PRODUCTION AND USE OF MONOCLONAL ANTIBODIES AGAINST A CONSERVED EPITOPE OF HEPATITIS B SURFACE ANTIGEN

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Abstract

The hepatitis B surface antigen (HBsAg) displays the major neutralising epitope of the hepatitis B virus (HBV) termed the $\alpha$ determinant. As well as its use as an immunogen in vaccines, HBsAg is the primary diagnostic marker for HBV, with its detection in serum being used for routine screening in diagnostic laboratories. Recently, mutations within the $\alpha$ determinant have been described, some of which have been linked to false-negative HBsAg reactions in the serum of HBV infected individuals. The inability of some assays to detect HBsAg mutants will result in the failure to monitor immune responses as well as the transmission of infection through blood or organs as a result of inaccurate diagnosis.

This thesis describes the use of monoclonal antibodies (MAbs) to define epitopes present on the wild-type (WT) HBsAg, that are preserved on HBsAg carrying mutations in the $\alpha$ determinant. The MAbs were raised against mutant HBsAg purified from two renal transplant patients. Following the screening process, those hybridomas which recognised the wild type and mutant HBsAg were selected. Characterisation studies using recombinant HBsAg further demonstrated that the MAbs were able to detect mutants ranging from codon 133 to 145. Once formatted into a solid phase radioimmunoassay, the MAbs were used to detect naturally-occurring in vivo mutant-virus infections.

The identification of the conserved epitope in the first loop of the $\alpha$ determinant, using oligopeptides, highlighted possible limitations of the monoclonal antibody which were later demonstrated in a study assessing the prevalence of HBsAg mutants in orthotopic liver transplant patients. Changes associated with natural genetic variation in the first loop resulted in the monoclonal antibody failing to recognise HBsAg in specimens from
patients infected with viruses of subtype ayw3 carrying changes in the putative binding site at residues 125 and 127.

An important, although maybe not pan-reactive, epitope has been identified which can be used to improve HBsAg detection assays as well as providing a strategy for studying the prevalence of HBsAg mutants in different populations.
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1.1.1 The hepatitis A virus

The identification of an epidemic type of infectious hepatitis (IH) was confirmed by Krugman et al., (1967). Two types of hepatitis with different clinical, epidemiological and immunological features were identified. This offered an explanation for the recurrence of hepatitis in previously infected patients. The patients with IH were immune to that type whilst remaining susceptible to serum hepatitis (hepatitis B). The IH virus was found to be the hepatitis A virus (HAV).

Hepatitis A virus was initially identified in stool and liver preparations of infected individuals by immune electron microscopy (Feinstone et al., 1973). Further work showed HAV to be a non-enveloped, 7.8 kb RNA virus whose size (27nm) and morphology led to its classification within the picornavirus group (Siegl et al., 1978).
The mode of transmission of HAV is primarily by the faecal-oral route, either person to person or food borne. The virus has a short incubation period of 15-45 days and generally causes a self-limiting infection where less than 5% of cases world wide are clinically recognised (Lemon et al., 1985). Infection with hepatitis A is indicated by the presence of IgM and IgG antibodies in serum (Feinstone et al., 1973).

The successful culture of HAV by Provost and Hilleman in 1979 led to the development of HAV vaccines (Gust, 1990). Pre- and post-exposure passive immunisation has been used successfully to prevent hepatitis A (Gust et al., 1988).

1.1.2 The hepatitis B virus

The terms hepatitis A and B were first introduced by MacCallum in 1947. The distinction between infectious hepatitis (hepatitis A) and serum hepatitis (hepatitis B) was initially based on epidemiological evidence. A study at an institution for the mentally handicapped by Krugman et al., (1967) showed that there were two types of hepatitis; hepatitis A which was characterised by a shorter incubation period and the parenterally transmitted hepatitis B which had an incubation period of more than three months.

The antigen, which was subsequently identified as the surface protein of the hepatitis B virus (the Australia antigen or HBsAg), was discovered as a result of a study carried out by Blumberg et al., (1965). In this study a serum sample taken from an Aborigine contained an antigen that reacted with serum taken from a multiply transfused haemophiliac. This antigen was originally thought to be associated with leukaemia and only later was the Australia antigen linked with post-transfusion viral hepatitis (Blumberg et al., 1967; Prince, 1968).
The availability of this marker led to the first identification of the hepatitis B virus (HBV) by immune electron microscopy (Dane et al., 1970). The 42nm virus particle, known as the Dane particle, was found to display the Australia antigen on its surface. Further work showed the Dane particle to comprise an inner core containing its own distinct antigen, designated hepatitis B core antigen (HBcAg), in addition to a small, circular, partially double stranded DNA molecule. A third antigen, designated hepatitis B e antigen (HBeAg) was subsequently described by Magnus and Espmark in 1972. This antigen was thought to be a marker of active viral replication and thus infectivity.

The development of specific laboratory tests for HBV has confirmed the importance of the parenteral routes of transmission. Since its initial identification the epidemiological concept that hepatitis is spread exclusively by blood and blood products has changed. Other routes of transmission such as maternal/perinatal, sexual and non-parenteral have since been identified.

HBV infection may occur as an icteric or an asymptomatic acute infection. In a minority of patients who are infected as adults and in the majority of those infected as infants, a chronic infection may develop and persist for years. In an acute infection viral replication generally ceases within 6 to 9 months.

1.1.3 The hepatitis C virus

Despite the identification of HAV and HBV there was still a relatively high proportion of patients who had undergone transfusion or used intravenous drugs who developed a non-A, non-B hepatitis (NANB hepatitis) (Feinstone et al., 1975;
Dienstag et al., 1983). This form of hepatitis was related to a transmissible agent present in blood.

In 1989, Choo et al., first isolated a viral, blood borne infectious agent from chimpanzee plasma. This cloned virus was called the hepatitis C virus (HCV). HCV is a positive stranded, 10Kb RNA virus which shows amino acid sequence homology to the flaviviruses (Miller et al., 1990).

Development of serological tests to HCV (Kuo et al., 1989) have shown that at least 50% of patients previously diagnosed as having post transfusion NANB hepatitis have tested positive for anti-HCV (Alter, 1989). In 1993, Makris and colleagues found that nearly 100% of haemophilia patients involved in their study had become infected with HCV due to contamination of clotting factor concentrates. The addition of anti-HCV testing to the screening of blood donors and the inactivation of pooled plasma products should significantly reduce the risk of transfusion associated hepatitis. Indeed in 1992, Donohue et al., demonstrated that the serological screening of blood donations had led to a dramatic reduction in the rate of post transfusion hepatitis, confirming HCV’s role as the major aetiological agent.

Groups at risk from HCV infection include patients receiving blood or blood products, intravenous drug users and children born to infected mothers. Sexual transmission of HCV is thought to be absent or rare (Gordon et al., 1992; Bresters et al., 1993). Although the infection rate is raised in sexually promiscuous groups, sexual partners of HCV antibody positive people are typically seronegative (Dusheiko et al., 1996).

Once infected the majority of HCV patients become chronic carriers. The course of infection is variable. HCV may cause a benign and asymptomatic disorder with an
indolent course, or it may cause progressive liver disease, cirrhosis and hepatocellular carcinoma. Morbidity is affected by many interacting factors including age at acquisition, co-infection with other viruses and the host immune response. To date there is no method of immunisation, either active or passive for the prevention of HCV infection.

1.1.4 The hepatitis D virus

The hepatitis D virus (HDV) (delta virus) was first detected as a new viral antigen in the hepatocytes and serum of HBsAg patients by Rizzetto et al., in 1977. The agent was later shown to be transmissible (Rizzetto et al., 1980). The genome of HDV consists of a single-stranded, 1.7kb RNA which is surrounded by a 35-37nm HBsAg envelope. Hybridisation techniques have shown no homology to exist between the HDV and HBV genome. HDV is thought to be a defective virus dependent upon the helper function of HBV for its replication (Rizzetto et al., 1985). The virus only infects persons carrying hepatitis B DNA as it replicates in HBV infected cells and acquires a HBsAg coat.

HDV infection may coincide with acute HBV infection or be a superinfection acquired later by HBV carriers. The prevalence of HDV in persons who have received multiple transfusions or in those who are intravenous drug abusers is low (Kumar et al., 1992; Smith et al., 1992). Once infection is established, however, it interferes with the expression of HBV gene products and reduces the concentration of hepatitis B markers. The incubation period varies from 2-12 weeks, being shorter in HBV carriers who are superinfected.
Persistence of HDV in a carrier can transform a mild, chronic HBV infection into a severe and more active form of chronic hepatitis (Nishioka et al., 1985).

Infection with HDV can be diagnosed by identifying an IgM anti-HDV response during the acute or chronic stage of the disease. There is no method of immunisation for HDV infection. However, prevention of HBV infection will provide an indirect method of control.

1.1.5 The hepatitis E virus

Investigations carried out on an enterically transmitted non A non B hepatitis outbreak in India led to the identification of the hepatitis E virus (HEV) (Wong et al., 1980). Initially the outbreak was thought to be due to HAV. However, further studies showed the causal agent to be antigenically distinct from HAV.

The spherical, non-enveloped virion of HEV resembles that of other caliciviruses. It has a 7.5 kb genome of single stranded RNA. The virus was identified as 23-27nm particles by Balayan et al., (1983) in the faeces of an HEV infected individual.

Hepatitis E has been transmitted to macaques by a virus obtained from human stools (Krawczynsky et al., 1989). HEV Ag was detected in the liver of the macaques and the agent could be recovered from the infected animal.

An enzyme immunoassay based on synthetic peptides, solid phase immune electron microscopy and the polymerase chain reaction have all been used to identify HEV infection (Humphrey et al., 1990; Lau et al., 1991).
1.1.6: Other ‘Non A Non B’ Agents

Despite the introduction of reliable diagnostic assays for the detection of HCV and HEV (Dawson et al., 1992; Kuo et al., 1989), there still remains cryptogenic cases of hepatitis of presumed viral origin (Alter, 1994; Thiers et al., 1993).

Hepatitis F virus (HFV) has been described in only a handful of cases with subsequent experimental transmission to primates. The agent(s) responsible in humans was serially transmitted in rhesus monkeys by intravenous inoculation of the stool extract from a patient. The novel agent was present as 27-37nm particles in the infectious stool extract. Hepatic lesions were noticed in infected monkeys during the acute phase of illness. The purified viral 27-37nm particles consist of a double stranded DNA of approximately 20kB and are detected in infected monkey liver. The virology, epidemiology, hepatotrophicity and clinical importance of HFV in humans remains quite uncertain (Deka et al., 1994).

Hepatitis G virus (HGV; also called hepatitis GB virus C or HGBV-C) was characterised in early 1996. HGV is a flavivirus and a close relative of HCV. The genomes of all three viruses have been cloned by polymerase chain reaction (Leary et al., 1995b; Simons et al., 1995b). An antibody test for HGV has recently been developed and should help elucidate the epidemiology of HGV infection.

The nature and frequency of HGV infection remain vague and so there is uncertainty about risk factors and means of prevention. Transmission through blood transfusion has been documented (Schmidt et al., 1996) and the virus has frequently been found in multiply transfused patients such as haemophiliacs (Linnen et al., 1996) and patients undergoing haemodialysis (Tsuda et al., 1996). It remains unclear what disease state HGV infection causes acutely and in the long term. There is however a
growing consensus that HGV is ‘a virus looking for a disease’ and may in fact prove not to be a cause of viral hepatitis. Although HGV RNA can be detected for years after infection in perhaps a minority of people who have been infected, there is no compelling evidence that HGV infection has important sequelae. There is also no evidence to suggest that HGV infection may worsen disease when co-infected with HBV and HCV.

1.2 Viral Hepatitis Type B

Of the many viral causes of human hepatitis few are of greater global importance than hepatitis B virus. Over 250 million people world-wide are persistently infected with HBV, and of these a significant minority develop severe pathologic consequences, including chronic hepatic insufficiency, cirrhosis and hepatocellular carcinoma.

1.2.1 Clinical features

Most HBV infections are subclinical particularly during childhood, but about one third of adult infections are icteric. The course of acute viral hepatitis can be divided into four clinical phases: a) the incubation period; b) a preicteric stage c) the icteric phase and d) the convalescent period. Following a long incubation period of 6-26 weeks, the preicteric phase commences, typically characterised by mild fever, lethargy, malaise, anorexia, myalgia, nausea and vomiting. Older children and adults have been reported to complain of right upper quadrant pain as a result of hepatomegaly (Ward et al., 1958). A minority of patients develop at this time a type
of serum sickness characterised by mild fever, urticarial rash, and polyarthritis, resembling a benign, fleeting form of acute rheumatoid arthritis.

The icteric phase is heralded by the appearance of dark urine due to bilirubinuria closely followed by pale stools and jaundice. The icteric phase gradually diminishes over 2 to 6 weeks and for the majority of patients this is followed by the convalescence period where complete regeneration of the damaged liver occurs. Occasionally, during acute viral hepatitis, more extensive necrosis of the liver occurs that leads to severe impairment of hepatic synthetic processes, excretory functions and detoxifying mechanisms. Designated as fulminant hepatitis, the process usually occurs during the first 8 weeks of illness and is characterised by the sudden onset of high fever, marked abdominal pain, vomiting and jaundice, followed by the development of hepatic encephalopathy associated with deep coma seizures (Adams, et al., 1953; Berk et al 1978). Death usually occurs in 70%-90% of patients due to ascites, a bleeding diathesis, renal dysfunction and decerebrate rigidity. Factors associated with the development of fulminant hepatitis B are thought to include genetic heterogeneity of HBV, coinfection or superinfection with other viral hepatitis agents and/or host immunological factors. The possible clinical outcomes following HBV infection is illustrated in figure 1.2.1.

Approximately 5-10% of patients progress to chronic hepatitis, defined as HBs antigenemia persisting for at least 6 months. This may take the form of an asymptomatic carrier state, of chronic persistent hepatitis or chronic active hepatitis causing progressive liver damage, which may lead eventually to cirrhosis and/or to primary hepatocellular carcinoma.
1.2.2: Epidemiology

The hepatitis B virus is a ubiquitous virus that is globally distributed with more than 200 million carriers world-wide. In the developing world, approximately 8-15% of individuals are chronic carriers, the majority of whom are infected at birth or in early childhood. Some 5-12% of parturient women are HBsAg positive, of whom nearly half are also HBV DNA positive, resulting in perinatal transmission with a 70-90% probability of the infant itself becoming a carrier. As the infant generally becomes HBsAg positive only 1-3 months after birth, it is considered that most perinatal infections result from contamination of the baby with blood during parturition, rather than transplacentally. In adults, transmission is principally by sexual intercourse; only 1-5% of infections acquired at this age progress to chronicity.
In the developed world, the carrier rate is generally less than 1%. Perinatal spread is less common, with sexual (including homosexual) transmission among adults being a significant risk. Percutaneous transmission represents the most common identifiable mode of spread, with injecting drug users constituting the largest cohort of carriers. Posttransfusion hepatitis B and infection of haemophiliacs by contaminated factor VIII have now almost disappeared as a result of routine screening of blood and organ donors. Hepatitis B, however, still represents an occupational risk for laboratory and health care workers.

1.3 Transmission

1.3.1 Maternal/Perinatal transmission

Perinatal transmission of hepatitis B virus is thought to represent the most important mechanism for maintenance of an HBV carrier population in certain areas of the world. It is estimated that at least 23% of the carriers in Asia and 8% of the carriers in Africa occur as a result of perinatal transmission (Kane et al., 1993). The age and route of infection is found to vary geographically (Botha et al., 1984).

In Asia, babies are more commonly infected at birth (Stevens et al., 1975 and 1979). The majority (85%) of those born to HBeAg positive mothers become persistently infected (Beasley et al., 1977). In contrast, 31% of the babies become carriers when the mother was HBeAg negative. The Beasley study also found a link between high HBsAg levels and transmission even in the absence of HBeAg.

The risk of perinatal transmission is greatest when the mother suffers an acute infection during the third trimester or the first two months post-partum (Merill et al., 1972). Infants are usually HBsAg negative at birth and develop antigenemia at 1-3
months of age. Although HBsAg can be detected in cord blood (Stevens et al., 1975), its presence does not correlate with the detection of HBsAg in venous blood in the infant at birth (Lee et al., 1978; Stevens et al., 1975). The incubation period coincides with that of if HBV infection has occurred at the time of delivery and indicates that perinatal transmission from mother to infant occurs during labour or delivery. The HBV could cross as the placental barrier breaks down and it has been shown that the foetus frequently swallows maternal blood during passage through the birth canal (Lee et al., 1978). The HBsAg found in the cord blood may represent contamination with maternal HBsAg.

Whilst in utero infection may be a rare event, the presence of relatively high levels of HBsAg in blood taken from the peripheral vein at birth was detected in 1.6% of infants examined in a study by Stevens et al., (1988) demonstrating that it can occur. Babies born to mothers who are anti-HBs positive seldom become infected and in most of these cases the HBV infection is transient.

In Africa, horizontal transmission is the more usual route of infection. Children are infected later in life (Botha et al., 1984) possibly by ingestion of breast milk and saliva. Transmission between familial contacts of HBsAg, HBeAg positive carriers is relatively common, particularly among the siblings of a carrier through close contact with blood or saliva (Szmuness et al., 1970).

### 1.3.2 Parenteral Transmission

Hepatitis B was recognised as a major problem after the introduction of parenteral therapy, mass immunisation campaigns and the extensive transfusion of blood and blood products (Mosley, 1965).
Long incubation periods, high incidence of asymptomatic infections and the occurrences of an infectious carrier state make HBV well suited to transmission by blood. The risk of infection has been reduced by a) screening of blood and blood products b) the use of an all volunteer blood donor population and c) the development of virucidal techniques to render blood components and derivatives safe.

However, some groups still remain at risk from infection. The stability of HBV and its retention of infectivity on mechanical surfaces, particularly those involved in haemodialysis (Bond et al., 1981) explains, in part, the high rate of infection that occurs in patients undergoing treatment in some haemodialysis units. This may also account for the high rate of transmission of HBV among drug abusers, associated with the sharing of contaminated syringes and needles. Healthcare workers are also at risk from HBV infection acquired from needlestick injuries and occupational exposure to blood or other body fluids (Maynard, 1978).

1.3.3 Non-Parenteral Transmission

Probable non-parenteral routes of HBV transmission are also likely though there is not much evidence. As well as occurring in serum, HBsAg has been demonstrated in a variety of other body fluids. These include sweat, breast milk, pleural fluid, vaginal secretions, saliva and semen, urine and faeces (Dobbin et al., 1985; Davison, 1987). The role of these fluids in the transmission of HBV is neither clear nor proven, but with HBV being present in such a diversity of bodily secretions, non-parenteral routes of transmission seem likely to occur.
1.3.4 Sexual Transmission

Evidence for the sexual transmission of HBV is provided by the strong epidemiological association between sexual behaviour in specific groups of patients and HBV infection.

In one study carried out by Fulford et al., (1973), HBsAg was detected in 15 of 658 men and 2 of 316 women attending the Department of Sexually Transmitted Diseases at the Middlesex Hospital. Anti-HBs was detected in 36 men and 11 women in the same group. There was a significant correlation between the presence of serum HBsAg and the country of origin of the patients, particularly those from Mediterranean and Middle Eastern countries. Similarly, a significant association between anti-HBs and a history of syphilis or gonorrhoea was found.

A significantly greater proportion of homosexuals and patients with three or more contacts had serum anti-HBs. However, there was no correlation between serum HBsAg and those two parameters. This led to the suggestion that HBV was transmitted sexually and when acquired in this way, was unlikely to lead to a chronic infection. In a later study carried out in the same department, (Gilson et al., 1990), anti-HBc was detected in 48% of the homosexual men tested, indicating a past or current HBV infection. In contrast, 7.5% of heterosexual men tested, were anti-HBc positive. The detection of the virus in semen and in asymptomatic rectal mucosal lesions (Reiner et al., 1984) indicated a possible mechanism for the transmission of HBV.
1.4. General Hepatitis B Virology

1.4.1 Hepatitis B virion structure

Electron microscopy of partially purified preparations of HBV shows the presence of three types of particles: a) 42nm double-shelled particles termed Dane particles, b) 22nm spheres present in excess over Dane particles and c) smaller quantities of filaments of 22nm diameter and variable length.

The Dane particle is the infectious virion of HBV (Dane et al., 1970); titres of Dane particles in the blood can range from $10^4$/ml to greater than $10^9$/ml (Almeida., 1972). The minimum infectious dose of the virions was determined by the inoculation of diluted sera from HBV infected patients into chimpanzees. It was shown that sera from HBV infected individuals diluted by up to $10^7$ remained infectious (Barker et al., 1975).

Three envelope related proteins have been associated with HBV: the hepatitis B surface antigen (HBsAg) which is displayed on the surface of the Dane particles (Alberti et al., 1984); the pre-S2 antigen/antibody system and the pre-S1 antigen/antibody system (Heerman et al., 1984). The 20nm spheres and filaments are composed exclusively of HBsAg and host-derived lipid. These particles lack nucleic acid but are highly immunogenic and efficiently induce a neutralising anti-HBs response.

Detergent treatment of the Dane particle reveals the nucleocapsid which has a diameter of 28nm (Almeida et al., 1970). The antigen associated with this structure is referred to as the core antigen (HBcAg) and its presence is indicated by a specific antibody in convalescent serum (anti-HBc) (Almeida et al., 1971).
In contrast to HBsAg, HBcAg is not readily detected free in serum (Magnius and Espmark., 1972) but has been identified in both the nuclei and cytoplasm of infected hepatocytes (Yamada and Nakane., 1977). Serum HBcAg is usually found as a complex with anti-HBc; specific treatments for the disruption of immune complexes are required to reveal HBcAg.

Within the core is the viral DNA (Robinson et al., 1974) and a DNA polymerase activity (Kaplan et al., 1973) which is involved in viral genomic replication. Purified cores also contain a protein kinase activity detected by its ability to phosphorylate C protein in vitro (Gerlich et al., 1982).

1.4.2 Organisation of hepatitis B genome

The structure of the HBV genome in the virion is unusual, consisting of a circular 3.2 kb partially double stranded DNA molecule. The long negative (L-) strand is complete but has a nick at a fixed position. The nick is usually found 1818 nucleotides from the EcoR1 cleavage site referred to as the origin (Sattler and Robinson., 1979). The existence of a complementary, shorter positively orientated strand (S+) was first demonstrated by restriction analysis (Summers et al., 1975). The S strand is incomplete, extending for a variable distance from a fixed 5’ terminus, which is some 250 base pairs 5’ to the nick in the L strand. The S strand is at least 50% of the length of the genome but after that point it is quite variable in length (Delius et al., 1983).

The long strand has a polypeptide covalently attached to the 5’ end (Gerlich and Robinson., 1980) which serves as a protein primer for (L-) strand synthesis. A basic protein bearing the HBV DNA polymerase and RNase H activity is linked to the 3’
end of the (S+) strand (Bavand et al., 1989; Toh et al., 1983). A 19 nucleotide capped ribonucleotide is covalently attached to the 5' end of the (S+) strand, most likely serving as a primer for (S+) strand synthesis.

The HBV genome is maintained in a circular configuration by the presence of a complementary 224 base 5' terminus between the long and short strands. This region is flanked by two 11bp direct repeats, DR1 and DR2, which are thought to be also involved in the circularisation of the genome. Sattler and Robinson, (1979) showed that the denaturation of this region provided linear molecules which could be returned to the circular form under appropriate conditions.

The HBV genome is highly compact with very efficient use being made of the coding capacity. All of the genome appears to be translated where overlapping genes are translated in different reading frames. In addition, all the regulatory signal sequences are found within protein coding regions. This compactness suggests that the virus is highly evolved.

The genome is divided into four major open reading frames (ORF). The P gene, which compromises 80% of the genome, encodes a polymerase with three distinct enzymatic functions (DNA polymerase, reverse transcriptase and RNase H) and also encodes the terminal protein primer (Bosch et al., 1988). Gene X encodes a transactivating protein that upregulates transcription from all the viral and some cellular promoters (Rossner et al., 1992). The C gene has two initiation sites that divide it into a pre-core and core region, producing two distinct proteins, HBeAg and HBcAg (Pasek et al., 1978). The pre-S/S gene encodes for the viral envelope protein (Valenzuela et al., 1979). There are two additional, potential open reading frames for
which no translational products have been identified (Miller et al., 1989). Figure 1.4.2 shows a representation of the HBV genome.

1.5 Viral Replication

In 1982, Summers and Mason reported the landmark discovery that viral DNA replication proceeds not by conventional semiconservative DNA synthesis but by reverse transcription of an RNA intermediate. Their work centred around subviral particles prepared from duck hepatitis B virus infected liver (DHBV). These particles incorporated labelled deoxynucleotides (dNTP’s) into both plus and minus strands of viral DNA, by using the DNA polymerase, as would be anticipated. Importantly, the synthesis of minus strand DNA was resistant to actinomycin D, implying its template was not DNA; plus strand synthesis was sensitive to this compound. In addition, a portion of newly made minus strand DNA was found in the form of RNA-DNA hybrids. These observations suggested that minus strand DNA was made from an RNA template, by inference, the minus strand DNA whose RNA template had been removed. These experiments predicted the existence of full-length, unspliced RNA that would serve as the template for reverse transcription. Such an RNA was indeed identified soon thereafter (Buscher et al., 1985; Enders et al., 1985; Moroy et al., 1985) and designated pregenomic RNA. Fowler et al., (1984) showed that replication of HBV was asymmetric and that a reverse transcription replication strategy, similar to that described for DHBV, was followed by HBV.

The scheme proposed by Summers and Mason (1982) has been largely confirmed. Upon entering the hepatocyte the viral envelope is stripped and the core, containing
Figure 1.4.2: Diagram of HBV genome (modified from The Molecular Medicine of Viral Hepatitis, 1994 -Eds T.J. Harisson and A.J. Zuckerman). The HBV genome is represented in the centre with a minus-DNA strand and an incomplete plus-DNA strand as is found in mature viruses. The structural elements, direct repeats 1 and 2, are designated as DR1 and DR2. The open reading frames representing the polymerase, core and pre-core, X, preS1, pre S2 and finally the S domain are according to Galibert et al., 1979. In the outer region the transcribed HBV RNA’s are shown. The triangles represent the different 5’ ends of the RNAs; the common 3’ end is located at position 1921, followed by an approximate 300bp poly A sequence.
the partially double stranded DNA, transported to the nucleus. At this stage the HBV DNA is in the non-replicative, relaxed circular form.

The short positive strand (S+) is completed by the HBV DNA polymerase and the completed DNA converted to the covalently closed circular form, which is suitable template for transcription.

Transcription of the minus strand produces several RNAs which may vary in length. A 3.4 kb RNA is produced which codes for the major structural core protein (p22), the reverse transcriptase protein and the polypeptide primer required for minus strand synthesis. There are also a 2.4kb RNA and an approximately 2.1kb RNA which have heterogeneous 5' ends. All of these RNAs have fixed 3' poladenylated ends that are mapped to the start of the core gene. The 2.1kb RNA codes for the major and middle surface protein and the 2.4kb RNA for the large surface protein. The 3.4kb RNA is transcribed and serves as the template for HBV replication.

Junker-Niepmon et al., (1990) identified a short sequence at the 5' end of the RNA pregenome which is the signal for encapsidation. Bartenschlager et al., (1990) determined that a fully functional polymerase gene product is required for encapsidation. Once encapsidated the reverse transcriptase, primed by the virus-coded terminal protein at the DR1, transcribes a complementary (minus) strand of DNA. Meanwhile, the RNase H progressively degrades the RNA template from its 3' end leaving only a short 5' ribonucleotide.

This ribonucleotide is then cleaved and translocated to the DR2 on the minus strand DNA where it serves as a primer for the DNA polymerase to transcribe a DNA plus strand. Some of the core particles, containing newly synthesised viral DNA, are recycled back into the nucleus to amplify the pool of HBV genomes available for
transcription. The remainder are assembled into virions before the plus strand of the genome has been completed. The mature core particles are then packed into the HBsAg/pre-S in the endoplasmic reticulum and exported from the cell.

1.6 Viral Proteins

1.6.1 Envelope proteins

The coding region of the genome for the envelope protein is divided into three sections, pre-S1, pre-S2 and S. These regions each have an in-frame start codon. The surface glycoprotein of the HBV envelope are retained in a lipid bilayer. There are three proteins present in the Dane particle envelope, the major, the middle and the large proteins.

Initiation of translation at codon 1 in the pre-S1 region produces the large protein (L) which includes the pre-S1, pre-S2 and S regions. The pre-S2 region is translated along with the surface gene to produce the middle protein (M). Translation from the start codon of the surface gene results in the major protein (HBsAg). All three proteins contain a coterminus HBsAg at their carboxyl end.

The Dane particle envelope contains equimolar amounts of middle and large proteins and a ten-fold excess of the major protein (Heerman et al., 1984). The HBsAg can be present in serum in two non-virion associated forms, 22nm spheres or filaments. These envelope particles are present at much higher concentrations than complete Dane particles. The filamentous particles have about 20% L protein but spherical particles are almost entirely S protein. The M protein is found in about 10% of all forms (Tiollais et al., 1985).
The large protein contains 389-400 amino acids and may exist in a partially glycosylated form (P39) or a fully glycosylated form (gP42). The middle protein is smaller and comprises 281 amino acids. Again, the protein may be detected as P33 or the glycosylated form gP36.

The function of the L protein is unclear but is thought to relate to the secretion of virions (Persing et al., 1986) and formation of a receptor complex on the surface of the virus. It appears that the area between amino acids 21 and 47 of pre-S1 is necessary for binding to hepatocytes (Neurath et al., 1985). The function of pre-S2 is obscure, but it may also play a role in receptor binding and viral uptake into the cell (Machida et al., 1984).

Pre-S1 and pre-S2 antigens are detected very early in the course of acute infection and in those who go on to clear the virus, antibodies to pre-S1 and pre-S2 also appear early (Meyer zum Buschenfelde et al., 1986). Pre-S1 and pre-S2 generate strong immune responses at the B-cell and T-helper cell levels and both can potentiate anti-HBs responses; in vaccine non-responsive mice (Milich et al., 1985) and humans (Thoma et al., 1990), the addition of these epitopes results in stimulation of significant anti-HBs responses.

The major protein is 226 amino acids long and may be present in a non-glycosylated (P24) and also a glycosylated form (gP27). The HBsAg contains the major neutralising epitope of HBV, termed the \( \alpha \) determinant, which spans amino acids 124-147 and is common to all HBV isolates. Antibodies to the \( \alpha \) determinant confer protection to all the subtypes of HBV. The pepscan approach has been used to define the major HBsAg epitopes recognised by a hyperimmune rabbit antiserum raised against a recombinant hepatitis B vaccine. The results showed that the rabbit
antibodies bound exclusively to peptides representing the region between amino acids 139-147 (Steward et al., 1993). Substitution experiments in which each residue was in turn sequentially replaced by all other possible amino acids confirmed that residues KPTDG between codons 141 and 145 were essential for antibody binding.

The $a$ determinant is found in all isolates of HBV. Variation of other amino acids within and around the $a$ determinant gives rise to subtypes. Two pairs of mutually exclusive determinants, $d/y$ (Le Bouvier. 1971) and $w/r$ (Bancroft et al., 1972), enables the distinction of four major subtypes of HBsAg, $adw$, $adr$, $ayw$ and $ayr$. The important allelic substitutions associated with $d/y$ and $w/r$ expression are found at residues 122 and 160, respectively, and at both these sites the subtypic changes are mediated by a shift from Lys to Arg (Okamoto et al., 1987). Additional subdeterminants of $w$ have allowed the definition of serotypes of $ayw$ ($ayw1$, $ayw2$, $ayw3$ and $ayw4$) and of $adw$ ($adw2$ and $adw4$). The residue 127 is important for the subdeterminants of $w$ and is associated with Pro, Thr and Leu for $w1/w2$, $w3$, and $w4$ respectively. Either one or both of two substitutions, at positions 134 (Phe instead of Tyr) and 159 (Ala instead of Gly), were important for the serological difference between $ayw1$ and $ayw2$ (Norder et al., 1992b). The $q$ determinant, originally described as present on all subtypes apart from $adw4$ (Magnius et al., 1975), was later also found to be absent from $adr$ strains in the Pacific region, thus defining $adr$ as either $q^+$ or $q^-$ (Courouce-Pauty et al., 1978).

The geographical distribution of these subtypes has been determined. In northern Europe, America and Australia $adw$ predominates. However, the subtype $ay$ has been associated with patients infected through intravenous drug use in these regions. Subtype $ayw$ occurs in a broad zone which includes north and west Africa, the
eastern Mediterranean, eastern Europe, northern and central Asia and the Indian subcontinent. Adw and adr are found in Malaysia, Thailand, Indonesia and Papua New Guinea. Adr is predominant in Japan and the Pacific islands. Individuals are usually infected with a single subtype. However, combinations of subtypes have been observed such as adwr, adyw and adyr (Courouce et al., 1976).

1.6.2 Nucleocapsid proteins

The second HBV open reading frame encodes the major hepatitis B core antigen (HBcAg) polypeptide. The HBcAg is produced by translation from the second ATG codon in the core ORF and is the major component of the virus nucleocapsid. The predominant core protein is 183-185 amino acids in length and is 22 000 daltons (p22). It is not detectable circulating free in serum and is present mainly in nuclei of liver hepatocytes (Hruska et al., 1977).

HBcAg is a potent immunogen in humans and antibody and T-cell responses to it can be detected in almost all infected patients. It is therefore important in the elimination of hepatocytes replicating HBV in chronic infection. In 1986, Ferns and Tedder, identified a single immunodominant HBcAg epitope using monoclonal anti-HBc. Immunisation of chimpanzees with HBcAg purified from infected liver has been shown to protect against HBV challenge, indicating that this antigen can lead to a protective immune response (Murray et al., 1987). Thus, an HBV virion internal antigen that does not illicit antibody that neutralises viral infectivity is protective, probably via cellular immune response.

Translation from the first ATG codon of the core ORF results in a larger polypeptide containing an additional 29 amino acids that are predominantly hydrophobic. These
amino acids make up the precore region and target the polypeptide to the endoplasmic reticulum where proteolytic cleavage of 19 amino acids at the amino terminal occurs. Further cleavage of 34 amino acids occurs at the carboxyl terminal which results in the HBeAg molecule of 159 amino acids (Garcia et al., 1988). From this point HBeAg is either transported to the Golgi apparatus and secreted from the cell or to the nucleus (Ou et al., 1986; Ou et al., 1989; Uy et al., 1986). HBeAg was shown to be a non-essential protein for viral formation (Schlitt et al., 1987).

HBeAg is associated with continued virus replication and high infectivity. The fact that HBcAg and HBeAg are cross-reactive on the T cell level suggests that the production of HBeAg may partially block the cellular immune responses against HBcAg and promote viral persistence. Other evidence suggests that HBeAg may be an important immune target since seroconversion from HBeAg to anti-HBe is commonly associated (though not always) with the clearance of circulating wild type HBV and the resolution of chronic liver disease.

1.6.3 The polymerase protein

Hepatitis B virus also contains a DNA-dependent polymerase protein (Robinson and Greenman., 1974). This is a large protein of up to 845 amino acids, which encodes for a terminal protein, an RNase H activity as well as the reverse transcriptase function. The reverse transcriptase is essential for replication. Sequence analysis of the reverse transcriptase revealed nucleotide homology with the corresponding pol gene of retroviruses (Toh et al., 1983). The polymerase amino terminus region encodes for the HBV DNA (Bartenschlager et al., 1988). The carboxyl-terminus
encodes RNase H which degrades the RNA template during viral replication (Radziwill et al., 1990).

**1.6.4: X protein**

The X gene is the smallest of the four, partially overlapping ORF’s of the HBV genome. It codes for an 154 amino acid polypeptide designated the X protein (Rossner, 1992). The X gene, the last of the HBV genes to be characterised, is thought to be an early gene (Wu et al., 1991) and has been hypothesised to be of cellular origin (Miller et al., 1986).

Experimental evidence has shown that when the X gene is mutated so that HBxAg polypeptides are not made, transfection of such mutated clones into human hepatoma cells resulted in a more than ten fold reduction in the levels of HBV transcripts, polypeptides and virus (Colgrave et al., 1989; Nakatake et al., 1993). Mutants of GSHV (Ganem et al., 1987) and WHV (Chen et al., 1993) with an inactive X gene failed to initiate a productive infection in their natural hosts suggesting that HBx is essential for establishment of infection or spread of the virus.

The main function of the X protein is as a transcriptional activator and it has been shown to transactivate a number of cellular and viral promoters (Rossner, 1992). Several mechanisms by which HBx could perform its transactivating function have been proposed. HBx does not bind directly to DNA and needs for its function the association with cellular proteins such as NFkB, TFIIC, AP2 (Maguire et al., 1991; Unger et al., 1990; Seto et al., 1990).

Due to sequence similarities with a Kunitz-type serine protease inhibitor, HBx was speculated to represent a protease inhibitor which may play a role in carcinogenesis.
(Takada et al., 1990). Moreover, evidence for a serine/threonine protein kinase activity of HBx has been reported recently and was speculated to play a role in hepatocarcinogenesis (Wu et al., 1990). HBx was shown to induce liver cancer in one line of transgenic mice (Kim et al., 1991), however no tumorigenesis was observed when using a second line (Lee et al., 1990).

Most patients infected by HBV develop antibodies to HBx. These antibodies, induced rather early in infection, may fall to undetectable levels in self-limited hepatitis, and reach the highest titres in chronic carriers with ongoing viral replication (Levrero et al., 1990).

1.7 Serodiagnostic Profiles Of Viral Hepatitis B

Several of the HBV serological markers provide a basis for monitoring the appearance of the virus and immune response during the course of infection. The profiles of these serological markers can be correlated with the course of disease and hence offer useful diagnostic and prognostic information.

1.7.1: HBV markers in acute infections

The average incubation period in naturally occurring infections is 3 months although in post-transfusion hepatitis B it maybe shorter. The first serologic marker to appear is HBsAg. This is typically detected some four weeks prior to the acute stage of illness, before declining to undetectable levels within 4 to 6 months.

DNA polymerase activity, which is representative of the viraemic stage of hepatitis B occurs early in the incubation period coinciding with the first appearance of HBsAg (Kaplan et al., 1974). HBeAg and HBV DNA can also be detected at this early stage.
With the onset of clinically apparent hepatitis, levels of DNA polymerase and HBeAg rapidly decline along with the serum HBsAg titre (Aldershvile et al., 1980). Antibody to HBcAg appears in the sera of all infected individuals approximately 2-4 weeks after the detection of HBsAg. Anti-HBc is the first antibody to appear in serum and is present as IgM class antibody (Cohen et al., 1978). Tedder et al., (1981) noted that levels of IgM anti-HBc remained higher in patients with an acute resolving infection than in patients who had developed chronic infection. As the illness progresses and clearance of HBsAg occurs, IgM antibody to HBcAg is replaced by IgG. The anti-HBc persists for many years and is the only marker of HBV infection between the disappearance of HBsAg and the development of anti-HBs. Anti-HBs is ultimately demonstrable in more than 90% of patients who experience a primary infection and once present it usually persists indefinitely and is protective against further attacks of wild type hepatitis B (Krugman et al., 1979).

The infectivity of a patient is related to the HBeAg/anti-HBe status. The presence of HBeAg in the serum is generally regarded as being consistent with a high level of infectivity and correlates well with the presence of HBV DNA in patients serum as detected by HBV DNA dot blot (Krugman et al., 1974; Krugman et al., 1979; Hoofnagle et al., 1981). HBeAg is rapidly lost during recovery from an acute infection and anti-HBe develops early in the convalescent phase. The presence of anti-HBe in serum is generally taken as an indication of low infectivity particularly in carriers. The duration of this antibody response is shorter than that of anti-HBc and anti-HBs.
1.7.2: HBV markers in persistent infection

While the time course of the typical HBV infection is measured in months, in some patients the natural history of the disease evolves over many years. These chronically infected patients are termed HBV (or HBsAg) carriers and are typically identified by the persistence of HBsAg for more than 6 months. The initial development of the carrier state parallels that of the acute infection. Levels of HBsAg, HBeAg and anti-HBc rise but rather than HBsAg and HBeAg then declining, they may persist for some years. The anti-HBc response is much increased (Hoofnagle et al., 1975) with the production of mainly IgG and not IgM anti-HBc. Subsequently, HBeAg is lost and anti-HBe develops. This happens in approximately 10% of carriers per year (Weller et al., 1986). Associated with this change is a drop in HBsAg levels. After a further period HBsAg may become undetectable with subsequent development of anti-HBs. However, some patients do not develop anti-HBs after the disappearance of HBsAg, retaining anti-HBc as the only marker of their HBV infection.

The variability in clinical presentations in infected individuals confirms that HBV does not play a direct cytopathic role (Dudley et al., 1972). The inference being that the immune response to HBV causes the associated liver disease.

The target of cytotoxic T cells was shown to be the core antigen expressed on the surface of infected cells. It was shown that the cytotoxic response to hepatocytes expressing HBcAg could be blocked by the addition of monoclonal and polyclonal anti-HBc (Mondelli et al., 1982). This was further confirmed by Naoumov et al., (1984) where hepatocytes expressing surface antigen were shown generally not to be destroyed. While in some cases this process results in the clearance of hepatocytes containing replicating HBV, there are other cases where prolonged destruction of the
hepatocytes results in hepatic damage (Thomas et al., 1985). This includes severe chronic lobular hepatitis, chronic persistent hepatitis, and also extrahepatic diseases (Thomas et al., 1984). Infection with HBV has also been associated with hepatocellular carcinoma (Szmuness, 1978). In these cases, the HBV genome has been found to be integrated with the chromosomal DNA (Schaffitz et al., 1981).

1.8: Prevention Of Hepatitis B Infection

1.8.1: Active immunisation

The development of a naturally acquired immunity to HBV was demonstrated by Lander et al., (1971). They showed that the development of anti-HBs response in HBV infected individuals prevented reinfection. Therefore, efforts in the development of successful vaccines were directed towards the promotion of a high, sustained anti-HBs response.

Active immunisation against hepatitis B in humans was initially investigated by Krugman and colleagues between 1971 and 1973 using a crude immunogenic preparation of HBV containing plasma. These studies led to the development of more sophisticated vaccines which consisted of highly purified, formalin and/or heat inactivated hepatitis B subvirion particles (22nm) of HBsAg that were free of detectable nucleic acid (Buynak et al., 1976; Purcell et al., 1975). The antigen was harvested from the plasma of asymptomatic, apparently healthy human carriers of HBV.

The efficacy of the vaccine was demonstrated in a study carried out among staff and patients of a renal dialysis unit (Maupas et al., 1978). The study also confirmed the
increased efficacy of an alum precipitated adjuvant form of vaccine when compared with an aqueous form.

The first commercially licensed vaccine was produced by Merck, Sharp & Dohme (Hilleman, 1979). The vaccine prevented HBV infection in all six chimpanzees that were challenged with HBV subtype adw. Szmuness et al., (1980) also demonstrated the efficacy of the plasma derived, inactivated vaccine in a randomised double blind, placebo controlled trial in a high risk population of 1083 homosexual men.

The identification of HIV, which has been transmitted via blood products, raised concerns regarding the safety of plasma-derived vaccines. As a result, in 1986, the first yeast-derived recombinant vaccines were licensed. The recombinant vaccine contains the surface gene cloned into E.coli and expressed in yeast cells (Saccharomyces cerevisiae). The efficacy of the purified HBsAg was demonstrated in chimpanzees by McAleer et al., (1984). Cross-protection by different serotype vaccines against various HBV subtypes has been observed: thus subtypes are not of major concern in formulating the vaccines. Post exposure immunisation after an HBV challenge also has been found to be efficacious in chimpanzees.

1.8.2: Passive immunisation

The administration of hepatitis B immunoglobulin (HBIG) both pre- and post-exposure is effective in the prevention and neutralisation of HBV infection. Early studies with conventional immune globulin were inconclusive due to the lack of consistent levels of anti-HBs in preparations and by the possibility of the inclusion of HBsAg which would produce a compound active, passive immunisation (Seeff et al., 1978).
In 1974, Szmuness et al., produced HBIG that had been prepared from plasma preselected for a high titre of anti-HBs. The HBIG appeared to protect solely by passive immunisation and was licensed for use in 1977.

With the availability of a vaccine for hepatitis B, HBIG is rarely used for pre-exposure prophylaxis. However, there are several situations where post-exposure prophylaxis is essential. These include a) exposure of an individual to hepatitis B containing material by percutaneous inoculation, oral ingestion, or direct mucous membrane contact b) sexual contacts of an acutely infected patient as well as e antigen positive carriers c) perinatal exposure to mothers infected with hepatitis B.

Several random trials have been carried out to test the efficacy of HBIG administration in such situations (Seeff et al., 1978, Redeker et al., 1975, Beasley et al., 1983). The results of these studies concluded that HBIG was effective in the prevention of hepatitis B. The studies also showed that the timing of the initial dose was important. For successful prophylaxis, HBIG should be administered as soon as possible after exposure.

Most of the regimens used today combine passive immunisation with active immunisation, induced by vaccine, thereby providing immediate protection with more durable immunity.

1.9: Treatment Of Hepatitis B

The main goal of HBV treatment is to eliminate infectivity with a view to prevent transmission and to halt the progression of liver disease. Much work has centred around controlling viral persistence by using agents which inhibit viral replication such as interferon and nucleoside analogues.
Nucleoside agents such as acyclovir, ribavirin or adenine arabinoside monophosphate have not proven to be of significant therapeutic value (Alexander et al., 1987; Fried et al., 1992; Garcia et al., 1987; Hoofnagle et al., 1981; Hoofnagle et al., 1982). The potential of ganciclovir and foscarnet, to prevent recurrent hepatitis B after transplantation has been investigated, but these drugs have been shown to have several disadvantages and limited efficacy (De la Mata et al., 1995; Singh et al., 1995). Two new nucleoside analogues, lamivudine and famciclovir, have been shown to be potent inhibitors of HBV (Doong et al., 1991; Kruger et al., 1995). Studies have shown a transient decrease in serum HBV DNA in patients with chronic hepatitis B treated with lamivudine for up to 6 months (Dienstag et al., 1995; De Man et al., 1995). Famciclovir has been used to treat post-transplant HBV infection. In some patients, this treatment has resulted in HBV DNA clearance, as well as histological clearance, but viral surface antigen persisted in serum (Kruger et al., 1995).

To date clearance of circulating virus has been achieved with interferon therapies as indicated by HBeAg to anti-HBe seroconversion (Wong et al., 1984; Peters, 1989). However, the removal of integrated virus or the prevention of integration of the virus into the host genome has not been achieved.

1.10 Hepatitis B Virus Variants

Hepatitis B virus is unique among the DNA viruses in that it employs reverse transcriptase for its replication. The polymerase lacks proof-reading capability resulting in a high rate of nucleotide misincorporation during transcription in the HBV genome. It is estimated that at a single nucleotide position of HBV $1-3 \times 10^{-5}$
mutations occur in an infected individual each year (Okamoto *et al.*, 1987). In this way, mutants are introduced into the viral pool. Some of these will not be replication competent but others will replicate well, perhaps even more efficiently. Those that replicate more efficiently will become the predominant species. Alternatively, there may be an active selection process. The host may be presented with a number of genotypes of HBV that have arisen by random mutation and only one or a few of these will survive in the presence of a particular host factor. Selection of most viral variants is likely to be immune-driven, either on a population or an individual basis. Variants positively selected by forces such as the humoral or cellular immune response (Pircher *et al.*, 1990) are termed escape mutants. Of course, a precondition for selection is viral viability. If there is a situation where there are two isolates of HBV, both with substantially different antigenic epitopes, the immune response to the initial isolate will neutralise the particle and destroy cells infected with this virus. The genotype with a different epitope survives the immune selection process and becomes the predominant strain. In the variants of HBV described to date, it is believed that the immune response is the predominant factor in selecting these variants.

1.10.1: Pre-S mutants

Pre-S1 and pre-S2 both contain important neutralising peptide as demonstrated by the administration of a pre-S peptide to chimpanzees which protect against subsequent infection (Itoh *et al.*, 1986). Work in mice using truncated pre-S polypeptides and synthetic peptides identified a T cell recognition site within pre-S2 residues 30-55 and an adjacent B cell epitope within residues 14-24 (Milich *et al.*, 1986).
There maybe a strong immune selection pressure on these epitopes and mutations have been detected in many patients.

Deletions found in the pre-S1 region often overlap with the promoter region for the pre-S2 and S proteins. In one study (Chisari et al., 1987), a 183bp pre-S1 deletion was observed which resulted in drastically decreased middle and major protein production. Over expression of pre-S1 protein in relation to pre-S2/S proteins can lead to the retention of L proteins in the endoplasmic reticulum of the cell. The accumulation of these filamentous particles is potentially cytopathic to the cell and can even trigger the development of anaplasia and hepatocellular carcinoma in transgenic mice (Chisari et al., 1989). This deletion spared the receptor binding site and the pre-S1 T cell recognition sites. Some of the deletion mutants lack the pre-S2 translation initiation codon and therefore no pre-S2 can be synthesised by these variants. Although there is no particular clinical course associated with these variants they are found frequently in patients with chronic hepatitis (Santantonio et al., 1992). The fact that HBV lacking the whole pre-S2 protein can represent the dominant population in serum suggested that pre-S2 was unnecessary for virus replication.

1.10.2: S gene mutants

The development of anti-HBs after acute or chronic HBV infection is usually associated with recovery and a good prognosis. The HBsAg contains the major neutralising epitope of HBV, termed the \( \alpha \) determinant. The majority of anti-HBs found in convalescent and post-vaccination sera binds to this area, which has an as yet undefined structure. It is clear that the \( \alpha \) determinant is composed of conformationally dependent epitopes as denaturation of this area by alkylation or
reduction gives rise to HBsAg particles with greatly reduced antigenicity (Imai et al., 1974). One proposed structure for the \( a \) determinant is that of a double loop where disulphide bridges between cysteine residues are thought to be responsible for maintaining the correct conformation (figure 1.10.1.2).

Anti-HBs binds predominantly to the second loop (Howard et al., 1984). However, antibodies that bind to the first loop are influenced by amino acid changes in the second loop (Waters et al., 1992), inferring that epitopes are not strictly confined to one of the loops but that the whole sequence probably contributes to the antigenic structure. The epitope is conserved though there is a degree of amino acid variation in normal isolates of HBV. Recently, variants of this highly antigenic region have been described which are thought to be clinically significant. HBsAg variation has been associated with immunisation, monoclonal/polyclonal antibody therapy and cases of HBV infection difficult to detect in clinical laboratories.

The first HBsAg mutant with clinical significance was described in an immunised baby born to a carrier mother in Italy (Carman et al., 1990). The infant became HBeAg positive and has remained a chronic carrier despite achieving protective levels of anti-HBs. Immunological studies showed that the \( a \) determinant was poorly detectable and sequence analysis revealed a single point mutation at codon 145 which resulted in the amino acid change of arginine for glycine. Further work showed that the mother carried the wild type sequence. It was concluded that though the normal genotype had been transmitted, the infant selected a mutant that evaded the vaccine-induced immune response. This may have been due to the delay in immunisation and HBIG therapy which commenced at 3 months of age. It would
Figure 1.10.1.2: Proposed structure of a determinant. Disulphide bridging between cysteines are thought to be responsible for maintaining this highly conformational epitope. Figure is adapted from Wallace et al., 1994.
appear that a single change at position 145 was sufficient to cause loss of protection by vaccine induced anti-HBs.

This marked effect on antigenicity, has been shown by the failure of yeast-expressed HBsAg with 145R to bind monoclonal anti-a antibodies, vaccinee or convalescent antisera (Waters et al., 1991). The same mutation was subsequently described in Singapore (Harrison et al., 1991) and in Japan (Fujii et al., 1992). The cases in Singapore were particularly interesting. Prior to their description, all of the vaccine-associated mutants had had delayed administration of vaccine. However, one of these cases was given the normal course of vaccine and another received recombinant vaccine. Additional mutants described in Singapore included a double mutant of G145R with D144A and individual cases of D144A, Q129H and M133L. None of these mutations, except for M133L were found in their mothers’ serum.

G145R is stable, as evidenced by persistence of infection with the same variant for up to 8 years in children who have been followed up. G145R can give rise to high level viraemia (Carman et al., 1990). This is of some concern, as it may become established in the population. However, it is still unknown whether these variants are transmissible between people. It has been transmitted to chimpanzees.

Although routine use of hepatitis B vaccines as part of childhood immunisation programmes in endemic areas has been shown to be highly effective in the prevention of chronic HBV carriage, breakthrough infections have been reported in vaccinated children as evidenced by the appearance of anti-HBc. Of vaccinated children in the Gambia, 8.3% had sub-clinical HBV infections of which 37.5% had high levels of anti-HBs. The variant HBV infections detected were characterised by a glutamic acid substitution at position 141 of the S protein, in contrast to the lysine
residue that is normally present (Karthigesu et al., 1994). Again, the single point mutation at codon 141 was enough to allow the variant to escape immune recognition.

A recent survey in the USA of children born to carrier mothers reveals similar results to those from Singapore (Nainan et al., 1996). A number of amino acid changes were observed, the most frequent being the 145R. The onset of HBV infection was noted to be delayed (> 6 months) in these children suggesting that selection had to occur.

It is now standard practice to administer anti-HBs (either MAb or HBIG) after liver transplantation to prevent infection of the new liver. Therapy with antibodies will however, select mutants within HBsAg which are not neutralised by that antibody. Monoclonal antibodies clearly put a highly focused pressure on HBsAg, but it is a little surprising that polyclonal sera (HBIG) also select escape mutants. Nevertheless mutants have been detected in both cases.

McMahon et al., (1992) described the emergence of G145R in liver transplant patients given monoclonal anti-a to prevent infection of the graft. These patients had become HBV DNA and HBsAg positive after transplantation. In a study looking at sequences pre- and post-HBIG administration, amino acid substitutions appeared in all patients during the course of HBIG administration (Carman et al., 1992). Two published studies have shown a mixed or subpopulation of viruses present in the serum of patients pre-transplant, including R145 (Cariani et al., 1995; Hawkins et al., 1996). HBIG administered after a transplant resulted in the emergence of a variant HBsAg as the dominant population. Mutants do not appear commonly in infections which occur after withdrawal of HBIG or in patients who never received HBIG.
Diagnostic assays are designed to achieve high sensitivity and specificity. Most assays for HBsAg detection depend on an anti-HBs on the solid phase binding HBsAg in the patients serum; this complex is then recognised by an anti-HBs which is either enzyme or radiolabelled. If there is a significant mutation in the epitope and it is not recognised by the anti-HBs, then HBsAg will either not be detected or the assay will be very insensitive. Serosurveys world-wide have revealed discrepancies in HBsAg detection using commercial assays based on either polyclonal/monoclonal antibodies. In Papua New Guinea, 5% of HBsAg positive carriers would have been labelled negative using a widely available assay (Carman et al., 1997). Just over half of these cases had variants localised in two regions: around amino acids 110-118 just upstream of the d/y subtype defining residue and between amino acids 154-158, just upstream of the w/r defining region. A further example was seen in an Indonesian HBV carrier who was being treated for lymphoma with cytotoxic agents (Carman et al., 1995). He went into remission but then developed a severe acute hepatitis, after withdrawal of cytotoxic therapy. As HBsAg was undetected by the standard monoclonal based assay, an HBV DNA assay was performed which showed circulating virus. A polyclonal based HBsAg assay was then used and detected the antigen. Sequencing of the isolate revealed a two amino acid insertion downstream of amino acid 122 and an arginine at codon 145. Two similar cases are described in chapter 3 where further diagnostic problems have occurred.

Other insertions in the a determinant have been found (Hou et al. 1995). These have all derived from patients in the Far East and all appear to have been sporadic. The insertions range from two to eight amino acids and are located between amino acids 122 to amino acids 124. They have highly significant effects on recognition by anti-
HBs. This is not surprising as they must severely abrogate the usual epitope structure.

There is now little doubt that variants of S protein are clinically significant and are a cause for concern on several accounts. Hepatitis B virus with mutant HBsAg may infect individuals even though they have been previously immunised and have a protective anti-HBs response. As for their importance to the eradication campaign, it is possible that they can become the dominant population in an increasingly immunised world. The fact that G145R is stable and can replicate efficiently adds to this worry.

It is still unclear whether HBIG will continue to be used alone in prophylaxis of graft infection after liver transplantation. It may be appropriate to use a combination of HBIG with monoclonal antibodies or a nucleoside analogue. There is clearly a need to re-evaluate diagnostic assays. This may involve using mixtures of monoclonal antibodies or joint polyclonal/monoclonal antibody assays. Insensitivity of HBsAg detection assays may fail to identify HBV infected individuals making monitoring of immune responses and the accuracy of diagnosis difficult. On another account, failure to detect these mutants may lead to transmission of infection through donated blood or organs.

1.10.3: Core/Pre-Core Mutants

HBeAg has always been a useful marker of viraemia and hence active HBV replication. However, it is now recognised that correlation between anti-HBe and suppression of HBV replication is not absolute. In 1989, Carman and colleagues described a set of patients with anti-HBe who showed high titre viraemia as seen in
HBeAg positive patients and had severe liver disease. Sequencing analysis revealed a single base substitution, G to A, at codon 28 of the precore region converting the tryptophan (TGG) to a stop codon (TAG). The creation of this stop codon prevented the translation of HBeAg. Experiments on precore defective duck hepatitis B virus (DHBV) and woodchuck hepatitis (WHV), suggest that expression of precore-derived secretory protein is not essential for virus replication. Thus, the naturally occurring precore mutants are HBe-minus, replication competent variants of the wild-type virus. In the absence of membrane bound precore protein, hepatocytes supporting HBV replication are no longer recognised by anti-HBe and will not be eliminated. In this sense, the precore mutant behaves as an escape mutant.

The fact that patients infected with precore mutant usually have a preceding HBeAg positive phase suggests that the precore mutant could be derived from wild type virus in the course of infection. This was shown by Okamoto et al., (1990). By sequencing PCR clones at various time points, they observed a pure wild type population at the early HBeAg phase emergence of a small number of precore mutants at later HBeAg phase, and take-over by precore mutants at the anti-HBe phase.

The mutation at $G_{1896}$ (codon 28) is engaged in the formation of a stem and loop structure, which is called the $\varepsilon$ encapsidation signal and is required for the encapsidation of the pregenome into the wild type HBV genome of genotypes B, C, D and E. $G_{1896}$ makes a wobble base pairing with $T_{1858}$ in the stem structure. The mutation of $G_{1896}$ for $A_{1986}$ creates a new base pairing which consolidates the stem loop structure. Such a potentiation of the encapsidation signal might enhance viral replication of HBeAg minus mutants. HBV genomes of genotype A or F, however, have C at nucleotide 1858; it pairs with $G_{1896}$. As mutation of $G_{1896}$ to $A_{1896}$ destroys
the C-G pairing, $A_{1896}$ is not acceptable, and has not been detectable in these genotypes (Li et al., 1993). When $A_{1896}$ occurs in genotype A, it is accompanied by another point mutation at nucleotide 1858 from C to T, making a matched pair between $A_{1896}$ and $T_{1858}$ in a double mutant (Lok et al., 1994).

While the precore mutation is believed to enable continued HBV replication during the chronic stage of infection and to guarantee viral persistence, the relevance of this point mutation in the course of liver disease is still controversial. It has been suggested that the mutants cause severe liver disease and they have been implicated in several cases of fulminant hepatitis. However the same precore mutation is present in asymptomatic chronic carriers of HBV, and fulminant hepatitis can be induced by precore intact HBV strains.

The precore mutation at codon 28 is often accompanied by another G to A mutation at codon 29 changing glycine to aspartic acid. This second mutation may be implicated in an enhanced ribosomal binding for active translation of the C gene product (Carman et al., 1989). A number of further point mutations leading to initiation failure or premature termination, as well as deletions and insertions inducing frameshifts, have been described (Okamoto et al., 1990; Hsu et al., 1995). The most common of these are the point mutations at codon 1 leading to initiation failure.

1.10.4: X Mutants

The likely roles of HBxAg in the stimulation of virus gene expression and replication imply that mutations in this product would result in HBsAg negative infections characterised by low levels of viremia. Experimental evidence has shown that when
the X gene is mutated so that HBxAg polypeptides are not made, transfection of such mutated clones into human hepatoma cells resulted in a more than 10 fold reduction in the level of HBV transcripts, polypeptides and virus (Colgrove et al., 1989; Nakatake et al., 1993).

The X region contains many important elements for HBV proliferation including the core promoter, enhancer II and DR1 and DR2 which are implicated in the origin of HBV DNA synthesis. The most described mutation found in the X region is that of an 8 nucleotide deletion (Uchida et al., 1994). This deletion may affect the core promoter binding site overlapping enhancer II. Since even one nucleotide mutation in modular promoter and enhancer sequences significantly reduces transcriptional activity (Phares et al., 1991), the 8 nucleotide deletion may result in critical changes in these elements. The 8 nucleotide deletion also truncates the X protein by 20 amino acids from the C-terminal end where an essential domain is located (Takada et al., 1990). Accordingly, the 8 nucleotide deletion causes several negative effects on the replication and expression of the HBV DNA and thus may be responsible for the extremely low level of viral replication.

Recently, X gene mutants have been implicated in serologically ‘silent’ HBV infections. In a study by Fukuda et al., (1996), HBV DNA was identified in 70% of non B non C chronic hepatitis cases by nested PCR and 85.7% of HBV DNA had the characteristic 8 base pair deletion mutation. The study did not investigate the full genome sequence of HBV mutants and so it was not clear why anti-HBe, anti-HBe and HBsAg were missing from serum. However an extensive investigation of full genome sequence of HBV mutants infecting patients without serological markers by Uchida et al., (1995), reported that although HBV DNA was present at extremely
low levels, no amino acid substitutions that might cause a loss of antigenicity were
detected in either the HBc or HBs coding region. However it should be noted from
the Fukuda study that 14.3% of HBV DNA detected in non B non C chronic hepatitis
did not show the characteristic 8 nucleotide deletion in the X gene. The existence of
critical mutations in another part of the HBV genome was speculated although there
has been no report supporting this hypothesis.

As a result, the clinical and pathological significance of these mutants in non B non
C chronic hepatitis still needs to be clarified.

1.10.5: Polymerase mutants

Intervention strategies to control the global burden of chronic liver disease caused by
HBV include primary prevention through immunisation (Kane 1996) and through
antiviral therapy (Hoofnagle et al., 1997). Both of these strategies provide powerful
selection pressures on HBV, which can result in the emergence of escape viruses.
HBV variants with mutations in the polymerase gene have now been described
following long-term nucleotide analogue therapy (Bartholomeusz et al., 1997).

Lamivudine is a cytidine analogue that has been shown to be effective in inhibiting
HBV replication (Benhamou et al., 1995) and clinical trials have demonstrated it to
be a promising antiviral for the treatment of chronic HBV infection (Tyrrell et al.,
1993; Dienstag et al., 1995). However, the recent emergence of lamivudine-resistant
variants now poses a clinical problem.

A study by Ling et al., (1996) reported the selection of polymerase mutants during
antiviral therapy of transplant recipients with lamivudine. The classic lamivudine
resistance ‘signature mutation’ in the HBV polymerase is the substitution of either
valine or isoleucine for methionine in the highly conserved tyr-met-asp-asp (YMDD) motif, which comprises part of the active site (domain C) of reverse transcriptase (Toh et al., 1983; Larder et al., 1989). A similar substitution occurs in an identical motif associated with the emergence of resistance to lamivudine in human immunodeficiency virus (HIV).

Another nucleoside analogue presently being evaluated as an antiviral therapy for chronic hepatitis B infection is famciclovir. Aye et al., (1997) reported a breakthrough infection in a patient following liver transplantation. Sequence analysis revealed mutations in the polymerase gene at codon 519 where valine was substituted with leucine and at codon 526 where methionine replaced leucine. The amino acid changes were located within the B region of the reverse transcriptase and were therefore found upstream of the ‘YMDD’ motif in which changes were not detected. The B domain of the HBV polymerase coincides with amino acids 150 to 175 of HBsAg. This domain seems to undergo the selection of most of the mutations observed in the presence of either famciclovir or lamivudine. The effect of these changes on the B-cell epitope residues of the HBsAg has yet to be determined, and their role in anti-HBs neutralisation escape does require further investigation.

There may be valuable lessons to be learned from the therapeutic approaches used to manage HIV infection. For HBV, a combination of famciclovir and lamivudine may be more effective than either drug being used as monotherapy. Unfortunately, the lamivudine mutations associated with resistance in HBV often include changes in the B domain of the polymerase, which is the same region associated with breakthrough during famciclovir therapy. Thus, theoretically it would appear unlikely that lamivudine resistant HBV would be sensitive to famciclovir; however, a number of
lamivudine resistant HBV isolates are only associated with the YMDD changes and not to B domain substitutions, and these may still be famciclovir sensitive. Famciclovir resistant HBV appears to be initially sensitive to lamivudine (De Man et al., 1997) although lamivudine resistance does appear to emerge more rapidly (Locarnini et al., 1997).

1.11: Antigenic Structure Of HBsAg

The s protein contains B, T helper and CTL epitopes. Antibodies produced during infection and following immunisation are directed against multiple epitopes in the s protein. Both linear and discontinuous B cell epitopes are thought to exist on the HBsAg.

The spatial structure of HBsAg is not completely understood but computer analysis of topogenic elements has provided a model of the organisation of the protein (Prange et al., 1995; Prange and Streeck, 1995). These show that there are at least three hydrophobic and two hydrophilic domains. The first two hydrophobic domains are important for correctly anchoring the s protein to the membrane (Bruss et al., 1991). The third hydrophobic domain lies between amino acids 161-226. It has been shown that even an HBsAg protein with a 51 amino acid C-terminal deletion could be assembled and secreted (Prange et al., 1995). However, Bruss et al., (1991) demonstrated that the truncated protein could only be secreted with help from a full length s protein.

The two hydrophilic domains are found between amino acids 30-80 and amino acids 99-169. In both hydrophilic domains some amino acid variability is allowed without affecting assembly (Delpeyroux et al., 1987). The first hydrophilic region is thought
to lie on the inner surface of the viral envelope and thus may be involved in attachment and envelopment of the core particle during maturation of the virion (Prange et al., 1995).

The second hydrophilic domain, however, is exposed on the outer virion surface and contains the major group and subtype-specific antigenic determinants. The region has a highly complex structure and is very cysteine-dense: 8 of the 14 cysteine residues in HBsAg are located here, and all 8 are highly conserved among mammalian hepadnaviruses and HBV subtypes. There is, therefore, the potential for a high degree of secondary, tertiary and quaternary structure through the formation of disulphide bridges. Competing structural models of this region have been proposed. However, the exact structure remains unsolved; but it does seem clear that disulphide bonds play a crucial role in stabilising the conformation of this region.

One current model of this region is that of a double loop structure from residues 124-137 and from 139-147 formed by disulphide bridges. Further evidence for this model is inferred from studies of synthetic peptides, which show a significantly greater binding to antibodies when circularised (Brown et al., 1984; Waters et al., 1991). Presumably, circularisation mimics the effect of a disulphide bond in stabilising exposed loops. It is within this region that the major B cell epitope cluster referred to as the a determinant is found.

Recent reports describing variants of the HBsAg have led to a better understanding of this region as well as to suggestions of a more complex antigenic structure (Chen et al., 1996) which could be extended up and downstream to include the entire hydrophilic region (Wallace et al., 1997).
Employing techniques such as site-directed mutagenesis (Ashton-Rickardt et al., 1989) and using peptides in amino acid replacement assays (Steward et al., 1993) has allowed the effect of predicted structural changes upon the antigenicity of HBsAg to be studied. As a result, some important antigenic residues in the \(\alpha\) determinant have now been identified. The substitutions C124S, K141E, P142G, P142I and C147S were all found to significantly reduce reactivity with anti-HBs. The residues, cysteine or proline play an important role in the determination of polypeptide conformation in proteins. The cysteine residues are involved in stabilisation through disulphide bridges, and the proline would also be expected to be of conformational significance in dictating a turn in the polypeptide chain.

Substitution of the proline with isoleucine was shown to decrease antigenicity more than substitution with glycine. The bulky hydrophobic side chain of isoleucine may impede the turn more than glycine, which allows relatively free movement of the peptide (Garnier et al., 1978).

Results from the Ashton-Rickardt study (1989), further emphasised the importance of the cysteines at both positions 124 and 147. It was found that both were required for full \(\alpha\) antigenicity, but that at 147 is virtually indispensable suggesting that this residue participates in a disulphide bridge that is essential for maintenance of the correct spatial arrangement of the antigen. Mutations of each of the three adjacent cysteine residues, 137, 138 and 139, to serines have also been made and show that mutation of the central cysteine residue had little or no effect on antigenicity but mutations of either of the flanking residues led to a loss of detectable activity (Bruce et al., 1995).
The first and still probably the most significant of the escape mutants of HBV to be described involves an alteration of glycine to arginine at codon 145 (Carman et al., 1990), a substantial change in stereochemical terms. Glycine residues are frequently located at junctions between domains or turns in polypeptides; this particular residue lies within the postulated loop between cysteine residues 139 and 147 and appears to be critical for maintenance of the correct conformation.

Another interesting model based on antibody binding to a phage display system has been proposed (Chen et al., 1996). Three of four monoclonal antibodies tested recognised distinct discontinuous epitopes in the amino acid region 99-207. A fourth antibody only recognised the region including residues cysteine 121 to cysteine 124, indicating that a tight loop is formed between these two residues. Some monoclonal antibodies recognised epitopes that were up to 70 residues apart, which were thought to be spatially close when the molecule folded. Suggested disulphide bridges include C121 to C124, C139 to C147, C107 to C138 and C137 to C149, where the latter two were considered less important by Chen et al., (1996). However, Bruce et al., (1995) showed that C149 is important for antigenicity and therefore the fine structure of this region remains unresolved.

A recent review by Wallace et al., (1997) suggests an extension of the area of study to include not only the $a$ determinant but the entire major hydrophilic region (MHR) of amino acid 99-169. It was proposed that the epitopes of HBsAg be clustered into five regions, named HBs1 to 5: HBs1 would include the region upstream of amino acids 120; HBs2 between amino acids 120 and 123; HBs3 between amino acids 124 and 137; HBs4 between amino acids 139 and 147 and HBs5 from amino acids 148 up to 169.
This organisational scheme was proposed to offer a framework to categorise s gene mutations and to provide a basis for examining the functional differences of the observed variants in different regions. The authors also suggest a division of s gene mutants into those which arise naturally and those which occur during the course of specific therapies. A further report by Carman, (1997) does indeed categorise the described variants. Those variants selected by human intervention were generally found in the HBs4 region whereas naturally occurring variants were found in HBs2 and 3. This further enhances the fact that HBs4 is the neutralising epitope cluster. Two further clusters were described in this report; HBs1 and HBs5 were highlighted because of serologically non-reactive samples. However, there still remain many exceptions which do not fall neatly into these categories.

Variants of the HBsAg have now been found in several important clinical scenarios (section 1.10.1.2). As the HBsAg is thought to contain the major neutralisation epitopes, there will be constraints as to the extent of variation permitted and only those variants which allow survival of the virus will be propagated. The major hydrophilic region is not hypervariable and most of the variants described to date have an antigenic effect. These variants may in turn lead to a better understanding of the HBsAg structure.

1.12: Aims Of Study

The a determinant of the HBsAg is the major domain for both the diagnosis and immunoprophylaxis of HBV. While much work has focused on the a determinant, its fine structure and spatial arrangement brought about by the three dimensional folding of the amino acid backbone remains uncertain. Though most anti-HBs found in
convalescent and post-immunisation sera binds to the epitopes carried between amino acids 124-147 (Howard et al., 1984), it has been suggested that the flanking regions may influence the antigenicity of the immunodominant region. Structural predictions, use of peptides and phage library screening all provide stronger evidence for clusters of epitopes and a more complex structure (Prange et al., 1995; Steward et al., 1993; Chen et al., 1996).

Evidence has accumulated that HBsAg mutants, selected in the face of immunological pressure, lead to i) vaccine failure which is of particular concern in an increasingly immunised world where HBsAg mutants may spread and become established within communities (Carman et al., 1990; Harrison et al., 1991; Karthigesu et al., 1994) ii) the possibility of reinfection or breakthrough infections; iii) immunoprophylaxis failure in liver transplant recipients (McMahon et al., 1992; Carman et al., 1995; Ghany et al., 1995; Hawkins et al., 1996); and iv) loss of diagnostic accuracy as a result of failure to detect the mutants (Yamamoto et al., 1994; Carman et al., 1995; Hou et al., 1995).

Inconsistent serological results led to the identification of mutant HBsAg in two renal transplant patients. While being detectable on polyclonal or mixed monoclonal/polyclonal assays, samples from these patients were repeatedly negative on monoclonal based HBsAg detection systems. Point mutations revealed upon sequence analysis in both patients, appeared to have led to the abrogation of the typical epitope structure resulting in the mutant HBsAg not being recognised by the monoclonal antibodies used.
The identification of these mutant infections provided an opportunity to study the epitopes carried in native HBsAg purified from patient serum. Their further characterisation could be achieved using monoclonal antibodies.

Female Balb/c mice were immunised with HBsAg purified from the sera of both patients, and used to produce hybridomas. A screening strategy using the mutant HBsAg identified hybridomas producing antibodies against mutant specific and mutant independent epitopes. As those monoclonal antibodies which displayed reactivity against the WT and mutant HBsAg were thought to be diagnostically and therapeutically more important, much of the work described in this thesis is centred around them.

The production of a panel of monoclonal antibodies which display reactivity to various HBsAg epitopes will be beneficial in developing our understanding of the HBsAg on both a molecular and scientific level. Epitope mapping studies using the monoclonal antibodies in conjunction with oligopeptides may help to determine what regions of the HBsAg are accessible in the surface of 22nm particle. This in turn may give an indication of how the epitopes cluster on the three dimensional structure and how peptide backbones are folded. Further, using the mutant specific monoclonal antibodies may enhance our understanding on a molecular level of how the single point mutations, as in the case of G145R, cause perturbations in the α determinant.

Certainly, having monoclonal antibodies which recognise epitopes not abolished by HBsAg mutants will improve detection assays. This may play an important role in studying the epidemiology of these mutants as well as in the delineation of the vaccine escape problem.
2: MATERIALS AND METHODS

2.1: Production Of Monoclonal Antibodies

2.1.1: Identification of patients infected with the HBsAg mutant

The monoclonal antibodies described in this study were raised against mutant HBsAg isolated from two renal transplant patients, NP and MAM. Neither of the patients had been previously immunised and were identified as a result of their inconsistent HBV serological markers.

Patient MAM was an HBV carrier. He was initially, in 1986, HBsAg positive by the polyclonal based reverse passive haemagglutination assay (RPHA Murex Biotech Ltd.) but negative by a monoclonal enzyme immunoassay (EIA). In 1988, he underwent renal transplantation. When tested again, HBsAg became detectable by polyclonal antibody assay only. He was anti-HBc and HBeAg positive throughout his infection.

Patient NP underwent renal transplantation in 1985. In 1990 he was anti-HBc positive but HBsAg and anti-HBs were not detected. Haemodialysis was started in 1993 and later that year HBsAg was detected by polyclonal based assays only.

HBV DNA sequence analysis was carried out on both of the patients. The region encompassing the α determinant was determined by direct sequencing of the PCR product (section 2.6).

2.1.2: Animals

The female Balb/c mice used were bought and raised in the animal house in the University College London Medical School.
2.1.3: Purification of HBsAg

Purification of HBsAg required for immunisation of mice was based on the method described by Cameron et al., (1980).

High titre HBsAg positive serum was fractionated by passing 20ml volumes through a 100x5 cm diameter column of Sepharose 6B (Pharmacia Ltd) equilibrated with 0.9% NaCl (Tris/Sal/Az). The HBsAg rich fractions now free of albumin and IgG were concentrated to a volume of 9ml by ultrafiltration in a stirred 200ml ultrafiltration cell fitted with an XM 100 A membrane (Amicon Ltd.). To this 2.69g of solid CsCl was added and the volume adjusted to 10ml to give a density of 1.2g/cm$^3$. The material was then ultracentrifuged for three days at 124000g in two 5ml tubes in a SW50-L swinging bucket rotor (Beckman RIIC Ltd) at 20°C to give isopyknic banding in the self forming density gradient.

The main HBsAg band containing the 22nm small particles was removed from both tubes, pooled and made up to 5ml with a solution of CsCl in Tris/Sal/Az (2.885g plus 10ml) which gave a final density of 1.2g/cm$^3$. The material was ultracentrifuged under the same conditions as before in one 5ml tube to give the second isopyknic banding. The final HBsAg band was removed and dialysed in PBS to remove CsCl.

2.1.4: Immunisation of animals

Fifty microlitres of purified HBsAg was mixed in an equal volume of Titermax™ was injected subcutaneously into a female Balb/c mouse. Approximately two months later a dose of 30μl of HBsAg was given intraperitoneally (i.p) followed three months later by a 75μl dose also given i.p. A final 25μl of HBsAg was given intravenously three days before the fusion.
2.1.5: Culture of Myeloma cells

JK cells derived from P3-X63-Ag8653 (Kearney et al., 1979) were cultured in complete medium (Appendix 1) at 37°C in a 5%CO2 in air atmosphere at 100% humidity.

The cells were split 1:2 approximately three times weekly and maintained at a concentration of between 2x10^5 and 2x10^6 cells per ml. This was done to ensure that no overgrowth which could lead to cell death actually occurred. Cells only in the log phase of growth were used for fusion to splenocytes.

For the fusion, the myeloma cells were spun at 400g for 10 minutes and washed three times in the same volume of incomplete medium (appendix 1). The resuspended JK cells were diluted 1:10 in incomplete medium and the viability and concentration of the cells determined by counting the cells using a Neubauer chamber. The concentration of JK cells was found to be approximately 5.87x10^7 cells/ml.

2.1.6: Preparation of feeder cells

Mouse peritoneal exudate cells were used as a feeder cell layer for hybrid cells. These were prepared the day before the fusion was undertaken. Cold complete medium containing hypoxanthine, aminopterin and thymidine (HAT medium-Appendix 1) was used for preparation of the feeder cells. Five milliliters of the medium was injected into the peritoneal cavity of a freshly killed Balb/c mouse and after gentle agitation was removed aseptically now containing exudate cells. Following gamma irradiation, one hundred microlitres of the cells were added to each well of a sterile 96 well plate (4-5 plates were needed per spleen to be fused).
All plates were examined on the day of the fusion to ensure that they remained free from any visible bacterial or fungal contamination.

2.1.7: Preparation of spleen cells

Preparation of spleen cells from both MAM HBsAg immunised and the NP HBsAg immunised mice were carried out using the same method.

The spleen was carefully removed from the freshly killed mouse and placed in a petri dish containing 5mls of cold complete medium. The spleen was disrupted carefully with the blunt side of a scalpel blade thus teasing away the cells from the spleen connective tissue. The cell suspension was then transferred to a sterile universal container and the debris allowed to settle to the bottom. The supernatant was carefully removed and the cells pelleted at 400g for 10 minutes. The cells were washed three times in 10ml of incomplete medium and the final cell pellet resuspended in 10ml of incomplete medium. The yield and viability of the spleen cells was determined using a Neubauer chamber. Approximately $7 \times 10^7$ cells were harvested from the NP spleen whilst $10 \times 10^7$ cells were obtained from the first MAM spleen and $2.5 \times 10^7$ cells from the second MAM spleen.

2.1.8: Cell fusion

The splenocytes were mixed with the myeloma cells at a 4:1 ratio and the mixture centrifuged at 400g for 10 minutes. The supernatant was removed leaving a dry pellet of cells at the bottom the container. This was placed in a water bath at 37°C. Polyethylene glycol 1540 (PEG-Sigma) which had been previously sterilised by autoclaving was also warmed in the 37°C waterbath. The cell pellet was loosened in
the bottom of the universal and 1ml of the PEG solution added dropwise whilst the container was gently agitated. The cell/PEG mix was left at 37°C for one minute and then an additional 2ml of the warm incomplete medium added. Further incomplete medium was added slowly so that the volume doubled every minute until 25ml of incomplete medium had been added. The fused cells were then centrifuged at 400g for 10 minutes and the pellet resuspended in 10ml of HAT medium. The cell suspension was distributed evenly over five plates of feeder cells and then placed in a CO₂ incubator.

2.1.9: Culture of hybrid cells

Clusters of growing fused cells appeared after about a week. At this stage the cells were refed by replacing 100μl of old medium with fresh HAT medium. Approximately 14 days after the fusion, 100μl of the supernatant fluid from each well where viable hybridoma colonies were visible, were removed and assayed for the presence of anti-HBs. The supernatant was replaced with complete medium containing hypoxanthine and thymidine (HT medium-Appendix 1).

2.1.10: RIA for the detection of anti-HBs secreting hybridomas

Supernatant from viable hybridoma cultures were tested for anti-HBs on the basis of a reverse capture assay.

Round bottom Nunc maxisorb wells were coated with 100μl of a 1:1000 dilution in Tris buffer of anti-mouse IgG. After two days at room temperature the wells were washed with Tween Saline (Appendix 1) and blocked for one hour with 0.5% Bovine serum albumin in Tris buffer (Tris BSA buffer- appendix 1). The wells were then
sealed and stored moist at 4°C. All subsequent washings were done with Tween Saline.

Before the assay was carried out, the Tris BSA buffer was removed. One hundred microlitres of a 1:10 dilution of the hybridoma supernatant fluid in phosphate buffered saline (PBS- Appendix 1) were added to the coated wells and incubated at 37°C for one hour. After washing, 100µl of purified $^{125}$I-wild type HBsAg, (45nCi diluted in Tris buffer containing 2% BSA and 20% normal human serum (NHS) which was negative for all serological markers), were added and left in a moist box at room temperature overnight. The wells were then washed and bound reactivity measured in a sixteen channel gammacounter (NE 1600). Supernatant containing anti-HBs gave an increased binding of the label. All hybridoma supernatant fluid were subsequently retested by the same assay where $^{125}$I-MAM HBsAg and $^{125}$I-NP HBsAg were used as detector labels. Positive and negative controls were included in each assay. The monoclonal antibodies, D2H5 and H3F5 acted as positive controls. These monoclonals had been raised against and shown to react with the WT HBsAg (Tedder et al., 1987). An anti-HIV gag monoclonal antibody 3D3F2 (Ferns et al., 1987) and PBS were used as negative controls.

Results of these assays allowed parent colonies secreting anti-HBs to be chosen for further screening and cloning.

Once the cells were growing well, they were aspirated using a glass pasteur pipette and transferred aseptically to 24 well sterile plates which contained a layer of mouse peritoneal exudate cells (PEC) in HT medium.
2.1.11: Cloning by limiting dilution

Hybridomas identified as producing anti-HBs were cloned by limiting dilution. This is done to ensure that the antibody secreted is homogenous and monospecific as well as to prevent overgrowth of any negative clones which may result in the loss of secretors. For each hybrid culture, the volume of cell suspension containing 100 cells was calculated and this volume added to 10ml of complete medium. One hundred microlitres of this cell suspension (nominally containing one cell) were added to each of 48 wells of a 96 well plate containing feeder cells in complete medium. One hundred more cells were added to the remaining 5ml of complete medium and 100μl of this more concentrated cell suspension were added to 24 wells of the plate. Finally, a further 100 cells were added to the remaining cell suspension and this was distributed over the last 24 wells. Variation in the concentration of cells on each plate made allowance for inaccurate cell counts and low viability of some cultures. This also ensured the rescue of single antibody producing clones. The procedure was repeated for each selected parent hybridoma.

Approximately five days after cloning the plates were examined for wells where only a single colony was visible. One hundred microlitres of supernatant was removed from these wells and retested for anti-HBs activity using the reverse capture assay described previously (section 2.1.10).

Clones secreting anti-HBs were then expanded as before and refed as necessary by removal of 100μl of tissue culture supernatant and replaced with fresh complete medium.
2.1.12: Culture of hybrid clones

Once the colonies were large enough to cover the well they were expanded into 12cm$^2$ tissue culture flasks containing feeder cells and then further into 25cm$^2$ and 75cm$^2$ flasks. The cells were split 1:2 approximately every 2-3 days with fresh complete medium.

Once good growth had been established, some of the cells were frozen and stored under liquid nitrogen in incomplete medium supplemented with 10% dimethyl sulphoxide and 50% foetal calf serum.

2.1.13: Production of ascitic fluid

Female Balb/c mice, aged 12-20 weeks were primed with 0.5ml pristane (Aldrich Chemical Company). One to four weeks after priming, 1ml of cloned hybridoma cells, resuspended in incomplete medium, were injected intraperitoneally. Mice were sacrificed 1-3 weeks later and the ascitic fluid aspirated and separated from cells by centrifugation at 1000g for 10 minutes. The ascitic fluid was then stored at -20°C until required. A diagram illustrating the production of monoclonal antibodies is shown in figure 2.1.13

2.2: Characterisation of the monoclonal antibodies

2.2.1: Isotyping of monoclonal antibodies

Isotyping of the monoclonal antibodies was carried out using a kit from Serotec Ltd. specifically designed to identify the class and subclass of monoclonal antibodies in tissue culture supernatants. Used in accordance with the manufacturers recommendations, it readily characterised the mouse monoclonal antibodies.
Figure 2.1.13: Illustration of procedure for producing monoclonal antibodies (modified with permission from Immunology-second edition (1994) by Janis Kuby)
2.2.2: Serum protein electrophoresis

Ascitic fluid from each clone was tested for the presence of a monoclonal protein band by serum protein electrophoresis (SPE, Paragon Electrophoresis System, Beckman Ltd). Following the protocol provided, the kit conveniently identified those ascitic fluids containing high levels of monoclonal immunoglobulin. The kit is intended for the electrophoretic separation of proteins in a buffered agarose gel. After electrophoresis, the proteins in the gel were immobilised in a fixative solution and the gel dried to a film. The protein pattern is visualised by staining the film with a protein-specific stain and the pattern visually interpreted.

2.2.3: Immunoglobulin G preparation

Immunoglobulin G was prepared by ion exchange chromatography from the various ascitic fluids.

DE52 gel (Whatman Ltd), supplied pre-swollen was resuspended in a 0.2M phosphate buffer pH 8.0 (appendix 1). The gel was then dispersed in distilled water to give a 10mM buffer strength.

A K9 column (Pharmacia Ltd.) was packed with the DE52 gel to give a gel to sample volume ratio of 5:1. All columns were equilibrated with an equal volume of 10mM phosphate buffer (PB-Appendix 1). After overnight dialysis at 4°C in 10mM PB, the sample was layered on top of the columns and allowed to absorb over 30 minutes.

Immunoglobulin G from the ascitic fluid was recovered by stepwise elution with 10mM, 30mM and 60mM PB. Each buffer was allowed to run through the column and the optical density of the eluent was monitored and recorded at 280nm (Ultra Violet Spectrophotometre, LKB Ltd.). The three different eluates were collected
separately and assayed for anti-HBs activity in the reverse capture RIA. The eluate containing the majority of anti-HBs reactivity could be identified.

The protein concentrations of the ascitic fluid IgG were calculated by determining their absorbance at 280nm using an \( E_{1\text{cm}}^{1\%} \) value of 1.4 in a spectrophotometer.

### 2.2.4: Immunoglobulin A preparation

Protein A (Pharmacia Ltd.) was used for the purification of IgA. Once packed, the protein A column was washed through with 100mM phosphate buffer (PB) pH 8.0. Following overnight dialysis, 1ml of ascitic fluid was added to an equal volume of 200mM of PB and the sample loaded onto the column. The IgA was then eluted by running 0.1M citric acid pH 3.0 (Appendix 1) through the column. The optical density of the fall through was monitored at 280nm (Ultra Violet Spectrophotometer, LKB Ltd.) and the eluted peak collected and neutralised by the addition of 1M Tris-HCL pH 8.0. The eluate was then tested for anti-HBs reactivity in the reverse capture assay (section 2.1.10). The protein concentration of the eluate was determined as described above.

### 2.2.5: Radiolabelling procedure

Labelling of the immunoglobulin fractions was carried out by the iodogen method (Salacinski et al., 1979).

Clean 7.5x10cm glass tubes were initially coated with 5\( \mu \)g of chloroform. To the iodogen tubes 15\( \mu \)g of protein in PBS was added. Finally Na\(^{125}\)I (1miCi, Amersham International Plc) was added and the reaction in the tube allowed to proceed for 10
minutes on ice. The iodogen acts as a mild oxidising agent for the Na\(^{125}\)I to bind onto a tyrosine residue on the protein.

A K9 column was then packed with sephadex G-25 and equilibrated in Tris BSA buffer. Non-radioactive iodide (KI/Nal) in PBS was added to the G25 column as this reduced the tendency for free \(^{125}\)I to stick to the column and to prevent labelling of BSA. The reaction mixture was then removed from the iodogen tube and transferred to the column. Tris BSA buffer was then used for eluting and the eluent monitored. The \(^{125}\)I labelled protein fraction was collected from the first peak and stopped as the peak began to tail off. The labelled protein was then stored at 4°C in Tris saline buffer containing 5% BSA.

Elution was continued until free \(^{125}\)I had come through (i.e. the second peak). The height of this peak compared to the height of the first peak gave an estimate of the percentage of \(^{125}\)I bound.

2.2.6: Cross Competition Assays

Round bottom Nunc plates, coated with the polyclonal antibody to HBsAg, currently used in the Murex GE15 HBsAg detection kit, were a gift from Murex Biotech Ltd. One hundred microlitres of a 1:1000 dilution of purified wild type HBsAg (1 mg/ml) in NHS, were added to the wells and left overnight at room temperature. The following day the wells were washed and purified immunoglobulin (5µg in 50µl of Tris BSA buffer) from each monoclonal antibody added separately to the wells. \(^{125}\)I labels prepared from the individual clones (50nCi in 50µl of Tris buffer containing 2% BSA and 20% NHS) were added to the wells. The assay was therefore set up so each monoclonal antibody competed with itself and each of the other antibodies. The
wells were incubated for four hours at room temperature, washed and the bound radioactivity measured. The assays were repeated with the MAM HBsAg and the NP HBsAg.

2.3: RIA to determine the binding specificity of the monoclonal antibodies

The following RIA was designed to test the binding specificity of the raised monoclonals. We obtained a purified recombinant protein, kindly donated to us by SmithKline Beecham, which was expressing the s region only of the envelope protein. The recombinant protein was labelled with $^{125}$I as is described in section 2.2.5. The assay was performed as before (section 2.1.10) testing the panel of hybridoma supernatant fluids with the labelled recombinant HBsAg.

2.4: Optimisation of Solid Phase Assay For HBsAg Detection

2.4.1: Homologous assay

Round bottom Nunc wells were coated with 100µl of a 1:1000 dilution in Tris buffer of purified P2D3 immunoglobulin. After being left at room temperature overnight, the wells were washed with Tween Saline and blocked for one hour with Tris-BSA buffer (Appendix 1)

Before the assay was carried out, the Tris-BSA buffer was removed. One hundred microlitres of half log dilutions of the purified NP, MAM, and WT HBsAg were added separately to the wells and incubated overnight in a moist box at room temperature. After washing, 100µl of $^{125}$I-P2D3 IgG (50nCi diluted in label diluent-Appendix 1) were added and the wells incubated for four hours at room temperature.
The wells were then washed and bound reactivity measured in a sixteen channel
gammacounter (NE 1600)

2.4.2: Heterologous Assay

A second assay, assessing the ability of P2D3 to detect HBsAg was carried out.
Wells coated with the polyclonal antibody, GE15, were supplied by Murex Biotech
Ltd. and used to capture half log dilutions of purified NP, MAM and WT HBsAg.
The remainder of the assay was then undertaken as described above. This assay is
referred to as the ‘conserved’ assay.

2.4.3: WT assay

One hundred microlitres of a 1:1000 dilution of purified H3F5 IgG were coated onto
round bottom Nunc wells. Following overnight incubation at room temperature, the
wells were washed with Tween Saline and free binding sites quenched at 4°C with
Tris-BSA buffer (Appendix 1).

After removing the Tris-BSA buffer, 100µl of a 1:1000 dilution of WT HBsAg
(1mg/ml) were added to the wells and incubated overnight in a moist box at room
temperature. The wells were then washed in Tween Saline. One hundred microlitres
of $^{125}$I D2H5 IgG (50 nCi diluted in label diluent) were added and the wells left at
room temperature for four hours. After undergoing a final wash, the wells were
placed in a sixteen channel gammacounter (NE1600) and bound reactivity measured.
2.5 Amplification using the Polymerase Chain Reaction (PCR)

The PCR method described below was developed and optimised for the efficient
detection of HBV DNA (Hawkins et al., 1994). The techniques are adaptations of the
original PCR methods used for \textit{in vitro} amplification of specific DNA sequences
(Mullis et al., 1987).

2.5.1 Primer design and synthesis

Targets for PCR amplification included the whole of the \textit{s} region as well as the
specific amplification of the region encompassing the \textit{a} determinant alone. All
selected primers were assessed by comparing published HBV sequences using the
Microgenie sequence analysis database (Genbank Issue 59, Beckman Instruments
Inc). All primers were designed to be between 18 and 25 nucleotides in length and
have as near as possible a 50\% G/C content. The primers were synthesised by Oswel
(University of Southampton). All the primers used in this study are listed in table
2.5.1

2.5.2 Serum DNA preparation by heat treatment

Twenty microlitres of serum were added to 20\m of PBS (Appendix 1). The sample
was placed in a boiling water bath and incubated for 10 minutes to denature serum
components and lyse DNA particles. The sample was then pelleted by centrifugation
at 400g for 15 minutes. Ten microlitres of the supernatant were transferred to a 0.5ml
Sarstedt tube containing 40\m of PCR reaction mix.
<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Orientation</th>
<th>Primer Position</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
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<td>1740-1760</td>
<td>CTCATTTCTTGTGGTTTCTT</td>
</tr>
<tr>
<td>H4069</td>
<td>antisense</td>
<td>2073-2054</td>
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<td>antisense</td>
<td>2157-2140</td>
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</tr>
</tbody>
</table>

Table 2.5.1: Sequences and Positions of PCR primers. (Nucleotide positions as in the sequence published by Pugh et al., 1986).

### 2.5.3: DNA Amplification

The target DNA was amplified using a nested PCR. The reaction conditions for the first round were 50mM Tris HCL (pH 9.0), 10mM KCL, 0.01% Triton X-100, 2mM MgCl₂, 0.2mM dNTP’s (Promega), 120ng of each external primer and 2U of *Taq* polymerase (Promega) in a volume of 40μl. Each reaction mixture was overlaid with 50μl of light white mineral oil. Ten microlitres of prepared sample were added to the reaction mixture, passing through the mineral oil, to give a final volume of 50μl.

The thermocycling programme for the first round was preceded by a pre-melting step of 94°C for 4 minutes. The conditions thereafter were 94°C for 1 minute, 45°C for 2
minutes and 72°C for 3.5 minutes over 35 cycles with a final extension for 7 minutes at 72°C.

For the second round, 1µl aliquots were transferred from the first round to 99µl reaction mixes prepared with fresh reagents and 120ng of each internal primer and 1U of Taq polymerase. Cycling parameters were 94°C for 1 minute, 50°C for 1 minute and 72°C for 1.5 minutes over 35 cycles with a final extension step of 72°C for 7 minutes. Amplification reactions were performed on a Techne PHC-1 thermocycler. The PCR products were analysed on a 2% agarose gel containing ethidium bromide and electrophoresed in 1xTAE buffer (Appendix 1). The gel was run at 80-120 volts for 45-60 minutes and photographed under ultra violet illumination.

2.6: Sequencing

Sequencing of HBV DNA was undertaken using an in-house version of the Sequenase™ 2 kit (United States Biochemicals, Winship 1989). The protocol was based on capturing biotinylated PCR products onto a streptavidin-coated solid phase which allowed single stranded DNA to be generated. Direct sequencing of the DNA could then be carried out by the dideoxy sequencing procedure (Sanger et al., 1977).

2.6.1: Biotinylation of PCR Products

Inclusion of a biotinylated version of the 3’ antisense internal primer in the second round of PCR, allowed the resulting DNA to be tagged with biotin at its 3’ end.
2.6.2: Purification of biotinylated PCR products

Biotinylated PCR products were captured onto streptavidin coated magnetic beads (Dynabeads\textsuperscript{TM}). The beads were initially prepared as according to the manufacturers instructions. Sixty microlitres of PCR product were then carefully removed from reaction tubes, avoiding any uptake of mineral oil, and added to 20\(\mu\)l of beads. To this a further 60\(\mu\)l of 2x Bind and Wash solution (Appendix 1) were added. The mixture was incubated at room temperature with gentle agitation for 15 minutes. The captured DNA was then pelleted by a magnetic field using a specifically designed rack (Dynal MPC). Any unreacted nucleotides and the non-biotinylated primer were removed and the pellet washed in 40\(\mu\)l of 1x Bind and Wash solution.

The DNA duplex was melted by incubating for 10 minutes at room temperature with 8\(\mu\)l of freshly prepared 0.1M NaOH. The eluted, non-biotinylated strand was removed and neutralised by the addition of 4\(\mu\)l of 0.2M HCL and 4\(\mu\)l of TE pH 7.4 (Appendix 1) and stored at -20°C until required.

The biotinylated strand was then washed sequentially in 50\(\mu\)l of 0.1M NaOH, 40\(\mu\)l of 1x Bind and Wash solution and finally 50\(\mu\)l of TE buffer (pH 7.4). The TE buffer was discarded and the sample finally resuspended in 10\(\mu\)l of water.

2.6.3: Sequencing of single stranded DNA

Five microlitres of the DNA template were added to 100ng of the appropriate sequencing primer, 1\(\mu\)l of DMSO and 2\(\mu\)l of 5x sequenase buffer (United States Biochemicals, Winship 1989 - appendix 1). The annealing mixture was heated to 70°C for 5 minutes and allowed to cool to 25°C over a 25 minute period using a thermal cycling machine.
A labelling reaction was set up adding 2μl labelling mix, diluted 1/30 in distilled water (appendix 1), to the annealed template-primer in the presence of 1μl of 0.1M DTT, 0.5μl $^{35}$S dATP (Amersham plc) and finally 0.2μl of sequenase version 2 (United States Biochemicals, Winship 1989). This was mixed thoroughly and incubated at room temperature for five minutes.

The sequencing reactions were completed by pre-warming a 96 well microtitre plate containing 2.5μl of each termination mix (ddATP, ddGTP, ddCTP and ddTTP-appendix 1) at 50°C for 5 minutes. To this 3.5μl of labelling mix was added and the incubation continued for a further five minutes. Reactions were stopped by the addition of 4μl of stop solution containing deionised formamide in 10mM TE, pH 7.5, 0.3% (w/v) xylene cyanol and 0.3% (w/v) bromphenol blue. Reaction products were stored at -20°C until required.

### 2.6.4: Sequence electrophoresis

The sequences were resolved on a wedged, 8% polyacrylamide gel (Appendix 1). The glass plates were carefully cleaned by sequential washing with distilled water, 70% ethanol and acetone before use. After 5 minutes incubation at 90°C on a hot block, 3.5μl aliquots of sequenced products were loaded and the gel run at 70W for 2 hours in 1x TBE buffer (Appendix 1). Sodium acetate was then added to the cathode chamber to a final concentration of 1.0M and the run continued for a further one hour. Once finished, the gel was removed from between the plates and fixed on 3MM filter paper (Whatman) in 10% methanol, 8% acetic acid for two 15 minute intervals. Finally, the gel was dried under vacuum for 2 hours at 80°C using a slab gel-dryer (Biorad).
2.6.5: Autoradiography of sequences

The dried gel was placed in an autoradiograph cassette (Dupont) and exposed to X-ray film at room temperature for 24-48 hours. The autoradiograph was developed in an automatic developer in accordance with the manufacturers instructions (X-Ograph).

2.7: Production And Use Of Recombinant Mutant HBsAg

The mutant recombinant HBsAg vectors described in this section were produced by Dr. T. Harrison and colleagues at the Royal Free Hospital School of Medicine, London. Briefly, a fragment containing the open reading frame of the small gene was obtained from the plasmid pEcoB6 by digestion with BamH1 and Hpa1. The resulting 934bp fragment was then cloned into the Sma1 site on the vector pBluescript SK+ in accordance with the manufacturers instructions (Stratagene). The orientation of the inserts in miniprep DNA was determined by digestion with Xba1.

Clones in the correct orientation showed fragments of about 3400bp and 237bp whereas those in the wrong orientation showed bands of approximately 3000bp and 732bp. Single stranded DNA was prepared by superinfection of TG2 containing the plasmid with helper phage VCS13 in the presence of kanamycin followed by extraction of a PEG precipitate of the supernantant.

In vitro mutagenesis was carried out using the Amersham in vitro mutagenesis kit in accordance to the manufacturers instructions. The kit allowed mutations to be introduced by extension from a mutant oligonucleotide containing the desired mutation. Following transformation, DNA was extracted using QIAprep spin kits.
(Qiagen, Hilden Germany) and the insert sequenced with surface antigen primers. Clones containing the appropriate mutations were subcloned into pMEP4 using KpnI and HindIII sites. Clones containing inserts as verified by digestion with XbaI were grown up and DNA extracted with Qiagen maxi prep kits (Qiagen, Hilden Germany). The DNA was then transfected into HepG2 cells and incubated at 37°C, 5%CO₂. Once good growth had been established, the cells were frozen.

2.7.1: Culture of transfected HepG2 cells

The recombinant vectors (11 mutant HBsAg and 1 WT HBsAg) were kindly supplied frozen in 1ml vials. Once thawed, the cells were immediately placed in a 25cm² flask, containing 5mls of medium (Appendix 1). The flasks were then incubated in a CO₂ in air atmosphere at 37°C. Once the cells had attached to the surface of the flask, usually overnight, the medium was removed and replaced with medium containing 300U/ml of hygromycin B.

2.7.2: Trypsinisation of HepG2 cells

Once the cells had reached approximately 75% confluency, they were expanded into 75cm² flasks. All the medium was first removed, aliquoted and stored at -20°C until required. The cells were then washed twice in 2mls of versene (Gibco BRL) followed by two further washes with 2mls of Trypsin-EDTA (Gibco BRL). After the last wash, only 1ml of Trypsin-EDTA was removed and the flask incubated at room temperature with occasional agitation. After approximately 10 minutes, the cells began to detach from the surface of the flask. The cells were then resuspended in medium and split 1:10.
2.7.3: Analysis of extrachromosomal DNA

2.7.3.1: DNA extraction

DNA was extracted from the HepG2 cells using the Wizard Genomic DNA purification kit from Promega. Flasks containing 1-3x10^6 cells/ml were trypsinised (section 2.7.2) and the DNA extracted in accordance to the manufacturers’ instructions.

2.7.3.2: DNA amplification

Using the relevant primers, the region encompassing the α determinant was amplified from the extracted DNA samples. Ten microlitres of the DNA were added directly to the PCR reaction mixture and two rounds of amplification carried out as described in section 2.5. Ten microlitres of the amplicon were then analysed on a 2% agarose gel containing ethidium bromide.

2.7.3.3: Sequencing analysis of DNA

Sequencing of the PCR products were undertaken as is described in section 2.6 using the primer H4073 (see table 2.5.1).

2.7.4: Detection of recombinant HBsAg

Two HBsAg assays based on different formats were assessed for their ability to detect the recombinant HBsAg expressed. Supernatant fluid was tested in parallel in both the ‘conserved’ and the ‘WT’ assays as described in sections 2.4.2 and 2.4.3.
2.8: Liver Transplant Patient Study

2.8.1 Patients

Twenty-six patients (23 male/3 female) who underwent orthotopic liver transplantation (OLT) between 1985 and 1995 for HBsAg positive cirrhosis at the Institute of Liver studies, King’s College Hospital, London were studied. Seven of these patients were from the UK, 17 from the Mediterranean region and 2 from the Far East.

All serological studies were carried out by King’s College Hospital. All patients were tested for HCV, and HDV antibodies as well as for all HBV markers using commercially available assays. HBsAg, HBeAg and anti-e were tested in assays available from Abbott Diagnostics, Maidenhead, UK; antibodies for HCV were tested in the UBI EIA (United Biochemicals Inc., Lake Success, New York, USA) and anti-delta was tested using the kit supplied by Sorin Biomedica (Saluggia, Italy). Serum HBV DNA was measured using a quantitative liquid phase hybridisation assay (Genostics, Abbott Laboratories, Maidenhead, UK).

It was possible to divide the patients into three groups based on whether they had reininfected their liver grafts and if they were receiving HBIG immunoprophylaxis. Group A was comprised of 12 patients who reinfected their grafts despite receiving HBIG prophylaxis; group B included 7 patients who had not received HBIG and went on to reinfect their liver grafts; group C consisted of 7 patients who had received HBIG and did not reinfect their liver grafts.
2.8.2: RIA for the detection of HBsAg

A total of 52 pre and post transplant samples were tested in two separate radioimmunoassays for the presence of HBsAg. The assays were run in parallel. The first assay, using the monoclonal antibody P2D3, was termed the ‘conserved’ assay and is described in section 2.4.2. The second assay, the ‘WT’ assay was carried out as is explained in section 2.4.3.

2.8.3 HBV DNA amplification using PCR and sequence analysis

The region encompassing the $a$ determinant amplified from all pre and post OLT patient samples using the appropriate primers as is described in section 2.5. In 7 of these patients the whole of the $s$ region was amplified as well. The sequencing primers H4073 and 668 were used to analyse the amplified DNA using single stranded sequencing as described in section 2.7.3.

2.9 Phage Display Library Analysis

2.9.1: Introduction

Peptide libraries based on filamentous phage were used to determine the epitope recognised by the three way cross monoclonals. However, due to the initial failure of the technique, the procedure had to be repeated. The data from both the experiments are presented and are hereafter denoted experiment 1 and experiment 2. Essentially, the two experiments were similar with only some of the conditions being modified.
2.9.1.1: Experiment 1

In the first experiment, the P2D3 monoclonal antibody was screened against the 6-mer phage display library alone. The optimum conditions for biopanning the monoclonal antibody had not been determined. Therefore each step of the affinity purification process was run in parallel at 4°C and at room temperature. It was also deemed essential for washes to be carried out at optimal stringency to avoid non-specific phage being isolated and therefore being amplified. Therefore, two different concentrations of wash buffers, one at 0.5M NaCl and the other at 0.15M NaCl (see appendix 1) were compared at each of the temperatures.

2.9.1.2: Experiment 2

For the second experiment, both the 6-mer and the 15-mer libraries were used to screen the P2D3 antibody. Unfortunately due to time restrictions, it was not possible to carry out experiments assessing the different optimising conditions as described above. The biopanning procedure was carried out at the conditions as described in Germaschewski et al., 1995. Therefore, all reactions were carried out at 4°C with all unbound phage being washed at a concentration of 0.15M NaCl (see appendix 1).

2.9.2: Fusion phage display library and monoclonal antibody

The 6-mer and 15-mer phage display libraries used had been amplified from a primary library, consisting of approximately $2 \times 10^8$ independent transformants (Scott and Smith, 1990), and was provided by Prof. G.P. Smith, University of Missouri, USA. The monoclonal antibody used in the analysis was P2D3, the three way cross monoclonal with BSA being used as a negative control.
2.9.3: Biopanning procedure

A sheet of nitrocellulose membrane was soaked in 20mls of transfer buffer (Appendix 1) for 15 minutes. Using a dot blot apparatus, 20μl of P2D3/BSA (5μg/20μl) were washed through onto the prepared nitrocellulose membrane. The washed nitrocellulose discs containing the monoclonal antibody were then excised and blocked overnight in 400μl of TBS (appendix 1) containing 0.05% Tween-20 and 0.1% BSA. The following day, the blocked discs were transferred to a separate tube containing 5 x 10^10 transforming units of phage in TBS with 0.1% BSA and incubated for 4 hours. The discs were then washed 6 times with wash buffer leaving a 10 minute interval between each wash. The remaining bound phage were eluted in 400μl of elution buffer (appendix 1) and the eluates neutralised with 38μl of 1M Tris-HCl pH 9.5.

For the amplification of phage contained in the first eluate (438μl), the whole eluate was used and therefore was first reduced to 100μl by centrifugation in a Microsep spin filter (Filtron, USA) with 160kDa cut off size (Sorvall SS34 rotor; 5000rpm). For subsequent rounds, only 100μl (25%) of eluates were taken as the input for the amplification.

2.9.4: Amplification of phage

Amplification of the phage was carried out by adding 100μl of K91 Kan cells, grown to an O.D_{600} of 2.0 (appendix 1) to an equal volume of the first round eluate. The mixture was incubated at room temperature for 10 minutes. The cells were suspended in 20ml LB medium (appendix 1) containing tetracycline (20mg/ml) and
incubated at 37°C for 45 minutes with shaking. A 50μl aliquot was then removed and 20μl of tetracycline (20mg/ml) added to each flask with the incubation being continued overnight. The removed aliquot was titrated onto LB agar plates containing tetracycline (20mg/ml) and kanamycin (100ng/ml) (appendix 1).

2.9.5: Phage particle purification

Following overnight incubation, the 20ml cultures were centrifuged twice at 5000rpm for 10 minutes at 4°C. Phage, present in the supernatant, were precipitated following a 4 hour incubation at 4°C in PEG/NaCl (appendix 1) and subsequently centrifuged at 10 000rpm for 15 minutes. The resulting pellet was dissolved in 1ml TBS and centrifuged briefly. The supernatant was transferred to a fresh eppendorf tube containing 150μl of PEG/NaCl mix (appendix 1), inverted several times and then incubated at 4°C for at least an hour. The tubes were then spun for 10 minutes at room temperature and the pellet dissolved in 200μl of TBS/0.02% NaN₃, thus producing the amplified eluate.

To determine the total number of infected clones represented, the amplified phage was titred using K91kan cells. The phage were first diluted (10⁻⁷ and 10⁻⁸) in TBS containing gelatin (1g/l) and 10μl aliquots added to 10μl of K91 cells grown to O.D₆₀₀ = 0.2. The mixture was incubated at room temperature for 20 minutes after which time 1ml LB plus 0.2μg/ml tetracycline was added. The tubes were then shaken at 37°C for 40 minutes. Aliquots from each tube were spread onto L-agar plates containing tetracycline (20mg/ml) and kanamycin (100ng/ml) and incubated overnight at 37°C. Colonies were counted and the total number of infected clones in the first round eluate was calculated. The biopanning procedure was repeated three
times using $5 \times 10^{10}$ transducing units of the previous rounds amplified eluates as the input phage.

2.9.6: Sequencing analysis of phage DNA

fd-DNA (single stranded) was prepared from 3ml overnight cultures of K91kan cells infected with individual colonies using a QIA prep spin M13 kit (Qiagen, Hilden Germany). The kit was used in accordance to the manufacturer's instructions.

The eluted DNA was then purified by ethanol precipitation. One hundred microlitres of DNA were added to 100μl of phenol. The mixture was vortexed for 1 minute and the tubes were spun for two minutes at room temperature. Eighty microlitres of the aqueous layer were removed and added to 100μl of chloroform. After spinning, the aqueous layer was removed and added to 8μl of 3M NaOAc, pH 5.2 plus 200μl of ethanol. The mixture was incubated at -20°C for 30 minutes and then spun for 15 minutes at room temperature. The supernatant was aspirated off and 100μl of 70% ethanol added to each tube. The ethanol was removed by careful aspiration and the pellet allowed to air dry. The pellet was finally resuspended in 10μl of TE.

Sequencing of the single stranded phage DNA was carried out as described in section 2.6 using primer A319 5’ ACC GTA ACA CTG AGT TTC GTC 3’, kindly provided by Fiona Gray (University of Edinburgh). The primer lies at position 1840-1819 of the fUSE 5 vector, approximately 154 base pairs downstream from the cloning site.
2.10 Epitope Location Using Oligopeptides

2.10.1 Oligopeptide Synthesis

Six, 15-mer peptides were synthesised by Murex Biotech Ltd. Five of these peptides were produced to reflect sequences found in the HBsAg while the final peptide paralleled a region located in the pre-S2. Details of the peptides are listed in table 2.10.1.

Unfortunately only, PD1 and PD2 were synthesised successfully. Therefore the peptides, LT1, LT2, A2 and PS1 were remade by Genosys Biotechnologies Inc. The resynthesised peptides were biotinylated at the N’ terminal.

All peptides were supplied lyophilised and were redissolved using PBS or DMSO.

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Amino Acid Positions</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT1</td>
<td>121-135*</td>
<td>CKTCTTPAQGTSMPF</td>
</tr>
<tr>
<td>LT2</td>
<td>121-135*</td>
<td>CKTCMTTAQGTSMPF</td>
</tr>
<tr>
<td>PD1</td>
<td>83-97*</td>
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<tr>
<td>PD2</td>
<td>170-184*</td>
<td>FSWLSLLVPFVQWFV</td>
</tr>
<tr>
<td>A2</td>
<td>138-152*</td>
<td>CCTKPSDGNCTCIPI</td>
</tr>
<tr>
<td>PS1</td>
<td>21-35*</td>
<td>YFPAGGSSSGTLPNV</td>
</tr>
</tbody>
</table>

Table 2.10.1: Sequence and amino acid positions of oligopeptides. * denotes position in the s region; # denotes position in the pre-S2 region.

2.10.2: Peptide Assay

Round bottom Nunc plates were coated with 50µl (10µg/ml) of streptavidin diluted in Tris buffer (Appendix 1). The wells were left at room temperature overnight and
washed with Tween Saline (Appendix 1). One hundred microlitres (5µg/ml) of each peptide were then added to the wells separately and incubated at 37°C for one hour. After blocking using Tris buffer containing 3% bovine serum albumin, 100µl dilutions of P2D3 (2µg/ml) were added to the wells separately and left for 4 hours at 37°C. The wells were then washed in Tween Saline and 100µl of anti-mouse conjugated peroxidase (Dako, High Wycombe, UK) diluted 1:2500 in PBS containing 10% normal goat serum and 3% bovine serum albumin added for one hour at 37°C. Following the final wash, the bound enzyme activity was detected with tetramethyl benzidine (TMB) (Murex Biotech, UK). The reactions were then stopped after 20 minutes with 50µl of 2M Sulphuric Acid The optical densities at 450nm were then determined in an ELISA spectrophotometre. A schematic representation of the peptide assay is shown in figure 2.10.2. The above assay was repeated with the monoclonal antibodies D2H5 and H3F5.
Figure 2.10.2: Schematic representation of peptide assay
Chapter 3: Identification Of Mutant HBsAg In Two Renal Transplant Patients

3.1: Introduction

Detection of HBsAg in serum forms the initial investigation for hepatitis B infection. The majority of anti-HBs found in convalescent and post-immunisation sera binds to the \( a \) determinant (Howard et al., 1984) and therefore antibodies to epitopes within this region are used in HBsAg detection assays.

However, mutations in the \( a \) determinant which bring about conformational alterations to this immunodominant region have now been described. These mutations result in the HBsAg not being recognised by the antibodies leaving many assays unable to detect these HBsAg mutants.

Discrepancies in HBsAg detection using commercial assays have been associated with mutations in both the first and the second loop (Waters et al., 1992; Carman et al., 1993; Yamamoto et al., 1994; Chiou et al., 1997) of the \( a \) determinant. Amino acid insertions ranging from 2-8 amino acids have also been described just downstream from the \( a \) determinant, between codon 122-123 and 123-124 which resulted in the patients' HBsAg not being detected on a monoclonal antibody-based ELISA, but were strongly reactive by a polyclonal-based assay (Carman et al., 1995; Hou et al., 1995).

This chapter describes the identification of HBsAg mutants in two renal transplant patients and their reactivity in different HBsAg detection assays.
3.2: Patients

Patients NP and MAM were initially identified as carrying mutant HBsAg due to their discordant results in a number of HBsAg assays. Results of the two patients’ HBsAg reactivity in five commercial assays over a period of time are shown in table 3.2. All serological and sequencing work presented in this chapter were carried out by Dr. Anna Hawkins.

<table>
<thead>
<tr>
<th></th>
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<tbody>
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<td>0.086</td>
<td>0.080</td>
<td>2.569</td>
<td>0.056</td>
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<td>c/o=0.125</td>
<td>c/o=0.183</td>
<td>c/o=0.062</td>
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<td>1:512</td>
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<td>N/T</td>
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<td>c/o=0.125</td>
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</tr>
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</table>

Table 3.2: Reactivity of sera from patients MAM and NP on 4 HBsAg assays at different time points

Sequence analysis of the region encompassing the \( a \) determinant identified multiple point mutations resulting in amino acid substitutions in both patients. No differences were observed in the sequence data from each patient at the various time points. Mutations were found at surface codons 133, 134 and 144 in HBV DNA amplified from patient NP and at codons 133, 134, 142, 144 and 145 in patient MAM. The mutations are listed in table 3.3 and illustrated in figure 3.4.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Codon</th>
<th>Wild Type Codon</th>
<th>Wild Type Amino Acid</th>
<th>Mutant Codon</th>
<th>Mutant Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>133</td>
<td>ATG</td>
<td>Methionine</td>
<td>ATT</td>
<td>Isoleucine</td>
</tr>
<tr>
<td></td>
<td>134</td>
<td>TAT</td>
<td>Phenylalanine</td>
<td>CAT</td>
<td>Histidine</td>
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<tr>
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<td>GAT</td>
<td>Aspartic Acid</td>
<td>GTC</td>
<td>Valine</td>
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<tr>
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<td>Glycine</td>
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</table>

Table 3.3: Amino acid substitutions revealed upon sequence analysis of amplified DNA from patients NP and MAM

Discussion

Prior to 1975, polyclonal antibodies (PAb) formed the main basis for most serological assays. While the specificity of polyclonal antibodies provided a way of overcoming the complexity of biological material, the production of highly specific antisera was difficult and often unreliable. The process required highly purified antigen and uncertainties about the specificity of polyclonal antisera remained. However, with the development of monoclonal antibodies, (Kohler et al., 1975) it was now possible to produce unlimited quantities of highly specific antibodies, regardless of the purity of the immunogen. The fine specificity and affinity of these antibodies allowed them be used for individual purposes.

Nevertheless, monoclonal antibodies have not completely replaced polyclonal antibodies in serological assays. Ironically, sometimes it is the monospecificity of monoclonal antibodies which may be a liability rather than an asset, and in such cases, polyclonal antibodies are the preferred reagent. Many HBsAg detection assays employ either polyclonal or monoclonal antibodies and in some cases both are used.
Figure 3.4: Sequence analysis of the \( a \) determinant of patients NP and MAM

pHBV 130 is subtype \( adyw \) as described by Pugh et al., 1986. Line 1: Consensus amino acid sequence; 2: Consensus nucleotide sequence; 3/4: recognised variant sequence.
Both the VIDAS (Vitek Immuno Diagnostic Assay) and the VK21 assays employ monoclonal antibodies for the HBsAg capture and probe stages. In the case of the VIDAS, the same monoclonal antibody is used on the solid phase as for antigen detection. The monoclonal antibodies used in both of these assays have been raised against WT HBsAg and have been shown to have only weak reactivity for the α determinant. Nevertheless, serum samples from patients NP and MAM (excluding the 1989 MAM sample) were not reactive on either of these assays. The amino acid changes found in these patients will bring about conformational changes to the HBsAg. These changes may be sufficient to incur the loss of the single epitope recognised by the monoclonal antibody resulting in the HBsAg not being captured and/or detected.

This problem can be overcome by using polyclonal antibodies as they present a number of sites to which the antigen can bind and therefore will not be as restricted by conformational changes. Although some discrepancies have been described associated with HBsAg mutants, (Carman et al., 1997), most polyclonal antibody based assays have been shown to detect mutants. Indeed, HBsAg from both patients NP and MAM was reactive in the polyclonal based RPHA (reverse capture haemagglutination assay) and the GE15 HBsAg detection assays.

In the RPHA, highly purified antibody, isolated from horse antiserum to HBsAg, is attached to turkey erythrocytes to yield a sensitised cell suspension that will agglutinate in the presence of HBsAg. The GE15 assay also uses polyclonal antibody specific to HBsAg on the solid phase to capture the antigen. This complex is then
recognised by a mixture of two monoclonal antibodies specific for two different epitopes of HBsAg.

DNA sequencing confirmed the presence of HBsAg mutants in these two patients. Several point mutations in both loops of the \( a \) determinant resulting in amino acid substitutions were revealed upon analysis. It is interesting to note that in patient MAM, the nucleotide sequences did not vary with change in HBsAg specificity. It was initially hypothesised that the patient was harbouring the mutant virus in 1986 which had been replaced by the wild type in 1989. However, the sequencing data did not confirm this. The normal EIA reactivity in 1989 was thought to have been due to the emergence of wild type virus present at less than 15-20% of the total viral load, the limit of detection by direct sequence analysis.
Chapter 4: Production Of Anti-HBs Secreting Monoclonal Antibodies

4.1: Introduction

The development of a technique for the production of monoclonal antibodies by Kohler and Milstein in 1975 provided a powerful tool for use in many biological fields. The creation of an immortal clone of cells which manufacture a single antibody of defined specificity is significant for both diagnostic and research purposes. The monoclonal antibodies act as highly specific probes which can be used to define individual antigenic sites.

Monoclonal antibodies have been raised against the HBcAg (Takahashi et al., 1983; Tedder et al., 1982b) and the HBeAg (Ferns et al., 1984). Several groups have also produced monoclonal antibodies against HBsAg (David et al., 1981; Goodall et al., 1981; Tedder et al., 1982b). The monoclonal antibodies raised have been used not only to characterise the antigenic determinants, but also to develop more sensitive and specific assays.

Recently, numerous studies have reported that many of the current antibodies which have been raised against the WT HBsAg do not detect the mutant HBsAg. The \( \alpha \) determinant has been shown to be a conformationally dependent epitope and it is therefore not surprising that amino acid changes brought about by the point mutations will disrupt this structure. It has been shown that a single point mutation in the immunodominant \( \alpha \) region is sufficient to alter the conformation such that it is no longer recognised by these monoclonal antibodies.

The principle aim of this study was to raise monoclonal antibodies against the mutant HBsAg previously identified in two patients (section 2.1.1). It was hoped that these monoclonal antibodies would act as useful probes when detecting these mutant
surface antigens as well as being used to further the understanding of these novel epitopes.

4.2 Identification And Distribution Of Monoclonal Antibodies

Hybridomas secreting anti-HBs were identified using a reverse capture radioimmunoassay (section 2.1.10). Approximately ten days after the fusions, supernatant from all wells containing clusters of growing cells were tested for anti-HBs activity.

All hybridomas arising from the two fusions were tested for their reactivity against the MAM HBsAg, NP HBsAg and lastly the WT HBsAg. In the first instance, hybridomas from the MAM fusion were tested for anti-HBs activity against the MAM surface similarly hybridomas from the NP fusion were tested for reactivity with the NP HBsAg. It was therefore possible to identify hybridomas secreting antibodies reactive to their homologous surface antigens. Results of the initial screening assays showed that of the 320 MAM hybridomas tested, 32 (10%) were positive for reactivity against the MAM HBsAg while 16 (8%) of the 200 NP hybridomas were positive with the NP HBsAg.

With the results obtained from the primary screening, it was possible to identify those hybridomas secreting anti-HBs which were recognising specifically either the MAM or the NP HBsAg. To determine if the hybridomas raised against the MAM HBsAg would cross react with the NP HBsAg and vice versa, the hybridomas were assayed in the reverse capture RIA, the NP hybridomas were tested for reactivity with $^{125}$I MAM and the MAM hybridomas with the $^{125}$I NP HBsAg. It was found that
4 of the MAM hybridomas recognised and bound to the NP HBsAg while 5 of the NP hybridomas recognised the MAM HBsAg.

Although the hybridomas were derived from mutant HBsAg fusions, it was important to establish if there was any reactivity against the WT HBsAg. Therefore clones from both the NP and MAM fusions, regardless of whether they were positive in the previous assays were also tested in the RIA using $^{125}$I labelled WT HBsAg. Of the 320 MAM hybridomas tested 19 (5.9%) gave positive results while 8 (4%) of the NP hybridomas were found to recognise the WT HBsAg.

Positive and negative controls were included in every screening RIA. The monoclonal antibodies, D2H5 and H3F5 had been previously raised against the WT HBsAg (Tedder et al., 1983) and were used as positive controls. As expected both showed reactivity against the $^{125}$I WT HBsAg. However, D2H5 only recognised the labelled NP HBsAg while H3F5 showed positive results against the MAM HBsAg alone. Mutations in the MAM HBsAg eliminate the epitope for D2H5 while those in NP eliminated the H3F5 epitope (Watts et al., 1994).

Results of the initial screening assays allowed the positive secreting hybridomas to be categorised in accordance with their HBsAg specificity. The possible monoclonal antibody groups are listed below.

1) MAM specific
2) NP specific
3) MAM/NP cross
4) MAM/WT cross
5) NP/WT cross
6) NP/WT/MAM cross
Eighteen hybridomas (eight NP and ten MAM) which fell under one of the categories listed above were chosen for further investigation. The hybridomas were cloned by limiting dilution to prevent overgrowth by non-producing clones which may result in the loss of secretors. This also ensured that the antibody secreted was both homogenous and monospecific. Approximately fourteen days after cloning, wells containing only single colonies were retested in the reverse capture assay for anti-HBs activity. Six of the eight NP hybridomas cloned successfully and were found to retain positivity for anti-HBs. However, cloning of the MAM hybridomas was more difficult with only six of the ten chosen hybridomas being successfully cloned. It has been shown that lymphoid cells often grow poorly or die when grown at low density. It was noted that the majority of MAM parent hybridomas were slow growers despite the addition of feeder cells to the culture medium. A list of the successfully cloned hybridomas and their specificities are shown in table 4.2.1. The results are expressed as binding ratios (p/n). (The prefix M before the name of a clone defines a MAM derived hybridoma while P describes a NP derived hybridoma).

The positive hybridomas derived from the MAM fusion were found to be unstable. For example, retesting of the M3C9 clone as it was passaged gave negative results for anti-HBs activity. This loss of reactivity was thought to be attributed to the overgrowth of non producer cells. After several passages, M3A10 proved to the only stable anti-HBs secreting clone derived from the MAM fusion. Therefore, a second MAM fusion was undertaken.

This fusion was carried out as described above. However, the yield of spleen cells was only 25% of that recorded in the original fusion. After one week, wells
containing clusters of growing cells were tested for anti-HBs activity. As before, the cells were tested with each of the HBsAg labels, \(^{125}\text{I} \text{MAM}\), \(^{125}\text{I} \text{NP}\) and \(^{125}\text{I} \text{WT}\). Of the 68 hybridomas tested, only two produced positive results. One clone was shown to be MAM specific and the other was a NP/WT/MAM cross (table 4.2.2). Both of the hybridomas displayed good cell growth and were cloned by limiting dilution. Cloning on this occasion proved successful and wells containing single colonies were tested in the RIA for anti-HBs activity and found to be positive. The hybridomas were immediately recloned in order to reduce the probability of outgrowth by non producer cells. Table 4.2.3 shows the total number of positive parent hybridomas and their antibody specificities obtained from the NP and the two MAM fusions.

4.3 Propagation Of Monoclonal Antibodies

Once active, cloned hybridomas had been identified, at least 5 positive clones were chosen. Each of the clones was allowed to grow initially in T24 plates and then transferred first to \(25\text{cm}^2\) and finally to \(75\text{cm}^2\) tissue culture flasks. Thymocytes were used as feeder cells in each of the expansion steps to encourage good cell growth. The cloned cells were maintained at a concentration between \(5 \times 10^5\) and \(2 \times 10^6\) cells per ml and frozen down at regular intervals. The hybridomas were retested for anti-HBs activity at each expansion step and all remained positive.

Single clones which were anti-HBs positive and displayed good growth were chosen for further propagation as ascitic tumours in mice. Typical antibody levels in the culture supernatant range from 5-50\(\mu\)g/ml. However for generating larger amounts of antibody, the cells were grown as tumours in mice. The ascites will usually contain
<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Binding ratios of $^{125}$I HBsAg</th>
<th>Antibody Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>MAM</td>
</tr>
<tr>
<td>P2D3</td>
<td>42.0</td>
<td>61.8</td>
</tr>
<tr>
<td>P2C6</td>
<td>1.0</td>
<td>51.5</td>
</tr>
<tr>
<td>P2H6</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>P2H9</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>P4C11</td>
<td>0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>P3E4</td>
<td>10.5</td>
<td>0.8</td>
</tr>
<tr>
<td>M3A10</td>
<td>31.5</td>
<td>51.5</td>
</tr>
<tr>
<td>Control D2H5</td>
<td>105.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Control H3F5</td>
<td>105.8</td>
<td>72.2</td>
</tr>
</tbody>
</table>

Table 4.2.1: Binding Ratio Values Of Hybridomas Derived From The MAM And NP Fusion

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Binding ratios of $^{125}$I HBsAg</th>
<th>Antibody Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>MAM</td>
</tr>
<tr>
<td>M4H2</td>
<td>0.8</td>
<td>82.5</td>
</tr>
<tr>
<td>M4F5</td>
<td>105.2</td>
<td>92.7</td>
</tr>
<tr>
<td>Control D2H5</td>
<td>105.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Control H3F5</td>
<td>105.8</td>
<td>72.2</td>
</tr>
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</table>

Table 4.2.2: Binding Ratio Values Of Hybridomas Derived From The Second MAM Fusion
<table>
<thead>
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<th>Monoclonal Antibody</th>
<th>Binding ratios of $^{125}$I HBsAg</th>
<th>Antibody Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>MAM</td>
</tr>
<tr>
<td>P2D3</td>
<td>42.0</td>
<td>61.8</td>
</tr>
<tr>
<td>P2C6</td>
<td>1.0</td>
<td>51.5</td>
</tr>
<tr>
<td>P2H6</td>
<td>0.9</td>
<td>0.8</td>
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<td>P2H9</td>
<td>0.8</td>
<td>0.7</td>
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<td>P4C11</td>
<td>0.5</td>
<td>0.6</td>
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<tr>
<td>P3E4</td>
<td>10.5</td>
<td>0.8</td>
</tr>
<tr>
<td>M3A10</td>
<td>31.5</td>
<td>51.5</td>
</tr>
<tr>
<td>M4H2</td>
<td>0.8</td>
<td>82.5</td>
</tr>
<tr>
<td>M4F5</td>
<td>105.2</td>
<td>92.7</td>
</tr>
<tr>
<td>Control D2H5</td>
<td>105.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Control H3F5</td>
<td>105.8</td>
<td>72.2</td>
</tr>
</tbody>
</table>

Table 4.2.3: Final List Of Hybridomas Derived From The MAM and NP Fusion With Their Respective Binding ratios.
around 1000 times the antibody concentration in culture. The mice were primed with pristane a week prior to the cells being injected. Pristane irritates the lining of the peritoneal cavity and increases the success rate of tumour development and hence the probability of ascites formation. Mice carrying hybridomas as an ascitic tumour usually exhibited ascites 10-14 days after intraperitoneal inoculation of cells. As soon as the swelling was easily visible, the mice were sacrificed and the ascitic fluid harvested. All 9 of the clones successfully produced an ascitic tumour in mice. The quantity of ascitic fluid obtained ranged between 1.0ml and 8.0ml; in mice where the tumours grew as a solid mass only small volumes of fluid were produced. The yield of ascitic fluid did not appear to be a growth characteristic of individual clones as substantial variation was observed between mice injected with the same monoclonal cells.

4.4 SPE analysis and purification of anti-HBs from ascitic fluid

Monoclonal protein was demonstrated in the ascitic fluid of all of the clones by serum protein electrophoresis (Figure 4.4.1). The electrophoretic mobility of the monoclonal protein band varied slightly for each different monoclonal ascitic fluid, but was identical for different batches of ascitic fluid obtained from the same clone. The disparity in migration distance between clones was a result of the differing ionic charges on the monoclonal proteins. The intensity of staining observed in the SPE monoclonal band gave an indication of the quantity of monoclonal protein present. This varied between ascitic fluid which had been harvested from different clones.

Purification of immunoglobulin from the ascitic fluid was carried out as described in section 2.2.3 and 2.2.4. Immunoglobulin G was prepared by ion exchange
Lane 1: P2C6
Lane 2: P2H9
Lane 3: P4C11
Lane 4: P2H6
Lane 5: P3E4
Lane 6: P2D3
Lane 7: M3A10
Lane 8: M4H2
Lane 9: M4F5

Figure 4.4.1: Demonstration of monoclonal protein band in anti-HBS ascitic fluid by serum protein electrophoresis.
chromatography on DE52 while IgA was purified using a protein A column. Bound proteins were eluted and collected at each peak. The anti-HBs IgG in the different buffer concentrations and the eluted IgA were then measured in the reverse capture assay with $^{125}$I MAM HBsAg, $^{125}$I NP HBsAg and $^{125}$I WT HBsAg. The assay allowed a direct comparison of results between different buffer concentrations to be made. The fraction showing the highest anti-HBs activity was selected and the protein concentrations were determined (Table 4.4.1).

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Quantity of IgG/IgA from ascitic fluid (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4C11</td>
<td>4.00</td>
</tr>
<tr>
<td>P3E4</td>
<td>4.40</td>
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<tr>
<td>P2D3</td>
<td>1.14</td>
</tr>
<tr>
<td>P2H6</td>
<td>2.40</td>
</tr>
<tr>
<td>P2H9</td>
<td>0.90</td>
</tr>
<tr>
<td>P2C6</td>
<td>3.00</td>
</tr>
<tr>
<td>M4F5</td>
<td>1.00</td>
</tr>
<tr>
<td>M4H2</td>
<td>1.70</td>
</tr>
<tr>
<td>M3A10</td>
<td>2.00</td>
</tr>
</tbody>
</table>

Table 4.4.1: Quantity of IgG/IgA harvested from each ascitic fluid

4.5: Isotyping of monoclonal antibodies

Isotyping of the monoclonal antibodies was carried out using a kit from Serotec. The isotyping reagents consist of a panel of purified rat monoclonal antibodies specific
for single subclasses of immunoglobulin, coupled to sheep red blood cells. The coupled rat antibodies recognised the heavy chain portion of the mouse immunoglobulin. The principle of the test system was based on red cell agglutination where a positive agglutinated result is produced when highly specific antibody recognised and bound to the particular isotype to which it is directed. The binding formed a lattice on the bottom of the microtitre plate well. A negative result was produced when the reagent cells were put into a supernatant containing a class of antibody which they did not recognize. The reagent cells fell to the bottom of the well forming a small ‘button’. The isotypes of the monoclonals are shown in table 4.5.1.

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Immunoglobulin Class</th>
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<tbody>
<tr>
<td>P4C11</td>
<td>IgG1</td>
</tr>
<tr>
<td>P3E4</td>
<td>IgG1</td>
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<tr>
<td>P2H6</td>
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<tr>
<td>M4H2</td>
<td>IgG1</td>
</tr>
<tr>
<td>M4F5</td>
<td>IgG1</td>
</tr>
</tbody>
</table>

Table 4.5.1: Immunoglobulin classes of monoclonal antibodies
4.6: Discussion

A typical antibody response is directed at multiple sites on each antigen molecule. Benjamin et al., (1984) showed that in most cases the great majority of the exposed surfaces of proteins will be antigenic. However, some epitopes, referred to as immunodominant, induce a more pronounced immune response. Traditionally, antigenic determinants have been viewed as being either linear or conformational with the latter thought to be the more numerous.

The HBsAg has been identified as containing the major immunodominant epitope of HBV, termed the \(a\) determinant. The majority of anti-HBs found in convalescent and post-vaccination sera binds to this region (Howard et al., 1984). It has been demonstrated that the \(a\) determinant is composed of several conformationally dependent epitopes (Imai et al., 1974) which are sensitive to even minor changes. Immunological studies have shown that many of the described variants are poorly recognised by some monoclonal antibodies as a result of conformational changes in the epitope. Therefore when generating monoclonal antibodies against variant HBsAg, it is essential to ensure that the immunogen conformation is exact in order to reflect the properties of individual proteins.

Although the use of synthetic peptides as immunogens has been shown to be successful for the genesis of antibodies, it cannot be guaranteed that a peptide will represent the true conformation of the protein of interest. This is especially significant when considering complex conformational epitopes such as the \(a\) determinant.

The monoclonal antibodies described in this study were therefore generated against native antigen purified from the serum of two patients (MAM and NP) who were
shown to be harbouring mutant HBsAg. A further benefit of using native antigen is that all post translational modifications associated with the protein are correct.

In order to identify antibody secreting hybridomas, a solid phase radioimmunoassay was chosen. The assay provided a very sensitive and precise method for detecting antibodies. As well as being very easy to set up, the assay is capable of a very low background and a large dynamic range.

Mutations found in the NP and MAM HBsAg will result in a HBsAg conformation which is unique to those changes. By using the NP, MAM and WT HBsAg in the screening assays it was possible to identify those hybridomas secreting anti-HBs capable of detecting either one or combinations of the three HBsAg’s. Thus, the screening assays allowed the hybridomas to be divided into distinct categories.

The MAM and NP specific hybridomas bound to and recognised epitopes present on their respective HBsAg’s alone. These monoclonals showed no cross reactivity. The monoclonal P2C6 showed positive results with both the NP and the MAM HBsAg. The monoclonal antibody must therefore be binding to an epitope which is common to both the NP and MAM HBsAg but absent on the WT HBsAg. P3E4 showed recognition of both WT and the NP HBsAg. The monoclonal antibody is able to bind to a region found on the WT HBsAg which is not lost on the NP HBsAg despite the mutations. However, the region is abolished on the MAM HBsAg. Hybridomas were also identified which showed reactivity with the WT, NP and the MAM HBsAg. These monoclonal antibodies clearly recognise a region on the WT HBsAg which is preserved on the NP and MAM HBsAg despite the conformational changes brought about by the mutations.
The control monoclonal antibodies, D2H5 and H3F5 were originally raised against the WT HBsAg and, as expected, recognised and bound to the WT HBsAg used in the screening assays. Neither of these monoclonal antibodies have been previously mapped and it was not known if they would react against the two mutant HBsAg’s. D2H5 could be classed as a NP/WT monoclonal as it readily recognised the NP HBsAg but the epitope to which it was binding was not present in the MAM HBsAg. The reverse is found with H3F5, that is although positive with the MAM HBsAg, it showed no reactivity with the NP HBsAg at all.

Cloning of the MAM derived hybridomas was initially not successful. The principle of the cloning technique is that every 100μl should nominally contain 1 cell thus ensuring absolute single colonies. The MAM parent hybridomas were observed to be slow growers. Therefore growing the cells at such low densities could have led to cell death as has often been observed with lymphoid cells. Further attempts at recloning the hybridomas also proved unsuccessful despite increasing the quantity of cells used.

Culturing of the MAM derived hybridomas also showed them to be unstable antibody secretors. After cloning, the M3C9 hybridomas were found it to be negative for anti-HBs activity. This could have been due to either overgrowth of the initially positive hybridoma colony by a negative hybridoma colony or a spontaneous cessation of antibody production by the hybridoma.

The codon 145 glycine to arginine mutation in the HBsAg was the first and arguably still the most important change described. This variant has been reported in immunised populations, in liver transplant patients and poses problems in diagnosis because some HBsAg assays are unable to detect this variant. The MAM HBsAg
carries a mutation at codon 145; a change of glycine to lysine. The amino acid change is not the more common glycine to arginine substitution but the significance of the mutation at this position remains the same. Generating a monoclonal antibody which would specifically recognise this major variant was therefore thought to be important. Its use in monitoring the prevalence of the 145 variant in the general population and as a detector in serological assays could be quite important.

It was therefore deemed important to repeat the MAM fusion. With the poor yield of spleen cells with the second fusion only two positive clones were identified, one of which was a MAM specific monoclonal. Cloning on this occasion was more successful and the positive hybridomas were immediately recloned to eliminate any possible growth of non-producer clones.

Expansion of the hybridomas and propagation as ascitic fluid was achieved without difficulties and, upon retesting the hybridomas remained positive. In order to confirm the presence of monoclonal protein in ascitic fluid, SPE was performed. This technique also gave an indication of the quantity of monoclonal antibody produced.

Ion-exchange chromatography was used for the isolation of IgG. The principle of the method is based on proteins binding electrostatically onto an ion-exchange matrix bearing an opposite charge. Proteins are then eluted differentially by increasing the ionic strength of the medium. As the concentration of the buffer ions is increased they compete with the charged groups upon the ion-exchanger. DE52 is a weakly basic DE anion exchanger which allows all proteins to bind except IgG which is then eluted by increasing the ionic strength of the buffer. For the isolation of IgA, a protein A column was used. Protein A binds specifically to the Fc region of the
immunoglobulin. IgA was then eluted by decreasing the pH gradient using 0.1M citric acid (pH 3.0).
Chapter 5 Further Characterisation Of Monoclonal Antibodies

5.1: Introduction

The specificities of the raised HBsAg monoclonal antibodies had been determined by their reactivity against labelled WT HBsAg, MAM HBsAg and NP HBsAg. However the WT or mutant surface antigenic determinants recognised by the monoclonal antibodies were still unknown. Cross competition assays set up between the monoclonal antibodies were carried out to help ascertain this.

5.1: Cross-competition assays

Cross-competition assays were set up where all radiolabelled monoclonal antibodies were allowed to compete individually with cold, unlabelled IgG prepared from itself and each of the other clones. The monoclonal antibodies competed for binding sites on the MAM, NP and WT HBsAg. The results are expressed as percentage inhibition values and are shown in tables 5.1a, 5.1b, and 5.1c.

All unlabelled clones effectively inhibited the binding of label made from their own immunoglobulin. Broadly speaking, the pattern of inhibition divided the panel of monoclonal antibodies into the six groups as described previously (section 4.2). For example, the NP specific antibody P4C11, inhibited itself and other NP specific monoclonal antibodies only. This pattern was repeated in the other five categories. Within each group there was major cross inhibition but between the groups no inhibition was observed. All reactions were specific. No inhibition was found on the HBsAg to which the monoclonal antibody had shown no reactivity. It would appear that the pattern of inhibition represents the recognition of six separate HBsAg determinants.
The control monoclonals were also included in the inhibition experiments to assess their ability to compete with the panel of monoclonal antibodies. D2H5 and H3F5 inhibited themselves on the MAM and NP HBsAg respectively. H3F5 also cross competed with the MAM specific monoclonal antibody M4H2. As expected both the antibodies exhibited inhibition against themselves on the WT HBsAg with no cross inhibition observed at all.
Table 5.1a: Cross competition between unlabelled MAb (at a 1000 molar excess) with labelled MAb for binding to WT HBsAg.  
> 75% inhibition in bold; 50-75% inhibition in plain; • = < 50% inhibition; • = no specific binding
<table>
<thead>
<tr>
<th>Unlabelled Antibody</th>
<th>P4C11</th>
<th>P3E4</th>
<th>P2D3</th>
<th>P2H6</th>
<th>P2H9</th>
<th>P2C6</th>
<th>M4F5</th>
<th>M4H2</th>
<th>M3A10</th>
<th>D2H5</th>
<th>H3F5</th>
</tr>
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<td>•</td>
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<td>•</td>
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Table 5.1b: Cross competition between unlabelled MAb (at a 1000 molar excess) with labelled MAb for binding to MAM HBsAg. > 75% inhibition in bold; 50-75% inhibition in plain; - = < 50% inhibition; • = no specific binding
<table>
<thead>
<tr>
<th>Unlabelled Antibody</th>
<th>P4C11</th>
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<th>P2D3</th>
<th>P2H6</th>
<th>P2H9</th>
<th>P2C6</th>
<th>M4F5</th>
<th>M4H2</th>
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<th>H3F5</th>
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</tr>
<tr>
<td>P3E4</td>
<td>-</td>
<td>90</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>P2D3</td>
<td>-</td>
<td>-</td>
<td>95</td>
<td>-</td>
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<td>92</td>
<td>-</td>
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<tr>
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<td>-</td>
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<td>P2C6</td>
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<td>-</td>
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</tr>
<tr>
<td>M4H2</td>
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<td>•</td>
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</tr>
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<td>-</td>
<td>74</td>
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<td>-</td>
<td>73</td>
<td>-</td>
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<td>-</td>
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<td>98</td>
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<td>H3F5</td>
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<td>•</td>
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<td>•</td>
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</tr>
</tbody>
</table>

Table 5.1c: Cross competition between unlabelled MAb (at a 1000 molar excess) with labelled MAb for binding to NP HBsAg. > 75% inhibition in bold; 50-75% inhibition in plain; - = < 50% inhibition; * = no specific binding.
5.2: Recombinant protein assay

The cross-competition results identified those monoclonal antibodies which were recognizing the same epitope. However, to further establish where in the envelope these epitopes may be, we obtained a purified recombinant HBsAg from SKB. The recombinant protein was expressing the s region (subtype \textit{adw}2) only and therefore did not include any of the \textit{pre S1/pre S2} regions. The recombinant HBsAg was labelled with $^{125}$I and used as the detector in a reverse capture assay (section 2.1.10). The results of the assay are expressed as binding ratios (p/n) and are shown in table 5.2.

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Binding Ratios of $^{125}$I rec HBsAg</th>
<th>Antibody Specificity</th>
</tr>
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<tbody>
<tr>
<td>P2H9</td>
<td>1.0</td>
<td>NP only</td>
</tr>
<tr>
<td>P4C11</td>
<td>0.9</td>
<td>NP only</td>
</tr>
<tr>
<td>P2H6</td>
<td>0.1</td>
<td>NP only</td>
</tr>
<tr>
<td>P2C6</td>
<td>0.6</td>
<td>NP/MAM cross</td>
</tr>
<tr>
<td>P3E4</td>
<td>0.5</td>
<td>NP/WT cross</td>
</tr>
<tr>
<td>P2D3</td>
<td>378.0</td>
<td>NP/MAM/WT cross</td>
</tr>
<tr>
<td>M3A10</td>
<td>347.0</td>
<td>NP/MAM/WT cross</td>
</tr>
<tr>
<td>M4F5</td>
<td>330.0</td>
<td>NP/MAM/WT cross</td>
</tr>
<tr>
<td>M4H2</td>
<td>1.5</td>
<td>MAM only</td>
</tr>
<tr>
<td>Control D2H5</td>
<td>368.4</td>
<td>Control WT monoclonal</td>
</tr>
<tr>
<td>Control H3F5</td>
<td>40.0</td>
<td>Control WT monoclonal</td>
</tr>
</tbody>
</table>

Table 5.2: Reactivity of monoclonal antibodies derived from the MAM and NP fusion to $^{125}$I rec HBsAg in a RIA.
Only the three way cross monoclonals showed positive results with the labelled recombinant HBsAg, confirming that they were recognising and binding to an epitope in the s region. The remainder of the panel of monoclonal antibodies showed no reactivity against the recombinant protein at all.

5.3: Discussion

Cross competition experiments were used to characterise the monoclonal antibodies. Results of this work demonstrated six different patterns of reactivity amongst the anti-HBs monoclonal antibodies. These were assumed to represent the recognition of six different determinants on the HBsAg. The data was quite clear with no inhibition observed across the groups. With a 1000 molar excess of unlabelled antibody to labelled antibody, little inhibition of large quantities of antibody were observed and there therefore appeared no value in titrating out the antibodies. It can therefore be concluded that the monoclonal antibodies which fell into each of the six categories were recognising distinct epitopes.

Of the panel of raised antibodies, only the three way cross monoclonals demonstrated reactivity against the labelled recombinant HBsAg. It was expected that the remaining monoclonal antibodies, if they were indeed binding to determinants in the s region would not show any reactivity against the recombinant protein. The recombinant is based on the wild type and will therefore form its structure in accordance with that sequence. The monoclonal antibodies, however, have been raised against mutant HBsAg, the structure of which is known to be different to that of the WT. It has now been well described that even single amino acid substitutions can bring about dramatic changes in the conformation of the a
determinant. Several studies have shown that monoclonal antibodies raised against the WT HBsAg fail to recognise the mutant HBsAg as a result of conformational differences.

It is therefore not surprising that the monoclonal antibodies will not recognise the WT recombinant HBsAg. These monoclonals will be binding to novel epitopes brought about by amino acid changes found only in the mutant HBsAg.

It can be concluded from the results that the three way cross monoclonals are binding and recognising an epitope in the s region which is found on the WT and preserved on the mutant HBsAg.

The monoclonal antibody, P3E4 showed reactivity with both the WT and NP HBsAg. It would appear that the monoclonal was recognising a region common to the WT and NP HBsAg which was lost on the MAM HBsAg as a result of the mutations present. However, the monoclonal showed no reactivity with the recombinant HBsAg which may suggest that P3E4 is recognising an epitope present in the pre-S region.

The control monoclonals used, D2H5 and H3F5 both raised against the WT HBsAg, although of a different subtype (ayw3) showed positive results with the \(^{125}\text{I} \text{rec HBsAg} \) as expected.
Chapter 6: Optimisation and Characterisation of Solid Phase Assay for HBsAg.

6.1 Introduction

Monoclonal antibodies are by far the most highly selective yet versatile of all biochemical isolation tools. Their usefulness in the identification of particular molecules contained in extremely complex mixtures is unsurpassed. This chapter describes the formatting of the raised monoclonal antibodies into a highly practical, working radioimmunoassay. The assay was then assessed for its ability to detect both mutant and WT recombinant HBsAg.

6.2: Solid Phase RIA using P2D3

The three way cross monoclonal antibodies had shown their ability to detect both the WT and the studied mutant HBsAg and were therefore thought to be clinically and diagnostically the most significant of the panel of raised monoclonal antibodies. Work therefore concentrated on formatting these monoclonal antibodies into a radioimmunoassay.

Two basic assay formats employing different solid phases were compared for their ability to detect HBsAg. Both assays used $^{125}$I P2D3 as the detector monoclonal antibody.

6.2.1: Homologous assay

In the homologous assay, the MAb P2D3 was used for both the capture and detection of HBsAg. Initial experiments focused on optimising the P2D3 concentration on the solid phase to ensure maximum assay sensitivity.
6.2.1.1: Optimisation of solid phase

Purified P2D3 immunoglobulin (1mg/ml) was diluted to concentrations of 1:100, 1:300, 1:1000, 1:3000, 1:10000 and 1:30000 in Tris buffer, before being coated onto breakapart maxisorp wells. After incubating at room temperature overnight, the wells were washed in Tween Saline and then blocked with 3% BSA. One hundred microlitres of a control WT HBsAg (1mg/ml) diluted 1:1000 in NHS were added to the wells and left at room temperature overnight. After washing, 100μl of ^{125}I P2D3 in PBS containing 20% NHS and 2% BSA, were added to the wells. After four hours at room temperature, the wells were washed and the bound reactivity measured. The results of this assay are shown in figure 6.2.1.1. A 1:1000 dilution of P2D3 IgG proved optimal for the capture of HBsAg.

6.2.1.2: HBsAg detection using homologous based assay

P2D3 IgG (1mg/ml) diluted 1:1000 in Tris buffer was coated onto breakapart wells and incubated as described above. After washing with Tween Saline half log_{10} dilutions in NHS of the purified NP, MAM and WT HBsAg were added separately to the wells and incubated overnight at room temperature. The captured HBsAg was detected with ^{125}I P2D3 as outlined above.

6.2.2: HBsAg detection using heterologous based assay

The heterologous assay used a polyclonal anti-HBs on the solid phase to capture HBsAg. This polyclonal antibody is currently used in the Murex Biotech HBsAg detection kit GE15, and wells were supplied by the company pre-coated at the optimum concentration.
Dilution of P2D3 IgG in 0.02M Tris buffer (1mg/ml)

Figure 6.2.1.1: Optimisation of P2D3 solid phase for the homologous based assay using 125I P2D3 as the detector
Under the conditions used for the homologous assay, half log$_{10}$ dilutions of purified NP, MAM and WT HBsAg in NHS, were captured onto polyclonal anti-HBs. This complex was then detected by $^{125}$I P2D3 as described in section 2.4.2.

An illustration of the homologous and heterologous assays is shown in figure 6.2.1. The results of the homologous and heterologous assays are shown in figure 6.2.2 and indicate that the heterologous assay was more sensitive in detecting HBsAg. In any future work described in this study, the heterologous assay is described as the ‘conserved’ assay.

6.3: Optimisation of solid phase assay using MAb s H3F5 and D2H5

Another HBsAg detection assay was optimised using the monoclonal antibodies D2H5 and H3F5.

Purified H3F5 (1mg/ml) was diluted in Tris buffer to concentrations of 1:100, 1:300, 1:1000, 1:3000, 1:10000 and 1:30000 and coated onto breakapart maxisorp wells. The wells were incubated overnight and then washed and blocked as described in section 2.4.1. One hundred microlitres of WT HBsAg (1µg/ml) diluted in NHS were then added. Following overnight incubation, the wells were washed and 100µl of $^{125}$I D2H5 in PBS containing 20% NHS and 2% BSA, were added to the wells. After four hours at room temperature, the wells were washed and bound reactivity measured. The results of the assay are shown in figure 6.3 and clearly indicate that a dilution of 1:1000 was the optimum concentration for HBsAg capture. The H3F5 and D2H5 assay was subsequently termed the ‘WT’ assay.
Figure 6.2.1: Illustration of homologous and heterologous based HBsAg detection assays
Figure 6.2.2: Comparison of two RIA formats on their ability to detect serial dilutions of WT and mutant HBsAg in NHS
Dilution of H3F5 IgG in 0.02M Tris buffer (1mg/ml)

Figure 6.3: Optimisation of H3F5 solid phase for the WT assay using 125I D2H5 as the detector
6.4: Detection of recombinant HBsAg in solid phase assays

Once an optimised assay had been developed, it was important to assess its ability to detect a wider range of HBsAg mutants. Up until this point, only a limited number of HBsAg mutants, such as those found at codon 144 and 145, had been tested with the three way cross monoclonal antibodies.

Wild type and mutant recombinant HBsAg were obtained from Dr. T. Harrison at the Royal Free Hospital School of Medicine, London. Using site directed mutagenesis, single point mutations had been introduced into the small gene as described in section 2.7. Eight HBsAg mutants were produced with mutations ranging from codon 126 to codon 145. All the mutations introduced into the HBsAg conserved the overlapping polymerase gene thus ensuring virus viability.

Once good growth had been established, supernatant fluid from the transfected HepG2 cells was harvested. The 'conserved' and the 'WT' HBsAg assays were then run in parallel and assessed for their ability to detect mutant and WT HBsAg. Both assays included positive and negative controls. A control purified WT HBsAg (1μg/ml) was used as a positive control while supernatant fluid from untransfected HepG2 cells were used as a negative control. The results of these assays are expressed as binding ratios (p/n).

The RIA results shown in table 6.4 demonstrate the ability of the two assays to detect the recombinant HBsAg. Considering results with binding ratios above 1.0 to be positive, all the recombinant HBsAg’s present in the panel were detected in the heterologous assay using P2D3. In comparison, however, only T126S was found to
<table>
<thead>
<tr>
<th>Codon</th>
<th>Amino Acid Change</th>
<th>Nucleotide Changes</th>
<th>Binding ratio of $^{125}$I</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Conserved Assay</td>
</tr>
<tr>
<td>145</td>
<td>gly-arg</td>
<td>gga-aga</td>
<td>20.0</td>
</tr>
<tr>
<td>144</td>
<td>asp-ala</td>
<td>gat-gct</td>
<td>13.2</td>
</tr>
<tr>
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<td>pro-leu</td>
<td>cct-tct</td>
<td>14.0</td>
</tr>
<tr>
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<td>pro-ser</td>
<td>cct-tct</td>
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</tr>
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<td>aaa-gaa</td>
<td>14.3</td>
</tr>
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<td>atg-ttg</td>
<td>10.1</td>
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<tr>
<td>129</td>
<td>gln-his</td>
<td>caa-cac</td>
<td>2.9</td>
</tr>
<tr>
<td>126</td>
<td>thr-ser</td>
<td>act-agt</td>
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<td>-</td>
<td>30.6</td>
</tr>
<tr>
<td>Positive Control</td>
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<td>-</td>
<td>55.2</td>
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<tr>
<td>Negative Control</td>
<td>-</td>
<td>-</td>
<td>0.9</td>
</tr>
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</table>

Table 6.4: Comparison Of Binding Ratios Of WT And Mutant Recombinant HBsAg in the ‘conserved’ and ‘WT’ assays.
be positive on the monoclonal only based assay. As expected both assays recognised the recombinant WT HBsAg.

To confirm the presence of the single point mutations, sequence analysis was undertaken of each of the recombinant HBsAg’s. Initially, the region encompassing the \( a \) determinant was PCR amplified as described in section 2.5. Single stranded DNA sequencing of the biotinylated PCR product was then carried out (section 2.6). Results of the sequencing reconfirmed the presence of single amino acid changes in all the respective HBsAg’s. Analysis of the WT HBsAg revealed no changes in the DNA sequence as expected. The sequence alignments for the recombinant HBsAg’s are shown in figures 6.4a and 6.4b.

6.5: Discussion

Comparison of two different assay formats using the monoclonal antibody P2D3, established that the heterologous based assay was more sensitive when detecting either mutant or WT HBsAg. The polyclonal antibody presents a number of reiterative binding sites on the solid phase. This leads to an increase in antigen capture as a result of cooperative binding of multiple epitopes.

The newly developed HBsAg assay termed the ‘conserved’ assay was then assessed for its ability to detect both WT and a range of recombinant mutant HBsAg. The assay was run in parallel with a second RIA termed the ‘WT’ assay (section 2.4.3). The ‘WT’ assay was modelled on the commercially available Murex Biotech VK21 HBsAg detection kit, where monoclonal antibodies H3F5 and D2H5 are used for the capture and detection of HBsAg respectively. The VK21 kit has repeatedly demonstrated its inability to detect HBsAg mutants. Conformational changes brought
**Figure 6.4a: Sequence analysis around the a determinant of recombinant WT/mutant HBsAg**

<table>
<thead>
<tr>
<th>Codon</th>
<th>Amino Acid Sequence</th>
<th>Nucleotide Sequence</th>
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<td>leuileproglyserthrthrserthrglyprocythrcthrthrproala</td>
<td>gctaattccagagctctacaccaccacgcagccgaccgcgcagactctgtgcagagatactctctatgtctcccctcgt</td>
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<td>Wild Type</td>
<td>c</td>
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<tr>
<td>126S</td>
<td>a</td>
<td>.</td>
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<td>129H</td>
<td>a</td>
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<td>133L</td>
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<tr>
<td>142S</td>
<td>a</td>
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</tbody>
</table>

pHBV 130 is subtype adyw as described by Pugh et al., 1986. Line 1: Consensus amino acid sequence; 2: Consensus nucleotide sequence; 3/4: recognised variant sequence.
Figure 6.4b: Sequence analysis around the \( a \) determinant of recombinant WT/mutant HBsAg

pHBV 130 is subtype \( adyw \) as described by Pugh \textit{et al.}, 1986. Line 1: Consensus amino acid sequence; 2: Consensus nucleotide sequence; 3/4: recognised variant sequence.
about by the mutations result in the HBsAg variants not being detected and/or captured by one or both of the monoclonal antibodies hence leading to false negatives.

The strategic decision to test the recombinant HBsAg in both the ‘WT’ and the ‘conserved’ assay allowed a direct comparison of the assays ability to detect WT and mutant HBsAg.

Results of the radioimmunoassay demonstrate the ability of the monoclonal antibody P2D3 to detect the WT as well as the mutant HBsAg. The point mutations introduced will result in amino acid substitutions. As has now been demonstrated by several studies, amino acid changes in the α determinant severely disrupt this conformationally dependent region.

It is now recognised that mutations found in the second loop severely abrogate the usual epitope structure and result in the loss of immunogenicity. Anti-HBs has been shown to bind predominantly to the second loop (Howard et al., 1984). However, Waters et al., (1992), demonstrated that the epitopes were not strictly confined to one of the loops as antibodies that bind to the first loop were found to be influenced by amino acid changes in the second loop. This inferred that the whole sequence contributes to the antigenic structure. Therefore, it can be assumed that changes introduced into both the first and the second loop of the recombinant HBsAg will be conformationally significant.

Results of the RIA demonstrate that despite the conformational changes brought about by amino acid substitution in both loops, the monoclonal antibody P2D3 is still capable of recognising each of the recombinant HBsAg’s. These results confirm
that three way cross monoclonal antibodies are binding to a region which is maintained on the mutants studied.

In comparison, the second assay, which used the monoclonal antibodies D2H5 and H3F5, only detected T126S. Results from the cross competition assays (section 5.1) reveal that no inhibition was observed between D2H5 and H3F5 concluding that they must be binding to separate epitopes. Although the exact binding sites of D2H5 and H3F5 are not known, it would appear that the single point mutations introduced into the recombinant HBsAg are enough to distort the epitope conformation and therefore abolishing the monoclonal antibody binding sites. The change made at residue 126, however, appears to have only a minor influence on the epitope conformation. Though a reduced binding is observed, the change does not obliterate the epitope and therefore the monoclonal antibodies are still able to recognise the HBsAg.

Although the monoclonal antibody P2D3 detects each of the recombinant mutant HBsAg, a much reduced binding ratio was seen with the T126S and the Q129H changes when compared with the remainder of the recombinant HBsAg’s. The expression levels of the recombinant proteins were not known. The low binding results observed with T126S and Q129H could be attributed to the poor expression of these two particular proteins. However, this cannot be concluded from the study. It should also be noted that the actual binding site of the P2D3 monoclonal antibody had as yet not been identified. It can be speculated that the mutations introduced at codon 126 and/or 129 may result in conformational changes of the P2D3 epitope. These changes would appear to have an effect on the epitope resulting in a reduction in monoclonal binding.
Chapter 7 Liver Transplant Patient Study

7.1 Introduction

End stage liver disease and fulminant hepatic failure associated with hepatitis B virus (HBV) infection have a very poor prognosis and treatment options are limited. Orthotopic liver transplantation (OLT) is usually recommended in these situations; however reinfection of the graft is common, especially if there is a high viral load pre-OLT (Lauchert et al., 1987; Samuel et al., 1991). The prevention of reinfection is crucial or the long term outcome will remain poor in these patients (Benner et al., 1990). The main established treatment option to prevent reinfection is the administration of polyclonal hepatitis B immunoglobulin (HBIG). HBIG reduces the rate of reinfection from about 90% to less than 30% and improves the long term outcome of patients who underwent OLT for HBV related disease (Muller et al., 1991).

Recently, reinfection of liver grafts in some cases has been attributed to the presence of HBsAg escape mutants. HBV uses the error prone reverse transcriptase for its replication. It is thought that this leads to the generation of mutants which will be selected for under the immunological drive. The addition of either polyclonal or monoclonal antibodies to many virus cultures has been shown to result in isolates which are not neutralised by the added antibody (Lemon et al., 1990). It is therefore not surprising that patients given either polyclonal or monoclonal antibody therapy will select for escape mutants.

The \( a \) determinant is a target for the humoral immune response and a large proportion of the anti-HBs found in immune serum is directed against it (Howard et al., 1984). Mutations are selected in and around this determinant in patients who
have received active and passive immunisation against HBV (Carman et al., 1990; Harrison et al., 1991; Waters et al., 1992). Mutations have been seen in patients given both monoclonal anti-α (McMahon et al., 1992) and more surprisingly HBIG (Carman et al., 1996) after OLT. The generation of these HBsAg mutants therefore presents a major problem in the efficacy of HBIG post OLT and calls for the reassessment of HBIG immunoprophylaxis in liver transplant patients. Once established the HBsAg mutants may pose further problems associated with increased morbidity and diagnostic difficulties.

A series of patients who had undergone OLT were studied. A number of these patients went on to reinfect their grafts. To determine if this was due to the escape mutants selected by exposure to HBIG or therapy failure, both pre and post transplant samples from these patients were investigated for the presence of mutant or WT HBsAg by immunological and sequencing techniques. These results were then compared with those patients who had undergone similar immunosuppressive regimens but without HBIG treatment.

### 7.2 Patients

Twenty six patients underwent OLT for end stage HBV-related liver failure. At the time of transplantation, six patients were positive for HBeAg and HBV DNA. The remaining patients were HBeAg negative, anti-HBe positive, with serum HBV DNA detected, by the liquid phase hybridisation assay, in five. Seven of the patients had Delta superinfection, while all were negative for antibodies against HCV.

After transplantation, the patients received triple immunosuppression with prednisolone, cyclosporine and azathioprine. Corticosteroids were usually
discontinued between three to six months post-OLT. All patients, except seven who were transplanted before 1989, received HBIG immunoprophylaxis with 5000 IU weekly for the first month and then 2000 IU monthly to maintain an anti-HBs titre above 100 IU/ml. Immunoprophylaxis was discontinued after diagnosis of HBV reinfection defined as reappearance of the HBsAg in serum and liver graft.

Details of the patients HBV serological markers and HBIG prophylaxis are given in table 7.2.

It was possible to divide the patients into three groups based on whether they had reininfected their liver grafts and if they were receiving HBIG immunoprophylaxis. Group A was comprised of 12 patients who reininfected the graft despite receiving HBIG prophylaxis; group B included 7 patients who had not received HBIG and reininfected their grafts; group C consisted of 7 patients who had received HBIG and did not reinfect their liver grafts.

7.3 Monitoring of HBsAg

Two assays based on separate formats were used for HBsAg detection. Briefly, in the ‘conserved’ assay, the polyclonal antibody solid phase was used to capture HBsAg present in patients’ sera (chapter 6) which was then detected by radiolabelled P2D3. This assay had been shown previously, to be capable of recognising WT and a wide selection of mutant HBsAg. The second assay, termed the ‘WT’ assay was run in parallel with the ‘conserved’ assay and used monoclonal antibodies, D2H5 and H3F5 for capture and detection respectively as described in chapter 6. The results of each patient group are shown in tables 7.3a, 7.3b and 7.3c. The results are expressed as binding ratios (p/n) where a value over 1.0 is considered as positive.
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<thead>
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<th>Patient</th>
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<th>HBeAg</th>
<th>HBV DNA pg/ml</th>
<th>Anti-HDV</th>
<th>HBIg Received</th>
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<tbody>
<tr>
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<td>1:1600</td>
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<td>Yes</td>
</tr>
<tr>
<td>A8</td>
<td>1:12800</td>
<td>-</td>
<td>Neg</td>
<td>+</td>
<td>Yes</td>
</tr>
<tr>
<td>A9</td>
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<td>-</td>
<td>2.6</td>
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</tr>
<tr>
<td>A10</td>
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<td>-</td>
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</tr>
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<td>-</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>46</td>
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<td>131</td>
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<td>9</td>
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</tr>
<tr>
<td>B5</td>
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<td>66</td>
<td>+</td>
<td>No</td>
</tr>
<tr>
<td>B6</td>
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<td>-</td>
<td>Neg</td>
<td>-</td>
<td>No</td>
</tr>
<tr>
<td>B7</td>
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<td>-</td>
<td>72</td>
<td>-</td>
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</tr>
<tr>
<td>C2</td>
<td>1:1600</td>
<td>-</td>
<td>Neg</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>C3</td>
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<td>Yes</td>
</tr>
<tr>
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<tr>
<td>C5</td>
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<td>-</td>
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<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>C6</td>
<td>1:25600</td>
<td>-</td>
<td>Neg</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>C7</td>
<td>1:3200</td>
<td>-</td>
<td>Neg</td>
<td>-</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 7.2: HBV markers and prophylaxis data on 26 patients who underwent OLT
<table>
<thead>
<tr>
<th>Patient</th>
<th>Pre/Post Transplant Sample</th>
<th>Binding Ratio Of $^{125}$I Anti-HBs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Conserved Assay</td>
</tr>
<tr>
<td>A1</td>
<td>pre</td>
<td>33.8</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>34.8</td>
</tr>
<tr>
<td>A2</td>
<td>pre</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>0.9</td>
</tr>
<tr>
<td>A3</td>
<td>pre</td>
<td>41.6</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>38.7</td>
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<td>A4</td>
<td>pre</td>
<td>27.2</td>
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<td></td>
<td>post</td>
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<td>37.5</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>39.1</td>
</tr>
<tr>
<td>A6</td>
<td>pre</td>
<td>34.1</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>35.8</td>
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<td>27.6</td>
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<tr>
<td></td>
<td>post</td>
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</tr>
<tr>
<td>A8</td>
<td>pre</td>
<td>37.9</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>27.1</td>
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<td>A9</td>
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<td>35.7</td>
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<td>31.6</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>36.3</td>
</tr>
<tr>
<td>A11</td>
<td>pre</td>
<td>37.6</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>37.5</td>
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<td>post</td>
<td>41.5</td>
</tr>
<tr>
<td>PBS (neg control)</td>
<td>pre</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>-</td>
</tr>
<tr>
<td>Purified WT HBsAg</td>
<td>pre</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>-</td>
</tr>
<tr>
<td>Purified MAM HBsAg</td>
<td>pre</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>-</td>
</tr>
<tr>
<td>Purified NP HBsAg</td>
<td>pre</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 7.3a: HBsAg binding ratios in the 'conserved' and the 'WT' assay of pre and post-OLT samples in 12 patients in whom HBV reinfection occurred in spite of HBIG therapy.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Pre/Post Transplant Sample</th>
<th>Binding Ratio Of $^{125}$I Anti-HBs</th>
<th>Conserved Assay</th>
<th>Wild Type Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>pre</td>
<td>0.6</td>
<td>80.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>1.4</td>
<td>93.8</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>pre</td>
<td>0.9</td>
<td>106.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>1.3</td>
<td>116.8</td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>pre</td>
<td>36.4</td>
<td>60.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>35.7</td>
<td>55.7</td>
<td></td>
</tr>
<tr>
<td>B4</td>
<td>pre</td>
<td>37.4</td>
<td>71.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>36.8</td>
<td>65.2</td>
<td></td>
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<td>B5</td>
<td>pre</td>
<td>37.2</td>
<td>77.0</td>
<td></td>
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<td></td>
<td>post</td>
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<td>56.1</td>
<td></td>
</tr>
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<td>B6</td>
<td>pre</td>
<td>1.6</td>
<td>87.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>1.4</td>
<td>38.3</td>
<td></td>
</tr>
<tr>
<td>B7</td>
<td>pre</td>
<td>21.6</td>
<td>112.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>31.4</td>
<td>104.9</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>(neg control)</td>
<td>-</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Purified WT HBsAg</td>
<td>-</td>
<td>78.0</td>
<td>115.3</td>
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<td>Purified MAM HBsAg</td>
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<td>65.0</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Purified NP HBsAg</td>
<td>-</td>
<td>58.2</td>
<td>0.9</td>
<td></td>
</tr>
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</table>

Table 7.3b: HBsAg binding ratios in the ‘conserved and ‘WT’ assays of pre and post-OLT samples in 7 patients who did not receive HBIG and went on to reinfect their livers.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Pre/Post Transplant Sample</th>
<th>Binding Ratio Of $^{125}$I Anti-HBs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Conserved Assay</td>
</tr>
<tr>
<td>C1</td>
<td>pre</td>
<td>38.4</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>3.4</td>
</tr>
<tr>
<td>C2</td>
<td>pre</td>
<td>24.8</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>4.3</td>
</tr>
<tr>
<td>C3</td>
<td>pre</td>
<td>25.4</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>2.1</td>
</tr>
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<td>C4</td>
<td>pre</td>
<td>42.4</td>
</tr>
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<td></td>
<td>post</td>
<td>3.2</td>
</tr>
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<td>C5</td>
<td>pre</td>
<td>42.1</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>3.8</td>
</tr>
<tr>
<td>C6</td>
<td>pre</td>
<td>0.9</td>
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<td>1.0</td>
</tr>
<tr>
<td>Purified WT HBsAg</td>
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<td>78.0</td>
</tr>
<tr>
<td>Purified MAM HBsAg</td>
<td>-</td>
<td>65.0</td>
</tr>
<tr>
<td>Purified NP HBsAg</td>
<td>-</td>
<td>58.2</td>
</tr>
</tbody>
</table>

Table 7.3c: HBsAg binding ratios in the 'conserved' and 'WT' assays of pre and post-OLT samples in 7 patients who received HBIG and did not reinfect their livers.
Of the 12 patients found in group A, pre and post OLT samples from 7 were positive on both the ‘conserved’ and ‘WT’ HBsAg assays confirming infection by HBV expressing the WT HBsAg. In four patients (A5, A7, A8 and A12), post OLT specimens were reactive on the ‘conserved’ assay only, showing no reactivity on the ‘WT’ assay. It is known that the monoclonal antibodies used in the ‘WT’ assay will not recognise mutant HBsAg and therefore confirms the presence of mutant HBsAg in these patients. In one of these patients, (A12), both pre and post OLT specimens were reactive only on the conserved assay suggesting that an HBsAg mutant was present in the patient before and persisted after transplant and through the follow up period.

In the remaining patient in group A (A2), pre and post OLT specimens were reactive on the ‘WT’ HBsAg assay indicating infection with WT HBsAg. However, both specimens were repeatedly negative on the ‘conserved’ HBsAg assay.

Group B consisted of 7 patients who did not receive HBIG after OLT. All of these patients went on to reinfect their liver grafts. All pre and post OLT sera reacted in the WT assay showing that all the patients were infected with WT HBsAg. However, in the pre OLT samples of 3 patients (B1, B2 and B6), no reactivity was observed on the ‘conserved’ assay. As with patient A2, the samples were retested, but as before the HBsAg was only detectable on the ‘WT’ assay.

The final group, C, is made up of 7 patients who received HBIG and did not reinfect their grafts after transplantation. RIA results on pre OLT samples reconfirmed that all patients were harbouring virus with WT HBsAg. However, one of these patients was only positive on the ‘WT’ assay, being unreactive on the ‘conserved’ assay. As
was expected, no reactivity was seen on either assay when testing the post-OLT samples.

Sequence analysis of the region encompassing the α determinant was undertaken in the post OLT samples of patients from group A and B, and pre-OLT samples of group C who were reactive in both the 'conserved' and 'WT' HBsAg assays. The sequencing alignments are shown in figures 7.3.1, 7.3.2 and 7.3.3.

Sequencing was also carried out on both pre and post OLT samples of patients who were negative on the 'WT' assay only. The results reconfirmed the presence of HBsAg mutants in post OLT samples from patients A5, A7 and A12; in patient A8 the mutant was found in both pre and post OLT samples. A single point mutation in patients A5 and A8 resulted in an amino acid change at codon 145 of glycine for arginine while patient A7 revealed a change at codon 144 of aspartic acid to glycine. However, the sequence of patient A12 showed a double mutant where glutamic acid replaced aspartic acid at codon 144 and glycine was substituted for arginine at codon 145. Sequence alignments illustrating the mutations are shown in figure 7.3.4. All the mutations identified were present in the second loop of the α determinant.

The entire s region from post-OLT samples of the five patients who were unreactive on the 'conserved' assay were also sequenced. It was thought that these five patients may harbour a virus strain carrying a common mutation in the region to which the P2D3 monoclonal is binding. These changes may have altered the P2D3 epitope but have no effect on the conformation of the immunodominant region. However, sequences generated from these patients revealed no significant changes in the s region. The sequence analysis of the α determinant of these patients is shown in figure 7.3.5.
Figure 7.3.1: Sequence alignments of patients’ post OLT samples reactive on both ‘conserved’ and ‘WT’ assays (Group A)

pHBV 130 is subtype \textit{adyw} as described by Pugh \textit{et al.}, 1986. Line 1: Consensus amino acid sequence; 2: Consensus nucleotide sequence; 3/4: recognised variant sequence.
Figure 7.3.2: Sequence alignments of patients’ post OLT samples reactive on both ‘conserved’ and ‘WT’ assays (Group B)

**pHBV 130**

<table>
<thead>
<tr>
<th>Codon</th>
<th>Amino Acid Sequence</th>
<th>Nucleotide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>122</td>
<td>Leuileglyserserthrthrserthr</td>
<td>caatatccaggatcatcaaccaccagcagcgtggacctgcaagaacctgactctgctctgactccctcctgt</td>
</tr>
<tr>
<td>160</td>
<td>Cyscysthrlysproserasp</td>
<td>tgctgtacaaaccttcggatggaaactgcacctgtattcccatcccatcatctgggctttcggaaaattcctatgggag</td>
</tr>
</tbody>
</table>

pHBV 130 is subtype *adyw* as described by Pugh *et al.*, 1986. Line 1: Consensus amino acid sequence; 2: Consensus nucleotide sequence; 3/4: recognised variant sequence.
Figure 7.3.3: Sequence alignments of patients' pre-OLT samples reactive on both 'conserved' and 'WT' assays (Group C)

pHBV 130 is subtype adyw as described by Pugh et al., 1986. Line 1: Consensus amino acid sequence; 2: Consensus nucleotide sequence; 3/4: recognised variant sequence.
Figure 7.3.4: Pre and post OLT a determinant sequence alignments of patients reactive on 'conserved' assay only (nucleotide substitutions resulting in amino acid changes are shown in red).

Sequence is subtype adyw as described by Pugh et al., 1986. Line 1: Consensus amino acid sequence; 2: Consensus nucleotide sequence; 3/4: recognised variant sequence.
Figure 7.3.5: Sequence alignments of patients sera reactive on the ‘WT’ assay only. (Residues in red indicate those amino acid changes which result in the loss of P2D3 activity)

<table>
<thead>
<tr>
<th>Codon</th>
<th>Amino Acid Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>122</td>
<td>Met &gt; Thr</td>
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</tbody>
</table>

**pHBV 130**

<table>
<thead>
<tr>
<th>Codon</th>
<th>Amino Acid Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>160</td>
<td>Cys &gt; Ser, Cys &gt; Thr, Lys &gt; Pro, Ser &gt; Asp, Gly &gt; Asn</td>
</tr>
</tbody>
</table>

pHBV 130 is subtype adyw as described by Pugh *et al.*, 1986. Line 1: Consensus amino acid sequence; 2: Consensus nucleotide sequence; 3/4: recognised variant sequence.
7.4: Discussion

HBV reinfection of the liver graft due to HBsAg escape mutants has already been defined as a clinical entity in the OLT setting (McMahon et al., 1992).

It is not surprising that the addition of monoclonal pharmaceuticals will lead to isolates that are not neutralised by that antibody. The change of viral antigenic structure required to avoid neutralisation involves the loss of a single epitope. However, HBIG contains polyclonal antibody and it would be reasonable to assume that patients treated with polyclonal antibodies would be under less pressure to select mutations in the surface antigen. Nevertheless, HBsAg mutants have been described in patients who have undergone orthotopic liver transplantation and were receiving HBIG (Carman et al., 1996).

In this study, the prevalence of HBsAg mutants in pre and post OLT samples of patients who have undergone HBIG immunoprophylaxis are investigated.

Two assays were employed for HBsAg detection as described previously. The monoclonal antibody P2D3 has been shown to recognise both the WT and mutant HBsAg as it binds to a region present on the WT HBsAg which is not affected by the described mutations in the HBsAg. This assay was run in parallel with the monoclonal antibody based ‘WT’ HBsAg assay. The assay is based on the Murex VK21 assay which is known not to detect virus bearing 144 and 145 mutations. The two monoclonal antibodies used in this assay had not been previously mapped but were known to recognise separate regions in the α determinant as observed by inhibition studies (chapter 5). However, conformational changes brought about by amino acid substitutions in the α determinant disrupt the epitopes to which these
monoclonals antibodies are directed. Therefore, HBsAg carrying mutations will not be captured and/or detected. Only WT HBsAg will be recognised. In the study described here, running the two assays in parallel seems to allow easy differentiation between WT and mutant infections. Results of the binding studies identified four patients (15%) who were confirmed to be infected with the HBsAg mutant. The pre-OLT samples of patients A5, A7 and A12 were positive for both the ‘conserved’ and ‘WT’ assays indicating the presence of the WT HBsAg in these samples. However, results of serum samples tested following transplant clearly shows the emergence of HBsAg mutants in all three patients. No HBsAg mutants were detected in the control group who had not received HBIG post transplantation but had undergone identical immunosuppressive regimens as the patients in group A. On the basis of these results it would appear that anti-HBs, in the form of HBIG, is contributing to the emergence of amino acid substitutions in treated OLT patients. Immunosuppression per se does not lead to mutations in the HBsAg, even with high levels of replication. However, in patient A8, HBsAg mutant was detected in both the pre and post OLT samples. The selection pressure in this case is harder to explain but HBsAg mutants have been described in patients who received neither vaccine nor HBIG (Yamamoto et al., 1994; Carman et al., 1995). It is thought that host immune pressure alone is able to drive the selection though the occurrence of this event is much more rare.

The use of HBIG has been recommended for all patients being transplanted for HBV associated liver disease. The major source of graft infection is thought to be due to residual circulating virions in blood during or soon after transplantation. Virions are also found replicating at extrahepatic sites. HBIG is thought to interfere with this process thus preventing virus from entering hepatocytes (Feray et al., 1990); the
main aim of HBIG administration being the abolishing of circulating virus. Evidence based on recent work and as shown in this study, demonstrated that some OLT patients undergoing HBIG administration will select escape mutants and therefore will go on to reinfect their liver grafts. In view of this, HBIG immunoprophylaxis in liver transplant patients needs to be reassessed.

Radioimmunoassays used in this study allow the easy discrimination between wild-type and mutant infections. The detection of HBsAg mutants post OLT will allow for better monitoring of patients and the use of alternative prophylaxis. Recent trials using nucleoside analogues after OLT have been encouraging (Boker et al., 1994; Grellier et al., 1995; Kruger et al., 1995). The possibility of identifying HBsAg mutants before transplantation provides a useful predictor for employing alternative appropriate treatment post OLT.

The presence of the HBsAg mutants was confirmed by sequence analysis of the region encompassing the \( \alpha \) determinant. All the mutations were found in the second loop of the immunodominant region at positions 144 and/or 145. These results are in keeping with the observation by Wallace et al., (1997) that the majority of mutations detected in OLT patients given anti-HBs (either anti-MAb or HBIG) are clustered in the second loop.

In 19 of the patients who went on to reinfect their liver grafts, HBV DNA was detectable in all pre-OLT samples, by a single round of PCR. In contrast, of the 7 patients who did not reinfect their liver grafts HBV DNA was detectable in their pre-OLT samples by a nested PCR only (250 copies/ml). Although the sample numbers are few it would appear that a high viral load before transplant is a key factor in determining the final outcome in OLT for HBsAg positive cirrhosis. It will be more
difficult for patients with a high viral load to clear the virus and therefore increases their probability of reinfecting their liver grafts. High viral load also indicates a rapid viral turnover which may result in the accumulation of polymerase errors in the HBV genome, some of which will be positively selected. Results from this study suggest that HBIG after OLT imposes a selection pressure in some patients on the s gene, and that mutations are one mechanism for reinfection while receiving HBIG. Initial analysis by the liquid phase hybridisation assay identified only 11 out of 19 of these patients who subsequently reinfections their grafts as having HBV DNA present in pre-OLT samples. The hybridisation assay is not a sensitive enough technique for the detection of HBV DNA.

It is interesting to note that the 'conserved' assay failed to identify 5 patients in spite of the WT phenotype. The patients are found across all three groups which is suggestive that the lack of reactivity observed was not associated with HBIG administration. All the samples were positive on the 'WT' assay confirming that these patients were harbouring the WT virus and therefore should have been detected by the 'conserved' assay.

It was speculated that these patients may carry changes at the region to which the monoclonal antibody recognises and binds to. These changes may be deleterious to the P2D3 epitope but have no effect on the conformation of the immunodominant region at all. This would account for the lack of reactivity observed on the 'conserved' assay. It had thus far been established that the three way cross monoclonals were indeed recognising an epitope found in the s region (chapter 5) but it was not known where exactly this region may be. It was therefore thought
necessary to analyse the complete s region sequence. The sequence of three patients who were positive on both assays were also analysed and acted as controls.

However, the sequence data generated on these patients were unremarkable. Upon analysis, only changes associated with described genotype variability were noted in the four patients. Alignment of these sequences against the three control sequences identified two amino acid changes found only in the patients which were being missed on the ‘conserved’ assay.

These two changes were located within the $\alpha$ determinant at amino acid positions 125 and 127. It was therefore possible to align the $\alpha$ determinant sequence from all the patients in the study to assess the frequency of these two changes. The results proved to be extremely interesting. Only the patients who were negative upon testing in the ‘conserved’ assay displayed both of the changes at amino acids 125 and 127 in their $\alpha$ determinants. These changes are associated with amino acid substitutions of threonine for methionine at codon 125 and proline for threonine at codon 127.

Figure 7.4 shows a multiple alignment of the six genotypes of HBV in the amino acid region of 101 to 180 as described by Norder et al., (1992). It would appear that these two changes are exclusive to the described variability associated with genotype D subtype $\text{ayw3}$. The patients A2, B1, B2, B6 and C6 were confirmed as being of genotype D subtype $\text{ayw3}$. None of the remaining patients in this study were of genotype D.

The entire nucleotide sequences of HBV genomes of various subtypes have been classified into genetic groups designated A to F based on an intergroup divergence of 8% or greater of the complete nucleotide sequence (Okamoto et al., 1988). The group D genome is found worldwide but appear to be predominant in the
Figure 7.4: Naturally occurring amino acid variation of the s gene from codons 101-180 (according to Norder et al., 1993). (Variation at codons 125 and 127 shown in blue)

<table>
<thead>
<tr>
<th>Genomic Group</th>
<th>Subtype</th>
<th>101</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>A adw</td>
<td></td>
<td>QGMLPVCPLIPGSTTTSTGPCKCTCTTAPAQGNSMFPSCCCTKPTDGNCTCIPSSWAFAKYLWEWASVRFSWLSLLVPFV</td>
<td></td>
</tr>
<tr>
<td>A adw2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B adw2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A ayw1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B ayw1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D ayw2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D ayw3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E ayw4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F adw4q-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C adrq-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C adrq+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C ayr</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Mediterranean area, the Near and Middle East (Norder et al., 1993). Genotype D encompasses the ayw 4 and ayw3 subtypes.

The monoclonal antibodies in this study have been raised against the mutant HBsAg of two patients, NP and MAM. The genotypes of these patients are C and A respectively and they therefore carry amino acids threonine and proline at codon 125 and 127. The folding of the protein as influenced by the amino acids present in this region will be different in genotype D. Epitopes present in genotype A and C may be lost in genotype D as a result of conformational changes and vice versa. This may explain why the P2D3 monoclonal antibody is unable to recognise patients infected with the genotype D virus. The RIA and sequencing results on these patients supports the argument that the three way cross monoclonal antibodies are binding to a region in the first loop of the α determinant.

Further evidence supporting the significance of these two changes was provided when testing two samples provided by Professor Colin Howard of the Royal Veterinary College. A 141 lysine to glutamic acid mutant had been introduced into a HBsAg backbone based on the ayw4 subtype. The mutant as well as the WT HBsAg were tested in both the ‘WT’ and the ‘mutant’ assay. Results of the RIA showed that the WT HBsAg alone displayed reactivity and only on the ‘WT’ HBsAg assay (data not shown). It was expected that the 141 mutant HBsAg would not be detected on the ‘conserved’ assay. Analysis of the sequence revealed that even though a threonine was found at codon 125, the proline at codon 127 had been replaced by a leucine; this sequence being typical of ayw4.

It would appear that this single change is sufficient to completely abolish the epitope and it is therefore no longer recognised by the monoclonal antibody. This however,
is not completely surprising as the change itself in terms of possible conformational alterations is quite significant as proline plays an important role in dictating turns in polypeptide chains.

In summary, it has been shown that the three way cross monoclonal antibodies are binding to an epitope present in the s region which is found on the WT HBsAg but preserved on mutant HBsAg. As revealed by sequence analysis of the patients who were not detected by P2D3, the region encompassing codon 125 and 127 in the first loop of the \(a\) determinant may form the epitope of the three way cross monoclonal antibodies. The evidence from this study supports this suggestion but this still remains to be reconfirmed.
Chapter 8: Phage Display Library Work

8.1: Introduction

Evidence based on the results obtained thus far indicated that the three way cross monoclonal antibody, P2D3 was binding to a region which is found on the WT HBsAg and is conserved on the studied mutant HBsAg. Random 6-mer and 15 mer fusion-phage libraries were screened to isolate phage that bound to the monoclonal antibody in an attempt to identify this region. Elucidation of the epitope sequence involved would assist in future understanding of the antigenic structure.

Peptide libraries on filamentous phage essentially provide an unbiased approach which has proved to be very powerful for determining native epitopes recognised by monoclonal antibodies (Scott et al., 1990; Cwirla et al., 1990; Felici et al., 1991). These studies show that the phage-display method can provide a simple route to high-resolution mapping, which yields information about the contribution of individual amino acids to the binding of the antibody which could not be obtained as readily with most methods based upon synthetic peptides or deletion analysis.

The procedure entails affinity screening the library against the antibody, isolating the phage that bind and determining the peptide sequence through DNA sequencing of the phage genome.

8.2: Phage Display Peptide Library

The filamentous virion consists of a stretched out loop of single stranded DNA sheathed in a tube composed of the major coat protein. Four minor coat proteins are found at the tips of the virion; the protein pIII being of particular interest. The gene III protein tolerates foreign amino acid inserts which are cloned into the phage gene
III, producing so-called ‘fusion phage’ that retain phage function and display the foreign amino acids on the surface (Smith 1985). Fusion phage whose displayed determinant binds an antibody can be selected from the background of non-binding phage by affinity purification.

Methods describing the production of the 6-mer and 15-mer libraries are detailed in Scott et al., 1990. Briefly, the epitope library was constructed using the vector fUSE 5 which is derived from fd-tet (Smith 1988). Phage fd-tet (Zacher et al., 1980) confers tetracycline resistance on the host and thus can be propagated like a plasmid independently of phage function. The filamentous phage do not kill their host, a single infection event suffices to give a detectable tetracycline resistant transductant clone, which can be propagated without further cycles of selection. The phage carry their inserts just downstream of the pIII signal peptide. These foreign inserts do not significantly debilitate pIII either during morphogenesis, when it is incorporated into the virion from the inner membrane or during infection when it binds the F pilus.

The library itself was made by ligating a synthetic 33 bp Bgl I fragment into fUSE 5 and transfecting E.coli cells with the ligation product by electroporation. Within the Bgl I fragment was the degenerate coding sequence (NNK)15; where N stands for an equal mixture of the deoxynucleotides G, A, T and C and K stands for an equal mixture of G and T. Thus the 33 bp fragment is an equal mixture of different nucleotide sequences. The fUSE 5 vector has two Sfi I sites along with a frameshift mutation engineered into gene III. The degenerate oligonucleotide inserts are ligated into the Sfi I sites. The inserts restore the reading frame, leading to a recombinant gene III protein that is incorporated as a ring of five group III molecules at the tip of the virion.
8.3: Affinity Purifying Target Clones: Biopanning

The biopanning carried out in this study can be divided into two experiments. Experiment 1 was designed to establish the optimum conditions for affinity purification. Each monoclonal antibody is unique and so by varying the temperature as well as the salt concentrations of the washes it was hoped to isolate high affinity phage binding specifically to monoclonal antibody P2D3. However, analysis of sequences generated from this study revealed that the fUSE 5 vector had appeared to have lost the random peptide inserts. It was speculated that the inserts may have been lost during the biopanning and subsequent propagation steps. However, further analysis of the primary library showed that the input phage also contained no insert. The reason for this loss cannot be explained.

It was therefore elected to repeat the investigation, using new 6-mer and 15-mer libraries (experiment 2). Unfortunately due to time restrictions the biopanning was undertaken at previously described conditions (Germaschewski et al., 1995). The data presented below is that generated from experiment 2.

Three rounds of biopanning were undertaken before propagating and sequencing individual clones. The initial step prior to each round involved immobilising the monoclonal antibody P2D3 and control BSA onto a nitrocellulose membrane as is described in section 2.9.3. In the first round, the immobilised antibody and BSA control were reacted with an aliquot of the primary library. Following incubation, any free phage were washed away and phage bound on the nitrocellulose membrane eluted in acid and then returned to neutral pH. The phage were shown to retain their infectivity under these conditions.
The first round eluates were grown up as described in 2.9.4. The titre of infective phage were determined by first adsorbing suitable dilutions of the phage onto *E.coli* K91 kan cells. The mixture was grown in selective medium for 45 minutes before being spread onto tetracycline/kanamycin plates. The next day, the colonies on the plates were counted and the phage yield from biopanning calculated. The results of the phage yield for the monoclonal antibody P2D3 and control BSA with both the 6-mer and 15-mer libraries in each of the rounds are shown in tables 8.3.1, 8.3.2 and 8.3.3.

The first round eluates were grown up overnight in 20ml cultures. The phage were then purified by PEG/NaCl precipitation yielding the amplified eluate. To determine the total yield of infected clones represented in the amplified eluate, 10µl of a suitable dilution of the purified phage were titred as before on K91 kan *E.coli* cells and are shown in tables 8.3.4, 8.3.5 and 8.3.6.

In contrast to the first round, subsequent rounds of biopanning used the amplified eluates from the previous rounds as the input phage.

### 8.4: Phage DNA Analysis

The final eluate was titred on K91 kan *E.coli* cells both to determine the yield and to provide clones for analysis by sequencing. Individual colonies were propagated in overnight cultures and fd-DNA prepared using a QIA prep spin M13 kit (Qiagen). The kit provides silica-gel membranes which bind to single stranded DNA. The DNA was then easily eluted using a low salt buffer i.e. 1mM Tris pH 8.5. Sequencing of the single stranded DNA was undertaken as described in section 2.9.6.
### Table 8.3.1: Phage Yield From First Round Of Biopanning

<table>
<thead>
<tr>
<th>Clone</th>
<th>Phage Library</th>
<th>Phage Yield (TU in 20ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2D3</td>
<td>15-mer</td>
<td>1.72 \times 10^3</td>
</tr>
<tr>
<td>BSA</td>
<td>15-mer</td>
<td>8.1 \times 10^4</td>
</tr>
<tr>
<td>P2D3</td>
<td>6-mer</td>
<td>7.5 \times 10^4</td>
</tr>
<tr>
<td>BSA</td>
<td>6-mer</td>
<td>1.81 \times 10^5</td>
</tr>
</tbody>
</table>

### Table 8.3.2: Phage Yield From Second Round Of Biopanning

<table>
<thead>
<tr>
<th>Clone</th>
<th>Phage Library</th>
<th>Phage Yield (TU in 20ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2D3</td>
<td>15-mer</td>
<td>8.9 \times 10^4</td>
</tr>
<tr>
<td>BSA</td>
<td>15-mer</td>
<td>2.2 \times 10^4</td>
</tr>
<tr>
<td>P2D3</td>
<td>6-mer</td>
<td>7.6 \times 10^4</td>
</tr>
<tr>
<td>BSA</td>
<td>6-mer</td>
<td>3.0 \times 10^3</td>
</tr>
</tbody>
</table>

### Table 8.3.3: Phage Yield From Third Round Of Biopanning

<table>
<thead>
<tr>
<th>Clone</th>
<th>Phage Library</th>
<th>Phage Yield (TU in 20ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2D3</td>
<td>15-mer</td>
<td>2.8 \times 10^3</td>
</tr>
<tr>
<td>BSA</td>
<td>15-mer</td>
<td>1.5 \times 10^3</td>
</tr>
<tr>
<td>P2D3</td>
<td>6-mer</td>
<td>2.6 \times 10^6</td>
</tr>
<tr>
<td>BSA</td>
<td>6-mer</td>
<td>2.5 \times 10^3</td>
</tr>
<tr>
<td>Clone</td>
<td>Phage Library</td>
<td>Yield Of Infected Clones (TU/ml)</td>
</tr>
<tr>
<td>-------</td>
<td>---------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>P2D3</td>
<td>15-mer</td>
<td>9.25 x 10^{12}</td>
</tr>
<tr>
<td>BSA</td>
<td>15-mer</td>
<td>5.4 x 10^{12}</td>
</tr>
<tr>
<td>P2D3</td>
<td>6-mer</td>
<td>1.7 x 10^{11}</td>
</tr>
<tr>
<td>BSA</td>
<td>6-mer</td>
<td>5.0 x 10^{9}</td>
</tr>
</tbody>
</table>

*Table 8.3.4: Total number of infected clones in first round amplified eluate*

<table>
<thead>
<tr>
<th>Clone</th>
<th>Phage Library</th>
<th>Yield Of Infected Clones (TU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2D3</td>
<td>15-mer</td>
<td>2.65 x 10^{12}</td>
</tr>
<tr>
<td>BSA</td>
<td>15-mer</td>
<td>3.15 x 10^{12}</td>
</tr>
<tr>
<td>P2D3</td>
<td>6-mer</td>
<td>5.3 x 10^{12}</td>
</tr>
<tr>
<td>BSA</td>
<td>6-mer</td>
<td>4.5 x 10^{11}</td>
</tr>
</tbody>
</table>

*Table 8.3.5: Total number of infected clones in second round amplified eluate*

<table>
<thead>
<tr>
<th>Clone</th>
<th>Phage Library</th>
<th>Yield Of Infected Clones (TU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2D3</td>
<td>15-mer</td>
<td>7.4 x 10^{12}</td>
</tr>
<tr>
<td>BSA</td>
<td>15-mer</td>
<td>7.6 x 10^{12}</td>
</tr>
<tr>
<td>P2D3</td>
<td>6-mer</td>
<td>1.35 x 10^{12}</td>
</tr>
<tr>
<td>BSA</td>
<td>6-mer</td>
<td>5.05 x 10^{12}</td>
</tr>
</tbody>
</table>

*Table 8.3.6: Total number of infected clones in third round amplified eluate*
Sixty P2D3 clones from the 6-mer and the 15-mer library eluates were sequenced. A further 25 clones of the control BSA were also sequenced.

Sequences were subsequently analysed using the computer programme CLUSTAL W version 1.6 (Washington University Institute for Biomedical Computing) aligning the 6-mer and 15-mer peptide sequences against the published HBV amino acid sequence of the S gene. The stringency of the alignment was kept at a minimum to increase the probability of finding a match.

8.4.1: 6-mer peptide alignments against P2D3

In the first instance, analysis concentrated on aligning the 6-mer peptide sequences against the s region of HBV. However, the matches with the 6-mer library appeared to be completely random with no consistency being observed at all. The percentage identity in a 6 amino acid overlap in any of the matches was found to be too low for them to be considered significant.

8.4.2: 15-mer peptide alignments against P2D3

Sequence alignments with the 15-mer peptides yielded a total of 42 matches. Grouping the sequences according to shared motifs on the published sequence showed that the majority of these matches were random being seen only once or twice. However, 12 of the matches (28.6%) were identified to occur overlapping in the region between amino acids 80-96 of the HBsAg. A second region was also
identified between amino acids 170-185 where 6 of the peptide sequences (15%) were aligning to. A diagram representing these two regions is shown in figure 8.4.2.

8.4.3: 6-mer and 15-mer peptide alignments against BSA

Sequence alignments on clones obtained from biopanning against BSA revealed no successful matches. The percentage identity in each alignment was too low to be considered significant.

8.5: Discussion

A random 6-mer and 15-mer peptide library displayed on fusion phage were used for the detection of the epitope recognised by the monoclonal antibody P2D3. Three rounds of biopanning against the monoclonal antibody were used to allow peptides similar to the epitope to be preferentially selected which can then be isolated for further analysis.

The yield of infective phage was found to be good and consistent after each round of biopanning. In the final round, phage titred at $1.35 \times 10^{12}$ for the 6-mer library and at $7.4 \times 10^{12}$ for the 15-mer library.

In spite of the low stringency used during the alignments, no selection was seen with the 6-mer library and further analysis was not considered to be justified.

Focus then turned to the 15-mer peptide sequence alignments. Although not as specific, the 15-mers would still be capable of giving a good indication of where the region of interest may be. As with the 6-mer library, majority of the matches were
Figure 8.4.2: A) and B) represent the two regions of the HBsAg identified by aligning the sequences obtained from the phage display analysis. • denotes the consensus sequence; • denotes those residues identical to the consensus sequence; • denotes related amino acids; • indicates unassociated amino acids.
random being seen only once or twice. However, two regions were identified as being dominant. Multiple matches were observed overlapping in the region between amino acids 79-96 and 170-180 of the HBsAg.

The interpretation of 15-mer alignments however, remain uncertain. Although several studies have been carried out on modelling the antigenic sites of HBsAg, much controversy still remains on their exact conformation. Both regions identified are rich in phenylalanine, isoleucine, leucine and valine. As a result these two regions may present a similar configuration. All antibodies are bivalent and recognise the overall conformation of an epitope rather than particular molecular species contained therein. It was considered that binding of the whole antibody could be mediated by Fab recognition of two spatially adjacent sites. However, a recent review by Carman et al. 1997 and the study carried out by Chen et al., 1996 using filamentous phage peptide libraries to identify discontinuous epitopes of HBsAg, reveal that these two regions do not lie in spatial proximity to each other. Therefore the initial model of the monoclonal antibody requiring both of these regions was not considered plausible

Why then do the 15-mer sequences repeatedly align against these two regions? Many of the studies which have used phage display libraries, immobilise the monoclonal antibody of interest by first biotinylating and then capturing it onto streptavidin coated plates. However, by omitting the antibody in the affinity purification procedure, Devlin et al, 1990 identified peptides that bound directly to streptavidin. A similar observation was noted where peptides which were histidine rich were found to bind directly onto the streptavidin (Fiona Gray - personal communication). The exact relation between strength of binding of the peptide to the streptavidin is
not clear but the presence of excipients on the solid phase presented to the phage may themselves select for sequences.

To overcome the problem presented by non-specific phage binding to streptavidin, the monoclonal antibody used in this study was immobilised on a nitrocellulose membrane. Unfortunately, the biopanning procedure was not carried out using the nitrocellulose membrane alone. It is therefore difficult to decipher if peptides will bind non-specifically directly to the nitrocellulose membrane. The two dominant regions are rich in leucine, phenylalanine and valine. It can be speculated that peptides rich in these residues may bind directly to the nitrocellulose membrane. However, this cannot be concluded from this study.

To avoid this occurring it is important to optimise phage incubation buffer conditions, to establish the correct temperature for carrying out the biopanning reactions and perhaps most importantly, to ascertain the ideal salt concentrations for the washes. Inefficient washing will result in low avidity phage being isolated and amplified and therefore being carried forward into subsequent rounds of biopanning. Bivalent binding may be essentially irreversible when the displayed peptide has a certain minimal threshold affinity for the binding protein. If so, affinity purification may be unable to distinguish peptides with moderately high affinity from peptides with very high affinity. It is therefore essential that washes are carried out at optimal stringency to ensure that all low avidity peptides are washed away.

Despite the multiplicity of possibilities, the monoclonal antibody should preferentially select peptides similar to the MHR epitope. However, it is possible that the isolated clones may not represent all tight binding peptides.
High affinity peptides may also be lost during phage amplification between rounds of affinity purification. This possibility is consistent with the observation made by Scott et al., 1990 where most clones analysed in the third round had identical sequences and therefore may have been derived from the same clone. Some bias may also be imposed on the library because of small differences in the growth rates of phage bearing different epitopes. Finally, the binding affinity of some peptides might be influenced, either positively or negatively, by pIII protein to which they are fused (Scott et al., 1990).

Filamentous phage libraries have been shown to be a powerful tool for determining native epitopes. Nevertheless, the results of this study fail to specifically identify the epitope recognised by the monoclonal antibody P2D3. Further work investigating these two regions of the HBsAg are described in chapter 9.
9: Epitope Location of MAb P2D3

9.1 Introduction

The three way cross monoclonal antibodies were binding to a region which was present on the WT HBsAg and which appeared to be preserved on the recombinant mutant HBsAg (chapter 6). Although results from the phage display library work identified two potential regions where the epitope might be located as discussed in chapter 8, the probability of the observed results identifying the monoclonal antibody epitope were small.

Another possible location for the P2D3 epitope was highlighted from the results of the liver transplant study (chapter 7). Four of the 26 patients studied, although harbouring virus with WT HBsAg sequence, carried serum HBsAg which was not detected by P2D3. Sequence analysis revealed two amino acid changes in the first loop of the \( \alpha \) determinant found only in these patients, implicating this region as the possible epitope.

Peptides to these regions were synthesised and assessed for their ability to bind to the monoclonal antibodies, P2D3, H3F5 and D2H5

9.2: Oligopeptide Synthesis

Six 15-mer oligopeptides were synthesised. Two of the peptides, PD1 and PD2 were constructed to reflect the sequences identified from the phage display library study (chapter 8). Two further peptides were made against part of the first loop of the \( \alpha \) determinant; the first peptide (LT1) mirrored the sequence found in genotype A of HBV while the second peptide (LT2) paralleled the sequence found in genotype D, subtype \( ayw3 \) as identified from the liver transplant study (chapter 7).
A fifth peptide, A2, was synthesised to reflect the second loop of the \( \alpha \) determinant from amino acid 138 to 152. This peptide was produced to allow a direct comparison of the binding of monoclonal antibody P2D3 to the first and second loop of the \( \alpha \) determinant. The final peptide (PS1) was made against a region of the pre S2 gene and acted as a negative control.

All oligopeptides used in this study were synthesised successfully except LT2. According to the manufacturers, Genosys Biotechnologies Inc, the peptide was found to collapse during the synthesis process between amino acids M and T. This was thought to be due to the amino acids being stearically hindered at this point. It could not be guaranteed if some or if any complete sequence had been synthesised and recovered. Therefore the peptide was still included in the assays and the results interpreted with caution.

The oligopeptides were supplied lyophilised. Initially attempts to redissolve them were carried out using PBS. All the peptides except PD1 and PD2 readily dissolved in PBS. Several attempts to redissolve these peptides in buffers ranging from acidic and basic solutions to alcohol solutions of varying concentrations, proved futile. Both peptides were rich in phenylalanine, isoleucine, leucine and valine residues and were therefore highly hydrophobic. However, even solutions containing DMSO failed to dissolve these peptides.

Work, therefore, concentrated on the peptides LT1, LT2, A2 and PS1

9.3: Peptide Assay

Capturing the biotinylated peptide onto streptavidin allowed the entire peptide to be exposed and also ensured that the movement of the peptide itself would not be restricted. Three monoclonal antibodies, D2H5, H3F5 and P2D3 were tested for their
ability to bind to each of the peptides LT1, LT2, A2 and the negative control PS1 as described in section 2.10.2. The results of these assays are shown in figures 9.3a, 9.3b, and 9.3c.

9.3.1 MAb P2D3
Analysis of the results with P2D3 showed that this monoclonal antibody bound only to the LT1 peptide. This peptide represented the region of the first loop from amino acid 121 to 135. The peptide sequence paralleled that found in genotype A and therefore crucially included amino acid threonine and proline at positions 125 and 127 respectively. P2D3 was also tested with and showed no reactivity against the LT2 peptide where amino acid positions 125 and 127 were substituted with methionine and threonine respectively. However in view of the difficulties in synthesising the peptide the negative result observed could also have been attributed to the presence of incomplete LT2 peptide sequence. Therefore, it was not possible to draw a definite conclusion from these results. The P2D3 monoclonal also showed no reactivity with the A2 peptide and the negative control PS1.

9.3.2 MAb H3F5
The monoclonal antibody, H3F5, did not demonstrate reactivity against any of the four peptides.

9.3.3 MAb D2H5
D2H5, however, did provide some interesting results. The monoclonal showed negative results with peptide LT1 and LT2 but did react against the peptide A2. A2 had been generated to mirror the sequence of the second loop from amino acid 138 to 152. As expected, D2H5 showed no reactivity with the negative control peptide PS1.
Figure 9.3a: Optical densities obtained when reacting MAb P2D3 with 4 peptides using a direct linked immunosorbant assay.
Figure 9.3b: Optical densities obtained when reacting MAb H3F5 with four peptides using a direct linked immunosorbant assay
Figure 9.3c: Optical densities obtained when reacting MAb D2H5 with four peptides using a direct linked immunosorbant assay.
9.4: Discussion

The aim of this study was to determine the binding epitopes of three monoclonal antibodies using oligopeptides.

The two peptides, PD1 and PD2, were synthesised against two regions identified as a result of the phage display library work (chapter 8). Although there was not enough evidence from the study to support either of these regions as being the probable epitope, peptides were constructed to assess their ability to bind to the monoclonal antibody, P2D3. However, attempts to redissolve the peptides once synthesised proved futile. Both peptides were found to be rich in hydrophobic residues making it impossible to dissolve the peptides even in solutions containing DMSO. The peptides could therefore not be included in the study.

The two sequences identified from the phage display work were highly hydrophobic and studies have predicted these regions as being located in the transmembrane (Stick et al., 1992; Prange et al., 1995). Based on these results, it was possible to exclude these two regions as being the probable epitope.

As clearly indicated in figure 9.3a, P2D3 only recognised peptide LT1. This confirms that the monoclonal antibody was indeed binding to a region in the first loop of the α determinant between residues 121 and 135. This region was initially identified as being a potential location for the P2D3 epitope as a result of sequence dependent loss of reactivity in WT HBsAg observed from the results of the OLT study (chapter 7). Sequence analysis of the α determinant in the serum of patients whose HBsAg was not detected by P2D3, revealed two amino acid substitutions of methionine for threonine and threonine for proline at positions 125 and 127.
respectively. It was speculated that these substitutions may bring about conformational changes in the protein resulting in the P2D3 epitope being abolished.

In order to confirm the specificity of P2D3 binding within the sequence defined by the LT1 a second peptide LT2 was synthesised so as to differ by only two amino acids; LT1 contained threonine and proline at amino acid positions 125 and 127 respectively while these residues were replaced with methionine and threonine in LT2. Results of the peptide assays showed that P2D3 was able to recognise and bind solely to the LT1 peptide. No reactivity was observed at all with peptide LT2. However, failure to bind to peptide LT2 could have been not only due to sequence divergence but also because the peptide sequence was incomplete as reported by the manufacturers. Although the results with the LT1 peptide clearly define where P2D3 is binding, the exact sequence required cannot be concluded, as would have been hoped, from the LT2 peptide results.

All the oligopeptides used in this study were linear which may account for the LT2 peptide synthesis collapsing. It was thought that the peptide was being stearically hindered at peptide positions 6 to 7 between the two threonine residues. Linear peptides do not allow for much flexibility between the amino acids and therefore will constrain the sequence which will lead to the peptide collapsing.

Ironically, the difficulties in synthesising the peptide LT2 when compared to LT1 does in itself illustrate the differences in the structure of these two regions. The peptides differ by only two amino acids. However, the changes themselves are significant in terms of protein stereochemistry and will influence the potential difference of secondary structure of these two sequences.
The monoclonal antibodies, D2H5 and H3F5 have been tentatively mapped by Murex Biotech Ltd using dot blot experiments with long synthetic peptides (Dr. Julian Duncan -personal communication). Data from this work showed that D2H5 reacted with a peptide which spanned amino acid 135-155 of the s region while H3F5 reacted, but only very weakly, with a second peptide covering amino acid 110-137. These findings, however, need as yet, to be confirmed. No cross reactivity was observed between these monoclonal antibodies reacting to these two peptides. This is not altogether surprising as cross competition studies have shown D2H5 and H3F5 to recognise separate epitopes (chapter 5).

H3F5 did not bind to any of the peptides assessed in the study as described in this chapter. From the data supplied by Murex Biotech Ltd, it was inferred that H3F5 might react with LT1. However, no binding was observed. This again supports the inhibition study results where P2D3 and H3F5 did not cross compete and appear to recognise separate epitopes (chapter 5).

A2 was synthesised to reflect the second loop of the a determinant between amino acids 138 to 152 and was shown to react with monoclonal antibody D2H5 alone. This observation supported the data produced by Murex Biotech Ltd indicating that the D2H5 epitope was indeed located in the second loop of the a determinant. D2H5, however did exhibit reduced binding avidity of the peptide as when compared to P2D3. The a determinant has been demonstrated to be a highly conformationally dependent epitope. It should be noted that the peptides used in this chapter were linear. It has been shown previously by Brown et al., (1984) and Waters et al., (1991) that synthetic peptides show a greater binding to antibodies when
circularised. Although D2H5 can still recognise the peptide A2, greater binding avidity might be achieved if the peptide was circularised.
Chapter 10: General Discussion

The identification of mutant HBsAg in two renal transplant patients NP and MAM provided useful immunogens against which hybridomas were raised. Monoclonal antibodies which recognised the WT, NP and MAM HBsAg, once formatted into a solid phase RIA were assessed for their ability to detect naturally-occurring and recombinant HBsAg mutants. Identification and characterisation of this epitope which appeared to be conserved on mutant HBsAg may enhance further our understanding of the ‘s’ antigen. Conceptually, any mutation which alters the hydrophilicity and therefore the 3 dimensional structure of the HBsAg, and conserves the overlapping polymerase function, can result in the appearance of variant HBV with altered expression of HBsAg reactivity and loss of immunoreactivity.

Point mutations resulting in amino acid substitutions have been described in the first, and more commonly, in the second loop of the $a$ determinant. More recently, amino acid insertions have also been reported occurring around codons 122, 123 and 124 (Hou et al., 1995; Carman et al., 1995). It has now been established that mutants of the highly antigenic $a$ determinant are clinically relevant. The HBsAg mutants have been identified and associated with immunisation and immunoprophylaxis failure and with cases of HBV infection difficult to diagnose using some conventional HBsAg detection assays.

Discordant results on different HBsAg detection assays led to the initial identification of HBsAg mutants in patients MAM and NP (chapter 3). While remaining reactive on polyclonal based assays, sera from both patients (bar one sample) were repeatedly unreactive on monoclonal based HBsAg detection assays. Similar findings of false negatives associated with HBsAg mutants have been reported. Where immunological
analysis had been carried out, the studies revealed that the $\alpha$ determinant was poorly detectable with commonly used monoclonal antibodies, implying that the WT epitope was no longer present (Waters et al., 1992).

Monoclonal antibodies used in detector assays provide pure specific IgG and so the loss of a single epitope is often sufficient to result in the antigen not being recognised by the monoclonal antibody. Therefore the poor reactivity observed on monoclonal based assays for HBsAg, as demonstrated by sera from patients NP and MAM, is not altogether surprising. In contrast, polyclonal based assays appear to provide a number of potential sites to which the HBsAg can bind. Subsequent detection using two monoclonal antibodies which recognise different epitopes, as is the case with the GE15 HBsAg assay (Murex Biotech Ltd.), also increases the probability of detecting the mutants.

HBsAg, purified directly from serum samples from patients NP and MAM were used as the preferred immunogen for monoclonal antibody production. Using native HBsAg ensured that the protein conformation and all associated post-translational modifications would be correct.

Following the fusion process, hybridomas secreting anti-HBs were identified using a reverse capture radioimmunoassay (chapter 4). By separately using the HBsAg prepared from NP, MAM and WT sera in the screening assays it was possible to identify those hybridomas secreting anti-HBs which were capable of detecting either one or more combinations of the three HBsAgs. The panel of monoclonal antibodies could therefore be categorised accordingly. Monoclonal antibodies were found which specifically recognised either the MAM or NP HBsAg, as well as those which were cross reactive
with the mutant HBsAg and/or the WT HBsAg. Hybridomas were then chosen from each category and cloned early by limiting dilution to prevent the overgrowth of positive secretors by non-producing clones. This also ensured that the antibody produced was homogenous as well as monospecific.

Cloning of the MAM derived hybridomas was initially unsuccessful. The slow growing hybridomas were very slow growing and appeared to die as a consequence of being grown at low cell densities. Further retesting after cloning found all but one of the MAM hybridoma cultures to be negative for anti-HBs activity. As a result, the MAM fusion was repeated. Cloning on this occasion was successful and all positive hybridomas were immediately recloned to eliminate any possible growth of non-secretors. Subsequent expansion of the NP and MAM hybridomas, and propagation as ascitic tumours was achieved without difficulty.

Inhibition assays were set up allowing the monoclonal antibodies to compete for binding sites on the MAM, NP and WT HBsAg (chapter 5). Purified immunoglobulin from each clone were tested at a 1000 molar excess for its ability to inhibit itself and all other clones. The observed pattern of inhibition neatly divided the panel of monoclonal antibodies into six groups. The control binding of heterotypic antigen to which the monoclonal antibodies had been shown to have no binding avidity, also demonstrated the specificity of the monoclonal antibodies to their respective HBsAgs. The pattern of inhibition was thought to represent the recognition of six distinct determinants found on the HBsAg.
The three way cross monoclonal antibodies which recognised the MAM, NP and WT HBsAg were tested for reactivity against a recombinant protein expressing the HBsAg only. Results of the assay confirmed that P2D3, M4F5 and M3A10 were indeed recognising an epitope present in the s region. As expected, the remaining monoclonal antibodies did not react with the recombinant HBsAg. These monoclonal antibodies recognise epitopes present on NP/MAM HBsAg which will not be found on the WT HBsAg. Surprisingly, the NP/WT monoclonal antibody, P3E4 also showed no reactivity with the HBsAg, which suggests that it is recognising an epitope in the pre-S region. As expected, the control monoclonal antibodies D2H5 and H3F5 reacted with the recombinant HBsAg.

The generation and identification of HBsAg mutants have brought to light the substantial differences in susceptibility between commercial HBsAg assays. This is an effect of test design, choice of polyclonal/monoclonal antibodies on the solid phase and the affinity of the antibodies for variants. Therefore, when considering HBsAg detection assays, not only is the sequence important but also the sensitivity of the assay where an otherwise non-reactive low level HBsAg sample may be reactive in a second assay.

The three way cross monoclonal antibodies had been shown to be capable of detecting both WT and the studied mutant HBsAg and were therefore thought to be clinically and diagnostically significant. As a result, work centred on formatting these monoclonal antibodies into a sensitive, accessible HBsAg detection radioimmunoassay (chapter 6).

A comparison of two assay formats found the heterologous based assay where, HBsAg was captured by polyclonal anti-HBs and then detected using labelled P2D3, to be the
most sensitive. The assay readily recognised the WT HBsAg and was termed the ‘conserved’ assay.

Setting up a second assay which recognised the WT HBsAg only, provided a useful comparative assay when samples were run in parallel with the ‘conserved’ assay. The format of this second assay, denoted the ‘WT’ assay, was based on the commercial VK21 HBsAg kit (Murex biotech Ltd). The commercial VK21 is a monoclonal antibody based assay and has been shown to miss the antigenically altered HBsAg mutants. Thus, running diagnostic specimens through the ‘conserved’ assay and the ‘WT’ assays in parallel would allow the easy identification of HBsAg carrying a determinant mutations.

Access to a wider range of recombinant mutant HBsAg allowed the performance of the ‘conserved’ assay be more fully assessed. HBsAg carrying mutations at sites ranging between codon 126 to 145 were tested in both the ‘conserved’ and the ‘WT’ assay. While the ‘conserved’ assay detected all the mutants ranging from codon 133 to 145, poor reactivity was observed with the 126 and 129 mutants. This may have been due to the poor expression of these two mutants or because P2D3 epitope had been abolished as a result of the mutations. The ‘WT’ assay did show minor reactivity against the 126 mutant only.

Mutations in the HBV s gene have been reported in OLT recipients who developed HBV reinfection despite prophylaxis with monoclonal or polyclonal anti-HBs. McMahon et al., (1992) found amino acid substitutions in the a determinant in all three patients who were reinfected despite monoclonal anti-HBs prophylaxis, while the incidence of mutations in the a determinant in OLT recipients who were reinfected
Despite HBIG prophylaxis varied from 0% to 33% (McNair et al., 1995; Hawkins et al., 1994; Carman et al., 1996).

In this thesis, a series of patients who had undergone OLT, a number of whom went on to reinfect their grafts, were studied (chapter 7). Both the 'WT' and 'conserved' assays were used to investigate the presence of mutant or WT HBsAg in pre and post OLT samples from these patients. Running these two assays in parallel allowed for the easy differentiation between WT and mutant infections.

The binding studies identified 4 out of the 12 patients who had undergone HBIG immunoprophylaxis post OLT to be infected with second loop mutants secondary to HBIG prophylaxis. The pre-OLT samples of 3 of these patients were positive on both the 'conserved' and 'WT' assays indicating the presence of the WT HBsAg in these samples. However, results of the post-OLT samples clearly demonstrate the emergence of HBsAg mutants in all three patients. In the fourth patient, the HBsAg mutant was detected in both pre and post OLT samples and it is thought that in this case, host immune pressure alone was sufficient to drive the selection process. Though this event is rare, it has been described in patients who received neither vaccine nor HBIG (Yamamoto et al., 1994; Carman et al., 1995). Indeed, both patients NP and MAM appear to fall into this category. No HBsAg mutants were detected in the control group of OLT patients who had undergone identical immunosuppressive regimens.

HBIG is now considered to be a mandatory prophylactic measure given to all patients being transplanted for HBV associated liver disease. This is a matter of concern when considering data generated from this study and other published work which demonstrate that some OLT patients undergoing HBIG administration will select escape mutants and therefore will go on to reinfect their liver grafts. Ghany et al., (1998) demonstrated a
significant correlation between the development of HBV s mutations and the duration of HBIG therapy, suggesting that these mutants were induced or selected by immune pressure exerted by prolonged exposure to high levels of anti-HBs. Further, the study found that 67% of the mutations reverted back to the pre-OLT sequences upon withdrawal of HBIG, suggesting that in the absence of immune pressure, virus bearing the wild-type/pre-OLT sequences have survival advantage over the mutants.

Evidence that HBV s mutants, that can escape neutralisation by anti-HBs, may play a role in HBV reinfection, calls for the use of HBIG immunoprophylaxis in OLT recipients to be reassessed. Prophylaxis using nucleoside analogues could provide an alternative option. Similarly, the combination of HBIG and new antiviral compounds such as lamivudine and famciclovir may result in decreased frequency of HBV s mutations. However, the issue whether mutants induced by one treatment will be susceptible to the other treatments will need to be initially addressed.

Sequence analysis confirmed the presence of HBsAg mutants in the four patients identified by the ‘conserved’ assay in our OLT study. All the mutations were found in the second loop of the immunodominant region at positions 144 and/or 145.

In total, 19 of the patients studied went on to reinfect their liver grafts, 12 of whom received HBIG post-OLT. HBsAg escape mutants were detected in only four of these patients. Clearly, other factors must be contributing to HBV reinfection.

All 19 patients had detectable HBV DNA in their pre-OLT samples by a single round of PCR (250 copies/ml). In contrast, of the 7 patients who did not reinfect their liver grafts, HBV DNA was detectable in their pre-OLT samples by nested PCR only. Therefore, a
high viral load before transplant may play an important role in determining the final outcome in OLT for HBsAg positive cirrhosis

The P2D3 monoclonal antibody has been shown to be capable of detecting both the WT and mutant HBsAg. It is therefore interesting to note that the 'conserved' assay failed to identify 5 patients in spite of the WT phenotype. As the samples were positive on the 'WT' assay, it was speculated that these patients may be mutated at the region which P2D3 recognises and binds to. These mutations may result in the loss of the P2D3 epitope.

However, sequence analysis of the entire s region of these patients revealed nothing unusual. Only changes associated with genotype variability were noted. Upon aligning these sequences against that of the control sequences, two amino acid changes at positions 125 and 127 were repeatedly found only in those patients being missed on the 'conserved' assay. The changes were associated with amino acid substitutions of threonine for methionine at codon 125 and proline for threonine at codon 127 and are exclusive to the described variability associated with genotype D subtype ayw3 (Norder et al., 1992).

The monoclonal antibody P2D3, was raised against the mutant HBsAg of patient NP who is genotype C and therefore carries amino acids threonine and proline at codon 125 and 127.

Is it possible that the two changes observed in the OLT patients will be sufficient to abolish the P2D3 epitope? If considered in terms of protein stereochemistry, it would not be surprising. The changes will alter the hydrophilicity of the protein and proline itself is known to play a role in dictating turns in polypeptide chains.
P2D3 was also found not to react with a recombinant WT and K141E mutant HBsAg obtained from Prof. C. Howard. Closer analysis showed that the HBsAg sequence was based on genotype D subtype ayw4 and rather than a proline, leucine was found at codon 127, this single change being sufficient to completely abolish the P2D3 epitope.

Collectively, the RIA and sequencing results lend weight to the suggestion that the three way cross monoclonal antibody P2D3 is binding to a region in the first loop of the determinant.

To pursue further the data generated from the OLT study, 15-mer oligopeptides were synthesised to reflect the sequence of the first loop from codon 121 to 135. One of the peptides (LT1) was made so as to mirror the sequence found in genotype C while the second peptide (LT2) paralleled the sequence found in genotype D subtype ayw3. Both of the peptides were assessed for their ability to bind to P2D3.

Results of the peptide assays confirmed that the monoclonal antibody P2D3 was indeed binding to a region in the first loop of the a determinant between residues 121 to 135. The precise sequence could however not be resolved by comparing with the binding to LT2. The LT2 peptide was found to collapse mid synthesis. Although, the peptide was included in the assays, the finding that P2D3 failed to bind LT2 needed to be treated with caution. This could have been not only due to sequence divergence but also because the peptide sequence, as reported by the manufacturers, was incomplete.
What, therefore, is the clinical and diagnostic significance of the identified epitope? Data generated from studies carried out during the course of the work described in this thesis, suggested that while the epitope is present on the WT HBsAg it was more importantly also preserved on the WT HBsAg. Binding studies using recombinant HBsAg from the Royal Free Hospital showed that the monoclonal antibody was capable of detecting mutants ranging from amino acid 133 to 145 of the immunodominant $\alpha$ determinant. It is difficult to conclude definitive results from the T126S and Q129H mutants. This may have been due to the poor expression of the recombinant proteins or because of P2D3 not being able to recognise these mutants. The first option is certainly probable and in light of the identification of the P2D3 epitope being in the first loop, the second option can also not be ruled out.

Amino acid substitutions in the $\alpha$ determinant have been described at positions 145, 144, 143, 142, 141, 134, 133, 131 and more recently at residues 129 and 126. As the $\alpha$ determinant is a highly conformational dependant epitope and because the polymerase gene overlaps this region it can be speculated (and maybe naively) that there are not too many significant mutants left to find.

The monoclonal antibody P2D3 showed no limitations in detecting mutants in the second loop and while the M133L mutant was also recognised, it is difficult to comment on the effect of other first loop mutants on the P2D3 epitope. Samples with mutations at positions 126, 129 and 131 need to be obtained or generated to address this issue.
Variants can be divided into two classes. The first class simply referred to as variants occur naturally and are selected over a long period. These include subtype and genotype related variants; the second class have been selected over a short period of time by either therapy or prophylaxis and are called mutants.

It has been demonstrated that a peptide sequence from amino acid 139 to 147 represents an essential part of the \( a \) determinant, eliciting antibodies cross reactive with both \( ad \) and \( ay \) subtypes (Bhatnager et al., 1982; Prince et al., 1982). It was further shown that the cyclic version of the peptide, in which there is a disulphide bond between cysteine 139 and cysteine 147 more closely resembles the native conformation of the \( a \) determinant.

Interestingly in a recent review, Carman, (1997) suggested dividing the epitopes of the HBsAg into 5 regions: HBs1 being upstream of amino acid 120; HBs2 between amino acid 120 and 123; HBs3 between amino acids 124 and 137; HBs4 between 139 and 145 and HBs5 from amino acid 148 up to 169. Based on this classification, mutants are generally found in region HBs4 indicating that this is a major neutralising epitope cluster. In contrast, variants are often seen in HBs2 and HBs3. Therefore, diagnostically an epitope in the HBs3, as is the case with P2D3, may be ideal. However, as with all simplifications, there are exceptions, with mutants being identified in the HBs3 region. As stated earlier, what effect these mutations have on the P2D3 epitope remains to be ascertained.

The fact that very few mutants have been described in the first loop of the \( a \) determinant does also suggest that the P2D3 epitope may not be neutralising. Experiments competing the monoclonal antibody with anti-HBs from convalescent and post-immunisation sera may help to ascertain this. The monoclonal antibodies raised in this
study were produced in mice, thus it was the murine response to the antigens that was monitored. Whether or not the antigen would have evoked a similar immune response in man is unknown.

Although a significant amount of data regarding HBsAg mutants has now been described, a major question relating to the prevalence of these s mutants in endemic and vaccinated situations remains unanswered. The 'conserved' and 'WT' assays which have been shown to have different susceptibilities to s mutations can be used to screen different human populations for the evidence of s escape mutants. The strategy has been proven to work in the OLT setting and can also be used to study immunised patients including those involved in HBV eradication programmes (The Gambia and other chosen sites), patients in STD and renal units and also people involved in the intravenous drug users outreach programmes. Where samples were found to contain HBsAg mutants, natural history studies could be undertaken including full HBV markers and characterisation of the infection. Further HBV DNA and transmission analysis may also help elucidate how many of these mutants are clinically relevant.

However, as illustrated in the OLT study, we must take account of the geographically related variation seen and implications for the diagnostic assay. P2D3 was found not to react with patients infected with the genotype D subtype ayw3 or ayw4 virus as a result of amino acid variation at codons 125 and/or 127. Based on the genotype classification as described by Norder et al., 1992, it is possible that P2D3 will also not recognise genotype F subtype adw4q- as the proline at position 127 is replaced with leucine.
The monoclonal antibody, P2D3 is currently being incorporated into the GE15 HBsAg assay by Murex Biotech Ltd. Initial results are promising (Julian Duncan- personal communication). The addition of P2D3 as one of the detector monoclonal antibodies does not appear to have decreased the specificity of the assay.

Although anti-HBs found in convalescent and post-immunisation sera is found to bind predominantly to the second loop (Howard *et al.*, 1984), it is thought that the sequence of both loops contributes to the antigenic structure. Using a cyclic synthetic peptide from amino acid 122-137, where a disulphide bond was found between cysteine 124 and cysteine 137, Dreesman *et al.*, (1982) demonstrated that this region contributed to the α determinant.

The α determinant is essentially conserved. However sequence alignments of the different subtypes demonstrate a greater degree of genetic variation in the first loop as compared to the second loop. This tolerance of variation observed in some subtypes may be functionally significant as the surface and polymerase genes do overlap.

In the scenarios where HBsAg mutants have been described, the region encompassing the identified P2D3 epitope of amino acids 125 to 127 has always remained conserved. The significance of the epitope conservation in the presence of immunological pressure has led to plans to produce monoclonal antibodies against genotype D, subtype ayw3 and genotype E, subtype ayw4.

As this thesis was being written, a patent was published identifying a conserved epitope in the HBsAg (Bridon *et al.*, 1997) which was present in all HBV subtypes. Using a peptide which corresponded to amino acid residues 117-128 of the HBsAg, the authors
had mapped an antigenic epitope of a monoclonal antibody, H166 which had been produced by Peterson *et al.*, in 1984. They further showed that a cyclic form of the peptide imparted an eight fold increase in affinity to the linear peptide for recognition by the monoclonal antibody. Based on these results, a previously unidentified disulphide bond between residues cysteine (cys) 121 and cysteine 124 was suggested.

In order to identify the important residues in the peptide (aa 117-128), single amino acid substitutions by alanine were carried out. Results showed residues cys121, thr123 and cys124 to be critical for H166 recognition. Minor effects were seen when the remaining residues of the peptide 117-128 were substituted with alanine. Therefore the CXTC motif was the important structural feature for antibody recognition. The residue X at position 122 represents the *ad* or *ay* subtype and so either amino acid lysine or arginine will be found. The monoclonal antibody, however is able to tolerate this change.

Alignment of the CXTC motif against 100 HBsAg sequences retrieved from the GenBank database, which included all the subtypes of HBsAg, revealed that despite sequence variation occurring between residues 101-160, the CXTC motif remained fully conserved. It is only with the 2 and 8 amino acid insertions described by Hou *et al.*, (1995) and Carman *et al.*, (1995) that the CXTC motif will be completely destroyed.

If the *a* determinant is analysed as a three loop structure, the region between amino acids 139-145, although highly immunogenic, is limited as a synthetic vaccine or diagnostic reagent due to the emergence and selection of mutants in this area under immunological pressure. A monoclonal antibody in the first loop (aa 124-137) as is the case with P2D3, may not be susceptible to the described mutants (although mutants in the first loop need further investigating), but remains sensitive to naturally occurring genetic variation as is described in this thesis. Just downstream from the identified
P2D3 epitope, the region between amino acids 121 and 124 provides an epitope which is common to all HBV subtypes and is also found to contribute to the a determinant. Ohnuma et al., (1990), demonstrated that 30% of human serum samples from HBsAg immunised individuals recognised the peptide 115-129, indicating that the peptide and therefore this region is immunogenic.

The emphasis of this thesis has centred around the three way cross monoclonal antibodies. It would however, be interesting to investigate the reactivity of the remainder of the panel of monoclonal antibodies generated in this study, with a series of mutants ranging from codon 124-147. These monoclonal antibodies may indeed be used to overcome the current problems encountered within genotype variability, especially when detecting the 144 or the 145 HBsAg mutants. Maybe more significantly, the monoclonal antibodies may help to further our understanding of the HBsAg conformation and the implications of individual mutations on the antigenicity of the a determinant.

Much work has concentrated on the identification of mutants in the a determinant. In contrast, there is very little data on the sequences in the rest of the s gene. Mutations have been described in patients who underwent HBIG prophylaxis by Ghany et al., 1998. The mutations tended to cluster in three regions around codons 40-45, 114-122 and 198-208. Carman et al., (1996) also noted clustering of mutations in codons 44-49. This region has been recently shown to contain a major histocompatibility class 1-restricted T-cell epitope of HBsAg (Tai et al., 1997). The 114-122 region is immediately upstream of the a determinant and changes in this region may alter the
conformation of the $a$ determinant. Amino acid insertions in this region abolish binding to anti-HBs (Hou et al., 1995). The significance of the mutation cluster at 198-208 remains unclear.

In the same report by Ghany et al., (1998) examined the effect of amino acid changes post-OLT on the predicted antigenicity of the $a$ determinant. They divided the HBsAg into four regions: 1) upstream region [codons 110-123]; 2) first loop [codons 124-137]; 3) second loop [codons 138-147]; 4) downstream region [codon 148-160] and examined the predicted changes based on mathematical modelling. The results are expressed as antigenic indices. Though this system may not necessarily reflect actual changes in antibody recognition, their results were nevertheless interesting. In most patients, the observed amino acid changes resulted in changes in the predicted antigenic index of their respective regions i.e. a mutation in region 2 demonstrated a change in that region only. However, it was found that the same mutation can induce different changes in predicted antigenicity depending on the rest of the s gene sequence as was evident in two patients who were both found to have the G145R mutant. The first patient had additional mutants at codons 40, 44, 49 and 204 and was found to have an antigenic index of +7% in the second loop while the second patient had a mutation at codon 103 and was found to have an antigenic index of -9%. Surprisingly, the most significant changes in the antigenic index in these two patients was not located at the site of the mutation but in the downstream region [148-160] where no mutants were found. The significance of these mutants outside the $a$ determinant need to be investigated further.

HBV mutants that are apparently resistant to famciclovir and lamivudine, two nucleoside analogues currently in clinical trials for the treatment of chronic hepatitis B
infection, have now been reported. As the polymerase gene completely overlaps the envelope protein, drug related mutations in the polymerase gene may have an impact on the envelope protein. The HBV polymerase encompasses five conserved domains A, B, C, D and E. The B domain of the HBV polymerase overlaps with amino acids 150 to 175 and appears to undergo the selection of most of the mutations observed in the presence of either of these two drugs.

Lamivudine-resistant HBV isolates have been shown to cause an amino acid substitution within the a determinant at residue 157 (Ling et al., 1996), whereas breakthrough isolates from patients administered famciclovir would contain an amino acid change at position 164. The classic lamivudine resistance YMDD mutation results in amino acid substitutions in the HBsAg at positions 195 and 196. Models of HBsAg structure, however, place these residues embedded in the lipid envelope and are therefore unlikely to affect antigenicity (Stirk et al., 1992).

An outline of work is currently being drawn up with Dr. S. Locarnini of the Victorian Infectious Diseases laboratory in Australia, to use our monoclonal antibodies to study the effect of these pol gene changes on the B-cell epitope residue of the HBsAg and their role in anti-HBs neutralisation.

In summary, of the panel of generated monoclonal antibodies, those which showed reactivity against the WT and mutant NP and MAM HBsAg were selected for investigation. Formatting these monoclonal antibodies into a solid phase RIA provided an accessible method for detecting WT and mutant HBsAg as was demonstrated by results obtained from the study using the recombinant HBsAg.
Running the ‘conserved’ and the ‘WT’ assays in parallel allowed the easy identification of HBsAg mutants in OLT patients. This highlights the importance of using monoclonal antibodies such as P2D3 to detect and therefore ascertain the prevalence of HBsAg mutants in clinical scenarios such as the OLT setting.

Results of the OLT study also led to the initial identification of the P2D3 binding site in the first loop of the \( a \) determinant. This region, while present on the WT HBsAg appeared to be preserved on the studied mutant HBsAg. Though the monoclonal antibodies demonstrated no limitations in detecting mutants found in the second loop of the \( a \) determinant, further work is required to assess their reactivity against mutants found in the first loop. Initial analysis from work carried out in this thesis suggests that this region does remain conserved in patients who are undergoing prophylaxis which is important if the monoclonal antibodies are to be used diagnostically. However, problems encountered as a result of natural genetic variation remain. Producing monoclonal antibodies against HBsAg of subtypes \( ayw3 \) and \( ayw4 \) may help address this issue and this remains to be ascertained.
References


major HBsAg: DNA and immunological analysis of recurrent HBsAg derived from monoclonal antibody-treated liver transplant patients. *Hepatology. 15*(5): 757-766


Appendix 1 Composition Of Reagents

Production of monoclonal antibodies

Complete medium

RPMI supplemented with 5mM Hepes buffer
2Mm L-glutamine
0.05M 2-Mercaptoethanol
20% v/v foetal calf serum
25μg/ml¹ Fungizone
10² units ml⁻¹ Penicillin
100μg ml⁻¹ Streptomycin

Incomplete medium

RPMI supplemented with 5mM Hepes buffer
2mM L-glutamine

HAT medium

Complete medium with 10⁻⁴ M Hypoxanthine
4 x 10⁻⁷ M Aminopterrin
1.6 x 10⁻⁵ M Thymidine

HT medium

Complete medium with 10⁻⁴ M Hypoxanthine
1.6 x 10⁻⁵ M Thymidine
**Tris BSA**

0.5% Bovine serum albumin in 0.02M Tris buffer

**Label diluent**

0.02M Tris buffer containing 2% bovine serum albumin and 20% normal human serum

**Freezing medium**

Incomplete medium supplemented with

- 10% dimethyl sulphoxide
- 50% foetal calf serum

**0.02M Phosphate buffer**

0.2M $\text{KH}_2\text{PO}_4$ (Solution A)
0.2 M $\text{Na}_2\text{HPO}_4$ (Solution B)
Take 200ml solution B. Adjust pH to 8.0 with solution A.

**Phosphate buffered saline**

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made up to 1 litre

**0.02M Tris buffer pH 7.6**

Sodium azide | 1.0g |
Tris (hyroxymethyl) methylamine | 2.4g |
distilled water | 900ml |

Adjust pH to 7.6 with concentrated HCL and make up to 1 litre

**Citric acid (pH 3.0)**

0.1M citric acid adjusted to pH 3.0 with NaOH.
**Medium used with recombinant HBsAg**

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</tbody>
</table>

Once cells have stuck down used above medium plus 300u/ml Hygromycin B
Phage Display Library Work

Transfer Buffer

15% (vol:vol) Methanol
25mM Tris Base
250mM Glycine

Blocking Buffer

10mg/ml BSA
0.5% Tween
0.02% NaN₃
50mM Tris.Cl pH 7.5
150mM NaCl

Incubation Buffer

0.1mg/ml BSA
0.5% Tween
in TBS

TBS

50mM Tris.Cl pH 7.5
150mM NaCl

Wash Buffer (1)

0.5% Tween
50mM Tris.Cl pH 7.5
0.15M NaCl

Wash Buffer (2)

0.5% Tween
50mM Tris.Cl pH 7.5
0.5M NaCl
**Elution Buffer**

0.1M HCL
Titrated to pH 2.2 by the addition of solid glycine

**Neutralising Buffer**

0.1M Tris.Cl pH 9.0
Titrate with HCL

**PEG/NaCl mix**

16.7% PEG 8000
3.3M NaCl

**TBS/Gelatin**

0.1g gelatin
in 100ml TBS

**Terrific Broth**

12g bacto-tryptone
24g yeast extract
4ml glycerol
in 900ml distilled water. Before use 10ml of K$_2$HPO$_4$ to 90ml aliquot.

**Potassium Phosphate Buffer**

0.17M KH$_2$PO$_4$
0.72M K$_2$PO$_4$

**L-Broth**

10g bacto-tryptone
5g yeast extract
5g NaCl
in 1 litre of distilled water
**L-agar**

10g bacto-tryptone  
5g yeast extract  
10g NaCl  
15g agar  
in 1 litre of distilled water

**K91 TB cells**

10ml of K91 kan cells in terrific broth + kan (100μg/ml) grown to OD$_{600}$ = 0.2.
**Sequencing reagents**

**Annealing reaction**
- 5μl DNA
- 2μl Primer (100ng/sample)
- 2μl Sequenase buffer
- 1μl 10% w.r.t. DMSO

**Sequencing reaction**
- 1μl 0.1M DTT
- 0.5μl α\(^{35}\)S dATP (1000 Ci/mM)
- 1μl Labelling mix, diluted 1:30 in water
- 1μl Sequenase (2 units)

**Labelling mix**
- 7.5mM dCTP (0.75μl 100mM dCTP in 10mls)
- 7.5mM 7-dGTP (7.5μl 1mM 7-dGTP in 10mls)
- 15mM dTTP (1.5μl 100mM dTTP in 10mls)

**Termination Mixes**
- dNTP mixture
  - 160mM dATP (16μl 100mM dATP in 10mls)
  - 160mM dTTP (16μl 100mM dTTP in 10mls)
  - 80mM dCTP (8μl 100mM dCTP in 10mls)
  - 40mM dGTP (4μl 100mM dGTP in 10mls)

- Modified termination mixtures
  - ‘A’ termination mix
    - 8mM ddATP (2.5mls dNTP mix + 4μl 5mM ddATP)
  - ‘T’ termination mix
    - 8mM ddTTP (2.5mls dNTP mix + 4μl 5mM ddTTP)
  - ‘G’ termination mix
    - 4mM ddGTP (2.5mls dNTP mix + 2μl 5mM ddGTP)
  - ‘C’ termination mix
    - 4mM ddCTP (2.5mls dNTP mix + 2μl 5mM ddCTP)

**Stop solution**
- 98% Deionised water
- 10mM EDTA (pH 8.0)
- 0.025% Xylene cyanol FF
- 0.025% Bromophenol blue
**TE buffer**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>10mM</td>
<td>Tris HCL pH 7.5</td>
</tr>
<tr>
<td>1mM</td>
<td>EDTA</td>
</tr>
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</table>

**TBE buffer**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 x</td>
<td>1M Tris base</td>
</tr>
<tr>
<td>1M</td>
<td>Boric acid</td>
</tr>
<tr>
<td>2.0mM</td>
<td>EDTA NaH₂O</td>
</tr>
</tbody>
</table>

**Polyacrylamide gel**

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>75g</td>
<td>Urea</td>
</tr>
<tr>
<td>10ml</td>
<td>TBE</td>
</tr>
<tr>
<td>20ml</td>
<td>Acrylamide mix (40%)</td>
</tr>
<tr>
<td>150mg</td>
<td>(NH₄)₂SO₄</td>
</tr>
<tr>
<td>15μl</td>
<td>TEMED</td>
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</tbody>
</table>

Final volume 150mls.

**PCR reagents**

**10 x reaction buffer**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>500mM</td>
<td>KCl</td>
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<tr>
<td>100mM</td>
<td>Tris HCL pH 8.3</td>
</tr>
<tr>
<td>0.01%</td>
<td>Triton X-100</td>
</tr>
</tbody>
</table>

**TAE buffer**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6M</td>
<td>Tris base</td>
</tr>
<tr>
<td>0.8M</td>
<td>Sodium acetate 3H₂O</td>
</tr>
<tr>
<td>40mM</td>
<td>EDTA-NaH₂O pH 7.2</td>
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</tbody>
</table>

**Phosphate buffered saline**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.17M</td>
<td>KH₂PO₄</td>
</tr>
<tr>
<td>0.72M</td>
<td>K₂PO₄</td>
</tr>
</tbody>
</table>