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LIST OF ABBREVIATIONS

ε  5’ epsilon loop of HBV pregenome
(NH₄)₂SO₄ 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 Chinese 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
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<td>Th</td>
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<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
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<td>URR</td>
<td>Upstream regulatory region</td>
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<tr>
<td>UV</td>
<td>Ultra violet</td>
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<td>WE</td>
<td>William’s Medium</td>
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<td>Woodchuck hepatitis virus</td>
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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; Magnus 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 \textit{et al.}, 1997; Mizokami \textit{et al.}, 1999), PCR with type-specific primers (Naito \textit{et al.}, 2001), commercial hybridization assay (Hou \textit{et al.}, 2001) and serologic genotyping assay (Usuda \textit{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