MOLECULAR AND CELLULAR STUDIES OF HUMAN HEPATITIS B VIRUS VARIANTS

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LIST OF ABBREVIATIONS

ε	5’ epsilon loop of HBV pregenome
$(NH_4)_2SO_4$	Ammonium sulphate
Anti-HBc	Antibody to HBcAg
Anti-HBe	Antibody to HBeAg
anti-HBs	Antibody to HBsAg
BCP	Basal core promoter
bp	Base pair
cDNA	Complementary DNA
CHO	Chineses hamster ovary
CTL(s)	Cytotoxic T lymphocyte(s)
DCs	Dendritic cells
DEPC	Diethyl pyrocarbonate
DHBV	Duck hepatitis B virus
DMEM	Dulbecco’s Minimal Essential Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Dideoxynucleotide triphosphates (dATP, dTTP, dCTP and dGTP)
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Fas-L	Fas ligand
FCS	Fetal calf serum
GRP94	94-kDa glucose-regulated protein
GSHV	Ground squirrel hepatitis virus
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
HBs/HBsAg	Hepatitis B major surface protein/surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCl	Hydrochloric acid
HHBV	Heron hepatitis B virus
HLA	Human leukocyte antigen
HNF-1	Hepatocyte nuclear factor-1
IFN	Interferon
IFN-α	Interferon-alpha
IFN-β	Interferon-beta
IFN-γ	Interferon-gamma
IFN-α/β	Interferon-alpha or beta
IgM	Immunoglobulin M
IKK	Inhibitor of κB kinase
IKK-α	Inhibitor of κB kinase-alpha

IKK-β	Inhibitor of κB kinase-beta
IL-1β	Interleukin-1-beta
IL-10	Interleukin-10
IL-18	Interleukin-18
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-8	Interleukin-8
kb	Kilobase
KCl	Potassium chloride
kDa	Kilodaltons
LHBs	Hepatitis B large surface protein
LMV	Lamivudine
MEM	Minimal Essential Medium
$MgCl_2$	Magnesium chloride
MHBs	Hepatitis B middle surface protein
MHC	Major histocompatibility complex
M-MLV-RT	Moloney murine leukaemia virus-reverse transcriptase
mRNA	Messenger RNA
NaCl	Sodium chloride
NF-κB	Nuclear factor κB
NK	Natural killer
nt(s)	Nucleotide(s)
ORF	Open reading frame
P/Pol	HBV polymerase gene/protein
PBMCs	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pgRNA	Pregenomic RNA
preC	Precore
preC/C	Precore and core
preS	Hepatitis B preS genes
preS/S	preS and surface
preS1	Hepatitis B preS1 gene
preS2	Hepatitis B preS2 gene
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Reverse transcription or reverse transcriptase
S	HBV surface region
SDS	Sodium dodecyl sulphate
TAE	Tris acetate EDTA buffer
Taq	Thermus aquaticus thermostable DNA
TE	Tris-EDTA buffer

Th	T helper
TNF-α	Tumour necrosis factor-alpha
URR	Upstream regulatory region
UV	Ultra violet
WE	William's Medium
WHO	World health organization
WHV	Woodchuck hepatitis virus

CHAPTER I

INTRODUCTION

Despite unceasing efforts of the medical community, hepatitis B remains, besides hepatitis C, the most serious type of viral hepatitis and one of the major problems of global public health. According to the latest World Health Organization fact sheets (2000), of the 2 billion people who have been infected with the hepatitis B virus (HBV), more than 350 million have chronic infections. These chronically infected persons are at high risk of death from cirrhosis of the liver and liver cancer, diseases that kill about 1 million persons each year (WHO, 2000).

The prevalence of HBV varies tremendously in different part of the world, with a much higher incidence in the Eastern than in the Western Hemisphere (WHO, 2001). High prevalence areas have been identified in Southeast Asia, China and Africa (reviewed by Lee, 1997). About 100 million carriers, making up 75% of the world's HBV carriers living in Asia, are from China (Tandon and Tandon, 1997). In Malaysia, voluntary testing carried out on 17 048 healthy volunteers indicated a HBsAg seropositivity of 5.24% (Merican *et al*., 2000).

HBV belongs to the *Orthohepadnavirus* genus of the *Hepadnaviridae* family, which is related to the large order of *Retroid* viruses (Kann and Gerlich, 1998). Within a size of only about 3.2 kb, its compact, partially double-stranded DNA genome is extremely small, bearing four highly overlapping open reading frames (ORFs), which encode at least seven proteins (Kann and Gerlich, 1998; Nassal, 1999; Seeger and Mason, 2000). Due to the use of a viral RNA-dependent polymerase without proofreading function, HBV has a higher mutational rate than other DNA viruses (Blum1995; Petzold *et al*., 1999). Thus, it is generally assumed that this reverse transcription step accounts for the majority of point mutations and deletions or insertions that can be observed in the HBV genome.

There are 2 major types of mutations in HBV. Firstly, there are genotype-specific mutations that allow the distinction of currently eight genotypes (A-H) (Norder *et al*., 1993; Stuyver *et al*., 2000; Arauz-Ruiz *et al*., 2002). These genotypes cluster geographically. Genotype A seems to represent the main European inland strain; genotype B and C, the Asian strain; genotype D, the Mediterranean basin strain; genotype E, the African strain; and genotype F, the New World strain (Norder *et al*., 1994; Magnius and Norder, 1995; Kidd Ljunggren, 1996). Genotype G was identified in France and United States (Stuyver *et al*., 2000) and genotype H was recently encountered in Nicaragua, Mexico and California (Arauz-Ruiz *et al*., 2002).

The second type of HBV variability concerns mutations that emerge in an individual during chronic infection. Several specific mutations of this type have been identified by a large number of longitudinal as well as cross-sectional studies conducted during the past decade (reviewed in Gunther *et al*., 1999). Most of the corresponding variants accumulate during infection and persist as a dominant population until the late phase. These mutants are clinically important. It is learned that the presence or emergence of specific mutations is associated with particular stages of chronic infections (Gunther *et al*., 1999).

In general, the enhancer II/core promoter and precore stop codon mutants appear to be associated with disease severity and progression (Lindh *et al*., 1999; Scaglioni *et al*., 1997; Pult *et al*., 1997; Takahashi *et al*., 1999). Mutations in the core antigen contribute strongly to immune escape at the T helper and cytotoxic T lymphocyte (CTL) level (Wakita *et al*., 1991; Chisari and Ferrari, 1995). Recent reports also revealed that mutations at basal core promoter (BCP) and precore/core (preC/C) mutations may influence the response rate to interferon-alpha (IFN-α) therapy (Fattovich *et al*., 1995; Zhang *et al*., 1996; Erhardt *et al*., 2000). Surface antigen mutants allow for escape from humoral immune responses and reduce the effectiveness of diagnostic tests and vaccination (Waters *et al*., 1992; Karthigesu *et al*., 1994; Carman *et al*., 1995,; Wallace and Carman, 1997; Hsu *et al*., 1999a).

HBV is a typical non-cytopathic virus that can induce tissue damage of variable severity by stimulating a protective immune response that can simultaneously cause damage and protection, by resolving intracellular virus through the destruction of virus infected cells (Ferrari *et al*., 2003). Therefore, immune elimination of infected cells can lead to the termination of infection when it is efficient, or to a persistent necroinflammatory disease when it is not.

Destruction of infected cells, however, is not the only mechanism implicated in the elimination of intracellular virus, as demonstrated by studies carried out in human hepatitis B showing the importance of cytokine-mediated, non-cytolytic mechanisms of antiviral protection. The first experimental evidence in favour of such mechanisms derives from studies performed in the transgenic mouse model (Guidotti and Ferrari, 2001). These studies showed that single stranded and relaxed circular double stranded HBV DNA replicative intermediates can be eliminated from the cytoplasm of HBV transgenic hepatocytes as a result of the antiviral effect of the interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) released within the transgenic liver primarily by infiltrating HBV-specific $CD8^+$ cells (Guidotti *et al*., 1996; Heise *et al*., 1999b) but also $CD4^+$ T cells (Franco *et al*., 1997).

Although the existence of genotypes is known for a long period of time, only very recently an association of genotype and clinical outcome was proposed (Kao *et al*., 2000a; Lindh *et al*., 1999). Recently, HBV genotypes have been partially clarified as

influencing the clinical manifestation of chronic liver disease in hosts. A higher disease-inducing capability of genotype C than genotype B has been observed in Asia (Orito *et al*., 2001a; Kao *et al*., 2000a; Lindh *et al*., 1999). Several studies, mostly from Taiwan and Japan, have shown that HBV genotype C is associated with the development of hepatocellular carcinoma (HCC) (Kao *et al*., 2000a; Ding *et al*., 2001; Fujie *et al*., 2001) and has a lower response rate to interferon therapy as compared to genotype B (Kao *et al*., 2000b). As for other HBV genotypes, most patients in Europe with genotype A have chronic hepatitis, whereas most patients with genotype D have acute hepatitis (Mayerat *et al*., 1999) and may predict the occurrence of HCC in young Indian patients (Thakur *et al*., 2002).

The genotype-related differences in HBV pathogenesis have been associated with the HBeAg/anti-HBe status. In the natural course of chronic HBV infection, early HBeAg/anti-HBe seroconversion usually associated with the cessation of virus replication and thus a favourable outcome (Chen, 1993). In contrast, late seroconversion of HBeAg after multiple episodes of reactivation and remission may accelerate the progression of chronic hepatitis B and thus have a poor clinical outcome (Perillo, 2001). Reports have revealed that the prevalence of HBeAg is more common in HBV genotype C than B. The reverse held true for the prevalence of anti-HBe, in that it is less common in genotype C than B (Ding *et al*., 2002; Chu *et al*., 2002; Kao *et al*., 2002; Orito *et al*., 2001a; Kobayashi *et al*., 2002; Yuen *et al*., 2003; Akuta *et al*., 2003).

Taken together, these data from different parts of the world have lent strong support to possible pathogenic differences among HBV variants. At present, those findings have been reported only in a few Asian countries. Moreover, the molecular virologic mechanisms that contribute to these clinical differences among HBV genotypes remain to be explored. The major limitation of previous studies is the lack of simple and efficient genotyping methods. Genotyping of viruses by sequencing and subsequent homology comparison or phylogenetic tree analysis is tedious and labour intensive and, therefore, not practical for diagnostic purposes. With the recent advances in molecular techniques, several novel genotyping methods, including polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Lindh *et al*., 1997; Mizokami *et al*., 1999), PCR with type-specific primers (Naito *et al*., 2001), commercial hybridization assay (Hou *et al*., 2001) and serologic genotyping assay (Usuda *et al*., 1999) have been introduced.

In Malaysia, where the incidence rate of HBV was 12.19 per 100,000 population in 2001 (Ministry of Health Malaysia, 2001), limited information on the molecular biology of the HBV is available. The prevalence of HBV genotypes and the clinical relevance of HBV variants have not been discussed. The studies from other areas may not apply worldwide because the HBV strains in various parts of the world are different, and thus the clinical outcome and the mechanisms responsible may be different in this country. This provided a strong motivation to investigate the molecular variants of HBV in our population and the immune response evoked by these HBV variants.

The objectives of this work were:

1. to determine the nucleotide sequences, nucleotide variations and amino acid substitutions of HBV BCP, preC/C and preS/S regions in sera of asymptomatic chronic HBV carriers in our population;

2. to investigate the prevalence of HBV genotypes using type-specific primers genotyping and preS amplicon restriction pattern analysis methods;

3. to evaluate the genotype-related differences in respect to HBeAg status in chronic HBV carriers;

4. to explore the genotype-related differences and BCP mutation in the expression of cytokines mRNA in *in vitro* HBV infection model.

CHAPTER II

LITERATURE REVIEW

2.1 Hepatitis B Infection

The hepatitis B virus (HBV) is an aetiological agent of both acute and chronic viral hepatitis. Chronic HBV infection remains a major public health problem worldwide with approximately 350 - 400 million chronic carriers, that is 5% of the world's population (WHO, 2000). Although clinical manifestations of the infection vary considerably, there is a strong correlation between chronic HBV infection and the risk of developing cirrhosis and hepatocellular carcinoma (HCC) (Robinson, 1994). Of the chronic carriers, 25-40% will eventually die of cirrhosis with or without HCC; the death rate being 50% for male carriers and 15% for female carriers (Lau *et al*., 1997).

The distribution of hepatitis B infection varies greatly throughout the world. In African countries, Southeast Asia and China, the incidence is high, while in the continental United States and in Western Europe the incidence is relatively low (Figure 2.1). The hepatitis B surface antigen (HBsAg) carrier rate varies from 0.1% to 20%. In Northern, Western and Central Europe, North America and Australia, the carrier rate is relatively low ranging from 0.2-0.5%, while the carrier rate is intermediate ranging from 2-7% in Eastern Europe, the Mediterranean, Russia and the Russian Federation, Southwest Asia,

Central and South America. In Southeast Asia, tropical Africa and parts of China, the carrier rates are between 8-20% of the populations (Zuckerman, 1996).

In Malaysia, about 1.2-1.6% of blood donors are HBsAg positive by EIA method in 2002 (Ministry of Health Malaysia). Another study has shown that 5% of healthy volunteers are positive for HBsAg in 1997 (Merican *et al*., 2000).

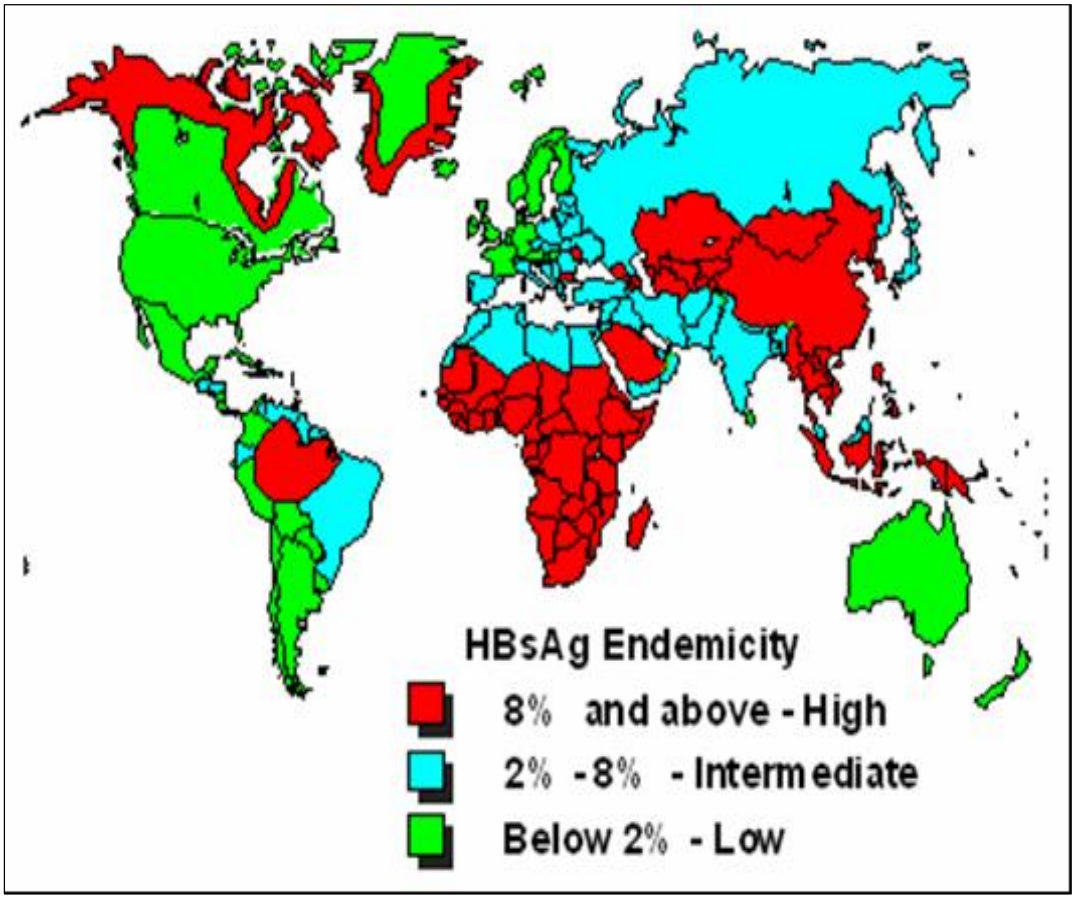

Figure 2.1: Geographical distribution of chronic hepatitis B virus infection (World Health Organization, 2001)

2.2 The Virus

HBV was first identified in 1965 by Blumberg as a new antigen in leukaemic sera of native Australians and was originally referred to as the "Australia antigen" (Blumberg *et al*., 1965). Only later was this antigen shown to be the hepatitis B virus surface antigen or HBsAg. In 1970, Dane managed to isolate an infectious complete particle and identified it by electron microscopy (later known as Dane particle) (Dane *et al*., 1970).

Human HBV represents the prototype of the *Hepadnaviridae*, a family of small DNA viruses that persistently infect liver cells and whose genome is the smallest known for mammalian viruses. HBV shares 70% sequence homology with mammalian hepadnaviruses discovered in woodchucks (WHV) (Summers *et al*., 1978) and in various ground squirrel species (GSHV) (Marion *et al*., 1980). Old and New World primates possess wild-type infection of HBV subvariants that may prove to be species specific (Takahashi *et al*., 2001). Human HBV is, however, capable of infecting chimpanzees, baboons and other great apes as well as various marsupials (Seeger and Mason, 2000). Avian hepadnaviruses, more distantly related viruses which share a similar genome structure, albeit with little sequence homology to the mammalian hepadnaviruses, have been found in ducks (DHBV) (Mason *et al*., 1980), wild herons (HHBV) (Sprengel *et al*., 1988) and recently in white storks (Pult *et al*., 2001). Domestic geese and other hosts are susceptible to infection from other avian hepadnaviral species (Marion *et al*., 1987).

2.3 Classification of HBV

Human hepatitis B virus can be serologically classified into four serotypes, also known as subtypes of HBsAg. These were initially defined a group-specific antigenic determinant *a* which is common to all, and two pairs of mutually exclusive subtype-specific determinant, *d*/*y* and *w*/*r* (Le Bouvier and McCollum, 1970). Ten subtypes, *ayw*1, *ayw*2, *ayw*3, *ayw*4, *adw*2, *adw*3, *adw*4, *ayr*, $adrq^+$ and $adrq^-$ were later identified following further subdivision of the *w* subdeterminant into *w*1 to *w*4 and the acquisition of the *q* determinant (Norder *et al*., 1992;1994; Arauz-Ruiz *et al*., 2002).

Based on nucleotide diversity of 8% or more in the genome, HBV of various subtypes have been classified into 8 genotypes, designated A-H (Norder *et al*., 1993; Stuyver *et al*., 2000; Arauz-Ruiz *et al*., 2002). The inter-relationship of subtypes to genotypes has been clarified (Orito *et al*., 1989). In general, genomes encoding *adw* are found in genotypes A, B, C, F and G, while the genomes encoding both *adr* and *ayr* occur in genotype C alongside with *adw* (reviewed in Kao, 2002). HBV genotypes have distinct geographical distribution (Magnius and Norder, 1995; Lindh *et al*., 1997; Arauz-Ruiz *et al*., 1997; Stuyver *et al*., 2000). Genotypes A and D are found in Africa, the Mediterranean area and Western Asia. Genotypes B and C prevail in Southeast Asia and the Far East, while genotype E circulates in Western sub-Saharan Africa (Lindh *et al*., 1997). Genotype F is indigenous to the Ameridian populations of the New World (Telenta *et al*., 1997; Arauz-Ruiz *et al*., 1997), and genotype G was identified in France Southeast Asia and the Far East, while genotype E circulates in Western sub-Saharan Africa (Lindh *et al*., 1997). Genotype F

is indigenous to the Ameridian populations of the New World (Telenta *et al*., 1997; Arauz-Ruiz *et al*., 1997), and genotype G was identified in France and United States (Stuyver *et al*., 2000). Genotype H was recently encountered in Nicaragua, Mexico and California, and was most likely split off from genotype F within the New World (Arauz-Ruiz *et al*., 2002).

2.3.1 HBV Genotype and Clinical Outcome

Differences in the natural history of HBV carriers and in the responses to interferon therapy of HBV infected patients have been described previously. Most studies from East Asia, have shown that HBV genotype C is associated with more severe clinical outcome (Kao *et al*., 2000a; Fujie *et al*., 2001). In Taiwan, genotype C is more prevalent in patients with cirrhosis compared to asymptomatic patients suggesting that the HBV genotype C is associated with a more severe liver disease (Kao *et al*., 2000a). Ding and colleagues (2001) have shown that genotype C is associated with the development of HCC, whereas genotype B has a relatively good prognosis in China. In Japan, the number of patients with liver cirrhosis or HCC increased with age in patients with genotype C, indicating that genotype C is also closely associated with the development of HCC (Orito *et al*., 2001a).

It has been shown that although the prevalence of HBsAg among blood donors in Okinawa is the highest in Japan, twice as high as the average for the whole country, the

mortality rates for both cirrhosis and HCC in Okinawa are the lowest in Japan (Sakugawa, 1992). Recently, a serologic genotyping study confirmed that genotype B is the most prevalent HBV genotype in Okinawa (Usuda *et al*., 1999).

As for other HBV genotypes, genotype A has been reported to be associated more frequently with chronic infection than genotype D in Europe (Mayerat *et al*., 1999). Recently, a prospective study was performed by Thakur and colleagues to determine the prevalence and clinical significance of HBV genotypes A and D in 130 histologically proven chronic HBV-infected Indian patients (Thakur *et al*., 2002). Their results showed that HBV genotype D is associated with a more severe liver disease and may predict the occurrence of HCC in young Indian patients.

2.3.2 HBV Genotype and Response to Antiviral Therapy

It has been reported that HBV genotype C, compared with genotype B, is associated with a higher frequency of core promoter mutation and a lower response rate to IFN-α therapy (Kao *et al*., 2000b). The data suggest that patients infected with HBV genotype B are predicted to have a better response to IFN-α. A similar situation has been observed between HBV genotype A and D patients. Hou and colleagues (2001) studied the relationship between HBV genotypes and IFN treatment response in a homogeneous group of 103 HBeAg positive patients with chronic hepatitis B recruited from 16