteeacetet aagagataet eaceeeact taactat gaa aaateagaet ttteatetee

jaa	g	aa	ct	aa	ca	g	g	a	gg	gg	aa	ca	tt	go	etc	tc	cg	ç c	ate	ca	ita	ac	aa
		••••				• • •	•••						• • •	•••		• • ••		•••			•••		• ••
•••			• • • •	•••			• ••	•••	• • • •		•••		• ••	••••	•••		•••	••••	••••		•••		
••••			• • • •	•••	•••		• ••	•••	• •• •	• • ••	•••		• ••	•••	•••	••••	• • • •	•••			• •	••••	••••

Figure 5.7. Serological and molecular profiles of WHV infection in woodchucks injected with virus obtained after serial passage of WHV derived from WM.C splenocytes in either lymphoid cells or hepatocytes. WF.1 and WF.2 woodchucks were injected (week 0) with the indicated amounts of WHV obtained after 13 passages in lymphoid cells (WF.1) or 12 passages in hepatocytes (WF.2). Serological results are depicted for WHsAg (WHsAg-positive, dotted grey bar; WHsAg-negative, open bar) and anti-WHc (anti-WHc-positive, hatched bar; anti-WHc- negative, open bar). Detection of WHV DNA in sequential serum, PBMC and liver samples are also shown. Estimated WHV vge levels detected in serum are as follows: 10 to 10³ vge/mL, open bars; >10³ vge/mL, black bars. WHV DNA quantities identified in PBMC are as follows: 0.005-5 vge/10⁴ cells, open bars; >5 vge/10⁴ cells, black bars. The levels of WHV DNA detected in liver biopsy or autopsy samples at the indicated time points (arrows) were shown as the estimated number of WHV vge per 10⁴ cells. Liver histology results are marked as follows: N, normal; AH, acute hepatitis; MIN, minimal (residual) hepatitis.







in the circulation, PBMC, and liver in both animals. Noticeably, the virus passaged in lymphoid cells induced AH in WF.1 with detectable WHsAg and anti-WHc in serum. On the other hand, WF.2, which was injected with WHV passaged in hepatocytes, did not show any serological markers of WHV infection. As we have previously reported (Section 4.3; Michalak *et al.*, 2004), the difference between serologically evident disease in WF.1 and the silent infection in WF.2 can be explained by the difference in the amount of virus administered, and not differences in the virus sequence (Figure 5.6). WF.1 was injected with a much larger dose (100-times) than that injected into WF.2. It is known that the dose of equal or greater than 10^3 WHV vge can induce symptomatic AH in woodchucks (Michalak *et al.*, 2004).

5.4 DISCUSSION

In this study, by employing both *in vitro* and *in vivo* investigations, we have shown that the infectivity of WHV remains intact after multiple passage in either lymphoid cells or hepatocytes. It was also found that a long-term passage of the virus in lymphoid cells is not

associated with the emergence of a distinct lymphoid cell-specific virus variant. Furthermore,

in the present study, for the first time, we have also been able to transmit WHV through

multiple passages in naive (untransformed) lymphoid cells and hepatocytes. Not only was the

resulting virus infectious *in vitro* to lymphoid cells and hepatocytes, but it was also infective and pathogenic to WHV-naive woodchucks.

It has been shown by others that HBV-infected human B cell hybridomas could

transmit the viral genome to normal lymphocytes in co-culture experiments (Colucci *et al.*, 1988). The B cells used in this study were obtained from a patient with acute HBV infection. However, it was not determined whether the virus originated from within the B cells or was adhered to the cell surface. Other studies have indicated that other subsets of lymphoid cells were also susceptible to HBV and are equipped with the cellular machinery capable of producing virus after *in vitro* infection (Romet-Lemonne et al., 1983; Elfassi *et al.*, 1984; Laure *et al.*, 1985; Yoffe *et al.*, 1986; Zeldis *et al.*, 1986; Theilmann and von Brunn, 1991; Muller *et al.*, 1992). However, again no cell surface treatments were included to remove extracellular adhered virions, making interpretation of these studies controversial.

So far, no specific mutations have been linked to lymphotropism of the hepadnavirus. However, very few experiments aimed to study this issue. One of these studies showed that the HBV sequences in serum and PBMC samples collected during SLAH did not show changes in the core promoter region (Laskus *et al.*, 1997). In the present study, WHV sequence analysis was focussed on determination of the sequence of the C and preS virus gene fragments, since the very low levels of virus did not enable amplification of the complete

WHV DNA genome. This caveat limited the size of the amplicons available for analysis.

However, we showed that no sequence variation from the wild-type WHV present in the

initial inoculum occurred in these samples either in the core gene or preS region, which is

known to be prone to variation.

Recently, it has also been shown that a human hepatoma cell line could support HBV infection when exposed to high concentrations ($\sim 4 \times 10^8$ vge) of hepatocyte-derived virus in

the presence of corticoids and DMSO (Gripon et al., 2002). These cells were derived from a patient with HCC induced by infection with hepatitis C. The unknown effect of HCV coinfection in these transformed cells on their susceptibility to HBV in vitro cannot be ascertained. There are major methodological differences between the studies mentioned above and our current study. They can be summarized as follows: (1) we used nontransformed, freshly isolated lymphoid cells and normal, although cultured, hepatocytes as virus targets; (2) we ensured detection of only intracellular WHV DNA sequences through cell surface enzymatic digestion and washing to remove any potentially attached WHV nucleic acids and virions before DNA extraction; (3) rather than showing only the susceptibility of the target cells to virus, we demonstrated that the produced virus can be serially transmitted for many passages; and (4) we proved that the virus passaged in either lymphoid cells or hepatocytes is infectious to healthy animals. Furthermore, the use of well characterized WHV as inoculum, in which the virus complete genome sequence has shown to be identical with that detected in the spleen and liver of the donor, helped to conclude that no specific variant emerged during the repeated multiple passage in lymphoid cells.

These results corroborate the data reported in Chapter 4, which demonstrated that occult, lymphatic-system restricted WHV infection was unlikely the result of invasion with a WHV variant preferentially infecting lymphoid cells (Michalak *et al.*, 2004). The current data support and expand these findings by showing that the induction of hepatitis was in fact related to the quantity of virus, but not to the virus origin. In one study of self-limited HBV infection in humans, it has also been found that the virus residing in the peripheral lymphoid

cells isolated 30 years after resolution of AH persisted without sequence variation (Bläckberg and Kidd-Ljunggren, 2001). Most recently, the study of HCC in patients with occult HBV infection showed that the majority of the viral species in the liver do not contain virus mutations (Pollicino *et al.*, 2004). In other viral infections, such as HCV infection in chimpanzees, it has been shown that the long-term persistence of virus was associated with low rate of virus genomic heterogeneity and amino acid substitutions (Fernandez *et al.*, 2004). Therefore, it is not surprising that low level replication of hepadnavirus persisting for an extended period of time may not be associated with the emergence of virus mutations. This could suggest that the genome of the persisting virus is probably the best fit for its long-term existence.

Despite these recent findings in occult infection models demonstrating that a low virus replication rate does not permit the emergence of viral variants, there are other studies that suggest that variations in hepadnavirus genome may occur during occult HBV infection. The hepadnavirus C gene is considered to be relatively stable and a few mutations in this gene have been clinically observed (see Section 1.7). In contrast, the preS1 genomic region is

relatively susceptible to sequence variations (Gunther *et al.*, 1998). The protein encoded by this region is thought to be involved in virus-host cell interactions, likely mediating virus binding to lymphoid cells and hepatocytes. Recently, it has been postulated that mutations in the preS1 sequence are prevalent in the virus circulating in patients with occult HBV infection (Minuk *et al.*, 2004). Similarily, during phases of low viremia in patients with chronic HBV infection, up to 37% of the HBV species in sera and liver samples contained

preS mutations (Fan *et al.*, 2001). This high frequency is greater than variation identified in other HBV gene sequences (Fan *et al.*, 2001). These studies suggested that when the virus is present at relatively low levels, preS1 variants may be more prevalent. Additionally, it has been previously shown that HBV mutants with various types of in-frame deletions in the preS1 region were replication competent *in vitro* (Melegari *et al.*, 1994; Gunther *et al.*, 1998). Since these preS1 gene deletions overlap the dispensable spacer domain of the P protein, generation of productive virus is possible, allowing these variants to accumulate at high levels in infected patients (Melegari *et al.*, 1994; Xu and Yen 1996; Pult *et al.*, 1997). Therefore, we analysed the WHV preS1 region to ascertain if a cell type-specific variant was generated or not during serial, low level WHV passage in lymphoid cells *in vitro*.

In our study, no sequence variation was found in the preS gene of WHV passaged in either lymphoid cells or hepatocytes. Additionally, no virus variants in the core and preS genes were detected in the virus recovered from WF.1 and WF.2 woodchucks, infected with WHV passaged in either lymphoid cells or hepatocytes, respectively. However, to fully elucidate whether minor WHV quasispecies could contribute to the induction of lymphotropic

WHV infection, the virus DNA fragments amplified from the cultured cells and supernatants,

and from WF.1 and WF.2, will have to be cloned and sequenced. These experiments are

currently underway. At this point, however, our direct sequencing results corroborate the

idea that lymphotropism is a natural propensity of WHV but not a consequence of the existence of a cell-type specific virus variant.

As we have previously shown, the pattern of WHV infection is dependent upon
the amount of invading virus. Doses above 10³ WHV vge induce serologically evident infection accompanied by AH, while those below this level induce serologically silent infection (i.e., POI). The amounts of WHV recovered after multiple passage in lymphoid cells or hepatocytes in this study were near this demarcating quantity. Therefore, development of AH in WF.1 injected with ~1.5 x 10^4 vge and POI in WF.2 injected with ~8.8 x 10^2 vge are not unexpected findings.

A recent study employing the SIV-macaque model of HIV showed the persistence of low levels of virus that evaded detection by conventional testing (i.e., 0.1-5.3 SIV DNA copies/10⁶ PBMC) (Zhu et al., 2004). The SIV envelope sequence remained homogeneous over a six year period in the lymphoid cells and these animals remained free of evident illness up to 10 years after inoculation. These data parallel the results of our study and suggest that low levels of virus replication do not normally permit the development of virus mutations and allows persistence of virus at levels which evade detection.

The current study provides strong support that hepadnavirus lymphotropism is an inherent property of wild-type virus and that the dose of the virus, but not the existence of a

particular virus variant, predetermines development of the lymphatic system-restricted infection. A high degree of similarity between WHV and HBV suggests that the same could be true for HBV.

CHAPTER 6: Induction of Occult, Lymphatic System-Restricted Infection by Direct Liver Transfection with Recombinant Complete WHV Genome

6.0 SUMMARY

Viral hepatitis can be induced in woodchucks by direct transfection of the liver with rWHV DNA. However, whether and to what extent the lymphatic system is involved in this artificially induced hepadnaviral infection has not been investigated. In order to examine if administration of naked rWHV DNA molecules to the liver can cause an infection of the lymphatic system, monomeric recircularized rWHV DNA derived from two different infectious WHV inocula were prepared in this study. One rWHV DNA construct was formed through ligation of the naturally occurring *Eco*RI restriction enzyme cleavage site in the virus preS1 genomic region, while another through ligation of a *Sap*I restriction enzyme site inserted in the nick region of the WHV genome. The results showed that, even though the rWHV DNA was transfected directly to the liver, the lymphoid cells were infected first, regardless of the source and dose of rWHV DNA administered. In animals injected with

rWHV DNA ligated through the *Sap*I restriction site, the liver was not infected, the infection was restricted to the lymphatic system, and serological markers of WHV infection were not detected. This pattern was identical to that of POI described in our previous studies. On the other hand, animals intrahepatically injected with rWHV DNA ligated through the natural *Eco*RI restriction enzyme site did develop serologically evident WHV infection and hepatitis, although their onsets were delayed by 4-6 weeks in comparison to those induced by wild-type

virions. These results demonstrate that WHV infection induced by transfection of the host with unenveloped (naked) recombinant virus genomes always involves the lymphatic system. They parallel our previous findings with intact virions and suggest that WHV uptake, cccDNA synthesis, and production of infectious virions in lymphoid cells precedes infection of the liver, and that it might be a prerequisite for establishing virus replication in hepatocytes.

6.1 INTRODUCTION

It is now well established that the lymphatic system is infected, without exception, during the course of natural WHV infection, independent of whether the primary infection is occult or serologically evident (Michalak et al., 1999; Coffin and Michalak, 1999; Lew and Michalak, 2000; Mulrooney and Michalak, 2003). Examination of other hepadnaviruses also suggests the lymphotropic nature of this family of viruses. For example, in the duck-DHBV model, it has been shown that DHBV replicates in the circulating lymphoid cells and in the lymphoid organs (Freiman et al., 1988; Jilbert et al., 1987; Hosoda et al., 1990; Walter et al., 1991). HBV DNA, viral transcripts, and replicative intermediates have been identified in

circulating lymphoid cells from patients with serologically evident, chronic infection (Pasquinelli et al., 1986; Davidson et al., 1987; Sugai and Okamoto, 1989; Bouffard et al., 1990; Feray et al., 1990; Mason et al., 1992). Evidence for the persistence of HBV in the PBMC of individuals who have resolved AH type B has also been shown (Michalak et al., 1994; Yotsuyanagi et al., 1998; Cabrerizo et al., 2000; Blackberg and Kidd-Ljunggren, 2000).

We recently discovered evidence that relates WHV hepatotropism to the amount of virus invading the host (Michalak *et al.*, 2004). The results of our studies indicate that regardless of the WHV dose administered, lymphoid cells are always infected. In the study described above (Chapter 4), we specifically identified that WHV doses greater than 10^3 vge tend to establish infection that engages the liver; otherwise, with lower virus doses ($<10^3$ vge), lymphatic system-restricted infection is initiated and persists for the lifetime (Michalak *et al.*, 2004). This fact, coupled with our previous findings that the putative virus binding site has a greater capacity for higher affinity interactions with lymphocytes than with hepatocytes (Jin *et al.*, 1996), suggests that lymphoid cells, not hepatocytes, may be primary targets of WHV invasion.

Over the past 20 years, various groups have reported the ability of naked, unenveloped viral DNA to induce infection when it is directly transfected to the natural host (Dubensky *et al.*, 1984). With respect to hepadnaviruses, HBV infection has been induced in chimpanzees by intrahepatic injection of large amounts of cloned HBV DNA (Will *et al.*, 1982). It has also been shown that large amounts of rWHV DNA injected into surgically

exposed livers of adult woodchucks or by percutaneous liver injection into neonates caused WHV infection (Chen *et al.*, 1992; Chen *et al.*, 1993; Girones *et al.*, 1989). The minimum dose of rWHV DNA required to induce AH in adult animals by intrahepatic injection has been determined to be as low as 50 ng (Chen *et al.*, 1998). All of these studies measured onset of WHV infection based solely on the detection of virus serological markers (*i.e.*, WHsAg, anti-WHc or anti-WHs) and none of them examined whether or not the lymphatic

system was involved.

Because of our interest in the elucidation of a role of lymphotropism in the initiation and persistence of hepadnaviral infection, we aimed to test, in this study, if unenveloped rWHV DNA of known sequence can establish infection in lymphoid cells. For this purpose, we constructed two different recombinant constructs containing complete sequences of the WHV genome, one formed through circularization using the endogenous *Eco*RI restriction enzyme cleavage site located in the WHV preS1 sequence and the second through ligation of a *Sap*I restriction enzyme site inserted by PCR in the nick region of the WHV genome. We expected that these two constructs may have different efficiencies in causing infection. Indeed, our findings showed that monomeric rWHV DNA recircularized through the endogenous *Eco*RI site was capable of inducing typical serologically evident infection and hepatitis, albeit 4-6 weeks later than that caused by natural virus. In contrast, constructs ligated using the *Sap*I restriction site induced serologically occult infection which did not involve the liver. We also found that regardless of the construct and amount of rWHV DNA administered, all animals infected by liver transfection initially displayed virus DNA and

cccDNA exclusively in the cells of the lymphatic system, before eventually being detected in

the liver. This finding parallels the results of our previous works in natural infection induced

with small WHV doses (see Chapter 4).

6.2 MATERIALS AND METHODS

6.2.1 Woodchucks

Liver transfection with rWHV DNA constructs and subsequent infection experiments were carried out in 2-3 year old healthy woodchucks. All animals were virus-free prior to the experiment, as described in Section 2.1.

6.2.2 Sample Collection

Samples were collected as outlined in Section 2.2. In brief, blood was collected in vials with no additives for serum isolation or with EDTA for PBMC isolation (Section 2.2.1). Liver biopsies were performed as described in Section 2.2.4.

6.2.3 Full-length genome amplification and ligation of rWHV DNA

Two different full-length rWHV DNA sequences were synthesized and recircularized for intrahepatic transfection. One of the constructs was developed from the WHV genome derived from WHV/tm3 inoculum (GenBank accession number AY334075; see Appendix

A.1; Michalak *et al.*, 2004). This was done using back-to-back PCR nucleotide primers incorporating the *SapI* restriction enzyme site and extended PCR amplification conditions, as described in Section 2.7.3 (Figure 6.1A).

The main reason for using PCR primers homologous to the sequence of the virus nick region sequence was to allow for amplification of full-length WHV DNA in one amplification reaction, since both of the virus DNA strands are open at this location. Therefore, primers Figure 6.1. Schematic representations of the rWHV DNA constructs used for liver transfection. (A) sp-rWHV DNA was generated by PCR using oligonucleotide primers spanning the virus nick region (indicated by a Z-line) which contained the *SapI* enzyme restriction site sequence. This enzyme cleaves outside its recognition site, leaving a 3-nucleotide sticky-end overlap (see Section 6.2). Ligation of these sticky ends produces a monomeric circular molecule. (B) ec-rWHV DNA was constructed by utilizing the natural *Eco*RI enzyme restriction site located in the preS1 sequence of the WHV genome, marked by a straight line. Cleavage using *Eco*RI resulted in a 5-nucleotide sticky-end (see Section 6.2). Ligation of these overlaps resulted in generation of a recircularized molecule.





A

B

Sapi TAAAT -WH	HV - TAAAT Sapi
	Sapl digestion
,	
AAAT - W A - W	HV - T HV - ATTT

Primers: FGM2:gctcttcATTTATGCCTACAGCCTCC (1907-1892) FGP2: gctcttcTAAATGCATGCGACTTCCG (1908-1925)



EcoRI site



EcoRI digestion



spanning this fragment would circumvent the discontinuities in the genome (Kock and Schlicht, 1993; Gunther *et al.*, 1995). The *SapI* enzyme site (GCTCTTCN_{1/4}) is a class II-S enzyme and is the only one that has the recognition site and the cleavage site spatially separated. Also, this enzyme site is not contained within the WHV genome. Therefore, placing the enzyme restriction site in the primer sequences allowed generation of WHV DNA with compatible non-palindromic sticky ends devoid of heterologous sequences. This approach enabled formation of ligated, recircularized WHV DNA molecules (see Figure 6.1A). The primers used for amplification of the full WHV genome were designed based on a previously described method for HBV (Gunther *et al.*, 1995) and WHV (Michalak *et al.*, 1999).

After generation of the amplicon containing the full sequence of WHV, as confirmed by EB-agarose gel electrophoresis (Section 2.9.1), the product was cloned using the TOPO-XL cloning system (Invitrogen), as outlined in Section 2.10.2. Then, plasmid DNA was isolated using the miniprep procedure summarized in Section 2.10.4. After screening for the presence of the proper DNA insert, positive colonies were expanded and the plasmids

containing the WHV DNA insert were extracted from maxipreps, as described in Section 2.10.5. Two WHV clones, each derived from a different colony, were sequenced and their sequences were found to be identical to each other and to WHV clones which were obtained in parallel from the liver and spleen of the WHV/tm3 donor, as previously reported (Michalak *et al.*, 2004).

To produce monomeric, circular rWHV DNA molecules, plasmid inserts were excised

using 10 U/µg of *Sap*I and incubation at 37°C for 12 h. After excision, WHV DNA was separated from the plasmid backbone by electrophoresis in 1% LMP-agarose gel and purified on a Wizard minicolumn, as outlined in Section 2.10.1. Monomeric WHV DNA molecules were ligated using 10 U/µg of T4 DNA ligase (Invitrogen) at 18°C for 14 h. rWHV DNA recircularized by ligation of the *Sap*I restriction site was designated sp-rWHV DNA (Figure 6.2).

As a second rWHV DNA source, a plasmid containing complete WHV (pWHV8; ATCC number: 45097) was used. In this construct, WHV DNA was originally inserted into the plasmid pBR325 using the *Eco*RI site (G \downarrow AATTC) naturally occurring in the virus genome (Cohen *et al.*, 1988). pWHV8 had been previously shown to be capable of inducing infection when directly injected to woodchuck liver (Girones *et al.*, 1989). The *Eco*RI site was utilized for both cloning and to remove the virus sequence from the plasmid (Figure 6.1B). In the first step, the lyophilized bacteria was reconstituted in LB broth, as per the manufacturers instructions. After expansion of the colony and plasmid DNA isolation, the insert was excised by digestion with *Eco*RI for 4 h, as outlined above. Circular WHV DNA

molecules were formed by incubation with T4 DNA ligase, as described above. Formation

of monomeric circular WHV DNA was confirmed by Southern blot hybridization (Figure

6.2). This construct was designated as ec-rWHV DNA (Figure 6.2).

Ligated DNA were stored at -20°C until the time of injection.

Figure 6.2. Southern blot hybridization analysis of the linearized plasmids and recircularized monomeric molecules of sp-rWHV DNA or ec-rWHV DNA. The linearized fragments (L), excised from the plasmid backbone of sp-rWHV DNA and ec-rWHV DNA, show 3308 and 3323 bp, respectively. The recircularized WHV molecules are also indicated (C). The absence of secondary bands in the recircularized (C) samples confirms the monomeric structure of these ligated molecules.







6.2.4 Intrahepatic injection

sp-rWHV DNA or ec-rWHV DNA were intrahepatically injected during laparotomy, as outlined in Section 2.2.4. Briefly, injections were performed using a 27-gauge needle ejecting 100-125 μ L of inoculum per site at 4-5 sites in the liver. After each insertion, the needle was kept in place for 10-15 sec to help prevent leakage and was slowly removed to reduce backflow, as described elsewhere (Chen *et al.*, 1998). In total, 4 animals were injected with rWHV DNA, *i.e.*, woodchuck WM1 with 500 ng sp-rWHV DNA, WM2 with 2.5 μ g sp-rWHV DNA, and WM3 and WM4, each with 3 μ g ec-rWHV DNA, based on OD 260/280 readings. Each of the indicated amounts of rWHV DNA was suspended in 500 μ L of sterile PBS.

6.2.5 Sample collection

Liver biopsy samples were collected before rWHV DNA administration and at 6-7 weeks post injection. WM1 was biopsied at 27 weeks p.i., WM2 at 19 weeks p.i., and WM3 at 14 weeks p.i. WM4 was euthanised at 14 weeks p.i. to examine to what extent the WHV

infection involved different lymphatic organs. Serum and PBMC samples were collected

before infection and at weekly intervals until 7-8 weeks p.i., then biweekly up to 27 weeks

p.i., and then monthly. Serological markers of virus infection were monitored as outlined in

Section 2.3.

6.2.6 Preparation of WHV inocula from PBMC obtained during serologically silent infection induced by rWHV DNA transfection

It was found that inoculation with sp-rWHV DNA induced POI, where PBMC were WHV DNA and cccDNA reactive in the absence of any detectable liver engagement (see below). To ascertain if the virus present in the PBMC was infectious, inoculum was prepared from these cells. For this purpose, PBMC were collected between 3-7 weeks p.i. from WM2. Similarly, PBMC collected from WM4 which was injected with ec-rWHV DNA were used Thus, approximately 6 x 10⁷ PBMC from each animal were treated with as controls. DNase/trypsin/DNase (Section 2.4) and cultured in the presence of 5 µg/mL concanavalin A (Con A; Pharmacia Fine Chemicals, Uppsala, Sweden) in 10 mL of RPMI-1640 supplemented with 10% FCS, 2 mM glutamine, and 1 X non-essential amino acids for 5 days. Then, the cells were spun down, washed, and cryopreserved for later analysis of WHV presence. The resulting culture supernatants were concentrated by ultracentrifugation at 40,000 rpm using a Beckman SW 50.1 rotor at 4°C for 20 h (Coffin and Michalak, 1999). The pellets were resuspended in 250 µL of sterile PBS and i.v. administered into two virus-naive woodchucks.

Thus, woodchuck WF5 received inoculum (~10⁵ vge) derived from PBMC obtained from

WM2 injected with sp-rWHV DNA, whereas WM6 was injected with virus (~10⁵ vge)

obtained from PBMC of WM4, which was injected with ec-rWHV DNA. Liver tissue

samples were obtained before infection and at 6 weeks p.i. Serum and PBMC samples were collected prior to and weekly p.i. for 10 weeks. Serological markers of virus infection were

monitored as outlined in Section 2.3.

6.2.7 Challenge with a liver pathogenic dose of wild-type WHV

WM1 and WM2, which both developed POI after intrahepatic transfection with sprWHV DNA were challenged with 1 x 10^{10} DNase-protected WHV vge of WHV/tm3 inoculum at 35 and 19 weeks p.i., respectively. The purpose of this experiment was to determine if these animals with POI induced by rWHV DNA were susceptible or not to the infection with wild-type virus at a dose known to be liver pathogenic (Michalak *et al.*, 2004). Following WHV challenge, animals were bled weekly to collect serum and PBMC, as described in Section 2.2. Liver biopsy was done at 6 weeks p.i. Serological markers of virus infection were monitored as outlined in Section 2.3. Both animals were euthanised at 13 weeks after the challenge and their tissues were preserved for analysis, as outlined in Section 2.2.5.

6.2.8 Preparation of monocyte-derived DC from animals injected with rWHV DNA

PBMC were isolated, as described in Section 2.2.3, and were subjected to DNase/trypsin/DNase digestion, as outlined in Section 2.4. Approximately 3 x 10⁷ cells were

resuspended in 5 mL of RPMI-1640 medium supplemented with 10% FCS, 2 mM glutamine

and 1 X non-essential amino acids. Monocytes were isolated from total PBMC by plastic

adherence for 3 h at 37°C in a 5% CO₂ atmosphere. Residual floating cells were removed

from the culture flask. 500 U/mL murine granulocyte-monocyte colony-stimulating factor

(GM-CSF; Roche Diagnostics) and 0.2 ng/mL human IL-4 (Roche Diagnostics) were used

to differentiate monocytes into DC during 7-days of culture. After this incubation, the

floating cells (immature DC) were removed from the adhered cells (mature DC), transferred to a new flask, and stimulated with 500 ng/mL liposaccharide (LPS; Sigma Chemical Co.) in the presence of the cytokines indicated above, to yield fully mature DC (Section 7.2.2). Total DNA was extracted from DC and used as template to detect WHV DNA and cccDNA by PCR (see below).

6.2.9 Detection of WHV DNA and cccDNA

Total DNA from serum, cells, culture supernatants, and tissues was extracted using the proteinase K/phenol/chloroform method as outlined in Section 2.5.1. WHV DNA was detected using 2-5 μ g DNA from PBMC or tissues or DNA extracted from 100 μ L of serum or from 200 μ L of culture supernatant equivalent. WHV C, S, and/or X gene-specific primers and cycling conditions established previously were employed, as described in Section 2.7.1. To detect WHV cccDNA replicative intermediates, 5 μ g of DNA from PBMC or lymphoid tissues was digested with mung bean nuclease, as outlined in Section 2.7.2.1, and then amplified using PCR primers spanning the nick region of the WHV genome (Section 2.7.2.2).

Contamination controls for DNA extraction and PCR amplifications were routinely included,

as outlined in Section 2.7.1.

6.3 RESULTS

6.3.1 Confirmation of recircularization of full-length monomeric rWHV DNA

Since we have previously determined, through complete WHV sequence analysis that

the inoculum, designated as WHV/tm3, contained wild-type homogenous WHV DNA sequence and that this inoculum was infectious to naive animals (Michalak *et al.*, 2004), we decided to use this virus as one of the sources of viral DNA to prepare recircularized monomeric rWHV DNA (*i.e.*, sp-rWHV DNA). We also prepared an ec-rWHV DNA construct which was derived from pWHV8 plasmid purchased from ATCC (Figure 6.1).

As illustrated in Figure 6.2, excision of each rWHV DNA sequence from both plasmids was highly efficient, yielding bands of 3308 bp and 3323 bp for sp-rWHV DNA and ec-rWHV DNA, respectively. After purification of the excised inserts, ligation of either 500 ng or 2.5 μ g of sp-rWHV DNA or 3 μ g of ec-rWHV DNA was done using T4 DNA ligase. Figure 6.2 shows that monomers were formed upon circularization and that no other bands were detected.

6.3.2 Intrahepatic injection with rWHV DNA induces infection of lymphoid cells

Figure 6.3 illustrates profiles of serological markers of WHV infection and WHV DNA found after transfection of WM1 with 500 ng and WM2 with 2.5 μ g of sp-rWHV DNA,

and WM3 and WM4 with 3 µg of ec-rWHV DNA each. Furthermore, the profiles of WHV

infection after challenge of WM1 and WM2 with a massive 1 x 10¹⁰ vge WHV dose are depicted.

WHsAg and anti-WHc were never detected in serum samples from WM1 and WM2 after injection with sp-rWHV DNA, until the animals were challenged with WHV (Figure 6.3

A). In either WM3 and WM4, serum WHsAg, anti-WHc or anti-WHs were not detected for

Figure 6.3. Serological and WHV DNA detection profiles and liver histology in four woodchucks after intrahepatic transfection with either sp-rWHV DNA or ec-rWHV DNA. (A) WM1 and WM2 were transfected with sp-rWHV DNA and subsequently challenged with WHV/tm3 inoculum at a dose of 1.1×10^{10} vge. (B) WM3 and WM4 were injected with ec-rWHV DNA. The profiles show the time of liver transfection and challenge, if applicable, and serum WHsAg and anti-WHc appearance and duration. The detection of WHV DNA in the serum, PBMC and liver biopsy samples is indicated. WHV DNA was detected by direct or nested PCR/NAH assay with WHC C gene-specific primers. Estimated WHV vge levels found in serum are depicted as follows: white (open) bars, 1 to 10 vge/mL; light-grey bars, 10 to 10^2 vge/mL; dark-grey bars, 10^2 to 10^3 vge/mL; and black bars, $>10^3$ vge/mL. Approximate WHV DNA quantities identified in PBMC are shown as follows: open bars, 0.005 to 0.5 vge/10⁴ cells; light-grey bars, 0.5 to 5 vge/10⁴ cells; dark-grey bars, 5 to 50 vge/10⁴ cells; and black bars, >50 vge/10⁴ cells. The levels of WHV DNA detected

in liver biopsy or autopsy samples at the time points indicated by arrowheads were shown as

the estimated number of WHV vge/10⁴ cells. Liver histology scores were based on a numeric

scale from 0-3 (0- normal liver; 3- severe hepatitis), as previously described (Michalak et al.,

1990; Hodgson and Michalak, 2001).

WM1		INOCULUM: sp-rWHV DNA 500 ng intrahepatic			CHA 1.1 x 10			
SERUM: PBMC: LIVER:	WHsAg anti-WHc WHV DNA WHV DNA WHV DNA histology				0		0	
		_	Lbx1	7	Lbx2	24	Lbx3	
WM2	2	۱۸ sp- 2.5 μ	NOCULU -rWHV D ag intrahe	IM: NA epatic		CHA 1.1 x 1	ALLENGE: 0 ¹⁰ WHV/t i.v.	: m3
SERUM:	SERUM: WHsAg anti-WHc WHV DNA							
PBMC: LIVER:	WHV DNA WHV DNA histology		0 0 ↑ Lbx1	0 0 ↑ Lbx2	0 0 ↑ Lbx3			200- 2 Lb
	WEEKS:	-2	0	5	10	15	20	:

A

LENGE: WHV/tm3 i.v.







psy	WHV/10 ⁴ cells:
een	0.5-5
ne marrow	0.5-5
nph node	>50
all intestine	e 5-50

up to 12 or 14 weeks p.i., respectively (Figure 6.3B). At these points, WHsAg appeared. Anti-WHc was not detected in the sera of either WM3, up to 21 weeks p.i., or WM4 at autopsy at 14 weeks p.i.

WHV DNA was found in sera from all 4 woodchucks by direct or nested PCR/NAH. Thus, WM1 and WM2 showed levels ~10² vge/mL beginning at 2 weeks after rWHV DNA administration and up until the time of challenge with WHV, when the virus levels escalated significantly. WM3 and WM4 carried low levels of WHV, as was the case in WM1 and WM2, until 10.5 and 12 weeks p.i., respectively, when virus DNA increased to ~10⁴ vge/mL. WHV DNA was detected in PBMC samples from all 4 animals as early as 2 weeks p.i. at levels not exceeding 0.5-5 vge/10⁴ cells, regardless of the construct administered. WHV DNA remained detectable, albeit occasionally, in the serum and PBMC for up to 35 weeks p.i. in WM1 and 19 weeks p.i. in WM2, at which time the animals were challenged with WHV (see below). In WM3 and WM4, who received ec-rWHV DNA, the levels of WHV DNA rose significantly to 5-50 vge/10⁴ PBMC at 9 weeks p.i. and persisted at these high loads until 24 weeks p.i. for WM3, and until the end of follow-up at 14 weeks p.i. for WM4.

Importantly, WHV DNA in circulation was not accompanied by the presence of the

WHV genome in any liver samples obtained after transfection with sp-rWHV DNA, but prior

to WHV challenge of WM1 and WM2. In the ec-rWHV DNA transfected animals, WHV

DNA was not found in the liver at 7 weeks p.i., even when up to 5 µg of liver DNA was

tested by PCR. Furthermore, evaluations performed using primer pairs specific for three non-

overlapping regions of the WHV genome, i.e., WHV C, X and S genes, confirmed the

absence of WHV DNA in hepatic tissue. In contrast, the livers of the same animals were WHV DNA reactive at 14 weeks p.i. (Figure 6.3). Autopsy of WM4 was done at 14 weeks p.i. to determine the involvement in the infection of the lymphatic system. At this time, WHV DNA was found in the liver, spleen, small intestine, bone marrow, lymph node, and PBMC (Figure 6.3 B).

In the early time period after transfection, when WHV DNA was solely detectable in the serum and PBMC, WHV cccDNA presence was examined in PBMC to test the replication competence of the detected virus. As shown in Figure 6.4, WHV cccDNA was found in PBMC from WM1 and WM2, and was detectable as early as 6 weeks p.i. and remained present for 29 and 13 weeks thereafter, respectively, at the time when the liver was WHV DNA non-reactive. Not surprisingly, WHV cccDNA was also detectable in PBMC samples from WM3 and WM4 which were collected before any evidence of liver infection, *i.e.*, as early as 4 weeks p.i..

Besides the lack of WHV DNA in the liver in WM1 and WM2, liver histology was normal in two samples obtained after rWHV DNA transfection but before challenge with a

high WHV dose. Also, liver biopsies from WM3 and WM4 showed no features of inflammation up to 14 weeks p.i. At this time, histological examination of liver biopsy from

WM3 showed early signs of AH, with edema of the portal areas.

In the time period after ec-rWHV DNA administration, similar results, in terms of WHV DNA detection in the serum and PBMC in the absence of WHV DNA in the liver, were found in WM3 and WM4. However, these animals showed delayed development of **Figure 6.4.** Detection of WHV C gene sequence and cccDNA in representative PBMC samples. Theses samples were obtained from woodchucks (A) WM1 and WM2 and (B) WM3 and WM4 after liver transfection with either sp-rWHV DNA or ec-rWHV DNA, respectively, prior to WHV DNA appearance in the liver. Viral DNA from PBMC acquired in the serologically silent phase of infection was detected by nested PCR/NAH with WHV C gene-specific primers. WHV cccDNA was identified as described in Section 6.2. As expected, the bands specific for the WHV C gene and cccDNA showed molecular sizes of 428-bp and 674-bp, respectively. Contamination controls consisted of water added instead of DNA in both direct (DW) and nested (NW) reactions, and a mock (M) extracted and treated as test DNA.







cccDNA



serologically evident infection, as determined by the appearance of WHsAg at 12-14 weeks p.i.. High levels of WHV DNA were present in the serum and PBMC at 10.5 and 12 weeks p.i. at the time when the liver was WHV DNA reactive.

In order to determine the susceptibility of WM1 and WM2 to challenge with a large pathogenic dose of WHV, both animals were injected with 1.1 x 10¹⁰ vge of WHV/tm3 inoculum at 35 and 19 weeks, respectively. After challenge, the animals developed similar WHV immunovirological profiles. Thus, the infection was associated with appearance of WHsAg and anti-WHc. WHV DNA was detected in the liver at 6 weeks after challenge. Therefore, these results demonstrated that the animals were not protected and developed typical AH which resolved and was followed by SOI.

6.3.3 Infectivity of WHV carried in lymphoid cells after animals were transfected with rWHV DNA

Because of the puzzling fact that even though the liver was the site of inoculation with rWHV DNA, the lymphoid cells, but not the liver, were actually the site of initial WHV infection, we decided to test if the detection of WHV DNA and cccDNA in the PBMC from

the initial phase of infection in fact reflected the existence of biologically competent

(infectious) virus. For this purpose, PBMC obtained from WM2 and WM4 between weeks

3 and 7 p.i. were cultured with Con A for 72 h. It has been previously shown that such

treatment enhances WHV replication in lymphoid cells (Coffin and Michalak, 1999). The

resulting cell culture supernatants were concentrated by ultracentrifugation and ~ 10⁵ WHV vge injected into virus-naive WF5 and WM6, respectively.

As shown in Figure 6.5, upon inoculation with the virus isolated from these culture supernatants, both animals developed serum WHsAg-positive infection at 5-6 weeks p.i. WHV DNA was detected in PBMC within 1 week of exposure to virus and cccDNA was detectable at 2-3 weeks p.i. In the serum, virus DNA was apparent within 2-3 weeks p.i. The liver biopsy samples, taken at 6 weeks p.i., from both WF5 and WM6, showed high levels of WHV DNA ($\geq 2 \times 10^3 \text{ vge}/10^4$ liver cells). Histology on these biopsies showed minimal inflammation, however, liver biopsy obtained at 13 weeks p.i. demonstrated very severe AH (grade 3) (for definitions of histological grades see Section 2.12).

Figure 6.6 shows the analysis of WHV DNA in monocyte-derived DC from WM2 and WM3 obtained after liver transfection with either sp-rWHV DNA or ec-rWHV DNA, respectively, prior to WHV DNA appearance in the liver. As shown by Southern blot analysis, DC from both animals contained WHV C gene sequences, as well as cccDNA. These results indicated that the DC were a reservoir of WHV during the POI phase of infection induced by intrahepatic transfection with rWHV DNA.

6.4 **DISCUSSION**

In this study, we have shown that POI, solely involving the lymphatic system, can be

induced in woodchucks receiving circularized recombinant WHV DNA molecules administered to the liver. It appears that this infection is initiated by the uptake of Figure 6.5. Profiles of serological and molecular markers of WHV infection in animals injected with inocula derived from PBMC obtained during the POI phase of infection induced by intrahepatic transfection with sp-rWHV DNA and ec-rWHV DNA. Virusnaive animals WF5 and WM6 were injected (week 0) with the indicated doses of PBMCderived WHV inocula. The diagram shows the appearance and duration of serum WHsAg and anti-WHc, and the detection of WHV DNA in sequential serum, PBMC, and liver samples. Approximate WHV vge levels displayed in serum and PBMC are identified, as described in the legend to Figure 6.3.



WM6

INOCULUM: sp-rWHV DNA ConA-PBMC super ~10⁵ vge i.v.



Figure 6.6. Detection of WHV C gene sequence and cccDNA in monocyte-derived DC samples (C) and their supernatants (S) obtained from WM2 and WM3 PBMC samples. These samples were collected after liver transfection with either sp-rWHV DNA or ec-rWHV DNA, but prior to WHV DNA appearance in the liver. Total DNA from DC was amplified by nested PCR/NAH with WHV C gene-specific primers. WHV cccDNA was identified as described in Section 6.2. As expected, the bands specific for the of WHV C gene and cccDNA amplicons showed molecular sizes of 428-bp and 674-bp, respectively. Contamination controls consisted of water added instead of DNA in both direct (DW) and nested (NW), reactions and a mock (M) extracted and treated as test DNA. DC derived from virus-naive PBMC (H) and from chronic WHV carrier (CH) animals served as negative and positive controls, respectively.



rWHV DNA and the synthesis of virions in the lymphoid cells. In this regard, the uptake of viral DNA by lymphoid cells residing in the liver or the leakage of rWHV DNA from the liver and harbouring viral DNA by extrahepatic lymphoid tissue needs to be considered. Using the rWHV DNA infection model, the only discernible indicator of WHV infection early after transfection was the presence of WHV DNA and cccDNA in the lymphoid cells, with the lack of serological markers of WHV infection and the absence of any evidence of liver engagement. We have previously identified this serologically silent form of WHV infection and we have established that it is caused by an exposure to a small quantity of WHV ($<10^3$ virions/dose), as described in Chapter 5. We have termed this form of hepadnaviral infection as primary occult infection, or POI (Michalak *et al.*, 2004).

Even though the dose of sp-rWHV DNA used for transfection experiments was moderately high (i.e., 500 ng and 2.5 μ g), in terms of natural virus DNA equivalents, it was evident that the recircularized DNA molecules have established an infection pattern which suggested that only a small quantity of infectious particles was assembled. This could be due to at least three possibilities: (a) the inevitable leakage of rWHV DNA inoculum from the liver, which may translate to a significantly smaller dose being actually administered to hepatocytes; (b) much higher susceptibility of lymphoid cells than hepatocytes to DNA transfection, and (c) the instability of the ligation through the *Sap*I enzymatic restriction site used for circularization.

This last point is of importance considering infectivity of the two rWHV DNA constructs used. Thus, ec-rWHV DNA has an intact virus nick region and was ligated

through the natural EcoRI site, whereas, the sp-rWHV DNA construct was formed through the excision of the SapI restriction site artificially inserted in the nick region. It is of note that the requirement for the completion of the virus nick region to generate intact double stranded DNA for the initiation of virus pgRNA transcription is absolute (Tuttleman et al., 1986). It is likely that the use of the sp-rWHV DNA construct, which in the first step required the closure of such a critical region, led to a less efficient generation of functional genomes than ec-rWHV DNA, in which the nick region was already closed. Additionally, the use of T4 ligase to enzymatically force the formation of monomeric circular WHV molecules, rather than re-ligation based on the dilution of linearized virus DNA (Chen et al., 1998), may have been a disadvantage. The use of the T4 ligase may possibly have led to formation of molecules with tertiary structures that might be less capable of transcription upon entry to cells, even though there was no evidence of the formation of secondary structures, as determined by Southern blot hybridization analysis (Figure 6.2). In future studies, we plan to generate WHV DNA monomers from WHV/tm3 inoculum using primers spanning the EcoRI region, which should generate rWHV DNA with replication efficiency comparable to

that of the ec-rWHV DNA construct.

Regardless of the final outcome of the infection induced by the constructs used, all animals investigated in this study developed, in the first instance, lymphatic system-restricted

occult infection. We have previously shown that lymphoid cells are invariably a site of WHV

replication in infection induced by wild-type virus and they carry WHV DNA, cccDNA, and

WHV RNA prior to the development of serologically and histologically evident hepatitis

(Coffin and Michalak, 1999; Mulrooney and Michalak, 2003; Michalak *et al.*, 2004). Based on the results of the current study, we now report that a similar WHV infection profile can be induced by transfection with rWHV DNA. Considering that the initial site of virus inoculation was the liver, the detection of lymphoid cell-restricted WHV presence was surprising, however, consistent with the hypothesis that the lymphatic system is the primary target and the initial site of hepadnavirus replication at low virus doses (Michalak, 2000). DC were specifically shown to carry virus in the infected animals. This does not exclude that WHV replicates in other lymphoid cell subsets after intrahepatic transfection. We have also clearly demonstrated that the virus formed in the lymphoid cells after transfection, but prior to liver involvement, has the ability to cause typical AH in virus-naive hosts.

In conclusion, we have demonstrated that, as in the case of natural infection, transfection with recombinant hepadnavirus genome leads, in the first instance, to the establishment of infection in the host's lymphatic system. We have also documented that transfection with recombinant virus DNA can cause POI, with serological and molecular profiles identical to those described in our previous study using low doses of wild-type WHV (Chapter 4; Michalak *et al.*, 2004). The availability of infectious WHV DNA constructs open

the possibility for a multitude of in vivo and in vitro investigations.

CHAPTER 7: WHV Persists in Dendritic Cells During Long-Term Symptomatic and Occult Infections

7.0 SUMMARY

Persistent viral infections can be accompanied by sustained virus replication in DC and they can modulate DC functions. A role of DC in occult persistent hepadnavirus infection is not yet determined. Therefore, we aimed to examine if, in the woodchuck model of HBV, DC are a site of persistent WHV replication. To accomplish this, we developed a DC derivation protocol using woodchuck monocytes. To confirm the phenotype of the derived cells, we applied a DC specific marker, CD209, of which a gene fragment derived from woodchuck PBMC was cloned and sequenced in this study. The results showed that DC harbour WHV DNA and, importantly, express its replicative intermediate cccDNA during both serologically evident chronic infection and years after resolution of AH (i.e., in SOI). This finding may have an implication for a better understanding of the mechanisms of hepadnaviral persistence. It provides further evidence supporting the conclusion that lymphotropism is an intrinsic property of WHV.

7.1 INTRODUCTION

It is well documented that DC function as a bridge between the innate and adaptive immune responses (Colonna, 2004). DC are the sentinels of the immune system that capture antigens at the infection site and migrate to the lymph nodes where they exhibit the function of antigen presentation and mediate T cell activation. They also have the ability to recognize foreign molecules through various non-clonotypic Toll-like receptors, by recognizing pathogen-associated molecular patterns, thereby canvassing both arms of the immune response (Colonna, 2004). The ability of pathogens to infect and modulate DC function could be a mechanism of immune evasion, potentially leading to persistent infection (Moll, 2003). Since the recognition and internalization of antigens stimulates the maturation of DC, induces their migration from the periphery to the T cell-rich areas of draining lymph nodes, and upregulates expression of cell surface MHC class II and co-stimulatory molecules, any disturbance in these processes due to pathogen invasion may have consequences for the immune response (Moll, 2003).

Many viruses use cell surface molecules on DC to enter these cells. The subsequent infection may cause virus-specific and potentially generalized immune suppression through

modulation of DC maturation. For example, DC-SIGN (DC-specific intracellular adhesion

molecule-grabbing nonintegrin), a C-type lectin, which is expressed on both myeloid- and

monocyte-derived DC, seemingly acts as a receptor for HIV-1 (Kwon et al., 2002), HCV

(Lozach et al., 2003), MCMV (Andrews et al., 2001), Ebola virus (Simmons et al., 2003),

and Dengue virus (Navarro-Sanchez et al., 2003). DC also are major targets of other viruses
known to cause generalized immunosuppression, such as EBV (Lindhout *et al.*, 1994), LCMV (Sevilla *et al.*, 2003), measles virus (Servet-Delprat *et al.*, 2003), HSV-1 (Mikloska *et al.*, 2001), vaccinia virus (Engelmayer *et al.*, 1999), and MCMV (Andrews *et al.*, 2001). Infection of DC by these viruses inhibit DC ability to stimulate T cells. In the case of HIV-1, infection of DC actually permits virus transmission to T cells (Kwon *et al.*, 2002). It has been recognized that these and other pathogens which interact with DC-SIGN frequently cause chronic infections that last a lifetime. This appears to be due to the manipulation of the Th1/Th2 immune balance through the pathogen action on DC, which, in turn, enables these pathogens to persist.

Because so many viruses take advantage of their ability to impair vital roles of DC in priming the immune system, it is reasonable to consider that hepadnaviruses, now known to persist for the lifetime of the host, may also employ this evasion strategy. So far, in the case of HBV, it has been shown that DC derived from patients chronically infected with HBV have an impaired capacity to stimulate Th1 cells that, in turn, leads to decreased virus-specific T cell responses (Arima *et al.*, 2003), due to compromised antigen presentation (Beckebaum *et al.*, 2003). This state can be partially restored *in vitro* by providing exogenous IL-12 (Lohr

et al., 2003). Other reports have shown the decreased potential of DC to stimulate mixed

lymphocyte reaction (MLR) in chronic HBV (Wang et al., 2001), indicating the existence of

a generalized immunosuppression. It was also demonstrated that the precursor DC frequency in circulation is lower in patients with chronic HBV infection than in healthy individuals. DC

propagated from these precursors showed lower expression of costimulatory molecules and

had an impaired allostimulatory capacity when compared to the cells from uninfected individuals (Beckebaum et al., 2002). However, the concept of the altered function of DC in HBV-infected patients remains controversial.

We have previously shown the persistence of the WHV genome and cccDNA in circulating lymphoid cells and in the lymphatic tissues in both serologically evident and occult WHV infections (Coffin and Michalak, 1999; Lew and Michalak, 2001; Mulrooney and Michalak, 2003; Michalak et al., 2004). We also recently have identified the persistence of HCV RNA in DC from patients with occult HCV infection after spontaneous or treatmentinduced resolution of hepatitis C (Pham et al., 2004). To test the involvement of DC in WHV infection, we first aimed to establish methods to derive woodchuck DC. Subsequently, we examined if WHV invades DC and, if so, whether it replicates in these cells and if DC may serve as a virus reservoir in either chronic symptomatic or occult infection. Our findings show that DC are a site of WHV persistence independent of the type of protracted infection. This may have implications for the ability of DC to process and present WHV antigens thereby impeding virus specific T-cell responses required for virus elimination. On the other hand, the persistence of minute quantities of virus in DC after apparent complete recovery

from symptomatic hepadnaviral infection may potentially serve as a constant immune trigger

to maintain efficient anti-viral responses during the lifetime of the host.

7.2 MATERIALS AND METHODS

7.2.1 Woodchucks

Samples of PBMC used for the derivation of DC were obtained from 3 groups of animals, as shown in Table 7.1. Only one PBMC sample was used for DC isolation per animal, usually at autopsy, due to the larger number of cells required.

Group 1 was constituted by 2 healthy, WHV-naive animals. They served as negative controls.

Group 2 included 5 woodchucks chronically infected with WHV, as confirmed by repeated detection of WHsAg in serum and histological examination of liver biopsy (see Section 2.12). All 5 animals acquired WHV infection in the wild and were serum WHsAg and anti-WHc reactive for 20-37.5 mo prior to PBMC collection. Serum WHV DNA loads were approximately 10¹⁰ vge/mL at the time of lymphoid cell isolation.

Group 3 included 10 animals that were experimentally infected with WHV and developed SLAH and then SOI that was followed between 10.5 and 41 mo p.i. At the time of PBMC collection, all animals were serum WHsAg and anti-WHs negative and anti-WHc reactive. Serum WHV DNA levels were below 10² vge/mL, as determined by nested

PCR/NAH.

7.2.2 Derivation of immature and mature DC from monocytes

PBMC were isolated by density centrifugation on Ficoll-Paque(Section 2.2.3). PBMC

were subjected to DNase/trypsin/DNase digestion, as outlined in Section 2.4. Approximately

Category of infection and animal no.	Time of PBMC collection (mo p.i.) ^a				Duration of serum WHV DNA positivity	Dendritic cells			
		Duration of serological markers (mo)				Immature	Mature		
		WHsAg	anti-WHs	anti-WHc	(mo)	WHV DNA (vge/µg)	WHV DNA (vge/µg)	WHV cccDNA (vge/µg)	WHV RNA (vge/µg)
GROUP 1: Healthy									
L75/F	n.a. ^b	n.a.	n.a.	n.a.	n.a.	n.d. ^c	n.d.	n.d.	n.a.
L17/M	n.a.	n.a.	n.a.	n.a.	n.a.	n.d.	n.d.	n.d.	n.a.
GROUP 2: Chronic WHV Hepatitis									
2641/F	37	37	n.a.	37	37	>50	>50	0.5-5	0.5-5
3228/M	24.5	24.5	n.a.	24.5	24.5	>50	>50	5-50	n.a.
3219/M	24	24	n.a.	24	24	>50	>50	5-50	n.a.
3231/M	22	22	n.a.	22	22	>50	>50	0.005-0.5	n.a.
3224/F	20	20	n.a.	20	20	>50	>50	0.5-5	0.5-5
GROUP 3: SOI									
L16/M	41	3	31	40.5	40.5	n.a.	0.5-5	0.5-5	n.d.
L14/M	39	3	26	37	38	n.a.	0.5-5	0.5-5	n.d.
L11/M	36	3.5	19	34	35.5	n.d.	0.5-5	n.a.	n.a.
L12A/F	21	3.5	n.t. ^d	19	20	0.005-0.5	n.d.	n.a.	n.a.
L12D/F	21	1	n.t.	19	20	0.005-0.5	n.d.	n.a.	n.a.
L18C/M	15	1	n.t.	14.5	14.5	0.005-0.5	n.d.	n.a.	n.a.
L75B/M °	14	n.t.	n.t.	11	10.5	n.a.	0.5-5	n.d.	n.a.
L19A/F °	14	1	n.t.	13.5	13.5	n.a.	n.d.	n.d.	n.a.
L75C/F °	14	n.t.	n.t.	11	10.5	n.a.	n.d.	n.d.	n.a.
L74B/M °	10.5	n.t.	n.t.	8	7	n.a.	0.5-5	n.d.	n.a.

Table 7.1 Immunovirological characteristics of chronic and SOI WHV infection in woodchucks studied and detection of molecular indicators of WHV infection in immature and/or mature DC derived from monocytes of these animals

^a mo p.i., From inoculation with WHV or from time of arrival at colony for animals with serologically evident, chronic WHV infection (Group 2) ^b n.a., not applicable ^c n.d., not detected ^d n.t., not tested

^e These animals were inoculated as neonates and serum samples were not available until 3 mo after WHV inoculation

 5×10^7 cells were resuspended at a concentration of 1×10^7 /mL in RPMI-1640 medium supplemented with 10% FCS, 2 mM glutamine and 1 X non-essential amino acids. Monocytes were removed by adherence to plastic for 3 h at 37°C in a 5% CO₂ atmosphere. Residual floating cells were removed from the culture flask. 500 U/mL murine granulocytemonocyte colony-stimulating factor (GM-CSF; Roche Diagnostics) and 0.2 ng/mL human IL-4 (Roche Diagnostics) were used to differentiate monocytes into DC during 7-days culture. After this incubation, the floating cells (immature DC) were removed, transferred to a new flask, and stimulated with 500 ng/mL LPS (Sigma Chemical Co.) in the presence of the cytokines indicated above. In some cases, the immature DC were cryopreserved without any further maturation. After a further 7 day incubation, the mature DC were harvested and cryopreserved in FCS/10% DMSO until the time of nucleic acid extraction (Section 2.2.3). Supernatants from the cultures of both immature and mature cells were stored at -20°C for further analysis.

Morphological examination of woodchuck DC showed that they were identical to human monocyte-derived DC which were derived using the same protocol. It is known that human DC show elevated expression of known DC markers, such as CD80, CD86, HLA-DR,

CD1a, and CD40, when compared to total PBMC. Since antibodies recognizing the surface

markers on woodchuck DC are not available, to confirm whether the cells obtained were truly

DC, we examined the expression of woodchuck CD209 gene, which transcribes CD209

protein specifically displayed on these cells (Geijtenbeek et al., 2000).

7.2.3 DNA and RNA Isolation

DNA was extracted from cells and supernatants using the proteinase K/phenol/chloroform method, as outlined in Section 2.5.1. Total RNA was prepared using TRIzol reagent (Invitrogen) (Section 2.5.2). In some cases, due to small numbers of DC available for analysis, DNA was isolated from the residual fraction after TRIzol extraction and RNA isolation, using 10% sodium acetate in absolute ethanol, as per the manufacturer's instructions (Invitrogen).

7.2.4 cDNA synthesis and PCR

cDNA was synthesized using 2-5 μ g of total RNA and random primers and amplified by PCR with WHV C-gene specific primers as outlined in Section 2.6. WHV DNA was detected by direct or nested PCR using primers specific for the WHV C gene (Section 2.7.1). WHV cccDNA was detected after mung bean digestion of 2-5 μ g of genomic DNA, as presented in Section 2.7.2. The PCR amplicons were identified by EB-agarose gel electrophoresis and Southern blot hybridization (Section 2.9).

7.2.5 Cloning of woodchuck CD209

CD209 (DC-SIGN) degenerate primers were synthesized based on the consensus nucleotide sequence obtained by comparison of published human and mouse CD209 molecules. The primers CD209 sense (5'-ATGAGTCAYTCYAAGGAA) and CD209 anti-

sense (5'-CYAGGSGYAGAGAKGG) were used to amplify cDNA transcribed from RNA

from woodchuck DC and PBMC. RNA from human DC and PBMC were used as positive controls. Amplification was done under the following PCR cycling conditions: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 42°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 10 min. Since it was unknown if the CD209 primers were intronspanning or not, RNA samples, which were not reverse transcribed (i.e., RT-), were included as controls to ensure that amplification was from a cDNA template and not from contaminating genomic DNA. After amplification, the product (676 bp) from the woodchuck DC cDNA, the fragment was excised and purified, as described in Section 2.10.1. The resultant purified DNA was cloned using the TOPO-TA cloning system (Invitrogen; see Section 2.10.2). After extraction of the plasmid DNA employing a miniprep isolation procedure, plasmids containing the proper size insert were identified by EB-agarose gel electrophoresis. The identity of the woodchuck CD209 sequence was confirmed by automated sequencing.

7.3 RESULTS

7.3.1 Woodchuck DC are phenotypically comparable to their human counterparts

In order to differentiate woodchuck monocytes into DC, a protocol used to derive

human DC from peripheral blood was adapted (Caux et al., 1992). Murine GM-CSF and

human IL-4 were added to the adherent cell aggregates to stimulate DC growth from

monocytes. After 7 day culture, the cells were morphologically identical to their human

counterparts, which were confirmed to be DC based on FACS analysis using antibodies

Figure 7.1. Expression of CD209 in woodchuck and human_DC. Degenerate primers were synthesized based on the consensus sequence from the mouse and human CD209 nucleotide alignment. cDNA from DC preparations and the corresponding PBMC, isolated from a healthy woodchuck and a human are shown. The PCR products were visualized by EB-agarose gel electrophoresis and yielded the expected size (676 bp), as indicated by arrows. Contamination controls consisted of water added instead of DNA in direct PCR (DW) and a mock (M) extracted and treated as test cDNA. Additionally, samples with (RT+) and without (RT-) reverse transcriptase were included for all cases to control for the potential of non-intron spanning primers to generate the proper size CD209 fragment from any possible contaminating genomic DNA in the RNA preparations.





specific for their cell surface markers, as described in Section 7.2.1. In order to provide further evidence that indeed woodchuck DC were generated, we cloned CD209 from woodchuck cDNA and examined expression of this marker in woodchuck DC. Figure 7.1 shows the expected size amplicon (676 bp) was obtained after RT-PCR when either cDNA from woodchuck or human DC was analysed. We further confirmed the identity of woodchuck CD209 by cloning and sequencing of the RT-PCR products. The woodchuck CD209 sequence determined has been submitted to GenBank (accession number AY842283; Appendix B). Woodchuck CD209 is comparable to CD 209 molecules from other species, with 68 % and 88 % homology to the sequence of corresponding human and mouse fragments, respectively. Therefore, we were confident that these cells were truly DC, based on their derivation, morphology, and the expression CD209.

7.3.2 DC from woodchucks with chronic hepatitis are a site of active WHV replication

In order to identify if DC from woodchucks with classical serum WHsAg-positive chronic infection carry WHV, DNA and RNA were isolated from DC cultures prepared from PBMC of the respective animals (see Table 7.1). To ensure that only intracellular WHV

genome was identified, PBMC were DNase/trypsin/DNase treated and extensively washed before monocyte isolation. Immature DC were collected after 7-day culture and mature DC harvested after maturation with LPS after a further 7-day culture. PCR was performed using WHV C gene-specific primers on DNA isolated from both immature and mature DC, and on cDNA transcribed from RNA isolated from mature DC. Additionally, the presence of WHV Figure 7.2. Detection of WHV DNA, cccDNA, and WHV RNA expression in immature and mature DC derived from 2 animals with serum WHsAg-positive, chronic WHV infection (2641 and 3224; see Table 7.1). This representative Southern blot illustrates the presence of WHV DNA (623 bp) and WHV cccDNA (674 bp), after mung bean nuclease digestion of template DNA, in both subsets of DC as determined by direct or nested PCR/NAH, respectively. WHV C-gene RNA intermediates were also detected by nested PCR/NAH in the mature DC RNA samples available for analysis (428 bp). Contamination controls consisted of water added instead of DNA in direct PCR (DW) or nested PCR (NW), and a mock (M) extracted and treated as the test samples. In the amplification of WHV RNA sequences, samples with (RT+) and without (RT-) reverse transcriptase were included for all cases to control for any amplification from potential contaminating WHV DNA in the RNA preparations. The positive control consisted of liver DNA isolated from a woodchuck with

WHsAg-reactive, chronic WHV hepatitis.





cccDNA was examined. Figure 7.2 illustrates the detection of WHV DNA, WHV cccDNA, and RNA, in DC samples collected from animals with chronic WHV infection lasting 24 or 24.5 months since arrival to the colony. This representative Southern blot illustrates the detection of WHV DNA in the immature and mature DC by direct PCR/NAH, while cccDNA and WHV RNA was identified in mature DC by nested PCR/NAH. Overall, 5 out of 5 preparations of monocyte-derived DC from woodchucks with chronic WHV infection revealed the presence of WHV DNA and cccDNA in DC (Table 7.1). Both of the mature DC samples available for study also were reactive for WHV RNA. This data provided convincing evidence that DC in serologically evident chronic WHV infection are a reservoir of persistent, actively replicating WHV.

7.3.3 DC from woodchucks with SOI persistently carry WHV

Because we could detect moderately high levels of WHV replicative intermediates in DC derived from woodchucks with chronic WHV infection, we wanted to next identify if the same situation exists in serologically silent SOI continuing after resolution of AH. After differentiation of DC using the protocol outlined above, sufficient numbers of cells for DNA extraction were available from 10 animals at various observation periods (10.5-41 mo) after recovery from AH (Table 7.1). As indicated in Table 7.1, both immature and mature DC were available for study from 4 of the 10 animals in this group. Of these 4, 3 displayed WHV DNA solely detectable in the immature DC subset, and one in the mature DC subset. In the

remaining 6 cases from which only mature DC were derived, 4 cases were reactive for WHV

Figure 7.3. Detection of WHV DNA and cccDNA in mature DC isolated from 2 animals with SOI persisting years after recovery from AH (L14 and L16, 39 and 41 mo p.i., respectively; see Table 7.1). Southern blot hybridization analysis confirms the size and specificity of WHV DNA C-gene sequence (428 bp) and WHV cccDNA (674 bp) in mature DC identified after amplification by nested PCR/NAH. Contamination controls consisted of water added instead of DNA in direct PCR (DW) or nested PCR (NW), and a mock (M) extracted and treated as the test DNA. The positive control consisted of DNA derived from mature DC of a woodchuck with WHsAg-reactive, chronic WHV infection.





DNA. WHV cccDNA was detected in 2 of the 6 cases from which sufficient amount of DNA was available for analysis. Interestingly, as shown in Figure 7.3, both of the WHV cccDNA reactive DC samples were derived from PBMC collected more than 3 years after resolution of AH. This figure shows WHV DNA and cccDNA detection in mature DC isolated at was available for analysis. Interestingly, both of the WHV cccDNA reactive DC samples were derived from PBMC collected more than 3 years after resolution of AH, as shown in Figure 7.3. This figure shows WHV DNA and cccDNA detection in mature DC isolated at 39 and 41 mo p.i. from woodchucks L14/M and L16/M, respectively. Taken together, the results showed that 80% of the animals with SOI showed evidence of WHV genome presence, while 33% of those available for testing exhibited evidence of active WHV replication in either the mature or immature DC subset.

7.4 DISCUSSION

We have shown that DC are infected by WHV and that the virus persists in these cells in both serologically evident chronic infection and years after resolution of acute WHV. In many infections, DC harbour pathogens that may impair specific immune responses which,

in turn, potentially affect general immune function (reviewed in Moll, 2003). Based on the detection of replicating WHV in DC, it is possible that this may also be the case for hepadnaviral infection.

We know that the detected virus was of an intracellular origin because DNase/trypsin/DNase digestion and extensive washing performed after cell isolation, which

efficiently removes traces of the cell surface adhered WHV and free WHV DNA (Mulrooney and Michalak, 2003), was employed. However, since DC were derived from monocytes, it is possible that WHV identified was a result of infection of monocytes or phagocytosis of the virus by these cells from other cell subsets infected, which were present in culture during monocyte adherence. To prove otherwise, DC would have to be directly purified ex vivo, which is not possible due to the very low number of circulating DC and the absence of reagents specific for identification of subsets of woodchuck lymphoid cells. Nevertheless, whether the virus detected in the DC was derived from monocytes or whether they were directly infected in vivo, this study shows that woodchuck DC are capable of harbouring WHV and can facilitate its replication. Because monocytes are naturally differentiated into DC in vivo, we can assume that WHV remains in the newly differentiated DC, even if virus was initially solely contained in the monocyte sub-population. It would not be surprising, however, that DC can be infected by WHV directly. DC display C-type lectins, like DC-SIGN, on their surface, which enable DC to recognize a broad range of pathogen carbohydrate patterns (Feinberg et al., 2001). This suggests that DC have the ability to bind

to many glycosylated molecules (reviewed in van Kooyk and Geijtenbeek, 2003), which could

be associated with the envelope of different viruses, including hepadnaviruses.

Perhaps the most significant finding from this work is that DC remain persistently

infected for a very long time after resolution of acute WHV infection. We have examined

animals for longer than 3 years after resolution of AH and were able to detect WHV

replication intermediates. This is the first time that a specific cell subset was clearly identified

as a reservoir of WHV in the woodchuck lymphatic system. As it was shown, virus replication remains active, although at very low levels in these cells. Based on the fact that certain subsets of DC can live for extremely long periods, up to months or years (Cremer *et al.*, 2002), the persistence of virus may also be coordinately prolonged. However, considering the percentage of WHV DNA reactive lymphoid cells detected in POI and SOI (as reported in Chapter 3), it is evident that lymphoid cell subsets other than DC are also infected.

It is likely that eradication of the virus from these cells never takes place, because of the unique role of DC in the lymphatic system and the longer life of antigen bearing DC (Cremer *et al.*, 2002). In regard to hepadnaviruses, it has been shown that even after antiviral treatment of chronic hepadnaviral infections leading to a decrease in HBV load in the circulation, viral cccDNA remains at stable levels in the liver (Mason *et al.*, 1998; Locarnini and Birch, 1999). This suggests that there is a reservoir, that may include DC, which is not susceptible to or accessible to the treatment.

The ability of DC to harbour minute quantities of virus may be reminiscent of the situation occurring in other infections, such as in tuberculosis and leishmaniasis (Belkaid *et*

al., 2002). It has been shown that small amounts of antigen remain at the site of initial insult

or in the DC in the draining lymph nodes. It has also been suggested that continuous, low

level stimulation of specific T cell immune responses may be required to maintain immunity

against particular agents, known as concomitant immunity (Belkaid et al., 2002). Therefore,

the low levels of persistent WHV may trigger anamnestic responses which enable lifelong

acquired antiviral immunity. This is not an unreasonable possibility in the case of WHV SOI, since high dose virus challenge of these animals does not induce infection and shows the maintenance of effective T-cell anti-viral immunity (Gujar and Michalak, manuscript in preparation). Furthermore, reports of detection of activated CTL specific for HBV antigens even up to 30 years after resolution of AH type B, suggest that there is harboured hepadnavirus, which, by continuous stimulation, may maintain HBV-specific responses (Penna et al., 1996; Rehermann et al., 1996b; Penna et al., 1997). This could be interpreted as evidence suggesting that the infection of DC may contribute to the mechanism sustaining memory virus-specific immune responses through the intermittent processing and presentation of hepadnaviral epitopes. This could constitute a negative feedback loop, where in the face of virus activation specific immune response increases, thereby keeping WHV production under control, yet maintaining virus at a level that maintains anamnestic antiviral responses, but that are not imminently harmful to the host. However, the long-term virus presence may eventually lead to the development of HCC in some cases (Korba et al., 1989; Michalak et al., 1999).

In this regard, it has been found that small amounts of HIV-1 are sufficient to infect

DC (Pope *et al.*, 1995), and that DC actually mediate virus transmission to T cells (Kwon *et al.*, 2002). Our recent findings in hepadnaviral POI also show that circulating lymphoid cells are initially infected when exposed to low levels of virus, without any engagement of the liver (Michalak *et al.*, 2004). Given the evidence for the essential involvement of DC in HIV-1 infection induced with low virus doses (Geijtenbeek *et al.*, 2000; Steinman, 2000), and the

proximity of DC to the site of infection, *i.e.*, the skin and mucous membranes, it could be considered that DC are involved in the uptake of hepadnavirus, particularly in cases of exposure to low virus dose. This could mediate the subsequent infection of lymphoid cells and possibly transmit the virus to the liver in the case of exposure to small amounts of virus. This notion could also explain the finding that when rWHV DNA was administered to the liver, WHV was first detected in the lymphoid cells but not in hepatocytes (see Chapter 6). The assumption could be made that DC can act as chaperones in the transmission of hepadnavirus to other cell types.

In summary, we have shown the persistence of WHV genome and its replication in monocyte-derived DC in woodchucks with SOI, as well as with serologically evident chronic WHV hepatitis. It remains to be determined if the immune functions of the infected DC remain intact. Future studies will examine if the virus produced by DC isolated from animals with occult WHV infection retain their pathogenic and oncogenic potential. Additionally, the ability of WHV-infected DC to stimulate T cell responses needs to be further examined. Nevertheless, the repercussions of DC infection by WHV, as it was shown in the case of other pathogens, could be significant in regard to the understanding of the mechanisms of initiation

and persistence of hepadnavirus infection, and further investigations into these mechanisms

are warranted.

CHAPTER 8: GENERAL DISCUSSION

The main purpose of the present series of studies was to investigate virological factors predisposing to hepadnavirus lymphotropism and how the lymphotropic nature of the virus relates to its persistence induced by either primary exposure to a low virus dose or due to residual virus presence after resolution of serologically evident infection. In particular, we focussed on the recognition of the significance of virus dose and the potential existence of specific virus genome variants to determine if they contribute to lymphoid cell infection. In the first study, we established a new approach to detect small amounts of WHV DNA in intact lymphoid cells using in situ PCR coupled with flow cytometry. We determined, for the first time, the extent to which circulating lymphoid cells are involved in hepadnavirus infection. It was found that a relatively large percentage of peripheral lymphoid cells were infected with WHV, regardless of whether the infection was serologically evident (up to 20%) or occult (up to 14%) (Chapter 3; Mulrooney and Michalak, 2003). We also examined if the quantity of WHV invading the host predetermines if the infection is exclusive to the lymphatic system. This study also showed that the lymphatic system engagement always preceded

infection of the liver, suggesting that it might be absolutely required to establish productive

infection in hepatocytes upon exposure to low virus doses. It was found that virus amounts

lower than 10³ vge invariably led to the lymphatic system-restricted infection which, with

time, could be transmitted to the liver (Chapter 4; Michalak et al., 2004). In the subsequent

study, we attempted to identify if the existence of cell-specific virus variants may determine

WHV lymphotropism. We employed multiple serial passage of splenocyte-derived WHV in virus-naive lymphoid cells or hepatocytes, aiming to enrich the detection of any potential lymphatic system-specific virus variants. The results of this study showed that the infection of lymphoid cells is unlikely due to the existence or emergence of a viral variant predisposed to propagate in lymphoid cells (Chapter 5). To confirm these findings and to expand the applicability of the WHV-woodchuck model for studies of hepadnaviral lymphotropism, we examined if rWHV DNA infected directly to the woodchuck liver can induce the infection of lymphoid cells. We discovered that transfection with unenveloped rWHV DNA always led to infection of lymphoid cells before the appearance of virus DNA in the liver. This supported our conviction that the lymphatic system, not the liver, is a primary target of hepadnavirus invasion, at least in situations when small amounts of virus infect a susceptible host (Chapter Since DC play an important role in both anti-viral immune responses and in the 6). establishment of persistence in other viral infections engaging the lymphatic system, we then investigated if the monocyte-derived subset of DC is involved in long-term WHV infection. We identified not only the WHV genome, but also its replicative intermediates in DC derived

from animals with either serologically evident or SOI (>3 years after recovery from AH)

(Chapter 7). Taken together, our studies unambiguously document that lymphotropism is a

natural propensity of hepadnavirus. They also suggest that virus replication in the lymphatic

system may be a prerequisite for infection of hepatocytes, especially when the host is exposed

to small amounts of virus. The validity of our observations was enhanced by the employment

of well-established and controlled molecular virology investigative protocols and virologic diagnostic procedures throughout the studies.

Overall, we showed that the lymphatic system is susceptible to and persistently maintains wild-type WHV infection. We clearly defined that occult hepadnavirus persistence has two distinct forms, *i.e.*, POI and SOI. POI occurs when the host is exposed to a low quantity of virus, generally below 10^3 vge. Other characteristics of POI include: (1) restriction to the lymphatic system, (2) association with low virus loads in the serum ($\leq 10^2$ vge/mL) and in peripheral lymphoid cells (≤ 50 vge/ 10^4 cells), and (3) the absence of classical serological markers of infection, including WHsAg, anti-WHc, and anti-WHs. However, with time, the liver may become infected in POI. We define SOI or residual infection as the long-term persistence of hepadnavirus after the clinical and serological resolution of hepatitis. This form of hepadnavirus persistence may also occur after primary serum surface antigen-negative infection, as we have recently described (Coffin *et al.*, 2004). In general, in SOI, antibodies to the virus core and surface antigens can be detected, and low levels of virus DNA can be found in the serum and lymphatic system at levels comparable to those occurring in POI (usually $\leq 10^2$ vge/mL in serum and ≤ 50 vge/ 10^4 PBMC). In addition, virus DNA is always

detectable in the liver ($\leq 2 \times 10^2 \text{ vge}/10^4 \text{ cells}$).

By optimizing a procedure that did not require nucleic acid extraction from PBMC,

combined with a rigorous protocol for the removal of potentially attached extracellular virions

and WHV DNA fragments, we were able to validate previous results that SOI and POI are associated with the existence of virus in the circulating lymphoid cells. Importantly, serum virus load did not correlate with the amount of virus in PBMC. Additionally, the number of infected PBMC is not stable during the long follow-up. This is consistent with observations made in infections with other lymphotropic viruses, *i.e.*, HIV (Re *et al.*, 1994) and HCV (Muratori *et al.*, 1996).

Overall, the data obtained imply that the current methods for HBV detection, by either identification of HBsAg or HBV DNA in sera, are not sufficient to diagnose HBV in individuals with occult infection, either POI or SOI. They also suggest that the identification of hepadnavirus DNA in lymphoid cells may be the most sensitive indicator of occult virus presence. The significance of this finding is unambiguous, as discussed later, considering the potential for undetected virus to be transmitted or reactivated in the host.

It was previously established that lymphoid cells are infected with WHV very early after inoculation with a high virus dose ($\sim 10^{10}$ vge) and that this occurs long before the development of symptomatic liver disease (Michalak, 1998; Michalak, 2000). Therefore, we aimed to identify the immunovirological and molecular profiles of WHV infection after injection with decreasing virus doses. We discovered that after inoculation with as little as 10 WHV vge, the lymphoid cells were infected in the absence of liver involvement (Chapter

4). The amount of virus required to induce liver disease was identified to be above 10^3

vge/dose. Therefore, we defined a virus threshold delineating induction of POI from

serologically evident infection accompanied by hepatitis.

It has been recently shown in chimpanzees that multiple injections of small doses of SIV had a cumulative effect on the induction of disease (McDermott *et al.*, 2004). Thus,

repeated weekly SIV injections with (30 TCID₅₀; *i.e.*, 50% tissue culture infectious doses) were capable of causing disease, resulting in the immunodeficiency syndrome, which could be also induced by a single high dose of virus (*i.e.*, 3000 TCID₅₀). Therefore, it is reasonable to expect that a similar situation may exist in hepadnavirus infection. Exposure to multiple low doses of HBV or WHV may, with time, cause symptomatic evident infection.

It is also noteworthy that POI, caused by low WHV dose, is not accompanied by the development of a protective immune response, since subsequent challenge with a massive dose of wild-type virus caused classical AH. However, it is possible that specific anti-viral immune responses develop and endure for a prolonged period in POI, but at a significantly lower magnitude as that induced by infection with higher amounts of virus. This could explain why the resolution of disease caused by challenge with a liver pathogenic virus dose in animals with POI always occurred and the infection did not progress to CH.

The absence of detectable genomic variations in WHV obtained from the liver and lymphoid cells in the aforementioned *in vivo* study led us to employ an *in vitro* infection system that should enrich potential cell type-specific viral variants. Thus, splenocyte-derived, wild-type WHV was passaged in either lymphoid cells or hepatocytes, and the virus derived

used to identify if a cell type-specific virus variant arose. Comparison of WHV sequences recovered after these multiple passages of WHV in lymphoid cells or hepatocytes with the sequence of wild-type WHV present in the inoculum revealed no cell type-specific variation when the preS region of the S gene sequence, which is most prone to variations, was analysed. Based on the works of others (*e.g.*, Nainan *et al.*, 2002), it is known that an altered

subpopulation would have to comprise more than 10% of the total virus population in order to be detected by direct sequencing. Therefore, it is reasonable to assume that even if a virus variant occurred at an undetectable level, its contribution to WHV infectivity towards lymphoid cells would be negligible. The results from this study also provided further evidence in regard to the lymphotropic nature of WHV by documenting that the virus can be serially transmitted in naive lymphoid cells without compromising its infectivity and without hepatocyte involvement.

Perhaps the most unusual evidence that hepadnaviral infection of lymphoid cells precedes infection of the liver was found in an artificial model of hepadnavirus infection employing intrahepatic injection of recombinant, complete WHV DNA. This approach has previously been shown to induce WHV infection in woodchucks (Chen et al., 1992; Chen et al., 1993). For the first time, we determined the kinetics of hepadnavirus infection in the lymphatic system in this model. We employed two different rWHV DNA constructs and found that they both caused POI, which was restricted to the lymphatic system, before the establishment of the infection in hepatic tissue, if it occurred at all. Thus, we showed that WHV DNA and cccDNA was initially found only in lymphoid cells and WHV DNA in the

sera in the animals transfected. This provides further evidence that WHV is in fact a

lymphotropic virus. Even though the inoculation site was the liver, only the lymphoid cells

were infected in the early phase after rWHV DNA transfection.

The fact that DC were persistently infected during SOI suggests potentially contradictory roles of these cells in viral persistence and host immunity. First, the continued

virus presence in these cells could, by modulating antigen processing and presentation, alter anti-viral immune responses, and possibly reactions against other antigens. On the other hand, the harboured virus in DC may serve as a constant trigger that maintains anti-viral memory T cell responses, as it has been shown in other infection models (Belkaid et al., 2002).

In summary, we have concluded that WHV invasion of lymphoid cells is an absolute requirement for propagation of the virus in the host and induction of disease. Thus, regardless of the dose of invading virus, the lymphatic system is always involved. Furthermore, the persistence of the virus in lymphoid cells for life after POI or after recovery from SLAH suggests that hepadnaviral clearance never truly happens (Michalak, 2000). Close virological and pathogenic similarities between WHV and HBV imply that the same could be true for HBV infection.

Until recently, it has been generally accepted that upon exposure to large amounts of HBV, hepatocytes are the first target of the virus invasion. With an incubation period of about 1-6 months, HBsAg is detectable in serum 1 week to 2 months after exposure. This is followed by the development of symptomatic hepatitis (Chisari and Ferrari, 1996). Thus, according to a previously accepted theory, de novo produced virus engages lymphoid cells

with little or no virus propagation occurring in this compartment. Furthermore, studies

examining the effect of exposure to low amounts of virus were not performed, as it was

assumed that minute quantities of virus were not able to induce infection and generate anti-

viral immune responses.

However, our results clearly indicate that not only are lymphoid cells infected and

support replication when exposed to small virus quantities, but they may actually be the preferential targets of hepadnavirus. Our current studies suggest that previously accepted concepts need to be revised, particularly when the host is invaded by a minute amount ($<10^3$) virions) of virus, or when rWHV DNA is used. We propose that WHV, as well as rWHV DNA is initially acquired by lymphoid cells, through either a virus-receptor interaction or phagocytosis by DC, monocytes, and resident macrophages in the lymphatic organs or in the liver (i.e., Kupffer cells) (see Figure 8.1). In the lymphoid cell, WHV replication then occurs. Subsequently, the produced virus can be transmitted between cells of the immune system. If the virus load increases over time, or a yet unknown mechanism modifies its ability to interact with hepatocytes, liver cells become infected. However, during infection induced by low doses, the spread of WHV from the lymphatic system to the liver may take a significant period of time, or not occur at all. Thus, the virus may remain solely in the lymphoid cell reservoir, as it is the case in POI. Besides the contribution of virus load, local tissue or cell factors could also influence the development of different infection profiles. These could include the predominant individual cytokine milieu, MHC haplotype, or the existence of other diseases or infections, which may modulate the response of the immune system to the virus.

In general, the potential of WHV to infect hepatocytes and cause liver disease appears to be a consequence of the amount of virus that the host is exposed to and the lapse of time since virus invasion (Michalak *et al.*, 2004). It appears that hepatocytes require more virus than lymphoid cells to be infected. Thus, WHV replication in lymphoid cells may increase the amount of virus to the level required to invade liver cells as time proceeds. This may explain Figure 8.1 Schematic diagram of the involvement of the lymphatic system in the perpetuation of hepadnavirus infection induced by low WHV doses ($\leq 10^3$ vge). Upon invasion of the host, WHV (intact virions or rWHV DNA) is initially acquired by extrahepatic lymphomononuclear cells (LMC) and/or dendritic cells (DC). Small quantities of *de novo* produced virus can be transmitted from one cell subset to another, including intrahepatic lymphocytes (IHL), or they may remain in a single lymphoid cell reservoir. This state is accompanied by the lack of serological and biochemical evidence of exposure to virus, albeit WHV DNA can be detected in circulating lymphoid cells and in serum. This form of occult infection is designated as POI. It is possible that POI can proceed to acute hepatitis, as the *de novo* produced virus reaches a level that can invade a substantial number of hepatocytes. Additionally, POI could potentially lead to SOI, resulting from virus engagement of the liver,





the long incubation period, lasting for 3-4 weeks for WHV and 6 weeks to 6 months for HBV infection. Based on these results and an escalating number of other studies, it is now clear that, although the symptoms of serologically evident disease arise from the liver, the absence of hepatitis does not mean that hepadnaviral infection does not exist (Chemin et al., 1992; Michalak et al., 1994; Penna et al., 1996; Rehermann et al., 1996a; Rehermann et al., 1996b; Coffin and Michalak, 1999; Michalak et al., 1999; Michalak et al., 2004).

The findings from our studies suggest that the existence of low-level (occult) HBV infection may have important epidemiological and pathogenic implications. Thus, the existence of occult HBV infection carries the potential risk of virus transmission through blood transfusion, hemodialysis, and organ transplantation. Although the rate of HBV infection is currently low (1/63,000), it remains much higher than that of HCV (Schreiber et al., 1996). In Canada, recent statistics on the relative risk of transfusion transmitted HBV due to undetected infection in blood donation is 13.88 per million donations (i.e., 1 in 72,000) (Chiavetta et al., 2003). This risk has not decreased over the past decade, in the face of seemingly improved blood screening procedures. Occult infection, coupled with the relatively limited sensitivity of currently used immunoassays which are based on detection of serum

HBsAg, may be responsible for this risk (Schreiber et al., 1996). In terms of organ transplantation, current literature indicates that the risk of acquiring HBV infection from liver donors ranges from 25% to 94% (Dickson et al., 1997; Uemoto et al., 1998). Importantly, serum, which is mainly used for screening suitable donors, is the poorest material for detection of occult hepadnavirus infection compared to circulating lymphoid cells and liver

tissue (Michalak et al., 1999).

Cryptogenic liver diseases, such as cirrhosis and HCC, may be explained, at least in part, by the existence of occult HBV infection (Hu, 2002). The detection of persistent HBV cccDNA and RNA in patients up to 5 years after recovery from SLAH type B indicated a prolonged period of low level virus replication that could, with time, be able to induce a HBV-related chronic liver disease (Mason et al., 1998). Retrospective analysis of non A-E hepatitis have indicated that 78.3% of patients with hepatitis of unknown aetiology carried low levels of HBV DNA in the liver, which were only detectable by specific nested PCR (He et al., 2003). Another study assessed 16 patients who had resolved AH type B for up to 30 years prior to enrollment (Blackberg and Kidd-Ljunggren, 2001). Even though serum HBV DNA remained negative, liver biopsies were found HBV DNA positive, and a mild ALT elevation was detected in one patient. This suggests a probable underestimation of the true number of HBV related liver disease cases, as occult HBV is not detectable by currently applied serological assays, as stated above.

HCC has developed in about 20% of woodchucks convalescent from acute WHV hepatitis (Michalak et al., 1999). Very little evidence is available to fully ascertain if occult

HBV infection is responsible for HCC of unknown origin in humans. Recently, however, the

prevalence of occult HBV DNA in the livers of HCC patients was determined by sensitive

PCR techniques comparable to those used in our studies (Pollicino et al., 2004). Surprisingly,

HBV DNA was detected in as many as 63.5% of cases of HCC. Also, HBV DNA has been

identified in tumour tissue (Paterlini et al., 1990), usually in an integrated form (Paterlini-

Brechot *et al.*, 2003). Another study found HBV integration in 9 of 9 HCC tumours studied, often in the human telomerase gene. Further, a study examining HBV/HCV co-infected individuals suggested that integrated HBV DNA may be required for the development of HCC in chronic HCV infection (Fukuda *et al.*, 1999). Thus, occult HBV infection cannot be ruled out in the pathogenesis of cryptogenic HCC and, therefore, could represent another potentially significant pathological consequence of this form of hepadnaviral persistence.

The other pathogenic consequence of occult HBV might be evident in the context of other viral infections, like HCV and HIV, or during immunosupression and/or chemotherapy. Because of the common routes of transmission, HBV/HCV co-infection is a common clinical occurrence. Up to 55% of patients with chronic HCV carry anti-HBc (Brechot *et al.*, 1998). We recently proved that, in the woodchuck model, anti-WHc is an indicator of continuing occult hepadnavirus replication and persistence (Coffin *et al.*, 2004). Similarly, up to 87% of the anti-HBc reactive patients demonstrate the presence of the HBV genome (Kazemi-Shirazi *et al.*, 2000). Clinically, the impact of HBV on HCV co-infection is unknown. Recently, HBV DNA was found to be integrated in PBMC DNA from patients with chronic active hepatitis B and in individuals after the clearance of HBsAg who are co-infected with

HCV (Murakami et al., 2004). Also of note is that hepatoma cells transfected with full-length

HBV cloned from patients with occult HBV infection co-existing with HCV infection showed

the replicative capacity of this HBV (Uchida et al., 1997).

The epidemiological and pathogenic consequences of occult HBV infection are not yet recognized. The results from studies using the woodchuck-WHV model suggest that the

transmission, pathogenicity, and oncogenicity of occult hepadnavirus might be important in the human disease situation. Therefore, it is evident that under no circumstance should blood or organs from individuals with occult HBV infection should be used for transfusion or organ transplantation.



CHAPTER 9: SUMMARY AND CONCLUSIONS

Over the course of this series of studies, we investigated the lymphotropic nature of hepadnavirus and to what extent infection of the lymphatic system may contribute to its long-term persistence. We applied the woodchuck-WHV infection system, which is the closest animal natural model of HBV infection and hepatitis B. The results of our research revealed new important aspects of the natural history of hepadnaviral infection and uncovered previously unknown properties of the virus. Investigations of woodchucks with different forms of experimentally induced WHV infection, frequently over the lifetime of the host, allowed for the collection of unique materials which would not be available from individuals infected with HBV. The results obtained in these studies can be summarized and concluded as follows:

1. Examination of the presence of WHV in lymphoid cells from animals with symptomatic chronic hepatitis and with primary occult infection (*i.e.*, POI) or secondary (residual) occult infection (*i.e.*, SOI) confirmed that the lymphatic system is invariably infected regardless of

whether WHV infection is serologically evident or occult. We have established a novel *in situ* PCR technique combined with flow cytometry to quantify WHV-infected cells without the necessity of nucleic acid extraction. The study demonstrated that a significant portion of circulating lymphoid cells carry the virus genome. Numbers of WHV DNA-positive cells ranged between 3.4 and 20.4% for symptomatic chronic infection and between 1.1 and 14.6%

for persistent occult infection. We also showed that the detection of WHV genome in peripheral lymphoid cells does not correlate with the serum WHV load. These results provide conclusive evidence that WHV infection continues indefinitely in the lymphatic system independently of the level at which the virus persists in the host.

2. We confirmed the existence of primary occult WHV infection (*i.e.*, POI) and discovered that its induction is determined by the dose of invading virus, but unlikely is due to the existence or emergence during infection of virus variant(s) specifically predisposed to infect lymphoid cells. Based on our detailed *in vivo* study examining the outcome of the infection induced by decreasing doses of a well-characterized, wild-type WHV, we established that the virus quantities lower than or equal to 10^3 vge cause infection restricted to the lymphatic system that does not engage the liver, *i.e.*, POI. We have also found that POI does not induce immune protection against challenge with a high, liver pathogenic (> 10^3 vge) virus dose. In contrast, large doses of WHV (*i.e.*, > 10^3 vge) cause infection involving both the lymphatic system and the liver, and enable the development of virus-specific protective immune responses. This study documents that the lymphatic system is the primary target of WHV

when small quantities of virions invade a susceptible host and that two distinctive forms of

occult hepadnavirus infection, POI and SOI, occur in nature.

3. By employing conditions allowing WHV serial passage in cultured woodchuck lymphocytes and hepatocytes, we demonstrated *in vitro* that the lymphotropic nature of the
virus is indeed not related to the emergence of a viral variant(s) preferentially infecting or replicating in lymphoid cells but is an intrinsic property of the wild-type virus. The virus sequences detected after serial passage of WHV in either lymphoid cells or hepatocytes (in total 13 passages in each cell type) were identical. Their sequences were the same as that of the wild-type virus present in inoculum. In addition, we clearly established that WHV can be maintained in culture, although at low levels, in both lymphoid cells and hepatocytes for prolonged periods of time without compromising the virus infectivity and pathogenic potential.

4. For the first time, we investigated the extent to which the lymphatic system is involved in WHV infection induced by intrahepatic transfection with unenveloped, recircularized, complete rWHV DNA. We have documented that the rWHV DNA induces, in the first instance, infection of the lymphatic system before eventually engaging the liver. In addition, we have shown that the virus produced by lymphoid cells during the early time period after transfection, prior to liver involvement, caused classical hepatitis when administered to healthy woodchucks, confirming that such derived virus is infectious and liver pathogenic.

Further, monocytic DC generated from animals with POI induced by transfection with rWHV

DNA harbored WHV DNA and its replicative cccDNA, illustrating the importance of DC in

the maintenance of POI.

5. Finally, by employing a protocol used for the derivation of monocytic DC from other mammals, we were able to establish DC from woodchucks having either chronic WHV hepatitis or SOI. We confirmed the DC identity by determining expression of the CD209 gene that encodes the CD209 molecule, specifically displayed on the surface of DC. For this purpose, we cloned and sequenced a portion of the woodchuck CD209 transcript. We demonstrated that DC are a reservoir of replicating WHV in animals with serologically evident chronic infection, by detecting WHV DNA, virus cccDNA and RNA replicative intermediates. Importantly, we also identified WHV DNA and cccDNA in DC derived from animals with SOI. These results strongly suggest that DC are a reservoir of persistent low-level WHV replication, regardless of whether the infection is serologically evident or occult.



CHAPTER 10: FUTURE DIRECTIONS

The results obtained in the course of these studies have identified several important aspects of hepadnaviral infection which warrant further investigations. These issues include:

1. How does hepadnavirus invade lymphoid cells? Further investigations should aim to elucidate the nature of the virus receptor(s) on lymphoid cells and to ascertain the mechanism(s) by which both intact virions and unenveloped rWHV DNA can be taken up and propagated in these cells. Experiments are needed to identify if there are different virus requirements for the infection of lymphoid cells and hepatocytes, for example, if a modification of viral surface proteins (envelope) is required prior to attachment to hepatocytes, while lymphoid cells can be infected by virus with a native unmodified envelope. Collection of lymphoid cells and hepatic tissue samples shortly after exposure to contrastingly different quantities of WHV and evaluations of virus presence in these samples, determined by sensitive molecular techniques, could delineate the time necessary after invasion to infect lymphoid cells. The role of lymphoid cells in virus transmission to the liver and the importance of the initial virus load in the development of serologically detectable infection

associated with hepatitis also needs to be investigated in future studies.

2. Is the life-long persistence of trace amounts of hepadnavirus in lymphoid cells required to

maintain virus-specific memory immune responses? As seen in other infections, the

persistence of virus traces may play a role in the continued re-stimulation of the immune system and in the perpetuation of anti-viral T cell and humoral immune responses.

3. Which lymphoid cell subsets support the long-term persistence of WHV and to what extent? The production of woodchuck-specific reagents to identify lymphoid cell surface markers and their utilization, in concert with other techniques (*e.g.*, *in situ* PCR for WHV DNA) could provide information about which lymphoid cells are preferentially infected by the virus and in which cell subset(s) virus replication persists. These findings may contribute to a better understanding of how the immune system is modulated by WHV infection.

4. How does infection of DC contribute to the establishment of WHV persistence and modification of the function of these cells? Studies examining DC function in various forms of WHV infection, *i.e.*, POI, SOI or CH, could indicate if DC are altered in their development and/or function.

5. Does the infection of the lymphatic system contribute to the final outcome of

hepadnavirus-induced disease? Since it is known that the induction of Th1 type responses is critical in evoking effective antiviral responses able to control infection, the potential infection of the lymphoid cells with WHV or the ability of virus proteins (*e.g.*, WHV envelope proteins) to interact with different immune cell subsets and modulate their function needs to be investigated. This could explain mechanisms of the development of serologically evident or occult WHV infections.

6. Are there any significant differences between POI and SOI in terms of the magnitude of induced WHV-specific immunity? Further studies to identify the induction of protective antiviral immune responses in these two distinct forms of hepadnaviral infection should be evaluated. Also, the cumulative effects of exposure to multiple small quantities of virus in the establishment of persistent infection are yet unknown and require examination.

7. The most important question, and most difficult to study, is the nature of the potential long-term consequences of primary occult hepadnavirus infection caused by unapparent exposure to and subsequent low level persistence of virus in seemingly healthy individuals. Determination of the occurrence of POI in humans is warranted based on our findings in the woodchuck-WHV model, since it is now evident that trace quantities of hepadnavirus are capable of replication and can evade complete eradication from lymphoid cells and hepatocytes. This suggests that, under certain circumstances, reactivation of apparently dormant virus may occur during POI or SOI. Further studies of individuals with multiple

exposure to small amounts of HBV (e.g., health care professionals) could ultimately

determine the worldwide rate of HBV exposure. This could further identify the mechanisms

and long-term consequences of HBV persistence, such as HCC and cirrhosis or proliferative

disorders of the lymphatic system.

REFERENCES CITED

Abbas A.K., Lichtman A.H., Pober.J.S. (2000): Cellular and Molecular Immunology, pp. 1-50, 4th ed. W.B. Saunders Company, Philadelphia, PA.

Ahmed R. (1992): Immunological memory against viruses. Semin. Immunol. 4:105-109.

Ahmed R. and Gray D. (1996): Immunological memory and protective immunity: understanding their relation. Science 272:54-60.

Akbar S.M., Horiike N., Onji M., Hino O. (2001): Dendritic cells and chronic hepatitis virus carriers. Intervirology 44:199-208.

Alcami A. and Koszinowski U.H. (2000): Viral mechanisms of immune evasion. Immunol. Today 21:447-455.

Ando K., Moriyama T., Guidotti L.G., Wirth S., Schreiber R.D., Schlicht H.J., Huang S.N., Chisari F.V. (1993): Mechanisms of class I restricted immunopathology. A transgenic mouse model of fulminant hepatitis. J. Exp. Med. 178:1541-1554.

Andrews D.M., Andoniou C.E., Granucci F., Ricciardi-Castagnoli P., Degli-Esposti M.A. (2001): Infection of dendritic cells by murine cytomegalovirus induces functional paralysis. Nat. Immunol. 2:1077-1084.

Angus P.W., Locarnini S.A., McCaughan G.W., Jones R.M., McMillan J.S., Bowden D.S. (1995): Hepatitis B virus precore mutant infection is associate with severe recurrent disease after liver transplantation. Hepatology **21**:14-18.

Arima S., Akbar S.M., Michitaka K., Horiike N., Nuriya H., Kohara M., Onji M. (2003): Impaired function of antigen-presenting dendritic cells in patients with chronic hepatitis B: localization of HBV DNA and HBV RNA in blood DC by *in situ* hybridization. Int. J. Mol.

Med. 11:169-174.

Asahina Y., Enomoto N., Ogura Y., Sakuma I., Kurosaki M., Izumi N., Marumo F., Sato C. (1996): Complete nucleotide sequences of hepatitis B virus genomes associated with epidemic fulminant hepatitis. J. Med. Virol. 48:171-178.

Atabani S.F., Byrnes A.A., Jaye A., Kidd I.M., Magnusen A.F., Whittle H., Karp C.L. (2001): Natural measles causes prolonged suppression of interleukin-12 production. J. Infect. Dis. **184**:1-9. Auffermann-Gretzinger S., Keeffe E.B., Levy S. (2001): Impaired dendritic cell maturation in patients with chronic, but not resolved, hepatitis C virus infection. Blood 97:3171-3176.

Bagasra O., Thikkavarapu S., Pomerantz R., Hansen J. (1995): *In situ* PCR and hybridization to detect low-abundance nucleic acid targets, pp. 14.8.18-14.8.24. In F. A. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (eds.), *Current Protocols in Molecular Biology*. John Wiley & Sons, Inc., New York, NY.

Bain C., Fatmi A., Zoulim F., Zarski J.P., Trepo C., Inchauspe G. (2001): Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. Gastroenterology **120**: 512-524.

Bancherau J. and Steinman R.M. (1998): Dendritic cells and the control of immunity. Nature **392**:245-252.

Bartenschlager R. and Schaller H. (1992): Hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal in the viral RNA genome. EMBO J. 11:3413-3420.

Bavand M.R. and Laub O. (1988): Two proteins with reverse transcriptase activities associated with hepatitis B virus-like particles. J. Virol. 62:626–628.

Beasley R.P. (1988): Hepatitis B virus: The major etiology of hepatocellular carcinoma. Cancer 61:1942–1956.

Beckebaum S., Cicinnati V.R., Dworacki G., Muller-Berghaus J., Stolz D., Harnaha J., Whiteside T.L., Thomson A.W., Lu L., Fung J.L., Bonham C.A. (2002): Reduction in the circulating pDC1/pDC2 ratio and impaired function of *ex vivo*-generated DC1 in chronic hepatitis B infection. Clin. Immunol. **104**:138-150.

Beckebaum S., Cicinnati V.R., Zhang X., Ferencik S., Frilling A., Grosse-Wilds H., Broelsch C.E., Gerken G. (2003): Hepatitis B virus-induced defect of monocyte-derived dendritic cells leads to impaired T helper type 1 response *in vitro*: mechanisms for viral immune escape. Immunology **109**:487-495.

Belkaid Y., Piccirillo C.A., Mendez S., Shevach E.M., Sacks D.L. (2002): CD4+ CD25+ regulatory T cells control Leishmania major persistence and immunity. Nature **420**:502-507.

Bendelac A., Rivera M.N., Park S.H., Roark J.H. (1997): Mouse CD1-specific NK1 T cells: Development, specificity, and function. Annu. Rev. Immunol. 15:535–562.

Bertoletti A., Costanzo A., Chisari F.V., Levrero M., Artini M., Sette A., Penna A., Giuberti T., Fiaccadori F., Ferrari C. (1994): Cytotoxic T lymphocyte response to a wild type hepatitis B virus epitope in patients chronically infected by variant viruses carrying substitutions within the epitope. J. Exp. Med. 180:933-943.

Biron C.A. (1997): Activation and function of natural killer cell responses during viral infections. Curr. Opin. Immunol. 9:24-34.

Biron C.A. and Brossay L. (2001): NK cells and NKT cells in innate defense against viral infections. Curr. Opin. Immunol. 13:458-464.

Bläckberg J. and Kidd-Ljunggren K. (2001): Occult hepatitis B virus after acute self-limited infection persisting for 30 years without sequence variation. J. Hepatol. 33:992-997.

Blum H.E., Liang E., Galun E., Wands J.R. (1991): Persistence of hepatitis B virus DNA after serological recovery from hepatitis B virus infection. Hepatology 14: 56-63.

Blumberg B.S., Gerstley B.J.S., Hungerford D.A., London W.T., Sutnick, A.I. (1967): A serum antigen (Australia antigen) in Down's syndrome, leukemia and hepatitis. Ann. Intern. Med. 66:924-931.

Borrow P., Lewicki H., Wei X., Horwitz M.S., Peffer N., Meyers H., Nelson J.A., Gairin J.E., Hahn B.H., Oldstone M.B., Shaw G.M. (1997): Antiviral pressure exerted by HIV-1specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat. Med. 3:205-211.

Bouffard P., Lamelin J.P., Zoulim F., Pichoud C., Trepo C. (1990): Different forms of hepatitis B virus DNA and expression of HBV antigens in peripheral blood mononuclear cells in chronic hepatitis B. J. Med. Virol. 31:312-317.

Brechot C. (1998): Molecular mechanisms of hepatitis B and C viruses related to liver carcinogenesis. Hepatogastroenterology 45 (Suppl 3):1189-1196.

Bréchot, C., Thiers V., Kremsdorf, D. Naplas B., Pol S., Paterlini-Bréchot P (2001): Persistent hepatitis B virus infection in subjects without hepatitis B surface antigen: clinically significant or purely "occult"? Hepatology 34:194-203.

Brunetto M.R., Stemler M., Schodel F., Will H., Ottobrelli A., Rizzetto M., Verme G., Bonino F. (1989): Identification of HBV variants which cannot produce precore derived HBeAg and may be responsible for severe hepatitis. Ital. J. Gastroenterol. 21: 151-154.

Burdin N. and Kronenberg M. (1999): CD1-mediated immune responses to glycolipids. Curr. Opin. Immunol. 11:326-331.

Burgert H.G., Ruzsics Z., Obermeier S., Hilgendorf A., Windheim M., Elsing A. (2002): Subversion of host defense mechanisms by adenoviruses. Curr. Top. Microbiol. Immunol. 269: 273-318.

Buti M., Jardi R., Cotrina M., Rodriguez-Frias F., Esteban R., Guardia J. (1998): Transient emergence of hepatitis B variants in a patient with chronic hepatitis B resistant to lamivudine. J. Hepatol. 28:510-513.

Cabrerizo M., Bartolomé J., Caramelo C., Barril G., Carreño V. (2000): Molecular analysis of hepatitis B virus DNA in serum and peripheral blood mononuclear cells from hepatitis B surface antigen-negative cases. Hepatology **32:**116-123.

Cacciola I., Pollicino T., Squadrito G., Cerenzia G., Orlando M. E., Raimondo G. (1999): Occult hepatitis B virus infection in patients with chronic hepatitis C liver disease. N. Engl. J. Med. **341**:22-2699.

Calmus Y., Marcellin P., Beaurain G., Brechot C. (1994): Distribution of hepatitis B virus DNA sequences in different peripheral blood mononuclear cell subsets in HBs antigenpositive and -negative patients. Eur. J. Clin. Invest. 24:548-552.

Canadian Consensus on the Management of Viral Hepatitis, Proceedings of a Consensus Conference, Montreal, Quebec, March, 1999, available at: www.lhsc.on.ca/casl/cont.htm

Canadian Communicable Disease Report, 2002, Blood-Borne Pathogens, available at: www.hc-sc.gc.ca/pphb-dgspsp/publicat/ccdr-rmtc/02vol28/28s2/index.html.

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

Carman W., Zanetti A.R., Karayiannis P., Waters J., Manzillo G., Tanzi E., Zuckerman A.J., Thomas H.C. (1990): Vaccine-induced escape mutant in hepatitis B virus. Lancet **336**:325-329.

Carman W.F., Zanetti A.R., Karayiannis P., Waters J., Manzillo G., Tanzi E., Zuckerman A.J., Thomas H.C. (1991): Association of a precore genomic variant of hepatitis B virus with fulminant hepatitis. Hepatology 14:219-222.

Carman W.F. and Thomas H.C. (1992): Genetic variation in hepatitis B virus. Gastroenterology. 102:711-719.

Carman W.F. (1996): Molecular variants of hepatitis B virus. Clin. Lab. Med. 16:407-428.

Carpenter P.A., Huang M.L., McDonald G.B. (2002): Activation of occult hepatitis B from a seronegative patient after hematopoietic cell transplant: a cautionary tale. Blood **99**:4245-4246.

Caux C., Dezutter-Dambuyant C., Schmitt D., Bancherau J. (1992): GM-CSF and TNFalpha cooperate in the generation of dendritic Langerhans cells. Nature **360**:258-261.

Cavanaugh V.J., Guidotti L.G., Chisari F.V. (1998): Inhibition of hepatitis B virus replication during adenovirus and cytomegalovirus infections in transgenic mice. J. Virol. 72:2630–2637.

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

Chemin I., Vermot-Desroches C., Baginski I., Saurin J.C., Laurent F., Zoulim F., Bernard J., Lamelin J.P., Hantz O., Rigal D., Trepo C. (1992): Selective detection of human hepatitis B surface and core antigen in peripheral blood mononuclear cells subsets by flow cytometry. J. Clin. Lab. Immunol. **38**:63-71.

Chemin I., Vermot-Desroches C., Baginski I., Lamelin J.P., Hantz O., Jacquet C., Rigal D., Trepo C. (1993): Monitoring of early events of experimental woodchuck hepatitis infection: studies of peripheral blood mononuclear cells by cytofluorometry and PCR. FEMS Immunol. Med. Microbiol. 7:241-249.

Chemin I., Zoulim F., Merle P., Arkhis A., Chevallier M., Kay A., Cova L., Chevallier P., Mandrand B., Trepo C. (2001): High incidence of hepatitis B infection among chronic hepatitis cases of unknown aetiology. J. Hepatol. **34**:447-454.

Chen H.S., Kew M.C., Hornbuckle W.E., Tennant B.C., Cote P.J., Gerin J.L., Purcell R.H., Miller R.H. (1992): The precore gene of the woodchuck hepatitis virus genome is not essential for viral replication in the natural host. J. Virol. 66:5682-5684.

Chen H.S., Kaneko S., Girones R., Anderson R.W., Hoenbuckle W.E., Tennant B.C., Cote P.J., Gerin J.L., Miller R.H. (1993): The woodchick hepatitis virus X gene is important for establishment of virus infection in woodchucks. J. Virol. 67:1218-1226.

Chen H.S., Miller R.H., Hornbuckle W.E., Tennant B.C., Cote P.J., Gerin J.L., Purcell R.H. (1998): Titration of recombinant woodchuck hepatitis virus DNA in adult woodchucks. J. Med. Virol. 54:92-94.

Chiavetta J. A., Escobar M., Newman A., He Y., Driezen P., Deeks S., Hone D.E., O'Brien S.F., Sher G. (2003): Incidence and estimated rates of residual risk for HIV, hepatitis C, hepatitis B and human T-cell lymphotropic viruses in blood donors in Canada, 1999-2000. CMAJ. 169:767-773.

Chisari F.V. and Ferrari C. (1995): Hepatitis B virus immunopathogenesis. Ann. Rev. Immunol. 13:29-60.

Chisari F.V. and Ferrari C. (1996): Viral hepatitis. pp. 745-778. In: R. Ahmed, K.V. Holmes, F. Gonzalez-Scarano, F.A. Murphy, D.E. Griffin, H.L. Robinson, (eds.) Viral pathogenesis. Lippincott-Raven Publishers, Philadelphia, USA.

Chisari F.V. (2000): Rous-Whipple Award Lecture. Viruses, immunity, and cancer: lessons from hepatitis B. Am. J. Pathol. 156:1117-1132.

Chu C.J. and Lok A.S. (2002): Clinical utility in quantifying serum HBV DNA levels using PCR assays. J. Hepatol. 36:549-551.

Chu C.M., Yeh C.T., Lee C.S., Sheen I.S., Liaw Y.F. (2002): Precore stop mutant in HBeAg-positive patients with chronic hepatitis B: clinical characteristics and correlation with the course of HBeAg-to-anti-HBe seroconversion. J. Clin. Microbiol. 40:16-21.

Churchill N.D. and Michalak T.I. (2004): Woodchuck hepatitis virus hepatocyte culture models. pp. 175-187, R.K., Hamatake and J.Y.N. Lau (eds.), In Methods in Molecular Medicine: Hepatitis B and D Protocols, vol. 2. Humana Press Inc., Totowa, NJ.

Coffin C.S. and Michalak T.I. (1999): Persistence of infectious hepadnavirus in the offspring of woodchuck mothers recovered from viral hepatitis. J. Clin. Invest. 104:203-212.

Coffin C.S., Pham T.N.Q., Mulrooney P.M., Churchill N.D., Michalak T.I. (2004): Persistence of isolated antibodies to woodchuck hepatitis virus core antigen is indicative of occult virus infection. Hepatology 40:1053-1061.

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

Cohen O.J. and Fauci A.S. (2001): Pathogenesis and medical aspects of HIV-1 infection, pp. 2043-2094, In D.M. Knipe and P.M. Howley, (eds.), *Field's Virology*, 4th ed. vol. 2. Lippincott, Williams, & Wilkins, Hagerstown, MD.

Colacino J. M. and Staschke K. A. (1998): The identification and development of antiviral agents for the treatment of chronic hepatitis B virus infection. pp. 259-321. In E. Jucker, (ed.), *Progress in Drug Research*. Birkhauser Verlag, Basel, Switzerland.

Colonna, M. (2004): Viral immunosuppression: disabling the guards. J. Clin. Invest. 113:660-662.

Colucci, G., Lyons P., Beazer Y., Waksal, S.D.(1988): Production of hepatitis B virusinfected human B-cell hybridomas: transmission of the viral genome to normal lymphocytes in cocultures. Virology **164**:238-244.

Combes B., Shorey J., Barrera A., Stastny P., Eigenbrodt E.H., Hull A.R., Carter N.W. (1971): Glomerulonephritis with deposition of Australia antigen-antibody complexes in glomerular basement membrane. Lancet 2:234-237.

Conjeevaram H. and Lok A. S. (2001): Occult hepatitis B virus infection: a hidden menace? Hepatology **34**:204-206.

Cote P.J., Korba B.E., Baldwin B., Hornbuckle W.E., Tennant B.C., Gerin J L. (1992): Immunosuppression with cyclosporin during the incubation period of experimental woodchuck hepatitis virus infection increases the frequency of chronic infection in adult woodchucks. J. Infect. Dis. **166**:628-631.

Cote P.J., Korba B.E., Miller R.H., Jacob J.R., Baldwin B.H., Tennant B.C., Gerin J.L. (2000a): Effects of age and viral determinants on chronicity as an outcome of experimental woodchuck hepatitis virus infection. Hepatology **31**:190-200.

Cote P.J., Toshkov I., Bellezza C., Ascenzi M., Roneker C., Ann Graham L., Baldwin B.H., Gaye K., Nakamura I., Korba B.E., Tennant B.C., Gerin J.L. (2000b): Temporal pathogenesis of experimental neonatal woodchuck hepatitis virus infection: increases initial viral load and decreased severity of acute hepatitis during the development of chronic viral infection. Hepatology **32**:807-817.

Cova L., Mehrotra R., Wild C.P., Chutimataewin S., Cao S.F., Duflot A., Prave M., Yu S.Z., Montesano R., Trepo C. (1994): Duck hepatitis B virus infection, aflatoxin B1 and liver cancer in domestic Chinese ducks. Br. J. Cancer 69:104-109.

Cremer I., Dieu-Nosjean M.C., Marechal S., Dezutter-Dambuyant C., Goddard S., Adams D., Winter N., Menetrier-Caux C., Sautes-Fridman C., Fridman W.H., Mueller C.G. (2002): Long-lived immature dendritic cells mediated by TRANCE-RANK interaction. Blood 100:3636-3655.

Croen K.D., Ostrove J.M., Dragovic L.J., Straus S.E. (1988): Patterns of gene expression and sites of latency in human nerve ganglia are different for varicella-zoster and herpes simplex viruses. Proc. Natl. Acad. Sci. USA 85:9773-9777.

Csepregi A., Rojkovich B., Nemesanszky E., Poor G., Hejjas M., Horanyi M. (2000): Chronic seropositive polyarthritis associated with hepatitis B virus-induced chronic liver disease: a sequel of virus persistence. Arthritis Rheum. 43:232-233.

Cui J., Shin T., Kawano T., Sato H., Kondo E., Toura I., Kaneko Y, Koseki H., Kanno M., Taniguchi M. (1997): Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. Science 278:1623-1626.

Curry M.P. and Koziel M. (2000): The dynamics of the immune response in acute hepatitis B: new lessons using new techniques. Hepatology **32**:1177-1179.

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

Davidson F., Alexander G.J., Anastassakos C., Fagan E.A., Williams R. (1987): Leukocyte hepatitis B virus in acute and chronic hepatitis B infection. J. Med. Virol. 22:379-385.

de Martino M., Rossi M.E., Muccioli A.T., Resti M., Vierucci A. (1985): Interference of hepatitis B virus surface antigen with natural killer cell function. Clin. Exp. Immunol. 61:90-95.

Demetris A.J., Todo S., Van Theil D.H., Fung J.J., Iwaki Y., Sysyn G., Ming W., Trager J.,

Starzl T.E. (1990): Evolution of hepatitis B virus liver disease after hepatic replacement. Practical and theoretical considerations. Am. J. Pathol. **137**: 667-676.

Diao J. and Michalak T.I. (1996): Composition, antigenic properties and hepatocyte surface expression of the woodchuck asialoglycoprotein receptor. J. Recept. Signal Transduct. Res. **16**:243-271.

Diao J. and Michalak T.I. (1997): Virus-induced anti-asialoglycoprotein receptor autoimmunity in experimental hepadnaviral hepatitis. Hepatotogy 25:689-696.

Diao J., Churchill N.D., Michalak T.I. (1998): Complement-mediated cytotoxicity and inhibition of ligand binding to hepatocytes by woodchuck virus-induced autoantibodies to asialoglycoprotein receptor. Hepatology **27**:1623-1631.

Diao J., Garces R., Richardson C.D. (2001a): X protein of hepatitis B virus modulates cytokine and growth factor related signal transduction pathways during the course of viral infections and hepatocarcinogenesis. Cytokine Growth Factor Rev. 12:189-205.

Diao J., Khine A.A., Sarangi F., Hsu E., Iorio C., Tibbles L.A., Woodgett J.R., Penninger J., Richardson C.D. (2001b): X protein of hepatitis B virus inhibits Fas-mediated apoptosis and is associated with upregulation of the SAPK/JNK pathway. J. Biol. Chem. 276:8328-8340.

Diao J., Slaney D.M., Michalak T.I. (2003): Modulation of the outcome and severity of hepadnaviral hepatitis in woodchucks by antibodies to hepatic asialoglycoprotein receptor. Hepatology **38**:629-638.

Dickson R.C., Everhart J.E., Lake J.R., Wei Y., Seaberg E.C., Wiesner R.H., Zetterman R.K., Pruett T.L., Ishitani M.B., Hoofnagle J.H. (1997): Transmission of hepatitis B by transplantation of livers from donors positive for antibody to hepatitis B core antigen. The National Institute of Diabetes and Digestive and Kidney Diseases Liver Transplantation Database. Gastroenterology **113**:1668-1674.

Dienstag J.L. and Purcell R.H. (1977): Recent advances in the identification of hepatitis viruses. Post. Grad. Med. J. 53:364-73.

Doherty D.G., Norris S., Madrigal-Estebas L., McEntee G., Traynor O., Hegarty J.E., O'Farrelly C. (1999): The human liver contains multiple populations of NK cells, T cells, and CD3+CD56+ natural T cells with distinct cytotoxic activities and Th1, Th2, and Th0 cytokine secretion patterns. J. Immunol. 163:2314-2321.

Dokun A., Kim S., Smith H.R., Kang H.S., Chu D.T., Yokoyama W.H. (2001): Specific and non-specific NK cell activation during virus infection. Nat. Immunol. 2:9551-956.

Domingo E., Diez J., Martinez M.A., Hernandez J., Holguin A., Borrego B., Mateu M.G. (1993): New observations on antigenic diversification of RNA viruses. Antigenic variation is not dependent on immune selection. J. Gen. Virol. 74:2039-2045.

Dubensky T.W., Campbell B.A., Villarreal L.P. (1984): Direct transfection of viral and plasmid DNA into the liver or spleen of mice. Proc. Natl. Acad. Sci. USA 81:7529-7533.

Dzwonkowski P. and Michalak T.I. (1990): Autoantibody pattern in a woodchuck model of hepatitis B. Clin. Invest. Med. 13: 322-328.

Elfassi E., Romet-Lemonne J.L., Essex M., McLane M.F., Haseltine W.A. (1984): Evidence of extrachromosomal forms of hepatitis B viral DNA in a bone marrow culture obtained from a patient recently infected with hepatitis B virus. Proc. Natl. Acad. Sci. USA **81**:3526-3528.

Embretson J., Zupancic M., Ribas J.L., Burke A., Racz P., Tenner-Racz K., Haase A.T. (1993): Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. Nature **362**:2359-362.

Engelmayer J., Larsson M., Subklewe M., Chahroudi A., Cox W.I., Steinman R.M., Bhardwaj N. (1999): Vaccinia virus inhibits the maturation of human dendritic cells: a novel mechanism of immune evasion. J. Immunol. 163:6762-6768.

Fan Y.F., Lu C.C., Chen W.C., Yao W.J., Wang H.C., Chang T.T. Lei H.Y., Shiau A.L., Su I.J. (2001) Prevalence and significance of hepatitis B virus (HBV) pre-S mutants in serum and liver at different replicative stages of chronic HBV infection. Hepatology **33**:277-286.

Feinberg H., Mitchell D.A., Drickamer K, Weis W.I. (2001): Structural basis for selective recognition of oligosaccharides by DC-SIGN and DC-SIGNR. Science 294:2163-2166.

Feitelson M.A., Duan L.X., Guo J., Horiike N., McIntyre G., Blumberg B.S., Thomas H.C., Carman W. (1995): Precore and X region mutants in hepatitis B virus infections among renal dialysis patients. J. Viral Hepat. 2:19-31.

Feray C., Zignego A.L., Samuel D., Bismuth A., Reynes M., Tiollais P., Bismuth H., Brechot C. (1990): Persistent hepatitis B virus infection of mononuclear blood cells without concominant liver infection. The liver transplantation model. Transplantation **49**: 1155-1158.

Fernandez J., Taylor D., Morhardt D.R., Mihalik K., Puig M., Rice C.M., Feinstone S.M., Major M.E. (2004): Long-term persistence of infection in chimpanzees inoculated with an infectious hepatitis C clone is associated with a decrease in the viral amino acid substitution rate and low levels of heterogeneity. J. Virol. 78:9782-9789.

Ferrari C., Penna A., Bertoletti A., Valli A., Antoni A.D., Giuberti T., Cavalli A., Petit M. A., Fiaccadori F. (1990): Cellular immune responses to hepatitis B virus-endoded antigens in acute and chronic hepatitis B infection. J. Immunol. 145: 3442-3449.

Flomberg P., Gutierrez E., Hogan K.T. (1994): Identification of class I MHC regions which bind to the adenovirus E3-19K protein. Mol. Immunol. **31**:1277-1284.

Fortuin M., Karthigesu V., Allison L., Howard C., Hoare S., Mendy M., Whittle H.C. (1994): Breakthrough infections and identification of a viral variant in Gambian children immunized with hepatitis B vaccine. J. Infect. Dis. 169:1374-1376.

Freiman J.S., Jilbert A.R., Dixon R.J., Holmes M., Gowans E.J., Burrell C.J., Wills E. J., Cossart Y.E. (1988): Experimental duck hepatitis B virus infection: pathology and evolution of hepatic and extrahepatic infection. Hepatology 8:507-513.

Fu L. and Cheng Y.C. (1998): Role of additional mutations outside the YMDD motif of hepatitis B virus polymerase in L(-) SddC (3TC) resistance. Biochem. Pharmacol. 55: 1567-1572.

Fukada R., Ishimura N., Niigaki M., Hamamoto S., Satoh S., Tanaka S., Kushiyama Y., Uchida Y., Ihihara S., Akagi S., Watanabe M., Kinoshita Y. (1999): Serologically silent hepatitis B virus coinfection in patients with hepatitis C virus-associated chronic liver disease: clinical and virological significance. J. Med. Virol. 58:201-207.

Funk A., Hohenberg H., Mhamdi M., Will H., Sirma H. (2004): Spread of hepatitis B viruses *in vitro* requires extracellular progeny and may be codetermined by polarized egress. J. Virol. **78**: 3977-3983.

Galibert F., Chen T.N., Mandart E. (1993): Nucleotide sequence of a cloned woodchuck hepatitis virus genome: Comparison with the hepatitis B virus sequence. J. Virol. 41:51-65.

Gavilanes F., Gonzalez-Ros J.M., Peterson D.L. (1982): Structure of hepatitis B surface antigen: Characterization of the lipid components and their association with the viral proteins. J. Biol. Chem. 257:7770–7777.

Geijtenbeek T.B., Kwon D.S., Torensma R., van Vliet S.J., van Duijnhoven G.C., Middel J., Cornelissen I.L., Nottet H.S., KewalRamani V.N., Littman D.R., Figdor C.G., van Kooyk Y. (2000): DC-SIGN, a dendritic cell specific HIV-1 binding protein that enhances transinfection of T cells. Cell **100**:587-597.

Geijtenbeek T.B., Torensma R., van Vliet S.J., van Duijnhoven G.C., Adema G.J., van Kooyk Y., Figdor C.G. (2000): DC-SIGN, a dendritic cell specific HIV-1 binding protein that enhances trans-infection of T cells. Cell 100:575-585.

Gerberding J.L. (1996): The infected health care provider. N. Engl. J. Med. 334:594-595.

Gerin J.L. (1990): Experimental WHV infection of woodchucks: an animal model of hepadnavirus-induced liver cancer. Gastroenterol. Jpn. 25 (Suppl 2):38-42.

Gerlich W.H., Heermann K.H., Lu X. (1992): Functions of hepatitis B surface proteins. Arch. Virol. Suppl. 4:129-32.

Girones R., Cote P.J., Hornbuckle W.E., Tennant B.C., Gerin J.L., Purcell R.H., Miller R.H. (1989): Complete nucleotide sequence of a molecular clone of woodchuck hepatitis virus that is infectious in the natural host. Proc. Natl. Acad. Sci. USA 86:1846-1849.

Gocke D.J., Hsu K., Morgan C., Bombardieri S., Lockshin M., Christian C.L. (1970): Association between polyarteritis and Australia antigen. Lancet 2:1149-53.

Gocke D. J. (1975): Extrahepatic manifestations of viral hepatitis. Am. J. Med. Sci. 270:49-52.

Gregory C.D., Murray R.J., Edwards C.F., Rickinson A.B. (1988): Downregulation of cell adhesion molecules LFA-3 and ICAM-1 in Epstein-Barr virus-positive Burkitt's lymphoma underlies tumor cell escape from virus-specific T cell surveillance. J. Exp. Med. 167:1811-1824.

Gripon P., Rumin S., Urban S., Le Seyec J., Glaise D., Cannie I., Guyomard C., Lucas J., Trepo C., Guguen-Guillouzo C. (2002): Infection of a human hepatoma cell line by hepatitis B virus. Proc. Natl. Acad. Sci. USA 99:15655-15660.

Grob P., Jilg W., Bornhak H., Gerken G., Gerlich W., Gunther S., Hess G., Hudig H., Kitchen A., Margolis H., Michel G., Trepo C., Will H., Zanetti A., Mushahwar I. (2000): Serological pattern "anti-HBc alone": report on a workshop. J. Med. Virol. 62: 450-455.

Guidotti L.G., Chisari F.V. (1996): To kill or to cure: Options in host defense against viral infection. Curr. Opin. Immunol. 8:478–483.

Guidotti L.G., Rochford R., Chung J., Shapiro M., Purcell R., Chisari F.V. (1999): Viral clearance without destruction of infected cells during acute HBV infection. Science **284**:825–829.

Guidotti L.G. and Chisari F.V. (2001): Non-cytolytic control of viral infections by the innate and adaptive immune response. Annu. Rev. Immunol. 19:65-91.

Gunther S., Meisel H., Reip A., Miska S., Kruger D.H., Will H. (1992): Frequent and rapid emergence of mutated pre-C sequences in HBV from e-antigen positive carriers who seroconvert to anti-HBe during interferon treatment. Virology 187:271-279.

Gunther S., Li B.C., Miska S., Kruger D.H, Meisel H., Will H. (1995): A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. J. Virol. **69**:5437-5444.

Gunther S., Fischer L., Pult I., Sterneck M., Will H. (1999): Naturally occurring variants of hepatitis B virus. Adv. Virus Res. 52:25-137.

He Z., Zhuang H., Wang X., Song S., Dong Q., Yan J., Buehring G.C., Luo G. (2003): Retrospective analysis of non-A-E hepatitis: possible role of hepatitis B and C virus infection. J. Med. Virol. **69**:59-65.

Hodgson P.D. and Michalak T.I. (2001): Augmented hepatic interferon gamma expression and T-cell influx characterize acute hepatitis progressing to recovery and residual lifelong virus persistence in experimental adult woodchuck hepatitis virus infection. Hepatology **34**:1049-1059.

Hollinger F.B., Troisi C.L., Pepe P.E. (1986): Anti-HBs response to vaccination with a human hepatitis B vaccine made by recombinant DNA technology in yeast. J. Infect. Dis. **153**:156–159.

Honkoop P., Niesters H.G., de Man R.A., Osterhaus A.D., Schalm S.W. (1997): Lamivudine resistance in immunocompetent chronic hepatitis B. Incidence and patterns. J. Hepatol. 26: 1393-1395.

Hoofnagle, J.H., Seeff L.B., Buskell-Bales Z.B., Zimmerman H.J., The Veterans Administration Hepatitis Cooperative Study Group. (1978): Type B hepatitis after transfusion with blood containing antibody to hepatitis B core antigen. N. Engl. J. Med. **298**:1379-1383.

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

Hoofnagle J.H., Carithers R.L.Jr., Shapiro C., Ascher N. (1995): Fulminant hepatic failure: summary of a workshop. Hepatology 21:240-252.

Hope-Simpson R.E. (1965): The nature of herpes zoster: a long term study and a new hypothesis. Proc. R. Soc. Med. 58:9-20.

Hosoda K., Omata M., Uchiumi K., Imazeki F., Yokosuka O., Ito Y., Okuda K., Ohto M. (1990): Extrahepatic replication of duck hepatitis B virus: more than expected. Hepatology 11:44-48.

Howard C.R. and Allison L.M. (1995): Hepatitis B surface antigen variation and protective immunity. Intervirology **38**: 35-40.

Hsu H.Y., Chang M.H., Ni Y.H., Lin H.H., Wang S.M., Chen D.S. (1997): Surface gene mutants of hepatitis B virus in infants who develop acute or chronic infections despite immunoprophylaxis. Hepatology 26:786-791.

Hu, K.Q. (2002): Occult hepatitis B virus infection and its clinical implications. J. Viral Hepatitis 9:243-257.

Hyman R.W., Ecker J.R., Tenser R.B. (1983): Varicella-zoster virus RNA in human trigeminal ganglia. Lancet 2:814-816.

Jamieson B.D., Somasundram T., Ahmed R. (1991): Abrogation of tolerance to a chronic viral infection. J. Immunol. 147:3521-3529.

Janeway C.A. Jr. and Medzhitov R. (2002): Innate immune recognition. Ann. Rev. Immunol. 20:197-216.

Jennings S.R., Rice P.L., Kloszewski E.D., Anderson R.W., Thompson D.L., Tevethia S.S. (1985): Effect of herpes simplex virus types 1 and 2 on surface expression of class I major histocompatibility complex antigens on infected cells. J. Virol. 56:757-766.

Jilbert A.R., Freiman J.S., Gowans E.J., Holmes M., Cossart Y.E., Burrell C.J. (1987): Duck hepatitis B virus DNA in liver, spleen, and pancreas: analysis by *in situ* and Southern blot hybridization. Virology. **158**:330-338.

Jin Y.-M., Churchill N. D, Michalak T.I. (1996): Protease-activated lymphoid cell and hepatocyte recognition site in the preS1 domain of the large woodchuck hepatitis virus envelope protein. J. Gen. Virol. 77:1837-1846.

Johnson D.C. and Huber M.T. (2002): Directed egress of animal viruses promotes cell-tocell spread. J. Virol. 76:1-8.

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

Jondal M., Klein G., Oldstone M.B., Bokish V., Yefenof E. (1976): Surface markers on human B and T lymphocytes. VIII. Association between complement and Epstein-Barr virus receptors on human lymphoid cells. Scand. J. Immunol. 5:401-410.

Kagi D., Ledermann B., Burki K., Seiler P., Odermatt B., Olsen K.J., Podack E.R., Zinkernagel R.M., Hengartner H. (1994): Cytotoxicity mediated by T-cells and natural killer cells is greatly impaired in perforin deficient mice. Nature **369**:31-37.

Kagi D. and Hengartner H. (1996): Different roles for cytotoxic T cells in the control of infections with cytopathic versus noncytopathic viruses. Curr. Opin. Immunol. 8:472-477.

Kagi D., Ledermann B, Burki K, Zinkernagel RM, Hengartner H. (1996): Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis *in vivo*. Annu. Rev. Immunol. 14:207-232.

Kajino K., Jilbert A.R., Saputelli J., Aldrich C.E., Cullen J., Mason W.S. (1994): Woodchuck hepatitis virus infections: very rapid recovery after a prolonged viremia and infection of virtually every hepatocyte. J. Virol. 68: 5792-5803.

Kakimi K., Guidotti L.G., Koezuka Y., Chisari F.V. (2000): Natural killer T cell activation inhibits hepatitis B virus replication in vivo. J. Exp. Med. 192:921-930.

Kanazawa S. and Matija-Perterlin B. (2001): Repression of MHC determinants in HIV infection. Microbes Infect. 3:467-473.

Kapoor P. and Frappier L. (2003): EBNA1 partitions Epstein-Barr virus plasmids in yeast cells by attaching to human EBNA1-binding protein 2 on mitotic chromosomes. J. Virol. 77:6946-56.

Karayiannis P., Alexopoulou A., Hadziyannis S., Thursz M., Watts R., Seito S., Thomas H.C. (1995): Fulminant hepatitis associated with hepatitis B virus e antigen-negative

infection: Importance of host factors. Hepatology 22:1628-1634.

Karthigesu V.D., Allison L.M., Fortuin M., Mendy M., Whittle H.C., Howard C.R. (1994): A novel hepatitis B virus variant in the sera of immunized children. J. Gen. Virol. 75:443-448.

Karthigesu V.D., Allison L.M., Ferguson M., Howard C.R. (1999): A hepatitis B virus variant found in the sera of immunized children induces a conformational change in the HBsAg "a" determinant. J. Med. Virol. 58:346-352.

Kazemi-Shirazi L., Petermann D., Muller C. (2000): Hepatitis B virus DNA in sera and liver tissue of HBsAg negative patients with chronic hepatitis C. J. Hepatol. **33**:785-790.

Kenna T., Golden-Mason L., Porcelli S.A., Koezuka Y., Hegarty J.E., O'Farrelly C., Doherty D.G. (2003): NKT cells from normal and tumor bearing human livers are phenotypically and functionally distinct from murine NKT cells. J. Immunol. **171**:1775-1779.

Kock J. and Schlicht H.J. (1993): Analysis of the earliest steps of hepadnavirus replication: genome repair after infectious entry into hepatocytes does not depend on viral polymerase activity. J. Virol. 67:4867-4874.

Kodoma K., Ogasawara N., Yoshikawa H., Murakami S. (1985): Nucleotide sequence of a cloned woodchuck hepatitis virus genome: evolutional relationship between hepadnaviruses. J. Virol. 56:978-86.

Koff R.S. and Galambos J.T. (1987): Viral hepatitis, pp. 457-581. In Schiff L. and Schiff E.R. (eds.), *Diseases of the Liver*. JB Lippincott, Philadelphia, PA.

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

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

Korba B.E., Wells F.V., Baldwin B., Cote P.J., Tennant B.C., Popper H., Gerin J.L. (1989): Hepatocellular carcinoma in woodchuck hepatitis virus infected woodchucks. Presence of viral DNA in tumor tissue from chronic carriers of animals serologically recovered from acute infections. Hepatology 9:461–470.

Kowalski D., Kroeker W.D., Laskowski M.Sr. (1976): Mung bean nuclease I: Physical,

chemical, and catalytic properties. Biochemistry. 15:4457-4463.

Krajcsi P., Dimitrov T., Hermiston T.W., Tollefson A.E., Ranheim T.S., Vande Pol S.B., Stephenson A.H., Wold W.S. (1996): The adenovirus E3-14.7K protein and the E3-10.4K/14.5K complex of proteins, which independently inhibit tumor necrosis factor (TNF)-induced apoptosis, also independently inhibit TNF-induced release of arachidonic acid. J. Virol. **70**:4904-4913.

Kwon D.S., Gregorio G., Bitton N., Hendrickson W.A., Littman D.R. (2002): DC-SIGNmediated internalization of HIV is required for *trans*-enhancement of T cell infection. Immunity 16:135-144.

Lanier L.L. (2003): Natural killer cell receptor signalling. Curr. Opin. Immunol. 15:308-314.

Laskus T., Wang L.F., Radkowski M., Vargas H., Cianciara J., Poutous A., Rakela J. (1997): Comparison of hepatitis B virus core promoter sequences in peripheral blood mononuclear cells and serum from patients with hepatitis B. J. Gen. Virol. 78:649-653.

Laskus T., Radkowski M., Wang L.F., Nowicki M., Rakela J. (1999): Detection and sequence analysis of hepatitis B virus integration in peripheral blood mononuclear cells. J. Virol. 73: 1235-1238.

Lau J.Y., Bird G.L., Naoumov N.V., Williams R. (1993): Hepatic HLA antigen display in chronic hepatitis B virus infection: relation to hepatic expression of HBV genome/gene products and liver histology. Dig. Dis. Sci. 38:888-895.

Laure F., Zagury D., Saimot A.G., Gallo R.C., Hahn B.H., Brechot C. (1985): Hepatitis B virus DNA sequences in lymphoid cells from patients with AIDS and AIDS-related complex. Science 229:561-563.

Leblebicioglu H., Turan D., Sunbul M., Esen S., Eroglu C. (2002): Transmission of human immunodeficiency virus and hepatitis B virus by blood brotherhood rituals. Scan. J. Infect. Dis. **35**: 210.

Lee P.I., Chang L.Y., Lee C.Y., Huang L.M., Chang M.H. (1997): Detection of hepatitis B surface gene mutation in carrier children with or without immunoprophylaxis at birth. J. Infect. Disease. 176: 427-430.

Lew Y-Y. and Michalak T.I. (2001): *In vitro* and *in vivo* infectivity and pathogenicity of the lymphoid cell-derived woodchuck hepatitis virus. J. Virol. 75:1770-1782.

Li D.H., Newbold J.E., Cullen J.M. (1996): Natural populations of woodchuck hepatitis virus contain variant precore and core sequences including a premature stop codon in the epsilon motif. Virology **220**: 256-262.

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

Liang T.J., Baruch Y., Ben-Porath E., Enat R., Bassan L., Brown N. V., Rimon N., Blum H. E., Wands J. R. (1991): Hepatitis B virus infection in patients with idiopathic liver disease. Hepatology 13:1044-1051.

Liang T.J., Bodenheimer Jr. H. C., Yankee R., Brown N. V., Chang K., Huang J., Wands J. R. (1994): Presence of hepatitis B and C viral genomes in US blood donors as detected by polymerase chain reaction amplification. J. Med. Virol. 42:151-157.

Lindhout E., Lakeman A., Mevissen M.L., de Groot C. (1994): Functionally active Epstein-Barr virus-transformed follicular dendritic-like cell lines. J. Exp. Med. 179:1173-1184.

Ling R., Mutimer D., Ahmed M., Boxall E.H., Elias E., Dusheiko G.M., Harrison T.J. (1996): Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. Hepatology 24:711-713.

Locarnini S. and Birch C. (1999): Antiviral chemotherapy for chronic hepatitis B infection: lessons learned from treating HIV-infected patients. J. Hepatol. 30:536-550.

Lohr H.F., Pingel S., Bocher W.O., Bernhard H., Herzog-Hauff S., Rose-John S. (2002): Reduced virus specific T helper cell induction by autologous dendritic cells in patients with chronic hepatitis B- restoration by exogenous interleukin-12. Clin. Exp. Immunol. 130:107-114.

Lok A.S., Liang R.H., Chiu E. K., Wong K. L., Chan T. K., Todd D. (1991): Reactivation of hepatitis B virus replication in patients receiving cytotoxic therapy: report of a prospective study. Gastroenterology 100:1432-1434.

Lowell J.A., Howard T.K., White H.M., Shenoy S., Huettner P.C., Brennan D.C., Peters M.G. (1995): Serological evidence of past hepatitis B infection in liver donor and hepatitis B infection in liver allograft. Lancet 345:1084-1085.

Lozach P.Y., Lortat-Jacob H., de Lacroix de Lavalette A., Staropoli I., Foung S., Amara A., Houles C., Fieschi F., Schwartz O., Virelizier J.L., Arenzana-Seisdedos F., Altmeyer R. (2003): DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. J. Biol. Chem. 278:20358-20366.

Mack D.H., Bloch W., Nath N., Sninsky J.J. (1988): Hepatitis B virus particles contain a polypeptide encoded by the largest open reading frame: A putative reverse transcriptase. J. Virol. 62:4786-4790.

Maini M.K., Boni C., Ogg G.S., King A.S., Reignat S., Lee C.K., Larrubia J.R., Webster G.J., McMichael A.J., Ferrari C., Williams R., Vergani D., Bertoletti A. (1999): Direct *ex vivo* analysis of hepatitis B virus-specific CD8(+) T cells associated with the control of infection. Gastroenterology 117:1386-1396.

Margolis H.S., Alter M.J., Hadler S.C. (1991): Hepatitis B: evolving epidemiology and implications for control. Semin. Liver Dis. 11:84-92.

Marion P.L., Oshiro L.S., Regnery D.C., Scullard G.H., Robinson W.S. (1980): A virus in Beechey ground squirrels that is related to hepatitis B virus of humans. Proc. Natl. Acad. Sci. USA 77:2941-2945.

Marion P.L., Trepo C., Matsubara K., Price P.M. (1991): Experimental models in hepadnavirus research: Report of a workshop, 866–874. F.B. Hollinger, S.M., Lemon, H.S. Margolis (eds.), In *Viral Hepatitis and Liver Disease*. Williams & Wilkins, Baltimore, MD.

Marusawa H., Uemoto S., Hijikata M., Ueda Y., Tanaka K., Shimotohno K., Chiba T. (2000): Latent hepatitis virus infection in healthy individuals with antibodies to hepatitis B core antigen. Hepatology **31**:488-495.

Maruyama T., McLachlan A., Iino S., Koike K., Kurokawa K., Milich D.R. (1993): The serology of chronic hepatitis B infection revisited. J. Clin. Invest. 9:2586-2595.

Maruyama T., Kuwata S., Koike K., Iino S., Yasuda K., Yotsuyanagi H., Moriya K., Maekawa H., Yamada H., Shibata Y., Milich D.R. (1998): Pre-core wild-type DNA and immune complexes persist in chronic hepatitis B after seroconversion: no association between genome conversion and seroconversion. Hepatology 27: 245-253.

Mason A.L., Xu L., Guo L., Kuhns M., Perrillo R.P. (1998): Molecular basis for persistent hepatitis B virus infection in the liver after clearance of serum hepatitis B surface antigen. Hepatology 27:1736-1742.

Mason A., Yoffe B., Noonan C., Mearns M., Campbell C., Kelley A., Perrillo R. P. (1992): Hepatitis B virus DNA in peripheral-blood mononuclear cells in chronic hepatitis B after HBsAg clearance. Hepatology 16:26-41.

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

Mason W.S., Cullen J., Moraleda G., Saputelli J., Aldrich C.E., Miller D.S., Tennant B., Frick L., Averett D., Condreay L.D., Jilbert A.R. (1998): Lamivudine therapy of WHV-infected woodchucks. Virology 245:18-32.

Maynard J.E., Kane M.A., Hadler S.C. (1989): Global control of hepatitis B through vaccination: role of hepatitis B vaccine in the Expanded Programme on Immunization. Rev. Infect. Dis. 11 (Suppl 3):S574-578.

McDermott A.B., Mitchen J., Piaskowski S., De Souza I., Yant L.J., Stephany J., Furlott J., Watkins D.I. (2004): Repeated low-dose mucosal simian immunodeficiency virus SIVmac239 challenge results in the same viral and immunological kinetics as a high-dose challenge: a model for the evaluation of vaccine efficacy in nonhuman primates. J. Virol. 78:3140-3144.

Melegari M., Bruno S., Wands J.R. (1994): Properties of hepatitis B virus pre-S1 deletion mutants. Virology 199:292-300.

Melegari M., Scaglioni P.P., Wands J.R. (1998): Hepatitis B virus mutants associated with 3TC and famciclovir administration are replication defective. Hepatology 27:628-633.

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

Menne S. and Tennant.B.C. (1999): Unravelling hepatitis B virus infection of mice and men (and woodchucks and ducks). Nat Med. 5:1125-1126.

Michalak T. (1978): Immune complexes of hepatitis B surface antigen in the pathogenesis of periarteritis nodosa: A study of seven necropsy cases. Am. J. Pathol. 90:619-632.

Michalak T. and Krawczynski K. (1981): Vascular lesions in hepatitis virus infection, pp. 36-50. E. Bartoli, L. Chaindussi, S. Sherlock (eds), In *Systemic Effects of HBsAg Immune Complexes*. Piccini Medical Books, Padova.

Michalak T.I. and Churchill N.D. (1988): Interaction of woodchuck hepatitis virus surface antigen with hepatocyte plasma membrane in woodchuck chronic hepatitis. Hepatology. 8:499-506.

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

Michalak T.I. and Bolger G.T. (1989): Characterization of the binding sites for glutaraldehyde-polymerized albumin on purified woodchuck hepatocyte plasma membranes. Gastroenterology **96**:153-166.

Michalak T.I., Lin B., Churchill N.D., Dzwonkowski P., Desousa J.R. (1990): Hepadnavirus nucleocapsid and surface antigens and the antigen-specific antibodies associated with hepatocyte plasma membranes in experimental woodchuck acute hepatitis. Lab Invest. **6**:680-689.

Michalak T.I., Pasquinelli C., Guilhot S., Chisari F.V. (1994): Hepatitis B virus persistence after recovery from acute viral hepatitis. J. Clin. Invest. 93:230-239.

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

Michalak T.I., Lau J.Y.N., McFarlane B.M., Alexander G.A.M., Eddleston A.L.W.F., Williams R. (1995): Antibody-directed complement-mediated cytotoxicity to hepatocytes from patients with chronic hepatitis B. Clin. Exp. Immunol. 100:227-232.

Michalak T. I. (1998): The woodchuck animal model of hepatitis B. Viral Hepatitis Rev. 4:139-165.

Michalak T.I., Pardoe I.U., Coffin C.S., Churchill N.D., Freake D.S., Smith P., and Trelegan C.L. (1999): Occult life-long persistence of infectious hepadnavirus and residual liver inflammation in woodchucks convalescent from acute viral hepatitis. Hepatology **29**:928-

938.

Michalak T.I. (2000): Occult persistence and lymphotropism of hepadnaviral infection: insights from the woodchuck viral hepatitis model. Immunol. Rev. 174:98-111.

Michalak T.I., Hodgson P.D., Churchill N.D. (2000): Posttranscriptional inhibition of class I major histocompatibility complex presentation on hepatocytes and lymphoid cells in chronic woodchuck hepatitis virus infection. J. Virol. 74:4483-4494.

Michalak T.I., Mulrooney P.M., Coffin C.S. (2004): Low doses of hepadnavirus induce infection of the lymphatic system that does not engage the liver. J. Virol. 78:1730-1738.

Mikloska Z., Bosnjak L., Cunningham A.L. (2001): Immature monocyte-derived dendritic cells are productively infected with herpes simplex virus type 1. J. Virol. 75:5958-5964.

Milich D.R. and McLachlan A. (1986): The nucleocapsid of hepatitis B virus is both a T-cell-independent and a T-cell-dependent antigen. Science 234:1398-1401.

Milich D.R., Jones J.E., Hughes J.L., Price J., Raney A.K., McLachlan A. (1990): Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance in utero? Proc. Natl. Acad. Sci. USA 87: 6599-6603.

Milich D.R., Schodel F., Hughes, J.L., Jones J.E., Peterson, D.L. (1997): The hepatitis B virus core and e antigens elicit different Th cell subsets: antigen structure can affect Th cell phenotype. J. Virol. 71: 2192-2201.

Milich D.R., Chen M.K., Hughes J.L., Jones J.E. (1998): The secreted hepatitis B precore antigen can modulate the immune response to the nucleocapsid: a mechanism for persistence. J. Immunol. 160:2013-2021.

Milich D. and Liang T.J. (2003): Exploring the biological basis of hepatitis B e antigen in hepatitis B virus infection. Hepatology **38**:1075-1086.

Miller D.M., Zhang Y., Rahill B.M., Waldman W.J., Sedmak D.D. (1999): Human cytomegalovirus inhibits IFN-alpha-stimulated antiviral and immunoregulatory responses by blocking multiple levels of IFN-alpha signal transduction. J. Immunol. **162**:6107-6113.

Minuk G.Y., Sun D.F., Greenberg R., Zhang M., Hawkins K., Uhanova J., Gutin A., Bernstein K., Giulivi A., Osiowy C. (2004): Occult hepatitis B virus infection in a North American adult hemodialysis patient population. Hepatology **40**:1072-1077.

Moll H. (2003): Dendritic cells and host resistence to infection. Cell. Microbiol. 5:493-500.

Mosmann T.R. and Sad S. (1996): The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol. Today 17:138-146.

Moyer L.A. and Mast E.E. (1994): Hepatitis B: virology, epidemiology, disease, and prevention, and an overview of viral hepatitis. Am. J. Prev. Med. 10 (Suppl):45-55.

Muller C., Bergmann K.F., Gerin J.L., Korba B.E. (1992): Production of hepatitis B virus by stably transfected monocytic cell line U937: a model for extrahepatic hepatitis B virus replication. J. Infect. Dis. 165:929-933.

Mulrooney P.M. and Michalak T.I. (2003): Quantitative detection of hepadnavirus-infected lymphoid cells by *in situ* PCR combined with flow cytometry: implications for the study of occult virus persistence. J. Virol. 77:970-979.

Murakami S. (1999): Hepatitis B virus X protein: structure, function and biology. Intervirology 42:81-99.

Murakami Y., Minami M., Daimon Y., Okanoue T. (2004): Hepatitis B virus DNA in liver, serum, and peripheral blood mononuclear cells after the clearance of serum hepatitis B surface antigen. J. Med. Virol. 72:203-214.

Muratori L., Gibellini D., Lenzi M., Cataleta M., Muratori P., Morelli M.C., Bianchi F.B. (1996): Quantification of hepatitis C virus-infected peripheral blood mononuclear cells by *in situ* reverse transcriptase polymerase chain reaction. Blood **88**:2768-2774.

Nainan O.V., Khristova M.L., Byun K., Xia G., Taylor P.E., Stevens C.E., Margolis H.S. (2002): Genetic variation of hepatitis B surface antigen coding region among infants with chronic hepatitis B virus infection. J. Med. Virol. 68:319-327.

Naumann U., Protzer-Knolle U., Berg T., Leder K., Lobeck H., Bechstein W.O., Gerken G., Hopf U., Neuhaus P. (1997): A pretransplant infection with precore mutants of hepatitis B virus does not influence the outcome of orthotopic liver transplantation in patients on high dose anti-hepatitis B virus surface antigen immunoprophylaxis. Hepatology **26**:478-484.

Navarro-Sanchez E., Altmeyer R., Amara A., Schwartz O., Fieschi F., Virelizier J.L., Arenzana-Seisdedos F., Despres P. (2003): Dendritic-cell-specific ICAM3-grabbing nonintegrin is essential for the productive infection of human dendritic cells by mosquito-cellderived dengue viruses. EMBO Rep. 4:1-6.

Ngui S.L., O'Connell S., Eglin R.P., Heptonstall J., Teo C.G. (1997): Low detection rate and maternal provenance of hepatitis B virus S gene mutants in cases of failed postnatal immunoprophylaxis in England and Wales. J. Infect. Dis. **176**:1360-1365.

Nguyen K.B., Watford W.T., Salomon R., Hofmann S.R., Pien G.C., Morinobu A., Gadina M., O'Shea J.J., Biron C.A. (2002): Critical role for STAT-4 activation by type 1 interferons in the interferon-gamma response to viral infections. Science **297**:2063-2066.

Niesters H.G., Honkoop P., Haagsma E.B., de Man R.A., Schalm S.W., Osterhaus A.D. (1998): Identification of more than one mutation in the hepatitis B virus polymerase gene arising during prolonged lamivudine treatment. J. Infect. Dis. 177:1382-1385.

Niewiesk S., Gotzelmann M., ter Meulen V. (2000): Selective *in vivo* suppression of T lymphocyte responses in experimental measles virus infection. Proc. Natl. Acad. Sci. USA 97:4251-4255.

Nowoslawski A., Krawczynski K., Brzosko W.J., Madalinski K. (1972): Tissue localization of Australia antigen immune complexes in acute and chronic hepatitis and liver cirrhosis. Am. J. Pathol. **68**:31-56.

Nowoslawski A., Krawczynski K., Nazarewicz T., Slusarczyk, J. (1975): Immunopathological aspects of hepatitis type B. Am. J. Med. Sci. 270:229-239.

Nuovo G.J. (1995): In situ PCR, pp. 235-248. In C. W. Dieffenbach and G. S. Dveksler (eds.), *PCR primer: A Laboratory Manual.*, Cold Spring Harbour Laboratory Press, New York, NY.

Ogg G.S. and McMichael A.J. (1998): HLA-peptide tetrameric complexes. Curr. Opin. Immunol. 10:393-396.

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

Ohba S., Kimura K., Mise N., Konno Y., Suzuki N., Miyashita K., Tojo A., Hirata Y., Uehara Y., Atarashi K., Goto A., Omata M. (1997): Differential localization of s and e antigens in hepatitis B virus-associated glomerulonephritis. Clin. Nephrol. 48:44-47.

Okada K., Kamiyama I., Inomata M., Imai K., Miyakawa Y. (1976): e Antigen and anti-e in the serum of asymptomatic carrier mothers as indicators of positive and negative

transmission of hepatitis B virus to their infants. N. Engl. J. Med. 294:746-749.

Okamoto H., Yotsumoto S., Akahane Y., Yamanaka T., Miyazaki Y., Sugai Y., Tauda F., Tanaka T., Miyakawa Y., Mayumi M. (1990): Hepatitis B viruses with precore region defects prevail in persistently infected host along with seroconversion to the antibody against e antigen. J. Virol. 64:1298-1303.

Okamoto H., Yano K., Nozaki Y., Matsui A., Miyazaki H., Yamamoto K., Tsuda F., Machida A., Mishiro S. (1992): Mutations within the S gene of hepatitis B virus transmitted from mothers to babies immunized with hepatitis B immune globulin and vaccine. Pediatr. Res. 32:264-268.

Oldstone M.B. (1989): Viral persistence. Cell. 56:517-520.

Oldstone M.B. and Rall G.F. (1993): Mechanism and consequence of viral persistence in cells of the immune system and neurons. Intervirology 35:116-121.

Oldstone M.B. (1998): Viral persistence: mechanisms and consequences. Curr. Opin. Microbiol. 1:436-441.

Oon C.J., Tan K.L., Harrison T., Zuckerman A. (1996): Natural history of hepatitis B surface antigen mutants in children. Lancet 348:1524.

Orito E., Mizokami M., Ina Y., Moriyama E. N., Kameshima N., Yamamoto M., Gojobori T. (1989): Host-independent evolution and a genetic classification of hepadnavirus family based on nucleotide sequences. Proc. Natl. Acad. Sci. USA **86**:7059-7062.

Ou J.H., Laub O., Rutter W.J. (1986): Hepatitis B virus gene function: The precore region targets the core antigen to cellular membranes and causes the secretion of the e antigen. Proc. Natl. Acad. Sci. USA 83:1578–1582.

Ozaki S., Ogasahara K., Kosaka M., Inoshita T., Wakatsuki S., Uehara H., Matsumoto T. (1998): Hepatosplenic gamma delta T-cell lymphoma associated with hepatitis B virus infection. J. Med. Invest. 44:215-217.

Pardoe I.U. and Michalak T.I. (1995): Detection of hepatitis B and woodchuck hepatitis viral DNA in plasma and mononuclear cells from heparinized blood by the polymerase chain reaction. J. Virol. Methods 51:277-288.

Pasquinelli C., Laure F., Chatenoud L., Beaurin G., Gazengel C., Bismuth H., Degos F., Tiollais P., Bach J.F., Brechot C. (1986): Hepatitis B virus in mononuclear blood cells. A frequent event in hepatitis B surface antigen positive and negative patients with acute and chronic liver disease. Hepatology **3**:95-103.

Paterlini P., Gerken G., Nakajima E., Terre S., D'Errico A., Grigioni W., Nalpas B., Franco D., Wands J., Kew M., Pisi E., Tollais P., Brechot C. (1990): Polymerase chain reaction to detect hepatitis B virus DNA and RNA sequences in primary liver cancers from patients negative for hepatitis B surface antigen. N. Engl. J. Med. **323**:80-85.

Paterlini-Brechot P., Saigo K., Murakami Y., Chami M., Gozuacik D., Mugnier C., Lagorce D., Brechot C. (2003): Hepatitis B virus-related insertional mutagenesis occurs frequently in human liver cancers and recurrently targets human telomerase gene. Oncogene 25: 3911-3916.

Paul W.E. and Seder R.A. (1994): Lymphocyte responses and cytokines. Cell 76:241-251.

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

Penna A., Del Prete G., Cavalli A., Bertoletti A., D'Elios M.M. Sorrentino D'Amato M., Boni C., Pilli M., Fiaccadori F., Ferrari C. (1997): Predominant T-helper 1 cytokine profile of hepatitis B virus nucleocapsid-specific T cells in acute self-limited hepatitis B. Hepatology 25:1022-1027.

Peterson D.L. (1981): Isolation and characterization of the major protein and glycoprotein of hepatitis B surface antigen. J. Biol. Chem. 256:6975–6983.

Petti L., Sample C., Kieff E. (1990): Subnuclear localization and phosphorylation of Epstein-Barr virus latent infection nuclear proteins. Virology 176:563-574.

Pham T.N.Q., MacParland S.A., Mulrooney P.M., Cooksley H., Naoumov N.V., Michalak T.I. (2004): Hepatitis C virus persistence after spontaneous or treatment-induced resolution of hepatitis C. J. Virol **78**:5867-5874.

Pircher H., Moskophidis D., Rohrer U., Burki K., Hengartner H., Zinkernagel R.M. (1990): Viral escape by selection of cytotoxic T cell-resistant virus variants *in vivo*. Nature **346**:629-633.

Pollicino T., Squandrito G., Cerenzia G., Cacciola I., Raffa G., Crax A., Farinati F., Missale

G., Smedile A., Tiribelli C., Villa E., Raimondo G. (2004): Hepatitis B virus maintains its pro-oncogenic properties in the case of occult HBV infection. Gastroenterology **126**:102-110.

Pontisso P., Poon M.V., Tiollais P., Brechot C. (1984): Detection of hepatitis B virus DNA in mononuclear cells. Br. Med. J. 288:1563-1566.

Pope M., Gezelter S., Gallo N., Hoffman L., Steinman R.M. (1995): Low levels of HIV-1 in cutaneous dendritic cells initiate a productive infection upon binding to memory CD4+ T cells. J. Exp. Med. **182**: 2045-2056.

Popper H., Shih W. W-K., Gerin D.C., Wong D.C., Hoyer B.H., London W.T., Sly D.L., Purcell R.H. (1981): Woodchuck hepatitis and hepatocellular carcinoma: correlation of histologic with virologic observations. Hepatology 1:91-98.

Prieto M., Gomez M. D., Berenguer M., Cordoba J., Rayon J. M., Pastor M., Garcia-Herola A., Nicolas D., Carrasco D., Orbis J. F., Mir J., Berenguer J. (2001): De novo hepatitis B after liver transplantation from hepatitis B core antibody-positive donors in an area with high prevalence of anti-HBc positivity in donor population. Liver Transpl. 7:51-58.

Protzer U., Goergen B., Hopf U., Neuhaus P., Knig V., Meyer zum Buxchenfelde K.H.M., Gerken G. (1996): Pre-core mutants of hepatitis B virus in patients receiving immunosuppressive treatment after orthotopic liver transplantation. J. Med. Virol. 50:135-144.

Pult I., Chouard T., Weiland S., Klemenz R., Yaniv M., Blum H.E. (1997): A hepatitis B virus mutant with a new hepatocyte nuclear factor 1 binding site emerging in transplanttransmitted fulminant hepatitis B. Hepatology 25:1507-1515.

Re M. C., Furlini G., Gibellini D., Vignoli M., Ramazzotti E., Lolli S., Ranieri S., La Placa M. (1994): Quantification of human immunodeficiency virus type-1-infected mononuclear cells in peripheral blood of seropositive subjects by newly developed flow cytometry analysis of the product of an in situ PCR assay. J. Clin. Microbiol. 32:2152-2157.

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

Rehermann B., Lau D., Hoofnagle J.H., Chisari F.V. (1996a): Cytotoxic T lymphocyte responsiveness after resolution of chronic hepatitis B virus infection. J. Clin. Invest. 97:1655-1665.

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

Roberts J.D., Bebenek K., Kunkel T.A. (1988): The accuracy of reverse transcriptase from HIV-1. Science 242:1171-1173.

Romet-Lemonne J.L., McLane M.F., Elfassi E., Haseltine W.A., Azocar J., Essex M. (1983): Hepatitis B virus infection in cultured lymphoblastoid cells. Science 221:667-669.

Salfeld J., Pfaff E., Noah M., Schaller H. (1989): Antigenic determinants and functional domains in core antigen and e antigen from hepatitis B virus. J. Virol. 63:798-808.

Saracco G., Macagno S., Rosina F., Rizzetto M. (1988): Serologic markers with fulminant hepatitis in persons positive for hepatitis B surface antigen. A worldwide epidemiologic and clinical survey. Ann. Intern. Med. 108:380-383.

Sayers T.J., Brooks A.D., Lee J.K., Fenton R.G., Komschlies K.L., Wigginton J.M., Winkler-Pickett R., Wiltrout R.H. (1998): Molecular mechanisms of immune-mediated lysis of murine renal cancer: differential contributions of perforin-dependent versus Fas-mediated pathways in lysis by NK and T cells. J. Immunol. 161:3957-3965.

Schlicht H.J., von Brunn A., Theilmann L. (1991): Antibodies in anti-HBe-positive patient sera bind to an HBe protein expressed on the cell surface of human hepatoma cells: implications for virus clearance. Hepatology 13:57-61.

Schmilovitz-Weiss H., Levy M., Thompson N., Dusheiko G. (1993): Viral markers in the treatment of hepatitis B and C. Gut 34 (Suppl 2):S26-35.

Schreiber G.B., Busch M.P., Kleinman S.H., Korelitz J.J. (1996): FTR-EDS. The risk of transfusion-transmitted viral infections. N. Engl. J. Med. 334: 1685-1690.

See D.M., Khemka P., Sahl L., Bui T., Tilles J.G. (1997): The role of natural killer cells in viral infection. Scand. J. Immunol. 46:217-224.

Servet-Delprat C., Vidalain P.O., Azocar O., Le Deist F., Fischer A., Rabourdin-Combe C. (2000): Consequences of Fas-mediated human dendritic cell apoptosis induced by measles virus. J. Virol. 74:4387-4393.

Servet-Delprat C., Vidalain P.O., Valentin H., Rabourdin-Combe C. (2003): Measles virus and dendritic cell functions: how specific response cohabits with immunosuppression. Curr.

Top. Microbiol. Immunol. 276:103-123.

Sevilla N., Kunz C., Mc Gavern D., Oldstone M.B. (2003): Infection of dendritic cells by lymphocytic choriomeningitis virus. Curr. Top. Microbiol. Immunol. **276**:125-144.

Shiao J., Guo L., and McLaws M.L. (2002): Estimation of the risk of bloodborne pathogens to health care workers after a needlestick injury in Taiwan. Amer. J. Infect. Control. **30**:15-20.

Shimizu H, Mitsuada T., Fujita S., Yokota S. (1991): Perinatal hepatitis B virus infection caused by antihepatitis B positive maternal mononuclear cells. Arch. Dis. Child. 66:718-721.

Shresta S., Pham C.T., Thomas D.A., Graubert T.A., Ley T.J. (1998): How do cytotoxic lymphocytes kill their targets? Curr. Opin. Immunol. 10:581-587.

Simmons G., Reeves J.D., Grogan C.C., Vandenberghe L.H., Baribaud F., Whitbeck J.C., Burke E., Buchmeier M.J., Soilleux E.J., Riley J.L., Doms R.W., Batges P., Pohemann S. (2003): DC-SIGN and DC-SIGNR bind Ebola glycoproteins and enhance infection of macrophages and endothelial cells. Virology **305**:115-123.

Slusarczyk J., Michalak T., Nazarewicz-de Mezer T., Krawczynski K., Nowoslawski A. (1980): Membranous glomerulopathy associated with hepatitis B core antigen immune complexes in children. Am. J. Pathol. **98**:29-43.

Soderberg-Naucler C., Streblow D.N., Fish K.N., Allan-Yorke J., Smith P.P., Melson J.A. (1997): Reactivation of latent human cytomegalovirus in CD14(+) monocytes is differentiation dependent. J. Virol. 75:7543-7554.

Spivack J.G. and Fraser N.W (1988): Expression of herpes simplex virus type I latencyassociated transcripts in the trigeminal ganglia of mice during acute infection and reactivation of latent infection. J. Virol. **62**:1479-1485.

Steinmann R.M. (2000): DC-SIGN: a guide to some mysteries of dendritic cells. Cell **100**:491-494.

Stemler M., Weimer T., Tu Z.X., Wan D.F., Levrero M., Jung C., Pape G.R., Will H. (1990): Mapping of B-cell epitopes of the human hepatitis B virus X protein. J. Virol. **64**:2802-2809.

Stevens J.G., Wagner E.K., Devi-Rao G.B., Cook M.L., Feldman L.T. (1987): RNA

complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. Science. 235:1056-1059.

Stoll-Becker S., Repp R., Glebe D., Schaefer S., Kreuder J., Kann M., Lampert F., Gerlich W.H. (1997): Transcription of hepatitis B virus in peripheral blood mononuclear cells from persistently infected patients. J. Virol. 71:5399-5407.

Straight S.W, Herman B., McCance D.J. (1995): The E5 oncoprotein of human papillomavirus type 16 inhibits the acidification of endosomes in human keratinocytes. J. Virol. 69:3185-3192.

Strauss W. (1997): Preparation of genomic DNA from mammalian tissue, pp. 2.21-2.23. FA. Ausebel, R. Brent, RE. Kingston, DD. Moore, JG. Seidman, JA. Smith, and K. Struhl (eds), In *Current Protocols in Molecular Biology*. John Wiley and Sons, New York, NY.

Sugai Y. and Okamoto H. (1989): State of hepatitis B virus DNA in peripheral blood mononuclear cells from persistently infected individuals: correlation with e antigen and viral DNA in the serum as well as activity of liver disease. Tohoku. J. Exp. Med. 158:73-84.

Sugata F., Chen H.S., Kaneko S., Purcell R.H., Miller R.H. (1994): Analysis of the X promoter of woodchuck hepatitis virus. Virology 205:314-320.

Summers J., Smolec J.M., Snyder R. (1978): A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. Proc. Natl. Acad. Sci. USA 75:4533-4537.

Summers J. and Mason W.S. (1982): Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell 29:403-415.

Swaminathan S. (2003): Molecular biology of Epstein-Barr virus and Kaposi's sarcomaassociated herpesvirus. Semin. Hematol. 40:107-115.

Tatti K.M., Korba B.E., Stang H.L., Peek S., Gerin J.L., Tennant B.C, Schinazi R.F. (2002): Mutations in the conserved woodchuck hepatitis virus polymerase FLLA and YMDD regions conferring resistance to lamivudine. Antiviral Res. 55:141-150.

Tennant, B.C. and J. L. Gerin. (1994): The woodchuck model of hepatitis B virus infection, pp. 1455-1466. I.M. Arias, J.L. Boyer, N. Fausto, W.B. Jakoby, D.A. Schachter and D.A. Shafritz (eds.), In *The Liver: Biology and Pathobiology*, Raven Press, New York, NY.

Thanavala Y.M., Browne S.E., Howard C.R., Roitt I.M., Steward M.W. (1986): A surrogate hepatitis B virus antigenic epitope represented by a synthetic peptide and an

internal image antiidiotype antibody. J. Exp. Med. 164:227-236.

Thisdale M., Kemp S.D., Parry N.R., Larder B.A. (1993): Rapid *in vitro* selection of human immunodeficiency virus type 1 resistant to 3'-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. Proc. Natl. Acad. Sci. USA 90:5653-5656.

Theilmann L and von Brunn A. (1991): Production of 22 nm HBsAg particles by human lymphocytes infected with a recombinant vaccinia virus containing the coding sequence for hepatitis B virus surface antigen. Biochem. Biophys. Res. Comm. **179**:1479-1484.

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

Uchida T., Kaneita Y., Gotoh K., Kanagawa H., Kouyama H., Kawanishi T., Mima S. (1997): Hepatitis C virus is frequently coinfected with serum marker-negative hepatitis B virus: probable replication promotion of the former by the latter as demonstrated by *in vitro* cotransfection. J. Med. Virol. **52**:399-405.

Uemoto S., Sugiyama K., Marusawa H., Inomata Y., Asonuma K., Egawa H., Kiuchi T., Miyake Y., Tanaka K., Chiba T. (1998): Transmission of hepatitis B virus from hepatitis B core antibody-positive donors in living related liver transplantation. Transplantation 65:494-499.

Uy A., Bruss V., Gerlich W.H., Kochel H.G., Thomssen R. (1986): Precore sequence of hepatitis B virus inducing e antigen and membrane association of the viral core protein. Virology 155:89-96.

van Doorn L.J., Capriles I., Maertens G., DeLeys R., Murray K., Kos T., Schellekens H., Quint W. (1995): Sequence evolution of the hypervariable region in the putative envelope region E2/NS1 of hepatitis C virus is correlated with specific humoral immune responses. J. Virol. **69**:773-778.

van Kooyk Y. and Geijtenbeek T.B.H. (2003): DC-SIGN: Escape mechanisms for pathogens. Nat. Rev. Immunol. 3:697-709.

Wain-Hobson S. (1996): Running the gamut of retroviral variation. Trends Microbiol. 4:135-141.

Walter E., Teubner K., Blum H.E., Offensperger W.B., Offensperger S., Gerok W. (1991): Duck hepatitis B virus infection of non-hepatocytes. Liver 11:53-62.

Wang F.S., Xing L.H., Zhu C.L., Lui H.G., Wang H.F., Lei Z.Y. (2001): Dysfunction of peripheral blood dendritic cells from patients with chronic hepatitis B virus infection. World J. Gastroenterol. 7:537-541.

Watanabe H., Miyaji C., Kawachi Y., Iiai T., Ohtsuka K., Iwanage T., Takahashi-Iwanaga H., Abo T. (1995): Relationships between intermediate TCR cells and NK1.1+ T cells in various immune organs. NK1.1+ T cells are present within a population of intermediate TCR cells. J. Immunol. 155:2972-2983.
Watkins B.A., Reitz Jr. M.S., Wilson C.A., Aldrich K., Davis A.E., Robert-Guroff M. (1993): Immune escape by human immunodeficiency virus type 1 from neutralizing antibodies: evidence for multiple pathways. J. Virol. 67:7493-7500.

Weimer T., Schodel F., Jung M.C., Pape G.R., Alberti A., Fattovich G., Beljaars H., van Eerd P.M., Will H. (1990): Antibodies to the RNase H domain of hepatitis B virus P protein are associated with ongoing viral replication. J. Virol. 64:5665-5668.

Weissman J.D., Brown J.A., Howcroft T.K., Hwang J., Chawla A., Roche P.A., Schiltz L., Nakatani Y., Singer D.S. (1998): HIV-1 tat binds TAFII250 and represses TAFII250dependent transcription of major histocompatibility class I genes. Proc. Natl. Acad. Sci. USA 95:11601-11606.

Welsh R.M., Zinkernagel R.M., Hallenbeck L.A. (1979): Cytotoxic cells induced during lymphocytic choriomeningitis virus of mice. II. "Specifities" of the natural killer cells. J. Immunol. **122**:475-481.

Will H., Cattaneo R., Koch H.G., Darai G., Schaller H. (1982): Cloned HBV DNA causes hepatitis in chimpanzees. Nature 299:740-742.

Whittle H.C., Inskip H., Hall A.J., Mendy M., Dowes R., Hoare S. (1991): Vaccination against hepatitis B and protection against chronic viral carriage in The Gambia. Lancet **337**:747-750.

World Health Organization, 2000, Hepatitis B fact sheet, October 2000 revision date. W.H.O./204. available at: http://www.who.int/inf-fs/en/fact204.html.

Wu H., Ceccarelli D.F., Frappier L. (2000): The DNA segregation mechanism of Epstein-Barr virus nuclear antigen 1. EMBO Rep. 1:140-144.

Wu T.T, Coates L. Aldrich C.E., Summers J., Mason W.S. (1990): In hepatocytes infected with duck hepatitis B virus, the template for viral RNA synthesis is amplified by an intracellular pathway. Virology 175:225-261.

Xu Z.C. and Yen T.S. B. (1996): Intracellular retention of surface protein by a hepatitis B virus mutant that releases virion particles. J. Virol. 70:133-140.

Yoffe B., Noonan C.A., Melnick J.L., Hollinger F.B. (1986): Hepatitis virus DNA in mononuclear cells and analysis of cell subsets for the presence of replicative intermediates of viral DNA. J. Infect. Dis. 153:471-477.

York I.A., Roop C., Andrews D.W., Riddell S.R., Graham F.L., Johnson D.C. (1994): A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. Cell 77:525-535.

Yotsuyanagi H., Yasuda K., Iino S., Moriya K., Shintani Y., Fujie H., Tsutsumi T., Kimura S., Koike K. (1998): Persistent viremia after recovery from self-limited acute hepatitis B. Hepatology 27:1377-1382.

Yotsuyanagi H., Shintani Y., Moriya K., Fujie H., Tsutsumi T., Kato T., Nishioka K., Takayama T., Makuuchi M., Iino S., Kimura S., Koike K. (2000): Virologic analysis of non-B, non-C hepatocellular carcinoma in Japan: frequent involvement of hepatitis B virus. J. Infect. Dis. 181:1920-1928.

Yuki N., Nagaoka T., Yamashiro M., Mochizuki K., Kaneko A., Yamamoto K., Omura M., Hikiji K., Kato M. (2003): Long-term histologic and virologic outcomes of acute self-limited hepatitis B. Hepatology 37:1172-1179.

Zeidler R., Eissner G., Meissner P., Uebel S., Tampe R., Lazis S., Hammerschmidt W. (1997): Downregulation of Tap1 in B lymphocytes by cellular and Epstein-Barr virusencoded interleukin-10. Blood **90**:2390-2397.

Zeldis J.B., Mugishima H., Steinberg H.N., Nir E., Gale R.P. (1986): In vitro hepatitis B virus infection of human bone marrow cells. J. Clin. Invest. 78: 411-417.

Zhou D.X., Taraboulous A., Ou J.H., Yen T.S.B. (1990): Activation of class I major histocompatability complex gene expression by hepatitis B virus. J. Virol. 64:4025–4028.

Zhu T., Hu S.L., Feng F., Polacino P., Liu H., Hwangbo Y., Learn G.H., Mullins J.I., Corey L. (2004): Persistence of low levels of simian immunodeficiency virus in macaques that were transiently viremic by conventional testing. Virology **323**:208-219.

APPENDICES

APPENDIX A

The following appendices denote the complete WHV sequences submitted to GenBank over the course of the thesis work.

Appendix A.1

LOCUS	AY334075	3308 bp DNA circular VRL 28-JAN-2004	
DEFINITION	Woodchuck hepa	titis B virus from sera, complete genome, WHV/tm3	
inoci	ulum		
ACCESSION	AY334075		
VERSION	AY334075.1 GI:33151075		
SOURCE	Woodchuck hepatitis B virus; Hepadnaviridae; Orthohepadnavirus.		
FEATURES	Location/Qualifiers		
source	13308		
	/organism="Woodchuck hepatitis B virus"		
	/mol type="genomic DNA"		
	/isolation_source="pooled sera of chronic WHV carrier		
	(WHV/tm3 inoc	ulum)"	
	/db_xref="taxon:	35269"	
CDS	join(24273308,1	1758)	
	/note="DNA polymerase, reverse transcriptase, RNase		
	activity"		
	/codon start=1		
	/product="polymerase protein"		
	/protein_id="AAP97418.1"		
	/db_xref="GI:331	51078"	
/translation="N	<i>IHPFSRLFRNIQSI</i>	LGEEEVQELLGPPEDALPLLAGEDLNHRVADALNLH	
PTADLOWVH	KTNAITGLYSNOA	AOFNPHWIOPEEPELHLHNELIKKLOOVEGPLTINE	

RKLQLNFPARFFPKATKYFPLIKGIKNNYPNFALEHFFATANYLWTLWEAGILYLRKNQ TTLTFKGKPYSWEHRQLVQHNGQQHKSHLQSRQNSSVVACSGHLLHNHLPSEPVSVST RDLSNNILGKSQNSTRTGLCSHKQIQTDRLEHLARISCGSKTTIGQQGSSPKTSSNFRNQ TWANNSSWNSGHTTWFSSASNSNKSRSREKAYSSNSTSKRYSPPLNYEKSDFSSPGVRG RIKRLDNNGTPTQCLWRSFYDSKPCGSYCIHHIVSSIDDWGPCTVTGDVTIKSPRTPRRI TGGVFLVDKNPNNSSESRLVVDFSQFSRGHTRVHWPKFAVPNLQTLANLLSTDLQWLS LDVSAAFYHIPISPAAVPHLLVGSPGLERFNTCLSYSTHNRNNSQLQTMHNLCTRHVYSS LLLLFKTYGRKLHLLAHPFIMGFRKLPMGVGLSPFLLAQFTSALASMVRRNFPHCVVFA YMDDLVLGARTSEHLTAIYTHICSVFLDLGIHLNVNKTKWWGNHLHFMGYVITSSGVL PQDKHVKKLSRYLRSVPVNQPLDYKICERLTGILNYVAPFTLCGYAALIPLYHAIASRTA FVFSSLYKSWLLSLYEELWPVVRQRGVVCSVFADATPTGWGIATTCQLLSGTFAFPLPI

ATAELIAACLARCWTGARLLGTDNSVVLSGKLTSFPWLLACVANWILRGTSFCYVPSAL NPADLPSRGLLPVLRPLPRLRFRPPTSRISLWAASPPVSPRRPVRVAWSSPVQNCEPWIPP"

CDS join(2992..3308,1..964)

/codon_start=1

/product="envelope protein"

/protein_id="AAP97419.1"

/db_xref="GI:33151079"

/translation="MGNNIKVTFNPDKIAAWWPAVGTYYTTTYPQNQSVFQPGIYQTTSLVNP KTQQELDSVLINRYKQIDWNTWQGFPVDQKLPLVNRDPPPKPAQTFEIKPGPIIVPGIRDI PRGLVPPQTPTNRDQGRKPTPPTPPLRDTHPHLTMKNQTFHLQGFVDGLRDLTTTERQ HNAYGDPFTTVSPVVPTVSTILSPPSTTGDPAQSPEMSPSSLLGLLAGLQVVYFLWTKIL TIAQNLDWWWTSLSFPGGIPECTGQNSQFQTCKHLPTSCPPTCNGFRWMYLRRFIIYLL VLLLCLIFLLVLLDWKGLIPVCPIQPTTETTVNCRQCTISVQDMYTPPYCCCLKPTAGNC TCWPIPSSWALGNYLWEWALARFSWLNLLVPLLQWLGGISLIAWFLLIWMIWFWGPAL LSILPPFIPIFVLFFLIWVYI"

CDS 1503..1928

/codon_start=1

/product="X protein"

/protein_id="AAP97416.1"

/db_xref="GI:33151076"

/translation="MAARLCCQLDPTRDVLLLRPFSSQSSGPPFPRPSAGSAASPASSLSASDES DLPLGRLPACFASASGPCCLVVTCAELRTMDSTVNFVSWHANRQLGMPSKDLWTPYIR DQLLAKWEEGSIDPRLSTFVLGGCRHKCMRLP"

CDS 1910..2587

/codon_start=1

/product="pre-core protein"

/protein_id="AAP97417.1"

/db_xref="GI:33151077"

/translation="MHATSVTMYLFHLCLVFACVPCPTVQASKLCLGWLWGMDIDPYKEFGS SYQLLNFLPLDFFPDLNALVDTATALYEEELTGREHCSPHHTTIRQALVCWDELTKLIA WMSSNITSEQVRTIIVNYVNDTWGLKVRQSLWFHLSCLTFGQHTVQEFLVSFGVWIRTP APYRPPNAPILSTLPEHTVIRRRGGARASRSPRRRTPSPRRRRSQSPRRRRSQSPSANC"

ORIGIN

1 aattegggac ataccaegtg gtttagttee geeteaaaet eaacaaate gagateaagg 61 gagaaageet acteeteeaa eteeacetet aagagataet eaeceacet taaetatgaa 121 aaateagaet ttteatetee aggggttegt ggaeggatta agagaettga eaacaaegga 181 aegeeaaeae aatgeetatg gagateettt taegaeagta ageeetggg tteetaetgt 241 ateeaceata ttgteteete eategaegae tggggaeeet geaeagteae eggagatgte 301 aeeateaagt eteetaggae teetegeagg attaeaggtg gtgtatttet tgtggaeaaa 361 aateetaaea atageteaga atetagattg gtggtggaet teteteagt tteeagggg 421 cataecagag tgeaetggee aaaattegea gtteeaaet tgeaaaeat tgeeaaeet

481 ctgtccaccg acttgcaatg gctttcgttg gatgtatctg cggcgtttta tcatatacct 601 acctgtctgt cctattcaac ccacaacaga aacaacagtc aattgcagac aatgcacaat 661 ctctgtacaa gacatgtata ctcctcctta ctgttgttgt ttaaaaccta cggcaggaaa 721 ttgcacttgc tggcccatcc cttcatcatg ggctttagga aattacctat gggagtgggc 781 cttagcccgt ttctcttggc tcaatttact agtgcccttg cttcaatggt taggaggaat 841 ttccctcatt gcgtggtttt tgcttatatg gatgatttgg ttttgggggc ccgcacttct 901 gagcatetta ecgceattta tacceatatt tgttetgttt ttettgattt gggtataeat 961 ttaaatgtta ataaaacaaa atggtggggc aatcatttac attttatggg atatgtaatt 1021 actagttcag gtgtattgcc acaagataaa catgttaaga aactttcccg ttatttacga 1081 tctgttcctg ttaatcaacc tctggattac aaaatttgtg aaagattgac tggtattctt 1141 aactatgttg ctccttttac gctgtgtgga tatgctgctt taatacctct gtatcatgct 1201 attgcttccc gtacggcttt cgttttctcc tccttgtata aatcctggtt gctgtctctt 1321 gcaaccccca ctggctgggg cattgccacc acctgtcaac tcctttctgg gactttcgct 1381 ttccccctcc cgatcgccac ggcagaactc atcgccgcct gccttgcccg ctgctggacg 1441 ggggctaggt tgttgggcac tgataattcc gtggtgttgt cggggaagct gacgtccttt 1501 ccatggctgc tcgcctgtgt tgccaactgg atcctacgcg ggacgtcctt ctgctacgtc 1561 ccttcagctc tcaatccagc ggacctccct tcccgaggcc ttctgccggt tctgcggcct 1621 ctcccgcgtc ttcgctttcg gcctccgacg agtcggatct ccctttgggc cgcctccccg 1681 cctgtttcgc ctcggcgtcc ggtccgtgtt gcttggtcgt cacctgtgca gaattgcgaa 1741 ccatggattc caccgtgaac tttgtctcct ggcatgcaaa tcgtcaactt ggcatgccaa 1801 gcaaggacct ttggactcct tatataagag atcaattatt agctaaatgg gaggagggca 1861 gcattgatcc tagattatca acatttgtat taggaggctg taggcataaa tgcatgcgac 1921 ttccgtaacc atgtatcttt ttcacctgtg ccttgttttt gcctgtgttc catgtcctac 1981 tgttcaagcc tccaagctgt gccttggatg gctttggggc atggacatag atccttataa 2041 agaatttggt tcatcttatc agttgttgaa ttttcttcct ttggacttct ttcctgatct 2101 taatgetttg gtggacaetg etactgettt gtatgaagaa gaactaacag gtagggaaca 2161 ttgctctccg catcatacaa ctattagaca agctttagta tgctgggatg aattaactaa 2221 attgataget tggatgagtt etaacataac ttetgaacaa gtaagaacaa teattgtaaa 2281 ctatgtcaat gatacctggg gacttaaggt gagacaaagt ttatggtttc atttgtcatg 2341 tctcactttc ggacaacata cagttcaaga atttttagta agttttggag tatggatcag 2401 gactccagct ccatatagac ctcctaatgc acccattctc tcgactcttc cggaacatac 2461 agtcattagg agaagaggag gtgcaagagc ttctaggtcc cccagaagac gcactccctc 2521 tectegeagg agaagatete aateacegeg tegeagaege teteaatete catetgecaa 2581 ctgctgatct tcaatgggta cataaaacta atgctattac aggtctttac tctaaccaag 2641 ctgctcagtt taacccgcat tggattcaac ctgagtttcc tgagcttcat ttacacaatg 2701 aattaattaa aaaattgcaa cagtattttg gtcccctgac tattaatgaa aagagaaaat 2761 tgcaattaaa ttttcctgca agatttttcc ccaaagctac taaatatttc cctttaatta 2821 aaggcataaa aaacaattat cctaattttg ctttagaaca tttctttgct accgcaaatt 2881 atttgtggac tttatgggaa gctggaattt tgtacttaag gaagaatcaa acaactttga 2941 cttttaaagg taaaccatat tcttgggaac acagacagct agtgcaacat aatgggcaac

3001 aacataaaag tcaccttcaa tccagacaaa atagcagcgt ggtggcctgc agtgggcact 3061 tattacacaa ccacttaccc tcagaaccag tcagtgtttc aaccagggat ttatcaaaca 3121 acatccttgg taaatcccaa aactcaacaa gaactggact ctgttctcat aaacagatac 3181 aaacagatag attggaacac ttggcaagga tttcctgtgg atcaaaaact accattggtc 3241 aacagggatc ctcccccaaa accagctcaa actttcgaaa tcaaacctgg gccaataata 3301 gttcctgg

LUCUS	AY 334076 3308 bp DNA circular VKL 28-JAN-2004
DEFINITION	Woodchuck hepatitis B virus from liver, complete genome.
ACCESSION	AY334076
VERSION	AY334076.1 GI:33151080
SOURCE	Woodchuck hepatitis B virus; Hepadnaviridae; Orthohepadnavirus.
FEATURES	Location/Qualifiers
source	13308
	/organism="Woodchuck hepatitis B virus"
	/mol_type="genomic DNA"
	/isolation_source="liver of chronic WHV carrier"
	/db_xref="taxon:35269"
CDS	join(24273308,11758)
	/note="DNA polymerase, reverse transcriptase, RNase
	activity"
	/codon_start=1
	/product="polymerase protein"
	/protein_id="AAP97422.1"
	/db_xref="GI:33151083"
/translation="M	IHPFSRLFRNIQSLGEEEVQELLGPPEDALPLLAGEDLNHRVADALNLHLP
TADLQWVHK	FNAITGLYSNQAAQFNPHWIQPEFPELHLHNELIKKLQQYFGPLTINEKRK
LQLNFPARFFI	PKATKYFPLIKGIKNNYPNFALEHFFATANYLWTLWEAGILYLRKNQTTL
TFKGKPYSWE	EHRQLVQHNGQQHKSHLQSRQNSSVVACSGHLLHNHLPSEPVSVSTRDL
SNNILGKSQN	STRTGLCSHKQIQTDRLEHLARISCGSKTTIGQQGSSPKTSSNFRNQTWA
NNSSWNSGHI	TTWFSSASNSNKSRSREKAYSSNSTSKRYSPPLNYEKSDFSSPGVRGRIK
KLDNNGIPIQ	CLWRSFYDSKPCGSYCIHHIVSSIDDWGPCTVTGDVTIKSPRTPRRITGG
VFLVDKNPNP	NSSESKLVVDFSQFSKGHIKVHWPKFAVPNLQILANLLSIDLQWLSLDV
SAAF I HIPISPA	HAVPHLLVUSPULERFNICLSISIHINKNINSQLQIMHINLUIKHVISSLLL
DDI VI GARTO	SEHI TAIVTHICSVEI DI GIHI NVNKTKWWGNHI HEMGVVITSSGVI DOD
KHVKKISRVI	RSVPVNOPI DYKICERI TGII NYVAPETI CGYAAI IPI VHAIASRTAEVES
INT THE DIGIT	

SLYKSWLLSLYEELWPVVRQRGVVCSVFADATPTGWGIATTCQLLSGTFAFPLPIATAE LIAACLARCWTGARLLGTDNSVVLSGKLTSFPWLLACVANWILRGTSFCYVPSALNPAD LPSRGLLPVLRPLPRLRFRPPTSRISLWAASPPVSPRRPVRVAWSSPVQNCEPWIPP"

CDS join(2992..3308,1..964)

/codon_start=1 /product="envelope protein" /protein_id="AAP97423.1" /db_xref="GI:33151084"

/translation="MGNNIKVTFNPDKIAAWWPAVGTYYTTTYPQNQSVFQPGIYQTTSLVNP KTQQELDSVLINRYKQIDWNTWQGFPVDQKLPLVNRDPPPKPAQTFEIKPGPIIVPGIRDI PRGLVPPQTPTNRDQGRKPTPPTPPLRDTHPHLTMKNQTFHLQGFVDGLRDLTTTERQ HNAYGDPFTTVSPVVPTVSTILSPPSTTGDPAQSPEMSPSSLLGLLAGLQVVYFLWTKIL TIAQNLDWWWTSLSFPGGIPECTGQNSQFQTCKHLPTSCPPTCNGFRWMYLRRFIIYLL VLLLCLIFLLVLLDWKGLIPVCPIQPTTETTVNCRQCTISVQDMYTPPYCCCLKPTAGNC TCWPIPSSWALGNYLWEWALARFSWLNLLVPLLQWLGGISLIAWFLLIWMIWFWGPAL LSILPPFIPIFVLFFLIWVYI"

CDS 1503..1928

/codon_start=1 /product="X protein" /protein_id="AAP97420.1" /db xref="GI:33151081"

/translation="MAARLCCQLDPTRDVLLLRPFSSQSSGPPFPRPSAGSAASPASSLSASDES DLPLGRLPACFASASGPCCLVVTCAELRTMDSTVNFVSWHANRQLGMPSKDLWTPYIR DQLLAKWEEGSIDPRLSTFVLGGCRHKCMRLP"

CDS 1910..2587

/codon_start=1 /product="pre-core protein" /protein_id="AAP97421.1" /db_xref="GI:33151082"

/translation="MHATSVTMYLFHLCLVFACVPCPTVQASKLCLGWLWGMDIDPYKEFGS SYQLLNFLPLDFFPDLNALVDTATALYEEELTGREHCSPHHTTIRQALVCWDELTKLIA WMSSNITSEQVRTIIVNYVNDTWGLKVRQSLWFHLSCLTFGQHTVQEFLVSFGVWIRTP APYRPPNAPILSTLPEHTVIRRRGGARASRSPRRRTPSPRRRRSQSPRRRRSQSPSANC"

ORIGIN

1 aattegggae ataceaegtg gtttagttee geeteaaaet eaaeaate gagateaagg 61 gagaaageet acteeteeaa eteeaeetet aagagataet eaeeeaett taaetatgaa 121 aaateagaet ttteatetee aggggttegt ggaeggatta agagaettga eaaeaaegga 181 aegeeaaeae aatgeetatg gagateettt taegaeagta ageeetggg tteetaetgt 241 ateeaeeata ttgteteete eategaegae tggggaeeet geaeagteae eggagatgte 301 aeeateaagt eteetaggae teetegagg attaeaggtg gtgtatttet tgtggaeaaa 361 aateetaaea atageteaga atetagattg gtggtggaet teteteagtt tteeaggggg

1081 tctgttcctg ttaatcaacc tctggattac aaaatttgtg aaagattgac tggtattctt 1141 aactatgttg ctccttttac gctgtgtgga tatgctgctt taatacctct gtatcatgct 1201 attgcttccc gtacggcttt cgttttctcc tccttgtata aatcctggtt gctgtctctt 1321 gcaaccccca ctggctgggg cattgccacc acctgtcaac tcctttctgg gactttcgct 1381 ttccccctcc cgatcgccac ggcagaactc atcgccgcct gccttgcccg ctgctggacg 1441 ggggctaggt tgttgggcac tgataattcc gtggtgttgt cggggaagct gacgtccttt 1501 ccatggctgc tcgcctgtgt tgccaactgg atcctacgcg ggacgtcctt ctgctacgtc 1561 ccttcagctc tcaatccagc ggacctccct tcccgaggcc ttctgccggt tctgcggcct 1621 ctcccgcgtc ttcgctttcg gcctccgacg agtcggatct ccctttgggc cgcctccccg 1681 cctgtttcgc ctcggcgtcc ggtccgtgtt gcttggtcgt cacctgtgca gaattgcgaa 1741 ccatggattc caccgtgaac tttgtctcct ggcatgcaaa tcgtcaactt ggcatgccaa 1801 gcaaggacct ttggactcct tatataagag atcaattatt agctaaatgg gaggagggca 1861 gcattgatcc tagattatca acatttgtat taggaggctg taggcataaa tgcatgcgac 1921 ttccgtaacc atgtatcttt ttcacctgtg ccttgttttt gcctgtgttc catgtcctac 1981 tgttcaagcc tccaagctgt gccttggatg gctttggggc atggacatag atccttataa 2041 agaatttggt tcatcttatc agttgttgaa ttttcttcct ttggacttct ttcctgatct 2101 taatgetttg gtggacaetg etactgettt gtatgaagaa gaactaacag gtagggaaca 2161 ttgctctccg catcatacaa ctattagaca agctttagta tgctgggatg aattaactaa 2221 attgataget tggatgagtt etaacataac ttetgaacaa gtaagaacaa teattgtaaa 2281 ctatgtcaat gatacctggg gacttaaggt gagacaaagt ttatggtttc atttgtcatg 2341 tctcactttc ggacaacata cagttcaaga atttttagta agttttggag tatggatcag 2401 gactccagct ccatatagac ctcctaatgc acccattctc tcgactcttc cggaacatac 2461 agtcattagg agaagaggag gtgcaagagc ttctaggtcc cccagaagac gcactccctc 2521 tcctcgcagg agaagatctc aatcaccgcg tcgcagacgc tctcaatctc catctgccaa 2581 ctgctgatct tcaatgggta cataaaacta atgctattac aggtctttac tctaaccaag 2641 ctgctcagtt taacccgcat tggattcaac ctgagtttcc tgagcttcat ttacacaatg 2701 aattaattaa aaaattgcaa cagtattttg gtcccctgac tattaatgaa aagagaaaat 2761 tgcaattaaa ttttcctgca agatttttcc ccaaagctac taaatatttc cctttaatta 2821 aaggcataaa aaacaattat cctaattttg ctttagaaca tttctttgct accgcaaatt 2881 atttgtggac tttatgggaa gctggaattt tgtacttaag gaagaatcaa acaactttga 2941 cttttaaagg taaaccatat tcttgggaac acagacagct agtgcaacat aatgggcaac 3001 aacataaaag tcaccttcaa tccagacaaa atagcagcgt ggtggcctgc agtgggcact 3061 tattacacaa ccacttaccc tcagaaccag tcagtgtttc aaccagggat ttatcaaaca 3121 acatecttgg taaateccaa aacteaacaa gaactggaet etgtteteat aaacagatae 3181 aaacagatag attggaacac ttggcaagga tttcctgtgg atcaaaaact accattggtc 3241 aacagggatc ctcccccaaa accagctcaa actttcgaaa tcaaacctgg gccaataata 3301 gttcctgg

LOCUS	AY334077 3308 bp DNA circular VRL 28-JAN-2004			
DEFINITION	Woodchuck hepatitis B virus from spleen, complete genome.			
ACCESSION	AY334077			
VERSION	AY334077.1 GI:33151085			
SOURCE	Woodchuck hepatitis B virus; Hepadnaviridae; Orthohepadnavirus.			
FEATURES	Location/Qualifiers			
source	13308			
	/organism="Woodchuck hepatitis B virus"			
	/mol type="genomic DNA"			
	/isolation source="spleen of chronic WHV carrier"			
	/db xref="taxon:35269"			
CDS	join(24273308,11758)			
	/note="DNA polymerase, reverse transcriptase, RNase			
	activity"			
	/codon start=1			
	/product="polymerase protein"			
	/protein id="AAP97426.1"			
	/db xref="GI:33151088"			
/translation="M	IHPFSRLFRNIQSLGEEEVQELLGPPEDALPLLAGEDLNHRVADALNLHLP			
TADLQWVHK	FNAITGLYSNQAAQFNPHWIQPEFPELHLHNELIKKLQQYFGPLTINEKRK			
LQLNFPARFF	PKATKYFPLIKGIKNNYPNFALEHFFATANYLWTLWEAGILYLRKNQTTL			
TFKGKPYSWE	EHRQLVQHNGQQHKSHLQSRQNSSVVACSGHLLHNHLPSEPVSVSTRDL			
SNNILGKSQN	STRTGLCSHKQIQTDRLEHLARISCGSKTTIGQQGSSPKTSSNFRNQTWA			
NNSSWNSGHT	TWFSSASNSNKSRSREKAYSSNSTSKRYSPPLNYEKSDFSSPGVRGRIK			
RLDNNGTPTQ	CLWRSFYDSKPCGSYCIHHIVSSIDDWGPCTVTGDVTIKSPRTPRRITGG			
VFLVDKNPNN	NSSESRLVVDFSQFSRGHTRVHWPKFAVPNLQTLANLLSTDLQWLSLDV			
SAAFYHIPISPA	AAVPHLLVGSPGLERFNTCLSYSTHNRNNSQLQTMHNLCTRHVYSSLLL			

307

FSSLYKSWLLSLYEELWPVVRQRGVVCSVFADATPTGWGIATTCQLLSGTFAFPLPIAT AELIAACLARCWTGARLLGTDNSVVLSGKLTSFPWLLACVANWILRGTSFCYVPSALN PADLPSRGLLPVLRPLPRLRFRPPTSRISLWAASPPVSPRRPVRVAWSSPVQNCEPWIPP"

LFKTYGRKLHLLAHPFIMGFRKLPMGVGLSPFLLAQFTSALASMVRRNFPHCVVFAYM

DDLVLGARTSEHLTAIYTHICSVFLDLGIHLNVNKTKWWGNHLHFMGYVITSSGVLPQ

DKHVKKLSRYLRSVPVNQPLDYKICERLTGILNYVAPFTLCGYAALIPLYHAIASRTAFV

CDS join(2992..3308,1..964)

/codon_start=1 /product="envelope protein" /protein_id="AAP97427.1" /db_xref="GI:33151089"

/translation="MGNNIKVTFNPDKIAAWWPAVGTYYTTTYPQNQSVFQPGIYQTTSLVNP KTQQELDSVLINRYKQIDWNTWQGFPVDQKLPLVNRDPPPKPAQTFEIKPGPIIVPGIRDI PRGLVPPQTPTNRDQGRKPTPPTPPLRDTHPHLTMKNQTFHLQGFVDGLRDLTTTERQ HNAYGDPFTTVSPVVPTVSTILSPPSTTGDPAQSPEMSPSSLLGLLAGLQVVYFLWTKIL TIAQNLDWWWTSLSFPGGIPECTGQNSQFQTCKHLPTSCPPTCNGFRWMYLRRFIIYLL VLLLCLIFLLVLLDWKGLIPVCPIQPTTETTVNCRQCTISVQDMYTPPYCCCLKPTAGNC TCWPIPSSWALGNYLWEWALARFSWLNLLVPLLQWLGGISLIAWFLLIWMIWFWGPAL LSILPPFIPIFVLFFLIWVYI"

CDS 1503..1928

/codon start=1 /product="X protein" /protein id="AAP97424.1" /db xref="GI:33151086"

/translation="MAARLCCQLDPTRDVLLLRPFSSQSSGPPFPRPSAGSAASPASSLSASDES DLPLGRLPACFASASGPCCLVVTCAELRTMDSTVNFVSWHANRQLGMPSKDLWTPYIR DQLLAKWEEGSIDPRLSTFVLGGCRHKCMRLP"

1910..2587 CDS

> /codon start=1 /product="pre-core protein" /protein id="AAP97425.1" /db xref="GI:33151087"

/translation="MHATSVTMYLFHLCLVFACVPCPTVQASKLCLGWLWGMDIDPYKEFGS SYQLLNFLPLDFFPDLNALVDTATALYEEELTGREHCSPHHTTIRQALVCWDELTKLIA WMSSNITSEQVRTIIVNYVNDTWGLKVRQSLWFHLSCLTFGQHTVQEFLVSFGVWIRT PAPYRPPNAPILSTLPEHTVIRRRGGARASRSPRRRTPSPRRRRSQSPRRRRSQSPSANC"

ORIGIN

1 aattcgggac ataccacgtg gtttagttcc gcctcaaact ccaacaaatc gagatcaagg 61 gagaaagcct actcctccaa ctccacctct aagagatact cacccccact taactatgaa 121 aaatcagact tttcatctcc aggggttcgt ggacggatta agagacttga caacaacgga 181 acgccaacac aatgcctatg gagatccttt tacgacagta agccctgtgg ttcctactgt 241 atccaccata ttgtctcctc catcgacgac tggggaccct gcacagtcac cggagatgtc 301 accatcaagt ctcctaggac tcctcgcagg attacaggtg gtgtatttct tgtggacaaa 361 aatcctaaca atagctcaga atctagattg gtggtggact tctctcagtt ttccaggggg 421 cataccagag tgcactggcc aaaattcgca gttccaaact tgcaaacact tgccaacctc 481 ctgtccaccg acttgcaatg gctttcgttg gatgtatctg cggcgtttta tcatatacct 601 acctgtctgt cctattcaac ccacaacaga aacaacagtc aattgcagac aatgcacaat 661 ctctgtacaa gacatgtata ctcctcctta ctgttgttgt ttaaaaccta cggcaggaaa 721 ttgcacttgc tggcccatcc cttcatcatg ggctttagga aattacctat gggagtgggc 781 cttagcccgt ttctcttggc tcaatttact agtgcccttg cttcaatggt taggaggaat 841 ttccctcatt gcgtggtttt tgcttatatg gatgatttgg ttttgggggc ccgcacttct 901 gagcatetta ecgceattta tacceatatt tgttetgttt ttettgattt gggtataeat 961 ttaaatgtta ataaaacaaa atggtggggc aatcatttac attttatggg atatgtaatt 1021 actagttcag gtgtattgcc acaagataaa catgttaaga aactttcccg ttatttacga

1081 tctgttcctg ttaatcaacc tctggattac aaaatttgtg aaagattgac tggtattctt 1141 aactatgttg ctccttttac gctgtgtgga tatgctgctt taatacctct gtatcatgct 1201 attgcttccc gtacggcttt cgttttctcc tccttgtata aatcctggtt gctgtctctt 1321 gcaaccccca ctggctgggg cattgccacc acctgtcaac tcctttctgg gactttcgct 1381 ttcccctcc cgatcgccac ggcagaactc atcgccgcct gccttgcccg ctgctggacg 1441 ggggctaggt tgttgggcac tgataattcc gtggtgttgt cggggaagct gacgtccttt 1501 ccatggctgc tcgcctgtgt tgccaactgg atcctacgcg ggacgtcctt ctgctacgtc 1561 ccttcagctc tcaatccagc ggacctccct tcccgaggcc ttctgccggt tctgcggcct 1621 ctcccgcgtc ttcgctttcg gcctccgacg agtcggatct ccctttgggc cgcctccccg 1681 cctgtttcgc ctcggcgtcc ggtccgtgtt gcttggtcgt cacctgtgca gaattgcgaa 1741 ccatggattc caccgtgaac tttgtctcct ggcatgcaaa tcgtcaactt ggcatgccaa 1801 gcaaggacct ttggactcct tatataagag atcaattatt agctaaatgg gaggagggca 1861 gcattgatcc tagattatca acatttgtat taggaggctg taggcataaa tgcatgcgac 1921 ttccgtaacc atgtatcttt ttcacctgtg ccttgttttt gcctgtgttc catgtcctac 1981 tgttcaagcc tccaagctgt gccttggatg gctttggggc atggacatag atccttataa 2041 agaatttggt tcatcttatc agttgttgaa ttttcttcct ttggacttct ttcctgatct 2101 taatgetttg gtggacaetg etaetgettt gtatgaagaa gaactaacag gtagggaaca 2161 ttgctctccg catcatacaa ctattagaca agctttagta tgctgggatg aattaactaa 2221 attgataget tggatgagtt etaacataac ttetgaacaa gtaagaacaa teattgtaaa 2281 ctatgtcaat gatacctggg gacttaaggt gagacaaagt ttatggtttc atttgtcatg 2341 tctcactttc ggacaacata cagttcaaga atttttagta agttttggag tatggatcag 2401 gactccagct ccatatagac ctcctaatgc acccattctc tcgactcttc cggaacatac 2461 agtcattagg agaagaggag gtgcaagagc ttctaggtcc cccagaagac gcactccctc 2521 tectegeagg agaagatete aateacegeg tegeagaege teteaatete catetgecaa 2581 ctgctgatct tcaatgggta cataaaacta atgctattac aggtctttac tctaaccaag 2641 ctgctcagtt taacccgcat tggattcaac ctgagtttcc tgagcttcat ttacacaatg 2701 aattaattaa aaaattgcaa cagtattttg gtcccctgac tattaatgaa aagagaaaat 2761 tgcaattaaa ttttcctgca agatttttcc ccaaagctac taaatatttc cctttaatta 2821 aaggcataaa aaacaattat cctaattttg ctttagaaca tttctttgct accgcaaatt 2881 atttgtggac tttatgggaa gctggaattt tgtacttaag gaagaatcaa acaactttga

2941 cttttaaagg taaaccatat tettgggaac acagacaget agtgeaacat aatgggeaac 3001 aacataaaag teacetteaa teeagacaaa atageagegt ggtggeetge agtgggeact 3061 tattacacaa ecaettacee teagaaccag teagtgttte aaccagggat ttateaaaca 3121 acateettgg taaateecaa aacteaacaa gaactggaet etgtteteat aaacagatac 3181 aaacagatag attggaacae ttggeaagga ttteetggg ateaaaaaet aceattggte 3241 aacagggate eteeceaaa aceageteaa aettegaaa teaaacetgg geeaataata 3301 gtteetgg

LOCUS	AY628095 3308 bp DNA circular VRL 15-JUN-2004				
DEFINITION	Woodchuck hepatitis B virus clone 1 isolation-source serum,				
	complete genome, WHV/tm2 inoculum.				
ACCESSION	AY628095				
VERSION	AY628095.1 GI:48526470				
SOURCE	Woodchuck hepatitis B virus; Hepadnaviridae; Orthohepadnavirus.				
FEATURES	Location/Qualifiers				
source	13308				
	/organism="Woodchuck hepatitis B virus"				
	/mol_type="genomic DNA"				
	/isolation source="serum; of a chronic WHV carrier"				
	/db xref="taxon:35269"				
	/clone="1"				
CDS	join(24273308,11758)				
	/note="DNA polymerase; reverse transcriptase; RNase				
	activity"				
	/codon_start=1				
	/product="polymerase protein"				
	/protein_id="AAT45432.1"				
	/db_xref="GI:48526473"				
/translation="N	HPFSRLFRNIQSLGEEEVQELLGPPEDALPLLAGEDLNHRVADALNLHL				
PTADLQWVH	KTNAITGLYSNQAAQFNPHWIQPEFPELHLHNELIKKLQQYFGPLTINEK				
RKLQLNFPAR	FFPKATKYFPLIKGIKNNYPNFALEHFFATANYLWTLWEAGILYLRKNQ				
TTLTFKGKPY	SWEHRQLVQHNGQQHKSHLQSRQNSSVVACSGHLLHNHLPSEPVSVST				
RDLSNNILGK	SQNSTRTGLCSHKQIQTDRLEHLARISCGSKTTIGQQGSSPKTSSNFRNQ				
TWAYNSSWN	ISGHTTWFSSASNSNKSRSREKAYSSNSTSKRYSPPLNYEKSDFSSPGVR				
GRIKRLDNNG	TPTOCLWRSFYDSKPCGSYCIHHIVSSIDDWGPCTVTGDVTIKSPRTPRR				

310

FAYMDDLVLGARTSEHLTAIYTHICSVFLDLGIHLNVNKTKWWGNHLHFMGYVITSSG VLPQDKHVKKLSRYLRSVPVNQPLDYKICERLTGILNYVAPFTLCGYAALMPLYHAIAS RTAFVFSSLYKSWLLSLYEELWPVVRQRGVVCSVFADATPTGWGIATTCQLLSGTFAFP LPIATAELIAACLARCWTGARLLGTDNSVVLSGKLTSFPWLLACVANWILRGTSFCYVP SALNPADLPSRGLLPVLRPLPRLRFRPPTSRISLWAASPPVSPRRPVRVAWSSPVQNCEP WIPP"

ITGGVFLVDKNPNNSSESRLVVDFSQFSRGHTRVHWPKFAVPNLQTLANLLSTDLQWL

SLDVSAAFYHIPISPAAVPHLLVGSPGLERFNTCLSYSTHNRNNSQLQTMHNLCTRHVY

SSLLLLFKTYGRKLHLLAHPFIMGFRKLPMGVGLSPFLLAQFTSALASMVRRNFPHCVV

CDS join(2992..3308,1..964) /codon_start=1 /product="envelope protein" /protein_id="AAT45433.1" /db_xref="GI:48526474" /translation="MGNNIKVTFNPDKIAAWWPAVGTYYTTTYPQNQSVFQPGIYQTTSLVNP KTQQELDSVLINRYKQIDWNTWQGFPVDQKLPLVNRDPPPKPAQTFEIKPGPIIVPGIRDI PRGLVPPQTPTNRDQGRKPTPPTPPLRDTHPHLTMKNQTFHLQGFVDGLRDLTTTERQ HNAYGDPFTTVSPVVPTVSTILSPPSTTGDPAQSPEMSPSSLLGLLAGLQVVYFLWTKIL TIAQNLDWWWTSLSFPGGIPECTGQNSQFQTCKHLPTSCPPTCNGFRWMYLRRFIIYLL VLLLCLIFLLVLLDWKGLIPVCPIQPTTETTVNCRQCTISVQDMYTPPYCCCLKPTAGNC TCWPIPSSWALGNYLWEWALARFSWLNLLVPLLQWLGGISLIAWFLLIWMIWFWGPAL LSILPPFIPIFVLFFLIWVYI"

CDS 1503..1928

/codon_start=1 /product="X protein" /protein_id="AAT45430.1" /db_xref="GI:48526471"

/translation="MAARLCCQLDPTRDVLLLRPFSSQSSGPPFPRPSAGSAASPASSLSASDES DLPLGRLPACFASASGPCCLVVTCAELRTMDSTVNFVSWHANRQLGMPSKDLWTPYIR DQLLTKWEEGSIDPRLSIFVLGGCRHKCMRLL"

CDS 1910..2587

/codon_start=1

/product="pre-core protein"

/protein id="AAT45431.1"

/db xref="GI:48526472"

/translation="MHATSVTMYLFHLCLVFACVPCPTVQASKLCLGWLWGMDIDPYKEFGS SYQLLNFLPLDFFPDLNALVDTATALYEEELTGREHCSPHHTAIRQALVCWDELTKLIA WMSSNITSEQVRTIIVNHVNDTWGLKVRQSLWFHLSCLTFGQHTVQEFLVSFGVWIRT PAPYRPPNAPILSTLPEHTVIRRRGGARASRSPRRRTPSPRRRRSQSPRRRRSQSPSANC"

ORIGIN

1 aattcgggac ataccacgtg gtttagttcc gcctcaaact ccaacaaatc gagatcaagg

- 61 gagaaagcct actcctccaa ctccacctct aagagatact cacccccact taactatgaa
- 121 aaatcagact tttcatctcc aggggttcgt ggacggatta agagacttga caacaacgga
- 181 acgccaacac aatgcctatg gagatccttt tacgacagta agccctgtgg ttcctactgt
- 241 atccaccata ttgtctcctc catcgacgac tggggaccct gcacagtcac cggagatgtc

901 gagcatetta ecgecattta tacceatatt tgttetgttt ttettgattt gggtataeat 961 ttaaatgtta ataaaacaaa atggtggggc aatcatttac attttatggg atatgtaatt 1021 actagttcag gtgtattgcc acaagataaa catgttaaga aactttcccg ttatttacga 1081 tctgttcctg ttaatcaacc tctggattac aaaatttgtg aaagattgac tggtattctt 1141 aactatgttg ctccttttac gctgtgtgga tatgctgctt taatgcctct gtatcatgct 1201 attgcttccc gtacggcttt cgttttctcc tccttgtata aatcctggtt gctgtctctt 1321 gcaaccccca ctggctgggg cattgccacc acctgtcaac tcctttctgg gactttcgct 1381 ttccccctcc cgatcgccac ggcagaactc atcgccgcct gccttgcccg ctgctggacg 1441 ggggctaggt tgttgggcac tgataattcc gtggtgttgt cggggaagct gacgtccttt 1501 ccatggctgc tcgcctgtgt tgccaactgg atcctacgcg ggacgtcctt ctgctacgtc 1561 ccttcagctc tcaatccagc ggacctccct tcccgaggcc ttctgccggt tctgcggcct 1621 ctcccgcgtc ttcgctttcg gcctccgacg agtcggatct ccctttgggc cgcctccccg 1681 cctgtttcgc ctcggcgtcc ggtccgtgtt gcttggtcgt cacctgtgca gaattgcgaa 1741 ccatggattc caccgtgaac tttgtctcct ggcatgcaaa tcgtcaactt ggcatgccaa 1801 gcaaggacct ttggactcct tatataagag atcaattatt aactaaatgg gaggagggca 1861 gcattgatcc tagattatca atatttgtat taggaggctg taggcataaa tgcatgcgac 1921 ttctgtaacc atgtatcttt ttcacctgtg ccttgttttt gcctgtgttc catgtcctac 1981 tgttcaagcc tccaagctgt gccttggatg gctttggggc atggacatag atccttataa 2041 agaatttggt tcatcttatc agttgttgaa ttttcttcct ttggacttct ttcctgatct 2101 taatgetttg gtggacaetg etactgettt gtatgaagaa gaactaacag gtagggaaca 2161 ttgctctccg catcatacag ctattagaca agctttagta tgctgggatg aattaactaa 2221 attgataget tggatgagtt etaacataac ttetgaacaa gtaagaacaa teattgtaaa 2281 ccatgtcaat gatacctggg gacttaaggt gagacaaagt ttatggtttc atttgtcatg 2341 tctcactttc ggacaacata cagttcaaga atttttagta agttttggag tatggatcag 2401 gactccagct ccatatagac ctcctaatgc acccattctc tcgactcttc cggaacatac 2461 agtcattagg agaagaggag gtgcaagagc ttctaggtcc cccagaagac gcactccctc 2521 tcctcgcagg agaagatete aatcaccgcg tcgcagacge teteaatete catetgecaa 2581 ctgctgatct tcaatgggta cataaaacta atgctattac aggtctttac tctaaccaag 2641 ctgctcagtt taacccgcat tggattcaac ctgagtttcc tgagcttcat ttacacaatg 2701 aattaattaa aaaattgcaa cagtattttg gtcccttgac tattaatgaa aagagaaaat 2761 tgcaattaaa ttttcctgca agatttttcc ccaaagctac taaatatttc cctttaatta 2821 aaggcataaa aaacaattat cctaattttg ctttagaaca tttctttgct accgcaaatt 2881 atttgtggac tttatgggaa gctggaattt tgtatttaag gaagaatcaa acaactttga 2941 cttttaaagg taaaccatat tcttgggaac acagacagct agtgcaacat aatgggcaac 3001 aacataaaag tcaccttcaa tccagacaaa atagcagcgt ggtggcctgc agtgggcact 3061 tattacacaa ccacttaccc tcagaaccag tcagtgtttc aaccagggat ttatcaaaca 3121 acatecttgg taaateccaa aacteaacaa gaactggact etgtteteat aaacagatae 3181 aaacagatag attggaacac ttggcaagga tttcctgtgg atcaaaaact accattggtc 3241 aacagggatc ctcccccaaa accagctcaa actttcgaaa tcaaacctgg gcctataata 3301 gttcctgg

LOCUS	AY628096 3308 bp DNA circular VRL 15-JUN-2004
DEFINITION	Woodchuck hepatitis B virus clone 2 isolation-source serum,
	complete genome, WHV/tm2 inoculum.
ACCESSION	AY628096
VERSION	AY628096.1 GI:48526475
SOURCE	Woodchuck hepatitis B virus; Hepadnaviridae; Orthohepadnavirus.
FEATURES	Location/Qualifiers
source	13308
	/organism="Woodchuck hepatitis B virus"
	/mol type="genomic DNA"
	/isolation source="serum; of a chronic WHV carrier"
	/db xref="taxon:35269"
	/clone="2"
CDS	join(24273308,11758)
	/note="DNA polymerase; reverse transcriptase; RNase
	activity"
	/codon start=1
	/product="polymerase protein"
	/protein id="AAT45436.1"
	/db xref="GI:48526478"
/translation="N	IHPFSRLFRNIQSLGEEEVQELLGPPEDALPLLAGEDLNHRVADALNLHL
PTADLQWVHI	KTNAITGLYSNQAAQFNPHWIQPEFPELHLHNELIKKLQQYFGPLTINEK
RKLQLNFPAR	FFPKATKYFPLIKGIKNNYPNFALEHFFATANYLWTLWEAGILYLRKNQ
TTLTFKGKPY	SWEHRQLVQHNGQQHKSHLQSRQNSSVVACSGHLLHNHLPSEPVSVST
RDLSNNILSKS	SQNSTRTGLCSHKQIQTDRLEHLARISCGSKTTIGQQGSTPKTSSNFRNQ
TWAYNSSWN	SGHTTWFSSASNSNKSRSREKAYSSNSTSKRYSPPLNYEKSDFSSPGVR
GRIKRLDNNG	TPTQCLWRSFYDSKPCGSYCIHHIVSSIDDWGPCTVTGDVTIKSPRTPRR
ITGGVFLVDK	NPNNSSESRLVVDFSQFSRGHTRVHWPKFAVPNLQTLANLLSTDLQWL
SLDVSAAFYH	IPISPAAVPHLLVGSPGLERFNTCLSYSTHNRNNSQLQTMHNLCTRHVY
SSLLLLFKTY(GRKLHLLAHPFIMGFRKLPMGVGLSPFLLAQFTSALASMVRRNFPHCVV

FAYMDDLVLGARTSEHLTAIYTHICSVFLDLGIHLNVNKTKWWGNHLHFMGYVITSSG VLPQDKHVKKLSRYLRSVPVNQPLDYKICERLTGILNYVAPFTLCGYAALMPLYHAIAS RTAFVFSSLYKSWLLSLYEELWPVVRQRGVVCSVFADATPTGWGIATTCQLLSGTFAFP LPIATAELIAACLARCWTGARLLGTDNSVVLSGKLTSFPWLLACVANWILRGTSFCYVP SALNPADLPSRGLLPVLRPLPRLRFRPPTSRISLWAASPPVSPRRPVRVAWSSPVQNCEP WIPP"

CDS join(2992..3308,1..964) /codon_start=1 /product="envelope protein" /protein_id="AAT45437.1" /db_xref="GI:48526479" /translation="MGNNIKVTFNPDKIAAWWPAVGTYYTTTYPQNQSVFQPGIYQTTSLVNP KTQQELDSVLINRYKQIDWNTWQGFPVDQKLPLVNRDPPPKPAQTFEIKPGPIIVPGIRDI PRGLVPPQTPTNRDQGRKPTPPTPPLRDTHPHLTMKNQTFHLQGFVDGLRDLTTTERQ HNAYGDPFTTVSPVVPTVSTILSPPSTTGDPAQSPEMSPSSLLGLLAGLQVVYFLWTKIL TIAQNLDWWWTSLSFPGGIPECTGQNSQFQTCKHLPTSCPPTCNGFRWMYLRRFIIYLL VLLLCLIFLLVLLDWKGLIPVCPIQPTTETTVNCRQCTISVQDMYTPPYCCCLKPTAGNC TCWPIPSSWALGNYLWEWALARFSWLNLLVPLLQWLGGISLIAWFLLIWMIWFWGPAL LSILPPFIPIFVLFFLIWVYI"

CDS 1503..1928

/codon_start=1 /product="X protein" /protein_id="AAT45434.1" /db_xref="GI:48526476"

/translation="MAARLCCQLDPTRDVLLLRPFSSQSSGPPFPRPSAGSAASPASSLSASDES DLPLGRLPACFASASGPCCLVVTCAELRTMDSTVNFVSWHANRQLGMPSKDLWTPYIR DQLLTKWEEGSIDPRLSIFVLGGCRHKCMRLL"

CDS 1910..2587

/codon_start=1 /product="pre-core protein" /protein_id="AAT45435.1" /db_xref="GI:48526477"

/translation="MHATSVTMYLFHLCLVFACVPCPTVQASKLCLGWLWGMDIDPYKEFGS SYQLLNFLPLDFFPDLNALVDTATALYEEELTGREHCSPHHTAIRQALVCWDELTKLIA WMSSNITSEQVRTIIVNHVNDTWGLKVRQSLWFHLSCLTFGQHTVQEFLVSFGVWIRT PAPYRPPNAPILSTLPEHTVIRRRGGARASRSPRRRTPSPRRRRSQSPRRRRSQSPSANC"

ORIGIN

l aattegggae ataceaegtg gtttagttee geeteaaate ceaaeaate gagateaagg

61 gagaaagcct actcctccaa ctccacctct aagagatact cacccccact taactatgaa

121 aaatcagact tttcatctcc aggggttcgt ggacggatta agagacttga caacaacgga

181 acgccaacac aatgcctatg gagatccttt tacgacagta agccctgtgg ttcctactgt

241 atccaccata ttgtctcctc catcgacgac tggggaccct gcacagtcac cggagatgtc

901 gagcatetta ecgecattta tacceatatt tgttetgttt ttettgattt gggtataeat 961 ttaaatgtta ataaaacaaa atggtggggc aatcatttac attttatggg atatgtaatt 1021 actagttcag gtgtattgcc acaagataaa catgttaaga aactttcccg ttatttacga 1081 tctgttcctg ttaatcaacc tctggattac aaaatttgtg aaagattgac tggtattctt 1141 aactatgttg ctccttttac gctgtgtgga tatgctgctt taatgcctct gtatcatgct 1201 attgcttccc gtacggcttt cgttttctcc tccttgtata aatcctggtt gctgtctctt 1321 gcaaccccca ctggctgggg cattgccacc acctgtcaac tcctttctgg gactttcgct 1381 ttcccctcc cgatcgccac ggcagaactc atcgccgcct gccttgcccg ctgctggacg 1441 ggggctaggt tgttgggcac tgataattcc gtggtgttgt cggggaagct gacgtccttt 1501 ccatggctgc tcgcctgtgt tgccaactgg atcctacgcg ggacgtcctt ctgctacgtc 1561 ccttcagctc tcaatccagc ggacctccct tcccgaggcc ttctgccggt tctgcggcct 1621 ctcccgcgtc ttcgctttcg gcctccgacg agtcggatct ccctttgggc cgcctccccg 1681 cctgtttcgc ctcggcgtcc ggtccgtgtt gcttggtcgt cacctgtgca gaattgcgaa 1741 ccatggattc caccgtgaac tttgtctcct ggcatgcaaa tcgtcaactt ggcatgccaa 1801 gcaaggacct ttggactcct tatataagag atcaattatt aactaaatgg gaggagggca 1861 gcattgatcc tagattatca atatttgtat taggaggctg taggcataaa tgcatgcgac 1921 ttctgtaacc atgtatcttt ttcacctgtg ccttgttttt gcctgtgttc catgtcctac 1981 tgttcaagcc tccaagctgt gccttggatg gctttggggc atggacatag atccttataa 2041 agaatttggt tcatcttatc agttgttgaa ttttcttcct ttggacttct ttcctgatct 2101 taatgetttg gtggacaetg etactgettt gtatgaagaa gaactaacag gtagggaaca 2161 ttgctctccg catcatacag ctattagaca agctttagta tgctgggatg aattaactaa 2221 attgataget tggatgagtt etaacataac ttetgaacaa gtaagaacaa teattgtaaa 2281 ccatgtcaat gatacctggg gacttaaggt gagacaaagt ttatggtttc atttgtcatg 2341 tctcactttc ggacaacata cagttcaaga atttttagta agttttggag tatggatcag 2401 gactccagct ccatatagac ctcctaatgc acccattctc tcgactcttc cggaacatac 2461 agtcattagg agaagaggag gtgcaagagc ttctaggtcc cccagaagac gcactccctc 2521 tectegeagg agaagatete aateacegeg tegeagaege teteaatete catetgecaa 2581 ctgctgatct tcaatgggta cataaaacta atgctattac aggtctttac tctaaccaag 2641 ctgctcagtt taacccgcat tggattcaac ctgagtttcc tgagcttcat ttacacaatg 2701 aattaattaa aaaattgcaa cagtattttg gtcccttgac tattaatgaa aagagaaaat 2761 tgcaattaaa ttttcctgca agatttttcc ccaaagctac taaatatttc cctttaatta 2821 aaggcataaa aaacaattat cctaattttg ctttagaaca tttctttgct accgcaaatt 2881 atttgtggac tttatgggaa gctggaattt tgtatttaag gaagaatcaa acaactttga 2941 cttttaaagg taaaccatat tcttgggaac acagacagct agtgcaacat aatgggcaac 3001 aacataaaag tcaccttcaa tccagacaaa atagcagcgt ggtggcctgc agtgggcact 3061 tattacacaa ccacttaccc tcagaaccag tcagtgtttc aaccagggat ttatcaaaca 3121 acatecttag taaateccaa aacteaacaa gaactggact etgtteteat aaacagatae 3181 aaacagatag attggaacac ttggcaagga tttcctgtgg atcaaaaact accattggtc 3241 aacagggatc cacccccaaa accagctcaa actttcgaaa tcaaacctgg gcctataata 3301 gttcctgg

3308 bp DNA circular VRL 15-JUN-2004 LOCUS AY628097 DEFINITION Woodchuck hepatitis B virus clone 1 isolation-source liver, complete genome. ACCESSION AY628097 VERSION AY628097.1 GI:48526480 Woodchuck hepatitis B virus; Hepadnaviridae; Orthohepadnavirus. SOURCE Location/Qualifiers FEATURES 1..3308 source /organism="Woodchuck hepatitis B virus" /mol type="genomic DNA" /isolation source="liver; of a chronic WHV carrier" /db xref="taxon:35269" /clone="1" CDS join(2427..3308,1..1758) /note="DNA polymerase; reverse transcriptase; RNase activity" /codon start=1 /product="polymerase protein" /protein id="AAT45440.1" /db xref="GI:48526483" /translation="MHPFSRLFRNIQSLGEEEVQELLGPPEDALPLLAGEDLNHRVADALNLHL PTADLQWVHKTNAITGLYSNQAAQFNPHWIQPEFPELHLHNELIKKLQQYFGPLTINEK RKLQLNFPARFFPKATKYFPLIKGIKNNYPNFALEHFFATANYLWTLWEAGILYLRKNQ TTLTFKGKPYSWEHRQLVQHNGQQHKSHLQSRQNSSVVACSGHLLHNHLPSEPVSVST RDLSNNILGKSQNSTRTGLCSHKQIQTDRLEHLARISCGSKTTIGQQGSSPKTSSNFRNQ TWAYNSSWNSGHTTWFSSASNSNKSRSREKAYSSNSTSKRYSPPLNYEKSDFSSPGVR GRIKRLDNNGTPTQCLWRSFYDSKPCGSYCIHHIVSSIDDWGPCTVTGDVTIKSPRTPRR ITGGVFLVDKNPNNSSESRLVVDFSQFSRGHTRVHWPKFAVPNLQTLANLLSTDLQWL SLDVSAAFYHIPISPAAVPHLLVGSPGLERFNTCLSYSTHNRNNSQLQTMHNLCTRHVY

FAYMDDLVLGARTSEHLTAIYTHICSVFLDLGIHLNVNKTKWWGNHLHFMGYVITSSG VLPQDKHVKKLSRYLRSVPVNQPLDYKICERLTGILNYVAPFTLCGYAALMPLYHAIAS RTAFVFSSLYKSWLLSLYEELWPVVRQRGVVCSVFADATPTGWGIATTCQLLSGTFAFP LPIATAELIAACLARCWTGARLLGTDNSVVLSGKLTSFPWLLACVANWILRGTSFCYVP SALNPADLPSRGLLPVLRPLPRLRFRPPTSRISLWAASPPVSPRRPVRVAWSSPVQNCEP WIPP"

SSLLLLFKTYGRKLHLLAHPFIMGFRKLPMGVGLSPFLLAQFTSALASMVRRNFPHCVV

CDS join(2992..3308,1..964) /codon_start=1 /product="envelope protein" /protein_id="AAT45441.1" /db_xref="GI:48526484" /translation="MGNNIKVTFNPDKIAAWWPAVGTYYTTTYPQNQSVFQPGIYQTTSLVNP KTQQELDSVLINRYKQIDWNTWQGFPVDQKLPLVNRDPPPKPAQTFEIKPGPIIVPGIRDI PRGLVPPQTPTNRDQGRKPTPPTPPLRDTHPHLTMKNQTFHLQGFVDGLRDLTTTERQ HNAYGDPFTTVSPVVPTVSTILSPPSTTGDPAQSPEMSPSSLLGLLAGLQVVYFLWTKIL TIAQNLDWWWTSLSFPGGIPECTGQNSQFQTCKHLPTSCPPTCNGFRWMYLRRFIIYLL VLLLCLIFLLVLLDWKGLIPVCPIQPTTETTVNCRQCTISVQDMYTPPYCCCLKPTAGNC TCWPIPSSWALGNYLWEWALARFSWLNLLVPLLQWLGGISLIAWFLLIWMIWFWGPAL LSILPPFIPIFVLFFLIWVYI"

1503..1928 CDS

> /codon start=1 /product="X protein" /protein id="AAT45438.1" /db xref="GI:48526481"

/translation="MAARLCCQLDPTRDVLLLRPFSSQSSGPPFPRPSAGSAASPASSLSTSDES DLPLGRLPACFASASGPCCLVVTCAELRTMDSTVNFVSWHANRQLGMPSKDLWTPYIR DQLLTKWEEGSIDPRLSIFVLGGCRHKCMRLL"

1910..2587 CDS

> /codon start=1 /product="pre-core protein" /protein id="AAT45439.1" /db xref="GI:48526482"

/translation="MHATSVTMYLFHLCLVFACVPCPTVQASKLCLGWLWGMDIDPYKEFGS SYQLLNFLPLDFFPDLNALVDTATALYEEELTGREHCSPHHTAIRQALVCWDELTKLIA WMSSNITSEQVRTIIVNHVNDTWGLKVRQSLWFHLSCLTFGQHTVQEFLVSFGVWIRT PAPYRPPNAPILSTLPEHTVIRRRGGARASRSPRRRTPSPRRRRSQSPRRRRSQSPSANC"

ORIGIN

l aattcgggac ataccacgtg gtttagttcc gcctcaaact ccaacaaatc gagatcaagg

61 gagaaagcct actcctccaa ctccacctct aagagatact cacccccact taactatgaa

121 aaatcagact tttcatctcc aggggttcgt ggacggatta agagacttga caacaacgga

181 acgccaacac aatgcctatg gagatccttt tacgacagta agccctgtgg ttcctactgt

241 atccaccata ttgtctcctc catcgacgac tggggaccct gcacagtcac cggagatgtc

301 accatcaagt ctcctaggac tcctcgcagg attacaggtg gtgtatttct tgtggacaaa 361 aatcctaaca atagctcaga atctagattg gtggtggact tctctcagtt ttccaggggg 421 cataccagag tgcactggcc aaaattcgca gttccaaact tgcaaacact tgccaacctc 481 ctgtccaccg acttgcaatg gctttcgttg gatgtatctg cggcgtttta tcatatacct 601 acctgtctgt cctattcaac ccacaacaga aacaacagtc aattgcagac aatgcacaat 661 ctctgtacaa gacatgtata ctcctcctta ctgttgttgt ttaaaaccta cggcaggaaa 721 ttgcacttgc tggcccatcc cttcatcatg ggctttagga aattacctat gggagtgggc 781 cttagcccgt ttctcttggc tcaatttact agtgcccttg cttcaatggt taggaggaat 841 ttccctcatt gcgtggtttt tgcttatatg gatgatttgg ttttgggggc ccgcacttct

901 gagcatetta ecgccattta tacccatatt tgttetgttt ttettgattt gggtatacat 961 ttaaatgtta ataaaacaaa atggtggggc aatcatttac attttatggg atatgtaatt 1021 actagttcag gtgtattgcc acaagataaa catgttaaga aactttcccg ttatttacga 1081 tctgttcctg ttaatcaacc tctggattac aaaatttgtg aaagattgac tggtattctt 1141 aactatgttg ctccttttac gctgtgtgga tatgctgctt taatgcctct gtatcatgct 1201 attgcttccc gtacggcttt cgttttctcc tccttgtata aatcctggtt gctgtctctt 1321 gcaaccccca ctggctgggg cattgccacc acctgtcaac tcctttctgg gactttcgct 1381 ttcccctcc cgatcgccac ggcagaactc atcgccgcct gccttgcccg ctgctggacg 1441 ggggctaggt tgttgggcac tgataattcc gtggtgttgt cggggaagct gacgtccttt 1501 ccatggctgc tcgcctgtgt tgccaactgg atcctacgcg ggacgtcctt ctgctacgtc 1561 ccttcagctc tcaatccagc ggacctccct tcccgaggcc ttctgccggt tctgcggcct 1621 ctcccgcgtc ttcgctttcg acctccgacg agtcggatct ccctttgggc cgcctccccg 1681 cctgtttcgc ctcggcgtcc ggtccgtgtt gcttggtcgt cacctgtgca gaattgcgaa 1741 ccatggattc caccgtgaac tttgtctcct ggcatgcaaa tcgtcaactt ggcatgccaa 1801 gcaaggacct ttggactcct tatataagag atcaattatt aactaaatgg gaggagggca 1861 gcattgatcc tagattatca atatttgtat taggaggctg taggcataaa tgcatgcgac 1921 ttctgtaacc atgtatcttt ttcacctgtg ccttgttttt gcctgtgttc catgtcctac 1981 tgttcaagcc tccaagctgt gccttggatg gctttggggc atggacatag atccttataa 2041 agaatttggt tcatcttatc agttgttgaa ttttcttcct ttggacttct ttcctgatct 2101 taatgetttg gtggacaetg etactgettt gtatgaagaa gaactaacag gtagggaaca 2161 ttgctctccg catcatacag ctattagaca agctttagta tgctgggatg aattaactaa 2221 attgatagct tggatgagtt ctaacataac ttctgaacaa gtaagaacaa tcattgtaaa 2281 ccatgtcaat gatacctggg gacttaaggt gagacaaagt ttatggtttc atttgtcatg 2341 tctcactttc ggacaacata cagttcaaga atttttagta agttttggag tatggatcag 2401 gactccagct ccatatagac ctcctaatgc acccattctc tcgactcttc cggaacatac 2461 agtcattagg agaagaggag gtgcaagagc ttctaggtcc cccagaagac gcactccctc 2521 tectegeagg agaagatete aateacegeg tegeagaege teteaatete catetgecaa 2581 ctgctgatct tcaatgggta cataaaacta atgctattac aggtctttac tctaaccaag 2641 ctgctcagtt taacccgcat tggattcaac ctgagtttcc tgagcttcat ttacacaatg 2701 aattaattaa aaaattgcaa cagtattttg gtcccttgac tattaatgaa aagagaaaat 2761 tgcaattaaa ttttcctgca agatttttcc ccaaagctac taaatatttc cctttaatta 2821 aaggcataaa aaacaattat cctaattttg ctttagaaca tttctttgct accgcaaatt 2881 atttgtggac tttatgggaa gctggaattt tgtatttaag gaagaatcaa acaactttga 2941 cttttaaagg taaaccatat tcttgggaac acagacagct agtgcaacat aatgggcaac 3001 aacataaaag tcaccttcaa tccagacaaa atagcagcgt ggtggcctgc agtgggcact 3061 tattacacaa ccacttaccc tcagaaccag tcagtgtttc aaccagggat ttatcaaaca 3121 acatecttgg taaateccaa aacteaacaa gaactggact etgtteteat aaacagatae 3181 aaacagatag attggaacac ttggcaagga tttcctgtgg atcaaaaact accattggtc 3241 aacagggatc ctcccccaaa accagctcaa actttcgaaa tcaaacctgg gcctataata 3301 gttcctgg

LOCUS	AV628098 3308 bp DNA circular VRL 15-IUN-2004			
DEFINITION	Woodebuck honotitis P virus clone 2 isolation source liver			
DEFINITION	woodchuck nepatitis D virus cione 2 isolation-source liver,			
	complete genome.			
ACCESSION	AY628098			
VERSION	AY628098.1 GI:48526485			
SOURCE	Woodchuck hepatitis B virus; Hepadnaviridae; Orthohepadnavirus.			
FEATURES	Location/Qualifiers			
source	13308			
	/organism="Woodchuck hepatitis B virus"			
	/mol type="genomic DNA"			
	/isolation source="liver; of a chronic WHV carrier"			
	/db xref="taxon:35269"			
	/clone="2"			
CDS	join(24273308,11758)			
	/note="DNA polymerase; reverse transcriptase; RNase			
	activity"			
	/codon start=1			
	/product="polymerase protein"			
	/protein id="AAT45444.1"			
	/db xref="GI:48526488"			
/translation-"N	AUDEODI EDNILOGI CEEEVOELI CODEDAL DI LACEDI NUDVADALN			

/translation="MHPFSRLFRNIQSLGEEEVQELLGPPEDALPLLAGEDLNHRVADALNLHL PTADLQWVHKTNAITGLYSNQAAQFNPHWIQPEFPELHLHNELIKKLQQYFGPLTINEK RKLQLNFPARFFPKATKYFPLIKGIKNNYPNFALEHFFATANYLWTLWEAGILYLRKNQ TTLTFKGKPYSWEHRQLVQHNGQQHKSHLQSRQNSSVVACSGHLLHNHLPSEPVSVST RDLSNNILGKSQNSTRTGLCSHKQIQTDRLEHLARISCGSKTTIGQQGSSPKTSSNFRNQ TWAYNSSWNSGHTTWFSSASNSNKSRSREKAYSSNSTSKRYSPPLNYEKSDFSSPGVR GRIKRLDNNGTPTQCLWRSFYDSKPCGSYCIHHIVSSIDDWGPCTVTGDVTIKSPRTPRR ITGGVFLVDKNPNNSSESRLVVDFSQFSRGHTRVHWPKFAVPNLQTLANLLSTDLQWL **SLDVSAAFYHIPISPAAVPHLLVGSPGLERFNTCLSYSTHNRNNSQLQTMHNLCTRHVY** SSLLLLFKTYGRKLHLLAHPFIMGFRKLPMGVGLSPFLLAQFTSALASMVRRNFPHCVV FAYMDDLVLGARTSEHLTAIYTHICSVFLDLGIHLNVNKTKWWGNHLHFMGYVITSSG VLPQDKHVKKLSRYLRSVPVNQPLDYKICERLTGILNYVAPFTLCGYAALMPLYHAIAS RTAFVFSSLYKSWLLSLYEELWPVVRQRGVVCSVFADATPTGWGIATTCQLLSGTFAFP LPIATAELIAACLARCWTGARLLGTDNSVVLSGKLTSFPWLLACVANWILRGTSFCYVP SALNPADLPSRGLLPVLRPLPRLRFRPPTSRISLWAASPPVSPRRPVRVAWSSPVQNCEP WIPP"

CDS

join(2992..3308,1..964) /codon_start=1 /product="envelope protein" /protein_id="AAT45445.1" /db_xref="GI:48526489" /translation="MGNNIKVTFNPDKIAAWWPAVGTYYTTTYPQNQSVFQPGIYQTTSLVNP KTQQELDSVLINRYKQIDWNTWQGFPVDQKLPLVNRDPPPKPAQTFEIKPGPIIVPGIRDI PRGLVPPQTPTNRDQGRKPTPPTPPLRDTHPHLTMKNQTFHLQGFVDGLRDLTTTERQ HNAYGDPFTTVSPVVPTVSTILSPPSTTGDPAQSPEMSPSSLLGLLAGLQVVYFLWTKIL TIAQNLDWWWTSLSFPGGIPECTGQNSQFQTCKHLPTSCPPTCNGFRWMYLRRFIIYLL VLLLCLIFLLVLLDWKGLIPVCPIQPTTETTVNCRQCTISVQDMYTPPYCCCLKPTAGNC TCWPIPSSWALGNYLWEWALARFSWLNLLVPLLQWLGGISLIAWFLLIWMIWFWGPAL LSILPPFIPIFVLFFLIWVYI"

CDS 1503..1928

/codon_start=1 /product="X protein" /protein_id="AAT45442.1" /db_xref="GI:48526486"

/translation="MAARLCCQLDPTRDVLLLRPFSSQSSGPPFPRPSAGSAASPASSLSASDES DLPLGRLPACFASASGPCCLVVTCAELRTMDSTVNFVSWHANRQLGMPSKDLWTPYIR DQLLTKWEEGSIDPRLSIFVLGGCRHKCMRLL"

CDS 1910..2587

/codon_start=1

/product="pre-core protein"

/protein_id="AAT45443.1"

/db_xref="GI:48526487"

/translation="MHATSVTMYLFHLCLVFACVPCPTVQASKLCLGWLWGMDIDPYKEFGS SYQLLNFLPLDFFPDLNALVDTATALYEEELTGREHCSPHHTAIRQALVCWDELTKLIA WMSSNITSEQVRTIIVNHVNDTWGLKVRQSLWFHLSCLTFGQHTVQEFLVSFGVWIRT PAPYRPPNAPILSTLPEHTVIRRRGGARASRSPRRRTPSPRRRRSQSPRRRRSQSPSANC"

ORIGIN

1 aattcgggac ataccacgtg gtttagttcc gcctcaaact ccaacaaatc gagatcaagg

61 gagaaagcct actcctccaa ctccacctct aagagatact cacccccact taactatgaa

121 aaatcagact tttcatctcc aggggttcgt ggacggatta agagacttga caacaacgga

181 acgccaacac aatgcctatg gagatccttt tacgacagta agccctgtgg ttcctactgt

241 atccaccata ttgtctcctc catcgacgac tggggaccct gcacagtcac cggagatgtc

901 gagcatetta ecgecattta tacceatatt tgttetgttt ttettgattt gggtataeat 961 ttaaatgtta ataaaacaaa atggtggggc aatcatttac attttatggg atatgtaatt 1021 actagttcag gtgtattgcc acaagataaa catgttaaga aactttcccg ttatttacga 1081 tctgttcctg ttaatcaacc tctggattac aaaatttgtg aaagattgac tggtattctt 1141 aactatgttg ctccttttac gctgtgtgga tatgctgctt taatgcctct gtatcatgct 1201 attgcttccc gtacggcttt cgttttctcc tccttgtata aatcctggtt gctgtctctt 1321 gcaaccccca ctggctgggg cattgccacc acctgtcaac tcctttctgg gactttcgct 1381 ttcccctcc cgatcgccac ggcagaactc atcgccgcct gccttgcccg ctgctggacg 1441 ggggctaggt tgttgggcac tgataattcc gtggtgttgt cggggaagct gacgtccttt 1501 ccatggctgc tcgcctgtgt tgccaactgg atcctacgcg ggacgtcctt ctgctacgtc 1561 ccttcagctc tcaatccagc ggacctccct tcccgaggcc ttctgccggt tctgcggcct 1621 ctcccgcgtc ttcgctttcg gcctccgacg agtcggatct ccctttgggc cgcctccccg 1681 cctgtttcgc ctcggcgtcc ggtccgtgtt gcttggtcgt cacctgtgca gaattgcgaa 1741 ccatggattc caccgtgaac tttgtctcct ggcatgcaaa tcgtcaactt ggcatgccaa 1801 gcaaggacct ttggactcct tatataagag atcaattatt aactaaatgg gaggagggca 1861 gcattgatcc tagattatca atatttgtat taggaggctg taggcataaa tgcatgcgac 1921 ttctgtaacc atgtatcttt ttcacctgtg ccttgttttt gcctgtgttc catgtcctac 1981 tgttcaagcc tccaagctgt gccttggatg gctttggggc atggacatag atccttataa 2041 agaatttggt tcatcttatc agttgttgaa ttttcttcct ttggacttct ttcctgatct 2101 taatgetttg gtggacaetg etactgettt gtatgaagaa gaactaacag gtagggaaca 2161 ttgctctccg catcatacag ctattagaca agctttagta tgctgggatg aattaactaa 2221 actgatagct tggatgagtt ctaacataac ttctgaacaa gtaagaacaa tcattgtaaa 2281 ccatgtcaat gatacctggg gacttaaggt gagacaaagt ttatggtttc atttgtcatg 2341 tctcactttc ggacaacata cagttcaaga atttttagta agttttggag tatggatcag 2401 gactccagct ccatatagac ctcctaatgc acccattctc tcgactcttc cggaacatac 2461 agtcattagg agaagaggag gtgcaagagc ttctaggtcc cccagaagac gcactccctc 2521 tectegeagg agaagatete aateacegeg tegeagaege teteaatete catetgecaa 2581 ctgctgatct tcaatgggta cataaaacta atgctattac aggtctttac tctaaccaag 2641 ctgctcagtt taacccgcat tggattcaac ctgagtttcc tgagcttcat ttacacaatg 2701 aattaattaa aaaattgcaa cagtattttg gtcccttgac tattaatgaa aagagaaaat 2761 tgcaattaaa ttttcctgca agatttttcc ccaaagctac taaatatttc cctttaatta 2821 aaggcataaa aaacaattat cctaattttg ctttagaaca tttctttgct accgcaaatt 2881 atttgtggac tttatgggaa gctggaattt tgtatttaag gaagaatcaa acaactttga 2941 cttttaaagg taaaccatat tcttgggaac acagacagct agtgcaacat aatgggcaac 3001 aacataaaag tcaccttcaa tccagacaaa atagcagcgt ggtggcctgc agtgggcact 3061 tattacacaa ccacttaccc tcagaaccag tcagtgtttc aaccagggat ttatcaaaca 3121 acatecttgg taaateccaa aacteaacaa gaactggact etgtteteat aaacagatae 3181 aaacagatag attggaacac ttggcaagga tttcctgtgg atcaaaaact accattggtc 3241 aacagggatc ctcccccaaa accagctcaa actttcgaaa tcaaacctgg gcctataata 3301 gttcctgg

LOCUS	AY628099 3308 bp DNA circular VRL 15-JUN-2004
DEFINITION	Woodchuck hepatitis B virus clone 1 isolation-source spleen,
	complete genome.
ACCESSION	AY628099
VERSION	AY628099.1 GI:48526490
SOURCE	Woodchuck hepatitis B virus; Hepadnaviridae; Orthohepadnavirus.
FEATURES	Location/Qualifiers
source	13308
	/organism="Woodchuck hepatitis B virus"
	/mol type="genomic DNA"
	/isolation_source="spleen; of a chronic WHV carrier"
	/db xref="taxon:35269"
	/clone="1"
CDS	join(24273308,11758)
	/note="DNA polymerase, reverse transcriptase, RNase
	activity"
	/codon_start=1
	/product="polymerase protein"
	/protein_id="AAT45448.1"
	/db_xref="GI:48526493"
/translation="N	MHPFSRLFRNIQSLGEEEVQELLGPPEDALPLLAGEDLNHRVADALNLHL
PTADLQWVH	KTNAITGLYSNQAAQFNPHWIQPEFPELHLHNELIKKLQQYFGPLTINEK
RKLQLNFPAR	RFPKATKYFPLIKGIKNNYPNFALEHFFATANYLWTLWEAGILYLRKNQ
TTLTFKGKPY	SWEHRQLVQHNGQQHKSHLQSRQNSSVVACSGHLLHNHLPSEPVSVST
RDLSNNILGK	SQNSTRTGLCSHKQIQTDRLEHLARISCGSKTTIGQQGSSPKTSSNFRNQ
TWAYNSSWN	ISGHTTWFSSASNSNKSRSREKAYSSNSTSKRYSPPLNYEKSDFSSPGVR
GRIKKLDNNC	STPTQCLWRSFYDSKPCGSYCIHHIVSSIDDWGPCTVTGDVTIKSPRTPRR
SI DVCA AEVI	UDISDA A VDIU I VOSDOJ EDENTOJ SVSTUDIDADISOJ OTNUDU OTDUDAV
SLUVSAAFYE	CDVI ULI AUDEIMCEDVI DMCVCI SDELI ACETSALASMVDDNEDUCVV
SSLLLLINIY	UNNERTERATIFFIIVIUFKNEPVIU VULSPFELAUF I SALASIVI V KKINPPHU V V

SSLLLLFKTYGRKLHLLAHPFIMGFRKLPMGVGLSPFLLAQFTSALASMVRRNFPHCVV FAYMDDLVLGARTSEHLTAIYTHICSVFLDLGIHLNVNKTKWWGNHLHFMGYVITSSG VLPQDKHVKKLSRYLRSVPVNQPLDYKICERLTGILNYVAPFTLCGYAALMPLYHAIAS RTAFVFSSLYKSWLLSLYEELWPVVRQRGVVCSVFADATPTGWGIATTCQLLSGTFAFP LPIATAELIAACLARCWTGARLLGTDNSVVLSGKLTSFPWLLACVANWILRGTSFCYVP SALNPADLPSRGLLPVLRPLPRLRFRPPTSRISLWAASPPVSPRRPVRVAWSSPVQNCEP WIPP"

CDS join(2992..3308,1..964) /codon_start=1 /product="envelope protein" /protein_id="AAT45449.1" /db_xref="GI:48526494" /translation="MGNNIKVTFNPDKIAAWWPAVGTYYTTTYPQNQSVFQPGIYQTTSLVNP KTQQELDSVLINRYKQIDWNTWQGFPVDQKLPLVNRDPPPKPAQTFEIKPGPIIVPGIRDI PRGLVPPQTPTNRDQGRKPTPPTPPLRDTHPHLTMKNQTFHLQGFVDGLRDLTTTERQ HNAYGDPFTTVSPVVPTVSTILSPPSTTGDPAQSPEMSPSSLLGLLAGLQVVYFLWTKIL TIAQNLDWWWTSLSFPGGIPECTGQNSQFQTCKHLPTSCPPTCNGFRWMYLRRFIIYLL VLLLCLIFLLVLLDWKGLIPVCPIQPTTETTVNCRQCTISVQDMYTPPYCCCLKPTAGNC TCWPIPSSWALGNYLWEWALARFSWLNLLVPLLQWLGGISLIAWFLLIWMIWFWGPAL LSILPPFIPIFVLFFLIWVYI"

CDS 1503..1928

/codon_start=1 /product="X protein" /protein_id="AAT45446.1" /db xref="GI:48526491"

/translation="MAARLCCQLDPTRDVLLLRPFSSQSSGPPFPRPSAGSAASPASSLSTSDES DLPLGRLPACFASASGPCCLVVTCAELRTMDSTVNFVSWHANRQLGMPSKDLWTPYIR DQLLTKWEEGSIDPRLSIFVLGGCRHKCMRLL"

CDS 1910..2587

/codon_start=1

/product="pre-core protein"

/protein id="AAT45447.1"

/db xref="GI:48526492"

/translation="MHATSVTMYLFHLCLVFACVPCPTVQASKLCLGWLWGMDIDPYKEFGS SYQLLNFLPLDFFPDLNALVDTATALYEEELTGREHCSPHHTAIRQALVCWDELTKLIA WMSSNITSEQVRTIIVNHVNDTWGLKVRQSLWFHLSCLTFGQHTVQEFLVSFGVWIRT PAPYRPPNAPILSTLPEHTVIRRRGGARASRSPRRRTPSPRRRRSQSPRRRRSQSPSANC"

ORIGIN

l aattcgggac ataccacgtg gtttagttcc gcctcaaact ccaacaaatc gagatcaagg

61 gagaaagcct actcctccaa ctccacctct aagagatact cacccccact taactatgaa

121 aaatcagact tttcatctcc aggggttcgt ggacggatta agagacttga caacaacgga

181 acgccaacac aatgcctatg gagatccttt tacgacagta agccctgtgg ttcctactgt

901 gagcatetta ecgecattta tacceatatt tgttetgttt ttettgattt gggtataeat 961 ttaaatgtta ataaaacaaa atggtggggc aatcatttac attttatggg atatgtaatt 1021 actagttcag gtgtattgcc acaagataaa catgttaaga aactttcccg ttatttacga 1081 tctgttcctg ttaatcaacc tctggattac aaaatttgtg aaagattgac tggtattctt 1141 aactatgttg ctccttttac gctgtgtgga tatgctgctt taatgcctct gtatcatgct 1201 attgcttccc gtacggcttt cgttttctcc tccttgtata aatcctggtt gctgtctctt 1321 gcaaccccca ctggctgggg cattgccacc acctgtcaac tcctttctgg gactttcgct 1381 ttcccctcc cgatcgccac ggcagaactc atcgccgcct gccttgcccg ctgctggacg 1441 ggggctaggt tgttgggcac tgataattcc gtggtgttgt cggggaagct gacgtccttt 1501 ccatggctgc tcgcctgtgt tgccaactgg atcctacgcg ggacgtcctt ctgctacgtc 1561 ccttcagctc tcaatccagc ggacctccct tcccgaggcc ttctgccggt tctgcggcct 1621 ctcccgcgtc ttcgctttcg acctccgacg agtcggatct ccctttgggc cgcctccccg 1681 cctgtttcgc ctcggcgtcc ggtccgtgtt gcttggtcgt cacctgtgca gaattgcgaa 1741 ccatggattc caccgtgaac tttgtctcct ggcatgcaaa tcgtcaactt ggcatgccaa 1801 gcaaggacct ttggactcct tatataagag atcaattatt aactaaatgg gaggagggca 1861 gcattgatcc tagattatca atatttgtat taggaggctg taggcataaa tgcatgcgac 1921 ttctgtaacc atgtatcttt ttcacctgtg ccttgttttt gcctgtgttc catgtcctac 1981 tgttcaagcc tccaagctgt gccttggatg gctttggggc atggacatag atccttataa 2041 agaatttggt tcatcttatc agttgttgaa ttttcttcct ttggacttct ttcctgatct 2101 taatgetttg gtggacaetg etactgettt gtatgaagaa gaactaacag gtagggaaca 2161 ttgctctccg catcatacag ctattagaca agctttagta tgctgggatg aattaactaa 2221 attgataget tggatgagtt etaacataac ttetgaacaa gtaagaacaa teattgtaaa 2281 ccatgtcaat gatacctggg gacttaaggt gagacaaagt ttatggtttc atttgtcatg 2341 tctcactttc ggacaacata cagttcaaga atttttagta agttttggag tatggatcag 2401 gactccagct ccatatagac ctcctaatgc acccattctc tcgactcttc cggaacatac 2461 agtcattagg agaagaggag gtgcaagagc ttctaggtcc cccagaagac gcactccctc 2521 tcctcgcagg agaagatctc aatcaccgcg tcgcagacgc tctcaatctc catctgccaa 2581 ctgctgatct tcaatgggta cataaaacta atgctattac aggtctttac tctaaccaag 2641 ctgctcagtt taacccgcat tggattcaac ctgagtttcc tgagcttcat ttacacaatg 2701 aattaattaa aaaattgcaa cagtattttg gtcccttgac tattaatgaa aagagaaaat 2761 tgcaattaaa ttttcctgca agatttttcc ccaaagctac taaatatttc cctttaatta 2821 aaggcataaa aaacaattat cctaattttg ctttagaaca tttctttgct accgcaaatt 2881 atttgtggac tttatgggaa gctggaattt tgtatttaag gaagaatcaa acaactttga 2941 cttttaaagg taaaccatat tcttgggaac acagacagct agtgcaacat aatgggcaac 3001 aacataaaag tcaccttcaa tccagacaaa atagcagcgt ggtggcctgc agtgggcact 3061 tattacacaa ccacttaccc tcagaaccag tcagtgtttc aaccagggat ttatcaaaca 3121 acatecttgg taaateccaa aacteaacaa gaactggaet etgtteteat aaacagatae 3181 aaacagatag attggaacac ttggcaagga tttcctgtgg atcaaaaact accattggtc 3241 aacagggatc ctcccccaaa accagctcaa actttcgaaa tcaaacctgg gcctataata 3301 gttcctgg

AY628100 3308 bp DNA circular VRL 15-JUN-2004 LOCUS DEFINITION Woodchuck hepatitis B virus clone 2 isolation-source spleen, complete genome. ACCESSION AY628100 VERSION AY628100.1 GI:48526495 Woodchuck hepatitis B virus; Hepadnaviridae; Orthohepadnavirus. SOURCE Location/Qualifiers FEATURES 1..3308 source /organism="Woodchuck hepatitis B virus" /mol type="genomic DNA" /isolation source="spleen; of a chronic WHV carrier" /db xref="taxon:35269" /clone="2" CDS join(2427..3308,1..1758) /note="DNA polymerase; reverse transcriptase; RNase activity" /codon start=1 /product="polymerase protein" /protein id="AAT45452.1" /db xref="GI:48526498" /translation="MHPFSRLFRNIQSLGEEEVQELLGPPEDALPLLAGEDLNHRVADALNLHL PTADLQWVHKTNAITGLYSNQAAQFNPHWIQPEFPELHLHNELIKKLQQYFGPLTINEK

RKLQLNFPARFFPKATKYFPLIKGIKNNYPNFALEHFFATANYLWTLWEAGILYLRKNQ TTLTFKGKPYSWEHRQLVQHNGQQHKSHLQSRQNSSVVACSGHLLHNHLPSEPVSVST RDLSNNILSKSQNSTRTGLCSHKQIQTDRLEHLARISCGSKTTIGQQGSTPKTSSNFRNQ TWAYNSSWNSGHTTWFSSASNSNKSRSREKAYSSNSTSKRYSPPLNYEKSDFSSPGVR GRIKRLDNNGTPTQCLWRSFYDSKPCGSYCIHHIVSSIDDWGPCTVTGDVTIKSPRTPRR ITGGVFLVDKNPNNSSESRLVVDFSQFSRGHTRVHWPKFAVPNLQTLANLLSTDLQWL SLDVSAAFYHIPISPAAVPHLLVGSPGLERFNTCLSYSTHNRNNSQLQTMHNLCTRHVY SSLLLLFKTYGRKLHLLAHPFIMGFRKLPMGVGLSPFLLAQFTSALASMVRRNFPHCVV FAYMDDLVLGARTSEHLTAIYTHICSVFLDLGIHLNVNKTKWWGNHLHFMGYVITSSG VLPQDKHVKKLSRYLRSVPVNQPLDYKICERLTGILNYVAPFTLCGYAALMPLYHAIAS RTAFVFSSLYKSWLLSLYEELWPVVRQRGVVCSVFADATPTGWGIATTCQLLSGTFAFP LPIATAELIAACLARCWTGARLLGTDNSVVLSGKLTSFPWLLACVANWILRGTSFCYVP SALNPADLPSRGLLPVLRPLPRLRFRPPTSRISLWAASPPVSPRRPVRVAWSSPVQNCEP WIPP" CDS join(2992..3308,1..964) /codon start=1 /product="envelope protein" /protein id="AAT45453.1" /db xref="GI:48526499"

/translation="MGNNIKVTFNPDKIAAWWPAVGTYYTTTYPQNQSVFQPGIYQTTSLVNP KTQQELDSVLINRYKQIDWNTWQGFPVDQKLPLVNRDPPPKPAQTFEIKPGPIIVPGIRDI PRGLVPPQTPTNRDQGRKPTPPTPPLRDTHPHLTMKNQTFHLQGFVDGLRDLTTTERQ HNAYGDPFTTVSPVVPTVSTILSPPSTTGDPAQSPEMSPSSLLGLLAGLQVVYFLWTKIL TIAQNLDWWWTSLSFPGGIPECTGQNSQFQTCKHLPTSCPPTCNGFRWMYLRRFIIYLL VLLLCLIFLLVLLDWKGLIPVCPIQPTTETTVNCRQCTISVQDMYTPPYCCCLKPTAGNC TCWPIPSSWALGNYLWEWALARFSWLNLLVPLLQWLGGISLIAWFLLIWMIWFWGPAL LSILPPFIPIFVLFFLIWVYI"

CDS 1503..1928

/codon_start=1 /product="X protein" /protein_id="AAT45450.1" /db_xref="GI:48526496"

/translation="MAARLCCQLDPTRDVLLLRPFSSQSSGPPFPRPSAGSAASPASSLSASDES DLPLGRLPACFASASGPCCLVVTCAELRTMDSTVNFVSWHANRQLGMPSKDLWTPYIR DQLLTKWEEGSIDPRLSIFVLGGCRHKCMRLL"

CDS 1910..2587

/codon_start=1

/product="pre-core protein"

/protein id="AAT45451.1"

/db xref="GI:48526497"

/translation="MHATSVTMYLFHLCLVFACVPCPTVQASKLCLGWLWGMDIDPYKEFGS SYQLLNFLPLDFFPDLNALVDTATALYEEELTGREHCSPHHTAIRQALVCWDELTKLIA WMSSNITSEQVRTIIVNHVNDTWGLKVRQSLWFHLSCLTFGQHTVQEFLVSFGVWIRT PAPYRPPNAPILSTLPEHTVIRRRGGARASRSPRRRTPSPRRRRSQSPRRRRSQSPSANC"

ORIGIN

1 aattcgggac ataccacgtg gtttagttcc gcctcaaact ccaacaaatc gagatcaagg

- 61 gagaaagcct actcctccaa ctccacctct aagagatact cacccccact taactatgaa
- 121 aaatcagact tttcatctcc aggggttcgt ggacggatta agagacttga caacaacgga
- 181 acgccaacac aatgcctatg gagatccttt tacgacagta agccctgtgg ttcctactgt
- 241 atccaccata ttgtctcctc catcgacgac tggggaccct gcacagtcac cggagatgtc

901 gagcatetta ecgecattta tacceatatt tgttetgttt ttettgattt gggtataeat 961 ttaaatgtta ataaaacaaa atggtggggc aatcatttac attttatggg atatgtaatt 1021 actagttcag gtgtattgcc acaagataaa catgttaaga aactttcccg ttatttacga 1081 tctgttcctg ttaatcaacc tctggattac aaaatttgtg aaagattgac tggtattctt 1141 aactatgttg ctccttttac gctgtgtgga tatgctgctt taatgcctct gtatcatgct 1201 attgcttccc gtacggcttt cgttttctcc tccttgtata aatcctggtt gctgtctctt 1321 gcaaccccca ctggctgggg cattgccacc acctgtcaac tcctttctgg gactttcgct 1381 ttcccctcc cgatcgccac ggcagaactc atcgccgcct gccttgcccg ctgctggacg 1441 ggggctaggt tgttgggcac tgataattcc gtggtgttgt cggggaagct gacgtccttt 1501 ccatggctgc tcgcctgtgt tgccaactgg atcctacgcg ggacgtcctt ctgctacgtc 1561 ccttcagctc tcaatccagc ggacctccct tcccgaggcc ttctgccggt tctgcggcct 1621 ctcccgcgtc ttcgctttcg gcctccgacg agtcggatct ccctttgggc cgcctccccg 1681 cctgtttcgc ctcggcgtcc ggtccgtgtt gcttggtcgt cacctgtgca gaattgcgaa 1741 ccatggattc caccgtgaac tttgtctcct ggcatgcaaa tcgtcaactt ggcatgccaa 1801 gcaaggacct ttggactcct tatataagag atcaattatt aactaaatgg gaggagggca 1861 gcattgatcc tagattatca atatttgtat taggaggctg taggcataaa tgcatgcgac 1921 ttctgtaacc atgtatcttt ttcacctgtg ccttgttttt gcctgtgttc catgtcctac 1981 tgttcaagcc tccaagctgt gccttggatg gctttggggc atggacatag atccttataa 2041 agaatttggt tcatcttatc agttgttgaa ttttcttcct ttggacttct ttcctgatct 2101 taatgetttg gtggacaetg etactgettt gtatgaagaa gaactaacag gtagggaaca 2161 ttgctctccg catcatacag ctattagaca agctttagta tgctgggatg aattaactaa 2221 actgataget tggatgagtt etaacataac ttetgaacaa gtaagaacaa teattgtaaa 2281 ccatgtcaat gatacctggg gacttaaggt gagacaaagt ttatggtttc atttgtcatg 2341 tctcactttc ggacaacata cagttcaaga atttttagta agttttggag tatggatcag 2401 gactccagct ccatatagac ctcctaatgc acccattctc tcgactcttc cggaacatac 2461 agtcattagg agaagaggag gtgcaagagc ttctaggtcc cccagaagac gcactccctc 2521 tcctcgcagg agaagatete aatcaccgcg tcgcagacge tetcaatete catetgecaa 2581 ctgctgatct tcaatgggta cataaaacta atgctattac aggtctttac tctaaccaag 2641 ctgctcagtt taacccgcat tggattcaac ctgagtttcc tgagcttcat ttacacaatg 2701 aattaattaa aaaattgcaa cagtattttg gtcccttgac tattaatgaa aagagaaaat 2761 tgcaattaaa ttttcctgca agatttttcc ccaaagctac taaatatttc cctttaatta 2821 aaggcataaa aaacaattat cctaattttg ctttagaaca tttctttgct accgcaaatt 2881 atttgtggac tttatgggaa gctggaattt tgtatttaag gaagaatcaa acaactttga 2941 cttttaaagg taaaccatat tcttgggaac acagacagct agtgcaacat aatgggcaac 3001 aacataaaag tcaccttcaa tccagacaaa atagcagcgt ggtggcctgc agtgggcact 3061 tattacacaa ccacttaccc tcagaaccag tcagtgtttc aaccagggat ttatcaaaca 3121 acatecttag taaateccaa aacteaacaa gaactggact etgtteteat aaacagatae 3181 aaacagatag attggaacac ttggcaagga tttcctgtgg atcaaaaact accattggtc 3241 aacagggatc cacccccaaa accagctcaa actttcgaaa tcaaacctgg gcctataata 3301 gttcctgg

APPENDIX B

The following appendix shows the cDNA sequence of the fragment of the woodchuck CD209 gene cloned during the study.

LOCUS	AY842283	676 bp mRNA	linear ROD 30-NOV-2004
DEFINITION	Marmota monax D	C-SIGN (CD209	9) partial sequence from immature
	dendritic cells.		
ACCESSION	AY842283		
SOURCE	Marmota monax.		
ORGANISM	Marmota monax		
FEATURES	Location/Qualifier	S	
source	1676		
	/organism="Marmota monax"		
	/db_xref="taxon:9	9995"	
	/tissue_type="dendritic cells"		
	/lab host="woodc	huck"	

ORIGIN

1 atgagtgact ccaaggaacc aagactgcag cagctgggcc tcctggagga ggaacagctg
61 acatccagcc acaccaggca ctccatcaaa ggccttggat tccaaacaaa ttctggattc
121 agtagcttca cagggtgtct tggccatggt ccctggtgcc tgcaactcct ctccttcacg
181 ctcttggctg ggctcctggt tgtccaagtg tccaaagttc cccagctcca taagtcagga
241 acaatccagg caagacgcga tctaccagaa ccctgaccca gtttaaagct ggcgtagatc
301 aactctcaga gacgctcctg cccctggaga tctaccagga gctgacccag ctgaaggcg
421 caacttgtgg tgcttccaga gaaatctaag ctgcagaact ttctaccaca gacttctaag
421 caacttgtgg tgcttccaga gaaatctaag ctgcagaact ttctaccaca gacttctaag
431 actagaggct acacttggta ggctccttg ccctgatga atgcaggaa gtctacatgg
431 actagaggct acacttggta ggctcctatt gaaaactaga atgcaaggaa gtctacatgg
541 tactgactag actgttcacc tctgactct agcttcatga agtatctaga gtaaaggaga
601 acctaccagg acctgggaga ggtagacggt gcagtagttc agagatgacg gctggaatgg
661 caccagatgt actaac





