MOLECULAR CHARACTERISATION OF HEPATITIS B VIRUS VACCINE ESCAPE MUTANTS IN SOUTH AFRICA

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DECLARATION

I, Penny Crowther, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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ABSTRACT

Since the introduction of vaccination against hepatitis B virus (HBV) infection in South Africa, at least one case of infection despite vaccination has occurred. The purpose of this study was to determine whether this infection was the result of mutations within the region of the surface (S) gene encoding the *a* determinant epitopes of the hepatitis B surface antigen, which permitted viral vaccine-escape. HBV DNA was extracted from the serum and liver tissue of the patient and amplified within the complete 3 215 bp genome and S gene, respectively. Following cloning, sequencing revealed a minor population displaying unique or uncommon S gene mutations that resulted in C138R, C139R, K141R, P142L, T143A, N146D, and T148A amino acid substitutions in the clones from the serum, and C139Y and D144N in the clones from the liver. Such isolates may represent South African HBV vaccine-escape mutants that caused chronic infection in the host prior to their reversion to wild-type.

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ABBREVIATIONS

3'A	3' deoxyadenosine
aa	amino acid
ALT	alanine aminotransferase
anti-HBc	hepatitis B core antibodies
anti-HBe	hepatitis B e antibodies
anti-HBs	hepatitis B surface antibodies
APC	antigen presenting cell
AST	aspartate aminotransferase
bp	base pair(s)
C	core
cccDNA	covalently closed circular deoxyribonucleic acid
ddNTP	2',3'-dideoxynucleoside triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DR	direct repeat
dsDNA	double-stranded deoxyribonucleic acid
EPI	Expanded Programme on Immunisation
ER	endoplasmic reticulum
HBc	hepatitis B core
HBcAg	hepatitis B core antigen
HBe	hepatitis B e
HBeAg	hepatitis B e antigen
HBIg	hepatitis B immunoglobulin

HBs	hepatitis B surface
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HBx	hepatitis B x
HBxAg	hepatitis B x antigen
HCC	hepatocellular carcinoma
kb	kilobase(s)
kDa	kiloDalton(s)
LB	Luria-Bertani
MHC	major histocompatibility complex
MHR	major hydrophilic region
mRNA	messenger ribonucleic acid
nt	nucleotide(s)
ORF	open reading frame
P	polymerase
PCR	polymerase chain reaction
pgRNA	pregenomic ribonucleic acid
PreC	precore
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase H	ribonuclease H
rpm	revolutions per minute
S	surface
V	volt(s)
vol	volume

Chapter 1: INTRODUCTION

Hepatitis B, a serious liver disease induced by the hepatitis B virus (HBV), is a major global public health problem with the virus infecting over two billion people, of which approximately 387 million are chronically infected [World Health Organisation, 2002]. Since the development of hepatitis B vaccines, the number of newly acquired HBV infections has decreased substantially, to a very small fraction of the number acquired prior to vaccine administration. Nevertheless, vaccination does not protect all individuals from infection, because of either weak immune responses from the host or viral mutations, both of which allow vaccine escape of the virus.

1.1 HEPATITIS B VIRUS

1.1.1 History

Epidemic jaundice was first described by Hippocrates in the fifth century BC, when he was undoubtedly referring to individuals infected with hepatitis B virus and other agents capable of infecting the liver [Mahoney, 1999]. Epidemics of jaundice have continued to occur throughout history, particularly during wars in the 19th and 20th centuries [Mahoney, 1999].

The existence of a form of hepatitis that was parenterally transmitted by direct inoculation of blood or blood products was first documented by Lurman in Bremen, Germany, in 1883,

during a smallpox vaccination campaign that utilised contaminated human lymph [Lurman, 1885]. Early in the 20th century, further outbreaks of parenterally acquired hepatitis occurred in various risk groups including patients who attended clinics for venereal diseases, diabetes, and tuberculosis; those who received blood transfusions; persons inoculated with mumps or measles serum; and military personnel who received yellow fever vaccines during World War II [Neefe *et al*., 1946; Mahoney, 1999].

Studies conducted during the 1930s and 1940s provided convincing evidence of a viral cause for hepatitis, with at least two etiologic agents [MacCallum and Bauer, 1944; Havens, 1946]. Subsequently, in 1947, MacCallum and Bauer (1947) proposed the nomenclature of hepatitis A for infectious hepatitis and hepatitis B for "homologous serum" hepatitis, terms which have since been adopted by the World Health Organisation (WHO) Committee on Viral Hepatitis. The distinctive seroepidemiologic features of hepatitis A and B were finally firmly established in a series of studies by Krugman and colleagues (1967) at the Willowbrook State School, New York, in the 1960s.

The viral agent associated with hepatitis B was first isolated during serological studies conducted independently by Prince *et al*. (1964) and Blumberg *et al*. (1965). In searching for serum protein polymorphisms linked to diseases, Blumberg and colleagues identified an antigen – termed Australia (Au) antigen – in serum from patients with leukaemia, leprosy, and hepatitis, although the relationship of the antigen to hepatitis was not fully recognised until 1967. Prince (1968) subsequently established that the Au antigen occurred specifically in the serum of hepatitis B patients, and in 1973 the antigen was renamed the hepatitis B surface antigen (HBsAg) by the WHO.

In 1970, Dane and colleagues (1970) were the first to detect complete hepatitis B virions, or Dane particles. The 42 nm double-shelled spheres consisted of an outer envelope composed of HBsAg and an inner core containing hepatitis B core antigen (HBcAg) and endogenous DNA. A third antigen related to infectivity, designated hepatitis B e antigen (HBeAg), was subsequently described in 1972 by Magnius and Espmark (1972).

In 1975, following these important breakthroughs in hepatitis research, hepatitis B vaccine studies were initiated using plasma-derived highly purified HBsAg particles, as it was found that antibodies targeting HBsAg were the neutralising antibodies for infectious HBV [Hollinger *et al*., 1974]. The safety and efficacy of these vaccines have since been established and continue to be maintained.

1.1.2 Natural History and Pathogenesis

The hepatitis B virus is one of the most successful viral pathogens of humans because of the nature of its infections. Although the hepatocytes are the principal site of viral infection, pathological features of HBV infection have been identified in extrahepatic tissues such as the kidneys, lymph nodes, spleen, gonads, thyroid gland, adrenal glands, and bone marrow [Hollinger, 1996; Hilleman, 2003]. The liver plays an essential role in protein, carbohydrate, and lipid metabolism, energy storage and conversion, blood homeostasis, chemical detoxification, and immunity to microbial infections [Seeger and Mason, 2000]. Disruption of this organ by HBV infection therefore leads to a varying array of acute and chronic disease manifestations.

The consequences of acute HBV infection are highly variable, ranging from subclinical and asymptomatic to clinically apparent disease [Robinson, 1994]. The incubation period of hepatitis B – the time from infection to the onset of signs or symptoms – ranges from six weeks to six months, with the development of clinical symptoms being highly dependent on the age of the host [Mahoney, 1999]. Clinical manifestations do not generally occur in neonates, while infection produces typical illness in only 5 to 15% of children aged one to five years [McMahon *et al*., 1985; Mahoney, 1999]. In contrast, 33 to 50% of older children and adults develop symptomatic infections, varying in severity from mild to fulminant hepatitis with extensive liver necrosis (*Figure 1.1*) [Mahoney, 1999]. The clinical signs and symptoms of acute HBV infection that result from hepatic inflammation, include fever, jaundice, malaise, nausea, vomiting, abdominal pain, and increased serum levels of the liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) [Francois *et al*., 2001]. Fulminant hepatitis, appearing suddenly, rapidly, and with great intensity, occurs in 1 to 2% of acute infections and has a case-fatality ratio of 63 to 93% [Mahoney, 1999].

The risk of developing chronic HBV infection – defined as the presence of HBsAg in the serum for longer than six months – decreases with age: approximately 90% of infected neonates, 50% of infants, 30% of children, and 10% of infected adults become HBV carriers [Hilleman, 2003]. Individuals with chronic hepatitis B infections generally have one of three types of disease – chronic persistent hepatitis, chronic active hepatitis, or cirrhosis [Mahoney, 1999]. The degree of histological injury is often not reflected by the symptoms, such that persons with severe chronic liver disease may remain asymptomatic until late in the course of their illness [Mahoney, 1999]. Chronic active hepatitis leads to the development of liver fibrosis and often to cirrhosis, an irreversible condition in which

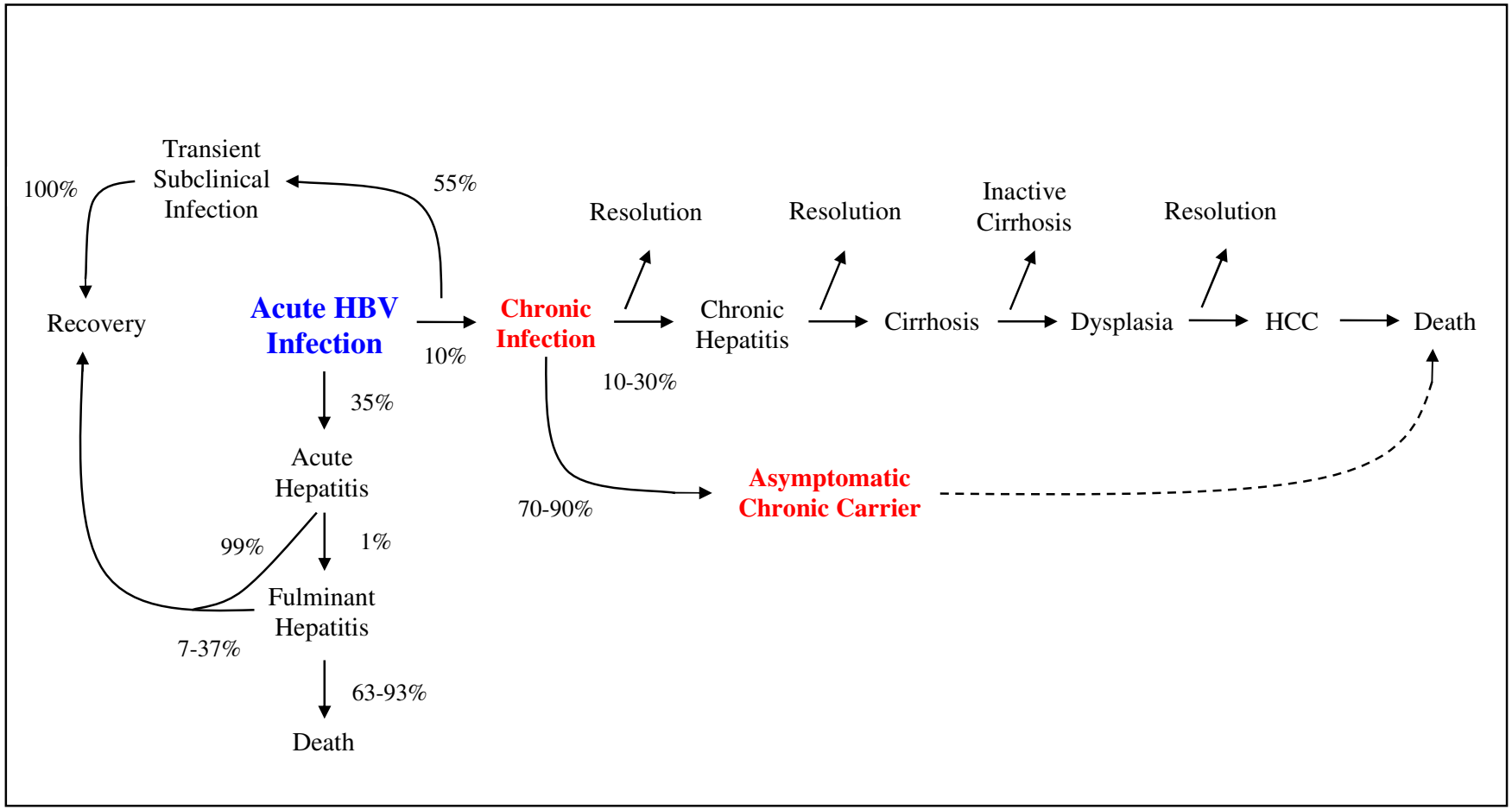

Figure 1.1 Schematic representation of the pathogenesis of hepatitis B virus infection in adults, indicating the percentage of infections that progress to various forms of the disease [Modified from Feitelson, 1994].

regenerative nodules and fibrosis cause severe liver injury [Mahoney, 1999; Ganem and Prince, 2004]. Because of its stimulating effect on hepatocyte regeneration, cirrhosis may subsequently lead to the development of hepatocellular carcinoma (HCC), a malignant liver tumour that typically appears 25 to 30 years after the onset of infection [Mahoney, 1999] and culminates in the death of the individual (*Figure 1.1*).

The pathogenesis of hepatitis B is extremely complex [Hilleman, 2003]. Although HBV causes hepatitis B, the virus itself is not directly cytopathic in humans and therefore causes only limited damage to the liver [Francois *et al*., 2001]. The clinical effects of the disease are, instead, the result of the destruction of infected hepatocytes by the host immune defence system [Hollinger, 1996]. Variations in the clinical outcome of HBV infection are therefore caused by differences in the host response and, possibly, by mutations in the HBV genome that result in the production of variant viruses [Feitelson, 1994; Kidd-Ljunggren, 1996].

Although the mechanism of liver injury in acute and chronic hepatitis B is not completely defined [Robinson, 1994], it is known that aggressive immune responses, involving both major histocompatibility complex (MHC) class II-restricted, CD4+ helper cells and MHC class I-restricted, CD8+ cytotoxic T-lymphocytes [Ganem and Prince, 2004], result in symptomatic acute hepatitis and viral clearance, while non-aggressive host responses that reflect weak T-cell-mediated immune responses lead to an asymptomatic chronic viral carrier state [Hilleman, 2003].

Of the approximately 387 million chronic carriers of HBV worldwide, 15-40% eventually develop cirrhosis, liver failure, or HCC [Lok, 2002]. In addition, HBV infection results in

500 000 to 1.2 million deaths each year, making it the tenth leading cause of death worldwide, while HCC is the fifth most common cancer, killing 300 000 to 500 000 people per year [Lavanchy, 2004].

1.1.3 Epidemiology

Present mainly in blood, saliva, and semen, hepatitis B virus is transmitted horizontally by percutaneous or permucosal exposure to infectious blood and body fluids, or perinatally from an infected mother to her infant during the perinatal period [Mahoney, 1999; Alter, 2003]. As a highly resilient virus, HBV is resistant to breakdown and can therefore also survive outside the body for longer than a week [Alter, 2003; Lavanchy, 2004].

The prevalence of HBV infection and patterns of transmission vary greatly in different geographic regions of the world and in different population subgroups (*Figure 1.2*) [Mahoney, 1999; Lavanchy, 2004]. Approximately 45% of the global population live in areas where the prevalence of chronic HBV infection is high (8% or more of the population is HBsAg positive), 43% live in areas with moderate prevalence (2 to 7% of the population is HBsAg positive), and 12% live in areas of low endemicity (less than 2% of the population is HBsAg positive) [Mahoney, 1999].

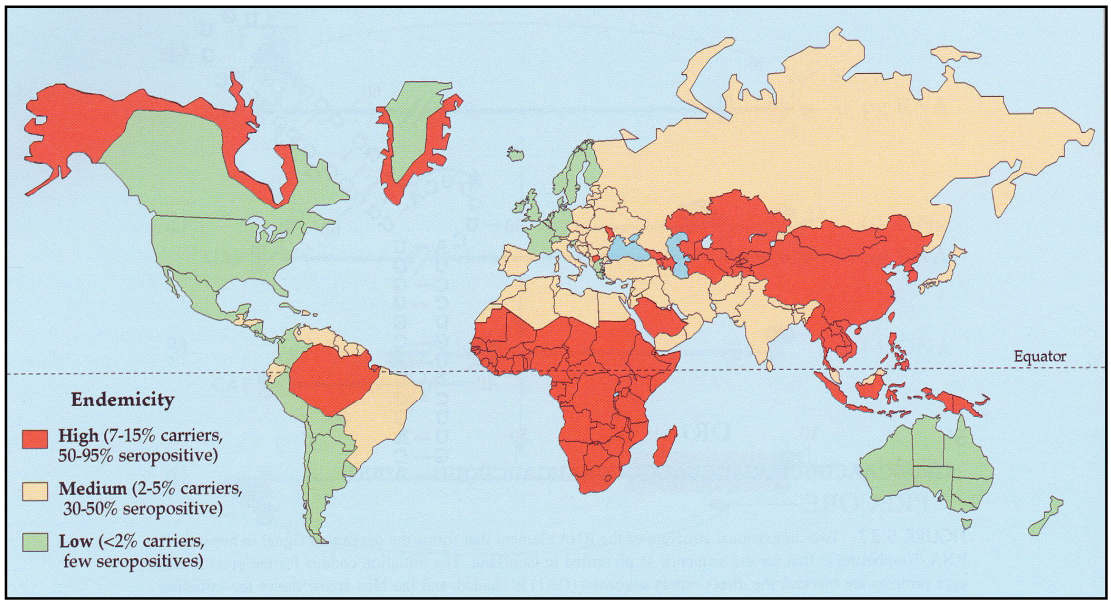

Figure 1.2 World map showing geographic distribution of chronic HBV infection [Strauss and Strauss, 2002].

In areas of high endemicity – including sub-Saharan Africa, most of Asia and the Middle East, the Amazon Basin of South America, the Pacific Islands, and other special population groups such as Native Alaskans, Australian Aborigines, and Maoris in New Zealand – the most common source of infection is through perinatal transmission or through infection acquired horizontally during early childhood [Mahoney, 1999; de Franchis *et al.*, 2003]. The high proportion of infectious HBV carriers explains the high rate of perinatal and childhood transmission, which perpetuate the high prevalence of HBV infections in these endemic countries [de Franchis *et al.*, 2003]. Thus, the lifetime risk of HBV infection in such highly endemic areas is greater than 60% [Mahoney, 1999].

In areas of moderate endemicity, such as North Africa, some of the Middle East, the southern parts of Eastern and Central Europe, the USSR, the Indian subcontinent, and part of Brazil, the current important modes of HBV transmission are needle sharing among

injecting drug users, nosocomial (hospital) transmission, tattooing, and body piercing [de Franchis *et al*., 2003]. In contrast to highly endemic countries, the lifetime risk of HBV infection in intermediate areas is 20 to 60% [Mahoney, 1999], with over 95% of new infections being acquired during adulthood [de Franchis *et al*., 2003].

Areas of low HBV endemicity include Northwest Europe, Australia, and North America [Mahoney, 1999; de Franchis *et al*., 2003]. In these regions, most HBV infections are acquired by horizontal transmission in early adulthood: through needle sharing among injecting drug users and through sexual contacts [de Franchis *et al*., 2003; Lavanchy, 2004]. Other rare sources of infection include contaminated surgical instruments, needle-stick injuries by hospital personnel, and blood transfusions [Alter, 2003]. The lifetime risk of infection in these developed countries is less than 20%, with most infections occurring amongst well-defined risk groups [Mahoney, 1999].

In South Africa, a country with intermediate HBV endemicity and pockets of high endemicity [Dusheiko *et al*., 1989a; Dusheiko *et al*., 1989b], more than 70% of the population has been exposed to HBV and approximately four million (almost entirely black people) are carriers [Kew, 1996; Tsebe *et al*., 2001]. Of these, an estimated 14 000 to 18 000 people die per annum [Mphahlele *et al*., 2002]. The majority of black carriers of HBV are infected in early childhood by horizontal transmission, probably by infected blood or saliva through skin abrasions, lesions, and bites, and tribal scarification or tattooing with unsterilised sharp instruments [Kew, 1996; Kiire, 1996]. Their main sources of infection are other family members, such as highly infectious siblings aged between one and five years, and playmates of the same age [Kew, 1996]. In contrast to it being the major source of HBV infection in Southeast Asia, maternal-infant perinatal infection is

relatively uncommon in black Africans [Kew, 1996]. The carrier rate of HBV differs regionally in South Africa, with higher rates of infection in rural areas (5 to 15%) than in urban areas (less than 5%) [Dusheiko *et al*., 1989a; Dusheiko *et al*., 1989b]; however, this difference cannot be explained by socioeconomic inequalities [Mphahlele *et al*., 2002].

1.1.4 Classification

The hepatitis B virus (HBV) was the first member to be discovered within the family of viruses designated the *Hepadnaviridae* (hepatotropic DNA viruses) [van Regenmortel *et al*., 2000]. This family was subsequently divided into two groups, the orthohepadnaviruses (mammalian hepadnaviruses), of which HBV is the prototype member, and the avihepadnaviruses (avian hepadnaviruses) [van Regenmortel *et al*., 2000].

Hepadnaviruses share many epidemiologic and biological features. As their names imply, all hepadnaviruses are hepatotropic and can cause hepatitis in their native host, and all exhibit a narrow host range that may be determined by the identity of the host hepatocyte receptor [Strauss and Strauss, 2002]. However, in contrast to orthohepadnaviruses, which mostly exhibit horizontal transmission, avihepadnaviruses are transmitted vertically [Strauss and Strauss, 2002].

1.1.5 Virion Structure

Each infectious HBV virion, or Dane particle, measuring 42 nm in diameter, consists of a spherical outer envelope that is 7 nm thick and encloses a 27-34 nm electron-dense, icosahedral core particle containing the viral nucleic acid and DNA polymerase (*Figure 1.3*) [Hollinger, 1996]. The envelope is composed of host-derived lipids, in which three types of different sized viral surface glycoproteins are embedded [Seeger, 1994]. In the blood of HBV-infected patients, titres of Dane particles can range from less than 10^4/ml to greater than 10^9/ml [Ganem, 1996].

In addition to complete Dane particles, sera of HBV infected individuals also contain two distinct subviral particles: spherical 20 nm particles and tubular or filamentous particles of 20 nm diameter and variable length (*Figure 1.3*) [Hollinger, 1996]. Both such particles are composed exclusively of viral surface glycoproteins (~70%) and host-derived lipids, such as phospholipids, cholesterol, and triglycerides [Ganem, 1996]. However, unlike Dane particles, subviral particles lack nucleic acid and are thus non-infectious, although they remain highly immunogenic [Ganem, 1996]. The most abundant of the three types of HBV particles, 20 nm spheres can reach titres as high as 10^{13}/ml, while filaments can reach titres of 10^{11}/ml [Ganem, 1996; Locarnini, *et al*., 2003]. As such, subviral particles comprise over 90% of the total HBV particles in the sera of infected individuals [Paran *et al*., 2003]. This excess of viral surface antigen particles is thought to adsorb neutralising antibodies, thereby helping to shield the infectious virions from host defences [Ganem, 1996].