



**STUDIES OF THE INTERACTION OF HEPATITIS B VIRUS WITH
HUMAN HAEMOPOIETIC CELLS**

Gerald James Atkins

BSc(Hons)

Department of Microbiology and Immunology
University of Adelaide
Adelaide
South Australia

**A thesis submitted to the University of Adelaide in fulfilment of the
requirements for the degree of Doctor of Philosophy**

May, 1997

CONTENTS

ABSTRACT

DECLARATION

ACKNOWLEDGMENTS

ABBREVIATIONS

CHAPTER 1: INTRODUCTION

1.1	HBV Epidemiology and Transmission	1
1.2	The Hepadnaviridae	2
1.3	HBV Morphology	4
1.4	The HBV Genome	5
	1.4.1 Structure and Organisation of the HBV Genome	5
	1.4.2 ORF-P and its Product	5
	1.4.3 ORF-X and its Function	7
	1.4.4 ORF-C and its Products	8
	1.4.5 ORF-S and its Products	9
	1.4.6 Serological Subtypes of HBV	11
1.5	Virus Particle Assembly	12
1.6	Hepadnavirus Genome Replication	16
	1.6.1 Reverse Transcription of the Minus DNA Strand	16
	1.6.2 Plus DNA Strand Synthesis	17
1.7	Transcriptional Control of the HBV Genome (and a molecular basis for hepatotropism)	19
1.8	Immunology and Pathogenesis of HBV Infections	20
	1.8.1 HBV is not Directly Cytopathic	20
	1.8.2 The Importance of Persistent Infections in the Hepadnavirus	

Life-Cycle	22
1.8.3 Infection Outcome is related to Immune System Development and Virus-Induced Immunological Tolerance	23
1.8.4 Infection Outcome is related to IR Efficacy	25
1.8.5 The role of inflammatory cytokines in HBV infection	27
1.9 Extrahepatic Tropism of the Hepadnaviridae	30
1.9.1 Infection of Bone Marrow Cells by HBV	31
1.9.2 Inhibition of Haemopoietic Cell Line Growth by HBV	33
1.9.3 The Presence of HBV Markers in PBL	34
1.9.4 The Significance of HBV Infection of Haemopoietic Tissue	37
1.10 Cellular receptors for HBV	39
1.10.1 B-cell epitopes of HBV surface proteins	39
1.10.2 Potential Receptors for HBV	42
a. Fc Receptors	42
b. Use of FcRs by Viruses	44
c. Receptors for complement fragments	44
d. Viruses that use complement receptors	44
1.10.3 Putative HBV Receptors	45
a. Receptor for polymerised human serum albumin	45
b. Receptors for Immunoglobulin A	47
c. Interleukin-6	49
d. Transferrin receptor (CD71)	50
e. Receptor for Small S protein	51
f. Apolipoprotein H	52
g. Endonexin II/Annexin V	53
h. Fibronectin	53

i.	Receptor and Cell Entry Studies of DHBV	54
j.	Conclusions	56
1.11	Approaches to studying Virus-Cell Interactions	56
a.	Monoclonal Antibodies with Transfection Techniques	56
b.	Molecular Biological Approach Without Monoclonal Ab	59
c.	Virus-Receptor Affinity Purification Techniques	60
d.	Anti-Idiotypic Antibodies	62
e.	Chromosomal Mapping of Receptor Genes	63
1.12	Thesis Aims	64

CHAPTER 2: MATERIALS AND METHODS

2.1	Purification of HBV from serum	65
2.1.1	Source of HBV-positive serum	65
2.1.2	Virus Centrifugation	65
2.1.3	Column Chromatography	65
2.1.4	Sucrose Gradient Purification of Virions and HBsAg Particles	66
2.1.5	Electron Microscopic Examination of Virus Preparations	66
2.1.6	Mock HBV Preparation	66
2.2	Immunoassays	67
2.2.1	Monoclonal Antibodies to Human Antigens	67
2.2.2	Preparation of Cells	67
2.2.3	Immunofluorescence Assay (IFA) on Slides	67
2.2.4	IFA for Flow Cytometry	68
2.2.5	ELISA	68
2.2.6	APAAP Staining of Intracellular Antigens	69
2.2.7	Protein A Purification of Immunoglobulin G	70

2.2.8 Iodination of IgG	71
2.2.9 Western Blotting	71
2.2.10 Immunoperoxidase staining for HBcAg	72
2.3 Nucleic Acid Hybridisation	73
2.3.1 Plasmid as RNA Probe Template	73
2.3.2 Large Scale Plasmid Preparation	73
2.3.3 Preparation of HBV DNA Standards	74
2.3.4 Nucleic Acid Probes	74
a. Riboprobe Synthesis	75
b. DNA Probe Synthesis	75
c. End-labelling Oligonucleotides	76
2.3.5 Spot Blot Preparation	76
2.3.6 Dot Blot Preparation	77
2.3.7 Prehybridisation / Hybridisation	77
2.3.8 Quantitation of HBV DNA	78
2.3.9 Preparation of Total DNA from Mammalian Cells	78
2.4 Tissue Culture	79
2.4.1 Cell Lines	79
2.4.2 Culture Media and Methods	79
2.4.3 Cryopreservation of Cells	79
2.4.4 Thawing Cryopreserved Samples	80
2.4.5 FMLP Stimulation of Human Neutrophils	80
2.4.6 Mitogenic Stimulation of Human Leucocytes	80
2.5 Virus Binding Assays	81
2.5.1 Source of Peptides and Antisera	81
2.5.2 Binding Assay on Microscope Slides	81

2.5.3 Preparation of PM from Human Leucocytes and Hepatocytes	82
a. Isolation of Leucocytes from Buffy Coats	82
b. Disruption of Cells	83
c. Isolation of Plasma Membranes from Cell Homogenate	83
d. Isolation of Internal Membranes	84
2.5.4 Assays for Membrane Purity	84
a. Alkaline Phosphatase Assay	84
b. Acid Phosphatase Assay	84
c. Modified Lowry Assay for Protein Quantitation	85
2.5.5 Virus - Plasma Membrane Binding Assay	85
2.5.6 Inhibition Assays	86
a. Monoclonal Antibody	86
b. Synthetic Peptides	86
c. Glycosaminoglycans (GAGs)	87
2.5.7 Virus - Whole Cell Binding Assay for Flow Cytometry	87
2.5.8 Enzyme Stripping Analysis	88
2.5.9 Tunicamycin Inhibition of <i>N</i> -Linked Carbohydrate Synthesis	89
2.6 HBV Infection Experiments	89
2.6.1 Detection of HBV by Polymerase Chain Reaction (PCR)	89
2.6.2 HBV Internalisation	90
2.6.3 Clonal Growth of Haemopoietic Cell Lines by ACD	91
2.6.4 Infection of Haemopoietic Cell Lines with HBV	91
2.7 Immunoprecipitation of HBV-Binding Proteins	91
<u>Chapter 3: Hepatitis B Virus Binding to Leucocyte Plasma Membranes</u>	
3.1 Introduction and Aims	93
3.2 Results	95

3.2.1 Column Purification of HBV Particles	95
3.2.2 Preparation of liver and leucocyte plasma membranes	96
3.2.3 HBV Binds Saturably to Leucocyte Plasma Membranes	97
3.2.4 Effect on HBV Binding of Blocking MAb to Opsonin Receptors	98
3.2.5 Specificity of HBV Binding	99
3.2.6 Involvement of GAGs in the HBV-Plasma Membrane Interactions	99
3.3 Discussion	100

Chapter 4: The Distribution of Potential Receptors for HBV on Liver and Haemopoietic Cells

4.1 Introduction and Aims	105
4.1.1 Aspects of the Experimental Design	106
4.2 Results	107
4.2.1 Immunofluorescence Detection of HBV Binding to Cell Lines - Timecourse and Temperature Dependence	107
4.2.2 Use of Confocal Imaging to Quantitate HBV Binding to Cells	109
4.2.3 Flow Cytometric Analysis of HBV Binding to K562	110
4.2.4 The Distribution of Potential Receptors for HBV on Cell Lines	111
4.2.5 Comparison of the Immunophenotypes of K562 and Receptor- Negative Cell Lines	112
4.2.6 Binding of HBV to cells of the Peripheral Blood	113
4.2.6.1 Binding of HBV to Neutrophils	114
4.2.6.2 Binding of HBV to Monocytes	114
4.2.6.3 Binding of HBV to Lymphocytes	115
4.2.7 Binding of HBV to Bone Marrow Sub-Populations	115
4.3 Discussion	116
4.3.1 HBsAg Displays L-Dependent Binding to K562, THP-1 and HepG2	

Cells	120
4.3.2 Surface Marker Comparison with K562 and Receptor-Negative Cell Lines	121
4.3.3 Sub-Populations of Peripheral Blood Leucocytes that Bind HBV	123
4.3.4 Binding of HBV to Activated Sub-Populations of PBL	125
4.3.5 Binding of HBV Bone Marrow Sub-Populations	126

Chapter 5: Characterisation of the HBV Binding Properties of Haemopoietic and HepG2 Cells

5.1 Introduction and Aims	129
5.1.1 Aspects of the Experimental Design	130
5.2 Results	130
5.2.1 Protease Stripping Analysis of the HBV Binding	130
5.2.2 Effect of De- <i>N</i> -Glycosylation of K562 on Virus Binding	131
5.2.3 Involvement of Sialic Acid in HBV Binding	132
5.2.4 Calcium/Magnesium Dependence of Binding	132
5.2.5 Effect of PI-PLC on HBV Binding	133
5.2.6 Candidate GPI-Linked Receptors	133
5.2.7 Effect of Acid Pre-Treatment on HBV Binding	134
5.2.8 Involvement of GAGs in the HBV-Cell Interactions	134
5.2.9 Molecular Size of the HBV Receptor on K562	135
5.3 Discussion	136
5.3.1 Protease Sensitivity of Binding	136
5.3.2 Involvement of Carbohydrate Moieties in Virus Binding	137
5.3.3 Cation Independence of HBV Binding	140
5.3.4 HBV Binding is not Mediated by Peripherally-Bound Molecules	141
5.3.5 PI-PLC Sensitivity of HBV Binding	141

5.3.6 The Involvement of GAGs in HBV Attachment	143
5.3.7 Molecular Size of the K562 HBV "Receptor"	146

Chapter 6: HBV Infection of Haemopoietic Cell Lines

6.1 Introduction and Aims	149
6.2 Results	155
6.2.1 The Effect of HBV on the Clonal Growth of Haemopoietic Cell Lines	155
6.2.2 Internalisation of HBV Assessed by PCR	156
6.2.3 Demonstration of HBV Internalisation by Immunocytochemistry	157
6.2.4 HBV Infection of K562 Cells	158
a. HBsAg	159
b. HBcAg	159
c. HBV DNA	160
6.3 Discussion	161
6.3.1 HBV Does Not Inhibit the Growth of Haemopoietic Cell Lines	161
6.3.2 K562 Cells can Internalise HBV Particles	163
6.3.3 The Fate of Internalised HBV particles in K562 Cells	166

Chapter 7: Concluding Remarks

Binding of HBV to Haemopoietic Cell Subsets	169
The HBV Receptor	175
Future Studies to Clone the Putative HBV Receptor on K562 Cells	178
<u>Bibliography</u>	181
Appendix I - Solutions	221
Appendix II	224

ABSTRACT

This thesis studied aspects of the interaction of hepatitis B virus (HBV) with haemopoietic cells and cell lines, to address the reported tropism of HBV for haemopoietic tissues. Emphasis was directed at demonstrating specific attachment of HBV to defined sub-populations of peripheral blood leucocytes (PBL) and bone marrow cells (BM), and the distribution of receptors for HBV on well-defined haemopoietic cell lines. Biochemical characterisation of the virus-cell interaction was also performed, and the question of infectivity of haemopoietic cell lines was addressed.

Firstly, a quantitative assay of HBV binding to liver plasma membranes (PM) was adapted to show that isolated PBL PM bound serum-derived HBV particles to a similar degree, based on their protein content. Using synthetic peptides representative of various amino acid sequences of the preS1 and preS2 regions of L HBsAg to inhibit HBV binding to the PM, it was found that peptide preS1(12-32) inhibited binding to PBL PM by 60-80% and peptide preS1(21-47) inhibited by 0-30% (depending on the source of PM), while peptides preS1(32-49) and preS2(120-145) did not inhibit binding. This contrasts with results obtained using liver PM, where peptide preS1(12-32) did not inhibit binding, while peptide preS1(21-47) inhibited by 70%, and preS1(32-49) inhibited by approximately 12%. Peptide preS2(120-145) had no effect on binding. Thus, different regions of the L surface protein appear to mediate attachment to PBL and hepatocytes.

HBV particles isolated from serum are complexed with serum proteins including IgG. To test the involvement of receptors for IgG and complement fragments (opsonins) in the HBV-PM interaction, a panel of ligand-blocking monoclonal antibodies (MAbs) to opsonin receptors was used, and it was shown that the three classes of receptors for IgG (Fc γ RI, Fc γ RII and Fc γ RIII) and CR3, are not major receptors for HBV on PBL or hepatocytes, as MAbs to these did not inhibit HBV binding. It was also shown that HBV does not utilise the receptor for IgA, Fc α R, for attachment to PBL, despite reported sequence homology between the large envelope protein of HBV and the Fc portion of human IgA. In contrast to a published report

that IL-6 mediates binding of HBV to hepatocytes, IL-6 was shown not to mediate attachment to either liver or PBL PM, by virtue of pre-incubation with a blocking polyclonal anti-serum to IL-6.

Glycosaminoglycans (GAGs) were found to influence HBV binding to PM: soluble heparin (HE) inhibited binding to liver PM by up to 80%, and to leucocyte PM by up to 40%; chondroitin sulphate C (CS-C) enhanced virus binding (approximately 1.5-fold) to leucocyte PM only. Chondroitin sulphate A and hyaluronate had no effect on binding to either PM, arguing that simple electrostatic properties of GAGs were not responsible for the observed effects. The incomplete inhibition by HE and enhancement by CS-C could indicate the presence of more than one class of binding site for HBV on the respective PM, and coupled with the differential pattern of inhibition in the presence of synthetic peptides, argues that receptors for HBV on PBL and hepatocytes may be either different, or altered forms of the same molecule(s).

To extend these studies, whole cell binding assays were developed in order to accurately define which subsets of PBL and BM cells could bind HBV. Using purified HBV particles as the first stage in an immunofluorescence-based detection system, followed by detection of bound HBV using anti-preS1 MAbs F35.25 or MA18/7, and a FITC-conjugated third-stage antibody, specific membrane staining of peripheral blood monocytes from 8/9 donors was observed. In addition, binding of HBV to the erythroleukaemia cell line K562 was observed, while other myeloid cell lines did not appear to bind virus. This assay was then adapted to a suspension cell assay with analysis by flow cytometry, using phycoerythrin as the detecting fluorochrome. The parameters of binding were optimised for K562 cells and these were then applied to analyse HBV binding to PBL and BM cells obtained from healthy volunteers, whose serum was free of HBV markers. Based on their light scatter characteristics, monocytes and neutrophils were the only cell types in the peripheral blood that bound HBV, while binding to lymphocytes was not observed. This was confirmed by two-colour immunofluorescence to simultaneously detect bound HBV and subset-specific leucocyte

markers. Similarly, in the BM, only monocytes bound HBV. Importantly, haemopoietic stem cells (CD34+) did not bind HBV. Binding was tested to 'activated' populations of lymphocytes (PHA-treated), monocytes (LPS-treated), and neutrophils (fMLP-treated). The pattern of HBV binding was not affected by these treatments. Monocytes cultured *in vitro*, bound significantly more virus than freshly isolated monocytes. Taken together, these results indicate that only monocytes, and to a lesser extent neutrophils, express potential receptors for HBV, and a differentiation-dependent upregulation of receptor sites for HBV is observed on monocytes.

The distribution of potential HBV receptors was determined on a number of haemopoietic cell lines, representative of various haemopoietic lineages. K562 (erythroid), and the monocyte cell line THP-1, were the only haemopoietic cell lines which bound HBV, while binding was also observed to the human hepatoma cell line HepG2. A number of other erythroid and monocyte cell lines, as well as T and B lymphoid, and a megakaryocytic line, all failed to bind HBV. A comparison of the surface immunophenotypes of all the cell lines tested excluded all known CD-classified molecules (including opsonin receptors), as candidate HBV receptors.

The biochemical characteristics of the interaction of HBV with all of these cell types were then examined. On K562 and THP-1, HBV binding was sensitive to the protease chymopapain but insensitive to trypsin, indicating that the molecule was a glycosylated protein. Pre-treatment of these cell lines with tunicamycin, to inhibit post-translational addition of *N*-linked carbohydrate to surface glycoproteins, did not influence HBV binding, indicating that these moieties are not important for virus attachment. Enzymatic removal of cell surface sialic acids with neuraminidase significantly enhanced HBV binding to K562 and THP-1 cells but did not confer binding to otherwise 'negative' cell lines. Binding of HBV to cultured monocytes and HepG2 cells was trypsin and chymopapain sensitive, and was not increased by neuraminidase pre-treatment. Cation chelation demonstrated that HBV binding of to all cell types was $\text{Ca}^{2+}/\text{Mg}^{2+}$ -independent, and acid elution of cells showed that binding

was not mediated by peripherally-bound molecules. Binding of HBV to monocytes and to HepG2 cells was significantly reduced by pre-treatment of the cells with PI-PLC, implying that the molecule responsible for binding to these cells is GPI-linked. In this case, a comparison with HBV binding to K562 was not informative due to the resistance of the GPI linkages on these cells, and possibly also on THP-1 cells (based on CD59 cleavage), to hydrolysis by PI-PLC. Thus, cells expressing potential receptor(s) for HBV, whose characteristics do not correlate with any other proposed candidate, have been identified.

Immunoprecipitation analysis using HBV particles covalently cross-linked to the surface of ¹²⁵I-labelled K562 cells, and anti-S MAb coupled to goat anti-mouse IgG-Sepharose, resulted in the identification of a 50 kDa species as a putative HBV receptor.

Contrary to reports that HBV inhibits colony formation by myeloid cell lines in semi-solid media, no inhibitory effect by HBV was seen on clonal cell growth in liquid culture. K562 cells were found to be able to efficiently internalise HBV particles, which accumulated in a perinuclear compartment. In infection studies, K562 cells positive for HBsAg after 2-4 days post-infection became enlarged, and their numbers decreased steadily over an 11 day period. It is not clear whether these cells represent a transient or differentiated cell type. Similarly, it appears that the level of HBV DNA in these cells declines steadily during the infection course.