THE MOLECULAR DIVERSITY OF THE HEPATITIS C VIRUS IN PATIENTS WITH HAEMOPHILIA

BY

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ABSTRACT

The hepatitis C virus (HCV) was first identified in 1989 and shown to be a major cause of non-A, non-B hepatitis. Haemophilic patients have particularly high rates of seroprevalence of HCV, and it is probable that all haemophilic patients treated with unsterilised multi-donor pooled clotting factor concentrates have been exposed to HCV. Infection with HCV is associated with a high rate of chronic liver disease, and a possible progression to cirrhosis and liver failure. The initial study assessed the patterns of HCV RNA in our patients. A minority treated with unsterilised concentrates were found to be persistently HCV RNA negative; these patients may not have been infected or may have cleared the virus. The second study assessed the HCV genotypes pre- and post-treatment with interferon, and found that interferon influenced the dominant, detectable genotypes. The third study assessed five patients from the interferon study in greater detail. The patients' samples were analysed by the four currently available methods for genotyping, and the HCV RNA was quantitated. This study showed no ideal method for genotyping patients with mixed-type infections, but restriction fragment length polymorphism analysis appeared to be the most useful. The final study assessed the evolution of two regions of the HCV genome (the highly conserved 5' untranslated region and an epitope in non-structural region 4) in two patients (one HIV positive and the other HIV negative) over 15 years. Comprehensive clinical information, and the infecting concentrate for the HIV positive patient, was available for both patients, and their clinical condition was compared to the molecular biology of their viruses, including sequence data, quantitation and genotype.
I would like to thank my supervisors, Dr C.A. Lee, Dr V.C. Emery and Dr G.M. Dusheiko for providing me with the opportunity to study for this thesis at the Royal Free Hospital School of Medicine. Their help and encouragement was invaluable to me throughout my study period. I would also like to thank the trustees of the Katharine Dormandy Trust who enabled this work to continue by providing the necessary funding.

I would like to thank Dr P.T. Telfer as without his clinical contribution this thesis would not have been possible; Mr D. Brown for teaching me the initial techniques required and for his assistance with the hepatitis C RNA quantitation; Dr C. Sabin for help with the statistics and Mr V. Jenkins whose technical assistance and encouragement enabled this thesis to be completed.

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LIST OF ABBREVIATIONS

AGE  agarose gel electrophoresis
AIDS  acquired immunodeficiency syndrome
ALT  alanine aminotransferases
APS  ammonium persulphate
AST  aspartate aminotransferases
bDNA  branched DNA
BPL  BioProducts Laboratory
BHK-21  baby hamster kidney cell line
C  core proteins of hepatitis C
CDC  Centres for Disease Control
CAH  chronic active hepatitis
C-J  Creutzfeldt-Jacob’s disease
CMV  cytomegalovirus
CNS  central nervous system
CPH  chronic persistent hepatitis
CTL  cytotoxic T lymphocyte
CV  coefficient of variation
DEPC  diethyl pyrocarbonate
DNA  deoxyribonucleic acid
ddNTPs  2', 3'-dideoxynucleoside 5'-triphosphates
dNTPs  2', 3'-deoxynucleoside 5'-triphosphates
DTP  dithiothreitol
E1/E2  envelope proteins of hepatitis C
EBV  Epstein Barr virus
EDTA  ethylenediaminetetra-acetic acid
ELISA/EIA  enzyme-linked immunosorbent assay
FDA  Food and Drug Administration
HAV  hepatitis A virus
HBsAg  hepatitis B surface antigen
HBV  hepatitis B virus
HCC  hepatocellular carcinoma
HCV  hepatitis C virus
HDV  hepatitis D virus
HEV  hepatitis E virus
HGV  hepatitis G virus
HGBV  hepatitis GB virus
HIV  human immunodeficiency virus
HLA  human leukocyte antigen
IFN  interferon
IgA, G, M  immunoglobulin A, G, M
II-2  interleukin 2
IRES  internal ribosome entry site
IU  international units
LB  Luria-Bertani medium
MHC  major histocompatibility complex
M-MLV  moloney-murine leukaemia virus
MU  million units
NAD  nicotinamide adenine dinucleotide
NADH  nicotinamide adenine dinucleotide reduced
NANB  non-A, non-B hepatitis
NHS  National Health Service
NS1-5  non-structural proteins of hepatitis C
OD  optical density
OPD  0-phenylenediamine
PAGE  polyacrylamide gel electrophoresis
<table>
<thead>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>QS</td>
<td>quantitation standard</td>
</tr>
<tr>
<td>rFVIII</td>
<td>recombinant factor VIII</td>
</tr>
<tr>
<td>RFHSM</td>
<td>Royal Free Hospital School of Medicine</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RIBA</td>
<td>recombinant immunoblot assay</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SIA</td>
<td>strip immunoblot assay</td>
</tr>
<tr>
<td>TBE</td>
<td>tris-borate buffer</td>
</tr>
<tr>
<td>TEMED</td>
<td>NNN’N’-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3’,5,5’-tetramethylbenzidine</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
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CHAPTER 1

INTRODUCTION

1.1 The History of Haemophilia and its Treatment

Haemophilia is an x-linked disorder which results in a deficiency of factor VIII (haemophilia A) or factor IX (haemophilia B). Queen Victoria was an obligate carrier of haemophilia as Prince Leopold, her youngest son, had haemophilia and died age 30 years from cerebral haemorrhage. Victoria’s daughters, Alice and Beatrice, were carriers, as was the daughter of Alice, the Tsarina (see Figure 1.1). A picture of the Tsarevitch, her son, shows him standing with his hip and knee bent because the joints were fixed in these positions. Untreated, haemophilia leads to crippling joint disease, wasting muscles and early death as shown in Table 1.1 (Birch, 1937).

The first written references to what was probably haemophilia are found in the Jewish writings of the 2nd century AD (Ingram, 1976). It was said that if a woman had two sons who had died from bleeding following circumcision then she should not have any other sons circumcised (Katzenelson, 1958; Rosner, 1969). At the end of the 18th century various descriptions appeared of families in which males suffered abnormally prolonged post-traumatic bleeding (Zoll, 1791; Consbruch, 1793; Rave, 1796; Otto, 1803). The first medical report which appeared in the literature was written by Otto in 1803 and described the transmission from a mother to her affected sons. Various names were used to describe the disorder, but ‘haemophilia’ (which means the love of blood) first appeared in a paper by Hopff in 1828.

Observations concerning the actual cause of haemophilia were first noted in the 1830s (Wardrop, 1835) and were established as a characteristic feature in 1893
Figure 1.1: The family tree of Queen Victoria
Table 1.1: Cause of death for 113 cases of haemophilia by Birch, 1937

<table>
<thead>
<tr>
<th>Cause of death</th>
<th>No. of cases</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Operations</td>
<td>25</td>
<td>Circumcision 15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tooth extraction 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vaccination 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lanced haematoma 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tonsillectomy 1</td>
</tr>
<tr>
<td>Trivial injuries</td>
<td>23</td>
<td>Cut lip, bitten tongue, injuries to forehead, finger, scalp, etc.</td>
</tr>
<tr>
<td>Epistaxis</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Haematuria</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Throat bleeding</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Cutting first tooth</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Fracture of leg</td>
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</tr>
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<td>Central nervous bleeding</td>
<td>7</td>
<td></td>
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<tr>
<td>Lung haemorrhage</td>
<td>5</td>
<td></td>
</tr>
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<td>3</td>
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<td>Gastric bleeding</td>
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<tr>
<td>Miscellaneous</td>
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<td>Birth trauma and umbilical bleeding</td>
<td>7</td>
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when the prolonged clotting time of haemophilic blood in a capillary tube was observed (Wright, 1893). Addis was the first to realise in 1911 that the addition of thrombin to haemophilic blood shortened the clotting time and so he concluded that it was caused by an abnormality in prothrombin. However, this was disproved and in 1937, Patek and Taylor discovered that a ‘globulin’ precipitated from normal plasma improved the clotting of haemophilic blood. This discovery allowed haemophilia to be redefined around the reduction in this anti-haemophilic globulin which was renamed ‘factor VIII’ in 1962 (Wright, 1962). The distinction between haemophilia A and B was made by Schulman and Smith, and Aggeler in 1952 when factor VIII was detected in plasma but not in serum. The immunological experiments of Ratnoff in 1971 led to haemophilia being defined as a sex-linked, recessive coagulation disorder in which the biological activity of factor VIII is reduced because the factor VIII molecule is functionally impaired (Ingram, 1976).

Many miscellaneous treatments have been described for the treatment of haemophilia, such as the administration of lime and the use of hydrogen peroxide or gelatin (United States Surgeon General’s Catalogue, Second Series, 1901). Even as late as 1942 treatments such as the application of bird’s muscle jostles with x-ray irradiation and autohaemotherapy appear in the Fourth Series of the United States Surgeon General’s Catalogue. However, references to the successful use of transfusion do appear as early as 1832 (Schonlein, 1832) and in 1840 a description of a 12 year old boy with haemophilia, who continued to bleed following surgery for a squint, being treated by the administration of 12 ounces of blood. However, it wasn’t until the 1930s that haemophilic bleeds were treated with transfusions more frequently, although a major trauma was far more difficult to treat.

The concentration and purification of factor VIII proceeded rapidly during the 1950s until 1965 when it was noted that the precipitate in fresh frozen plasma when thawed in the cold contained a high concentration of factor VIII (Pool and Shannon, 1965).
This development allowed widespread production of cryoprecipitate for rapid treatment of severely bleeding haemophiliacs. In the early 1970s the factor VIII from multiple units of fresh frozen plasma or cryoprecipitate was pooled for extraction, concentration and lyophilization. This revolutionised haemophilia treatment as factor VIII levels could be maintained for longer periods of time so allowing surgical procedures to be carried out under relatively safe conditions. The availability of high potency dried replacement products also led to the development of home and prophylactic treatment (Hilgartner, 1991) (see Figure 1.2). Low purity high volume products were replaced by intermediate purity and ultimately the high purity products which are used today.

The benefits provided to haemophilic patients by the introduction of easily available dried factor products were enormous; the overall effect being a lengthening of the life expectancy to that of the rest of the population. Unfortunately, this successful treatment was not without its problems. Some of the major drawbacks occurred as a result of the pooling of up to 25,000 plasma donations from which the lots of dried concentrates were made. The complications which arose from the use of multi-donor pooled concentrates (from paid as well as unpaid donors) included transfusion reactions due to white cells and platelets in fresh frozen plasma and cryoprecipitates, inhibitor development against the transfused factor, immune complex disease with renal disease and chronic joint disease, haemolytic anaemia and a possible decrease in immune function due to the heavy antigenic stimulation (Hilgartner, 1991).

The largest problem, however, occurred with the transmission of viral infections, such as hepatitis A and B, non-A, non-B hepatitis (hepatitis C), hepatitis D, Epstein-Barr virus, B19 parvovirus and the human immunodeficiency virus (HIV), type 1 (see Table 1.2). Due to this problem, the use of virucidal methods is now obligatory for licensed factor VIII and IX concentrates in most countries, and virus-inactivated products have been in use in the U.K. since 1985.
Figure 1.2: A haemophilic boy giving prophylactic treatment
One of the first methods of virus-inactivation tried in the early 1980s was based upon terminal heating of lyophilised concentrates at temperatures between 60°C and 68°C for periods of time between 24 and 72 hours (Mannucci, 1995). Unfortunately, it was shown that these dry-heated concentrates transmitted the hepatitis viruses with high frequency (Allain et al, 1986; Colombo et al, 1985; Preston et al, 1985), and there were also well-documented cases of transmission of the more heat-labile HIV (White et al, 1986; van den Berg et al, 1986). Therefore, these methods of viral inactivation have been largely abandoned. Dry-heating at a higher temperature (80°C for 72 hours) is used for factor VIII and factor IX concentrates manufactured by BioProducts Laboratory (BPL) and the Scottish National Blood Transfusion Service. No case of hepatitis has been recorded in 51 previously untreated haemophilic patients infused with these concentrates (Skidmore et al, 1990b; Bennett et al, 1993; Study Group of the UK Haemophilia Centre Directors, 1988).

Pasteurisation in solution at 60°C for 10 hours in the presence of glycine and sucrose, which stabilise factor VIII activity, is also used for several products ranging from intermediate-purity products to ultrapure products. A number of studies have shown that this method is effective in eliminating HIV (Schimpf et al, 1989) and hepatitis viruses (Schimpf et al, 1987; Mannucci et al, 1990a). However, five cases of hepatitis B or C related to the use of pasteurised concentrates in haemophilic patients not enrolled in prospective studies have been reported (Brackmann et al, 1988; Schulman et al, 1992; Gerritzen et al, 1992b). The significance of these results is not fully understood, but they do indicate that the risk of contracting hepatitis after receiving pasteurised concentrates is not absent.

A third method of virus-inactivation involves heating the concentrate, after its suspension in the organic solvent n-heptane, at 60°C for 20 hours. Two prospective
Table 1.2: Main blood borne viruses transmitted by factor concentrates

<table>
<thead>
<tr>
<th>Virus</th>
<th>Genome</th>
<th>Size (nm)</th>
<th>Lipid envelope</th>
<th>Solvent/detergent resistant</th>
<th>Heat resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human immunodeficiency virus, type 1</td>
<td>RNA</td>
<td>80-100</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>RNA</td>
<td>27</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
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<tr>
<td>Hepatitis B virus</td>
<td>DNA</td>
<td>42</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
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<tr>
<td>Hepatitis C virus</td>
<td>RNA</td>
<td>30</td>
<td>Yes</td>
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<tr>
<td>Hepatitis D virus</td>
<td>RNA</td>
<td>35</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>B19 parvovirus</td>
<td>DNA</td>
<td>20</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
studies (Kemoff et al, 1987; Carnelli et al, 1987) detected five cases of hepatitis, and as a result this method is now only used for two factor IX products.

A solvent/detergent virucidal method, which is based upon the addition to coagulation factor concentrates of an organic solvent, tri (n-butyl) phosphate (TNBP) and a detergent (sodium cholate, Tween 80 or Triton X-100), inactivates large amounts of hepatitis B and C viruses and HIV in vitro, with little or no loss in coagulation factor activity. This method is relatively simple, safe and maintains high yields of biological activity, and, therefore, has become the most widely used method of virus-inactivation among most concentrate manufacturers. Several clinical studies have shown that no cases of hepatitis B, C and HIV infection had occurred in a large number of previously untreated haemophilic patients (Horowitz et al, 1988; Noel et al, 1989; Mariani et al, 1993). However, although this method is highly effective at removing lipid-enveloping viruses, it does not inactivate non-enveloped viruses, for example, Parvovirus B19 infection developed in a number of haemophilic patients infused with a solvent/detergent concentrate (Azzi et al, 1992).

Another virucidal method consists of exposing the moistened lyophilised concentrate to hot vapour at 60°C for 10 hours at a pressure of 1190 mbar (vapour heating). An initial study showed that out of 28 previously untreated haemophilic patients, four developed clinical and/or serological evidence of hepatitis B infection (Mannucci et al, 1988), and one also contracted the hepatitis C virus (Mannucci et al, 1990b). In contrast, a subsequent multicentre study recorded no case of hepatitis or HTV infection (Mannucci et al, 1992b; Shapiro, 1992).

Since 1987, and the widespread adoption of the improved methods of virus-inactivation, only six cases of HIV seroconversion have occurred after infusion of a factor IX complex concentrate treated with beta-propiolactone and UV light (a procedure since abandoned) (Klein et al, 1990). The most frequently used virucidal
methods (dry-heated at 80°C, pasteurisation, solvent/detergent and vapour heating) have all been shown to be effective in eliminating the risk of transmission of both hepatitis B and C viruses.

Newer methods of concentrate purification include the use of a monoclonal antibody which is specific for the von Willebrand protein to selectively bind the von Willebrand factor (vWF) in cryoprecipitate (Lee, 1992). Factor VIII is also bound, but the extraneous plasma proteins and viruses remain free. The unbound proteins and viruses are eliminated by extensive washing. The factor VIII is next eluted, leaving vWF bound to the antibody. The eluted factor VIII is concentrated by ultracentrifugation and further purified by affinity chromatography.

Hemofil M (Baxter) uses factor VIII antibodies covalently bound to sepharose beads, which are then packed into a column and a solution of cryoprecipitate is applied. The dissolved cryoprecipitate containing the factor VIII flows down the column the antibodies fixed to the sepharose beads selectively absorb the factor VIII. The column is then washed with solvent to remove viruses, extraneous proteins and chemical impurities. An elution buffer releases the tightly bound factor VIII from the antibody and it is collected from the column and further purified by ion-exchange chromatography. This method is used to manufacture high-purity NHS factor VIII.

Recombinant factor VIII (rFVIII) is now being produced by transferring the factor VIII gene into a baby hamster kidney cell line (BHK-21) (Lee, 1994) under the control of an adenovirus major late promoter (Toole et al, 1984). In suitable culture conditions, the cells multiply and produce human FVIII along with the normal hamster cell products. The FVIII is released into the medium and purified by ion exchange, gel filtration and immunoaffinity chromatography (see Figure 1.3). To stabilise the FVIII, pasteurised human albumin is added and the product is freeze-
Figure 1.3: A schematic diagram of the manufacture of recombinant factor VIII
dried. The use of rFVIII will eliminate any risk of transmission of viruses. However, it is more expensive than traditional FVIII products and may cause the production of inhibitors, (antibodies to FVIII), therefore, its introduction is slow.

The UK Haemophilia Centre Directors’ Organisation Annual Returns 1994 showed that there are now 5,209 people with haemophilia A, 1,095 people with haemophilia B and 3,654 people with von Willebrand’s Disease (an autosomal clotting disorder) in the UK.

1.2 The History of Haemophilia and Viral Hepatitis

The first reported outbreak of jaundice was in 1883 and occurred amongst workers in a Bremen shipyard who were vaccinated against smallpox with human lymph derived from patients with vaccinia. 191 employees out of 1289 who were given this vaccine became ill with jaundice (Lurman, 1885). Other outbreaks included 41 cases of jaundice in a group of 109 children who had been inoculated with human convalescent plasma for measles (Propert, 1938) and in 1943 seven patients developed jaundice after receiving transfusions of whole blood or plasma (Beeson, 1943).

In the mid 1940s it was discovered that the jaundice associated with transfusions and vaccinations was caused by one of two types of hepatitis (MacCallum and Bradley, 1944 and Havens et al, 1945). The first type was known as homologous serum hepatitis and was transmitted by blood products and the second type was known as infective hepatitis and was transmitted via the oro-faecal route. Controversial transmission studies carried out at an institution for the mentally retarded showed two types of hepatitis (Krugman et al, 1967), one which resembled infective hepatitis with an incubation period of 30-38 days (hepatitis A) and the other which resembled serum hepatitis with an incubation period of 41-108 days (hepatitis B).
Hepatitis A was first described by Feinstone et al in 1973 and rarely causes post-transfusion hepatitis. HAV is an RNA virus which is mainly transmitted via the oro-faecal route. However, transmission of HAV by clotting factor concentrates was recently reported when hepatitis A virus infections occurred in 85 haemophilic patients from Italy, Germany, Ireland and Belgium (Mannucci et al, 1992a; Gerritzen et al, 1992a; Temperly et al, 1992; Peerlinck et al, 1993), all of whom were treated exclusively with solvent/detergent inactivated factor VIII concentrates. HAV, like parvovirus B19, is non-enveloped and, therefore, unaffected by the solvent/detergent procedure. For this reason manufacturers are introducing double processes of virus-inactivation, and vaccination against hepatitis A is recommended in those countries where the vaccine is licenced. HAV seroprevalence depends on the endemic prevalence within the population studied, but has been reported to be higher in younger haemophilic populations than in the general population (Evatt, 1995).

In 1965 Blumberg discovered the association of Australia antigen with Down’s syndrome, leukaemia and serum viral hepatitis which led to the development of antigen and antibody markers for hepatitis B virus (HBV). Dane et al described the HBV virus particles using electron microscopy in 1970 and in 1971 Almeida et al used detergent to separate the surface and core antigens. Magnius and Espmark described the ‘e’ antigen in 1972 after which it became mandatory to test all blood donors for HBsAg in the USA.

The majority of older haemophilic patients who have been treated with unsterilised clotting factor concentrates have serological evidence of previous hepatitis B virus infection (Lee et al, 1995). Most of these infections were acquired sub-clinically followed by acquired immunity as shown by the presence of surface antibody (anti-HBs). There is evidence that chronic carriers of HBsAg have suppression of other viral infections, particularly hepatitis C, and this is known as viral interference (Lim et al, 1991; Hanley et al, 1993). HBV infection has been virtually eliminated in
haemophilic patients due to the introduction of sterilisation procedures of the factor concentrates, and the use of the HBV vaccination.

Vaccines were derived from HBsAg carriers, but are now produced by recombinant technology in yeast. It was initially thought that a protective level of anti-HBs would usually be retained for at least five years, but there is now evidence that it may be lost much earlier at two or three years (Hadler et al, 1986; Jilg et al, 1984). In a further study it was estimated that the median time for anti-HBs levels to fall to 100 IU/l was between 36 and 42 months, with a shorter period for older or HIV-infected individuals (Pillay et al, 1994).

After the development of markers for hepatitis A and B it became clear that non-A, non-B (NANB) hepatitis was the major cause of post-transfusion hepatitis. A study by Alter et al in 1975 showed that 89% of the hepatic events following open-heart-surgery in multiply transfused patients were caused by NANB virus(es). In 1975, Craske et al highlighted this problem in the haemophilic population who had been treated with the new dried factor products by showing symptomatic NANB hepatitis developed in 52 out of 417 patients in the UK. Two other studies in haemophilic patients (Fletcher et al, 1983; Kernoff et al, 1985) both showed a 100% infection rate of non-A, non-B hepatitis after administration of unsterilised clotting factor concentrate.

Non-A, non-B hepatitis infected virtually 100% of haemophilic patients who had received unsterilised clotting factor concentrates or other blood products between 1965 and 1985 (Lee et al, 1993). In one study 232/241 patients who had received unsterilised clotting factor concentrates showed anti-HCV seropositivity (Telfer et al, 1994). Another study (Lim et al, 1991) assessed 58 patients following a first exposure to clotting factor concentrate, and showed that the median time to AST elevation was four weeks (range 1-7 weeks) and to anti-c100-3 seroconversion was
11 weeks (range 7.5-14.5 weeks). In the same group of patients it was shown that the time to anti-c22 seroconversion was 6.3 weeks (range 7.5-14.5 weeks) (Dourakis et al, 1992). These intervals to seroconversion were much shorter than those for transfusion recipients, and this may be due to a higher inoculum in the pooled concentrates. It is not clear if this high, and repeated, exposure has a deleterious effect in haemophilic patients.

In 1989 the major causative agent of NANB hepatitis was described and named hepatitis C virus (HCV) (Choo et al, 1989). Since then antibody tests have been developed which have enabled testing of all high risk populations. Haemophilic patients have a particularly high seroprevalence, reported to be between 59 and 85% (Makris et al, 1990), depending upon age, the duration of infection, unsterilised clotting factor usage and source of clotting factor.

Hepatitis D virus (HDV or Delta agent) was identified in 1977 and is an incomplete RNA virus which requires helper functions of HBV for its propagation (Rizzetto et al, 1980) as it uses the HBsAg as its coat. Among recipients of multiple blood transfusions and haemophilic patients there is a high risk of acquiring HDV infection (Rosina et al, 1985). Haemophilic patients with HDV infection have rapidly progressive liver disease and a higher rate of death from this infection.

Hepatitis E was first isolated in 1990 and is a self-limited, enterically transmitted acute viral hepatitis that occurs most frequently in epidemic outbreaks (Krawczynski, 1993). HEV is frequently spread by faecally contaminated drinking water and is particularly prevalent in the developing countries of India, Asia and Africa. HEV only has a high mortality rate in pregnant women, and is less infectious than hepatitis A. There have been no cases described where infection with HEV has been associated with the use of clotting factor concentrates.
In 1995 hepatitis G was isolated by Genelabs Technologies, US (unpublished). HGV was cloned from a patient infected with hepatitis C and then passaged in marmosets. It has a single open reading frame of 9393 nucleotides, and has a 26% amino acid homology to hepatitis C. No epitopes have been defined so far, but it can be amplified by polymerase chain reaction. Preliminary prevalence studies have shown that 1.5% are infected in the general population (200 tested), and of 49 haemophilic patients tested 18% were infected. HGV appears to develop into persistent infection and shows a similar response to interferon as observed for hepatitis C.

Hepatitis GB was also isolated in 1995 (unpublished). Named after George Barker, a surgeon who developed hepatitis in the mid 1970s, GB-A, GB-B and GB-C have been described. GB-A and GB-C are closely related, but all three are distinct from all hepatitis C genotypes. Preliminary prevalence studies have shown a 2% incidence amongst blood donors (200 tested) and a 14% incidence in 101 multi-transfused patients. The low incidence of hepatitis GB amongst multi-transfused patients is unexplained considering the high incidence amongst blood donors.

HIV is thought to have infected haemophilic patients from 1979 to 1985, with the first AIDS cases in haemophilic patients being reported in 1982. It has been estimated that 80% of patients over 10 years of age with severe haemophilia A in the USA are infected with HIV (Goedert et al, 1989). CD4 is a T-cell receptor. HIV has been shown to replicate in CD4+ T-cells, therefore the progression of HIV disease can be monitored by measuring the CD4 counts. It has been shown that the rate of progression to AIDS can be determined largely by differences in the rate of decline of the CD4 count (Phillips et al, 1991).

HIV can have a long latent period, up to approximately 20 years in some patients, and there are various prognostic markers of progression to symptomatic disease. It
has been shown that older age at the time of seroconversion is associated with an increased risk of AIDS in haemophilic patients (Eyster et al, 1987). A high titre of p24 antibody at seroconversion is associated with a longer time to the development of HIV-related disease in haemophilic patients (Cheingsong-Popov et al, 1991). Previous cytomegalovirus (CMV) infection is also associated with a more rapid progression to AIDS in haemophilic patients (Webster et al, 1989). The HLA haplotype A1 B8 DR3 was shown to distinguish amongst 18 haemophilic patients infected with HIV, those who would progress to AIDS (Steel et al, 1988). High CD8 counts and high IgA levels have also been associated with a more rapid loss of CD4 cells (Phillips et al, 1993).

It is likely that all haemophilic patients infected with HIV as a result of treatment with unsterilised clotting factor concentrates must also have been infected with HCV. There is increasing evidence that liver disease is more severe when there is co-infection with HIV and HCV (Eyster et al, 1993). It is possible that the immune deficiency of HIV infection enhances HCV replication.

Cytomegalovirus (CMV) transmission has been associated with the use of cellular products, but the risk can be reduced by using anti-CMV positive blood (Evatt, 1995). In HIV infection, acute CMV infection can produce serious morbidity. Therefore, CMV negative, HIV positive haemophilic patients should not be exposed to blood products from CMV positive donors.

National regulatory agencies have recently discussed the potential transmission of Creutzfeldt-Jacob’s disease (C-J) or other slow acting agents by blood or blood products (Evatt, 1995). These diseases are thought to be transmitted by prions or proteins which are capable of modifying similar cellular proteins. C-J disease has not yet been transmitted by blood except in some animal experiments where the blood is
injected directly into CNS tissue. These agents are extremely resistant to all known viral inactivation procedures.

Multiple viral infections occur in haemophilic patients, and, as a result, complex viral interactions occur. Concurrent infection with HCV and HBV interferes with the replication of both viruses and HCV PCR is often negative in patients who are also HBsAg positive (Hanley et al, 1993). Infection with HIV as well as HCV seems to increase the rate of progression to liver failure and AIDS (Eyster et al, 1993; Telfer et al, 1994). It is possible that the immunosuppressant effects of HIV infection facilitate increased HCV replication and increased liver damage.

1.3 Hepatitis C Virus

1.3.1 Virology

The hepatitis C virus (HCV) is a single-stranded, positive sense RNA virus with one large open reading frame of 9370-9481 nucleotides coding for approximately 3000 amino acids. The virus particles have a diameter of approximately 30nm and have a lipid envelope (see Figure 1.4). The open reading frame encodes a single polyprotein precursor which is subsequently cleaved into functional proteins by host and viral proteases (Alter HJ, 1995). It has no DNA intermediate and so cannot integrate into the host genome, but it does use a negative-strand RNA in its replication cycle within the liver. HCV is now considered to be a member of the Flaviviridae having the closest relationship to the pestiviruses that include hog cholera virus and bovine diarrhoeal virus.

The size and density of the HCV particles are highly variable (Jursch et al, 1994). The large particles, which are more than 40nm in diameter and have a low density of between 1.02 and 1.08g/ml, may be associated with lipoproteins which may be
Figure 1.4: An Artistic Representation Of The Hepatitis C Virus Particle

RNA
9033 nucleotides

Nucleocapsid

Envelope

5'

C
E1
E2
NS2
NS3
NS4
NS5
3'
involved in transporting the virus to the liver, and, therefore, these particles could be very infectious. The small particles range in size from 10-25nm and have densities from 1.03 to 1.21g/ml and they are probably defective virus particles. Free, replication competent HCV particles are thought to be between 25-33nm in diameter.

Shortly after the first HCV-specific clone was detected (Choo et al, 1989) the entire viral genome was sequenced and many of its structural and functional relationships were defined (Choo et al, 1991) (see Figure 1.5). The 5' untranslated region (UTR) is 329-341 nucleotides in length and is highly conserved between different HCV isolates. This suggests that it has a major regulatory role in transcription or translation. The 5' UTR has three to six start codons and is folded in a very complicated structure containing nine hairpins A-I (Bredenbeek et al, 1994). Deletion of the first hairpin (A) results in enhanced translation, whilst deletion of the first three hairpins (ABC) did not affect translation. Deletion of all the others (D-I) prevented translation. Therefore, all the hairpins from D to I are necessary for the internal ribosome entry site (IRES) which allows translation to proceed.

Downstream from the 5' UTR are the structural regions of the genome coding for the core (C) and the envelope glycoproteins (E1 and E2/NS1). It is unknown whether the NS1 region is part of the envelope region or the first non-structural (NS) region but the core region is relatively conserved whereas the envelope region shows high sequence variation between different strains. Further downstream from the structural regions are the non-structural regions NS2 to NS5. The function of NS2 is unknown, but it is thought that the 5' end sequence of NS3 codes for a protease (Bartenschlager et al, 1994) and another segment of NS3 codes for a helicase. NS4A is a cofactor of the proteolytic activity of NS3 and NS4B may have membrane binding properties. NS5A or NS5B are predicted to encode an RNA-dependent RNA polymerase. Finally, the 3'UTR shows considerable variation between isolates with some having a poly-(rU) tail and others having a poly-(rA) tail. Overall, the 5'
Figure 1.5: Schematic diagram of the hepatitis C virus genome and its putative proteins
UTR, core, NS3 and NS4 are relatively well conserved whereas E1, E2/NS1, NS2 and NS5 are the most variable regions.

HCV is a highly variable virus and various classification schemes have attempted to group isolates into genotypes (Simmonds et al, 1993a; Okamoto et al, 1992a). Genotyping of the virus is a useful tool in studying its evolutionary history; the effects of different genotypes on serological diagnosis; the development of protective antibodies; the effect of treatment on different genotypes and the epidemiology of spread. Phylogenetic analysis of different HCV isolates in the 5' UTR and NS5 regions showed clustering of sequences into six major groups (Simmonds et al, 1993a). Each major group contains a number of more closely related variants designated subtypes. Although different systems of nomenclature have been proposed the most widely used is that of Simmonds et al (1993a), where the major genotypes are designated 1 to 6 and the subtypes designated a, b, and c etc. HCV genotypes can now be easily determined by the use of either RT-PCR with type-specific primers or by performing RT-PCR followed by type-specific restriction endonuclease digestion.

At present, attempts to culture HCV have been unsuccessful. A group in the USA (Beach et al, 1994) have attempted to culture HCV in PK15 cells and have been able to propagate the virus for over 60 passages. Reverse transcription-polymerase chain reaction (RT-PCR) has shown that the virus is being propagated and sequence analysis has shown the virus to be identical to the original inoculum. Chimpanzees were inoculated with tissue culture derived HCV and became PCR positive but their aminotransferase levels remained normal over 200 days and no seroconversion was detected. Therefore, the virus appears to have become attenuated during tissue culture and further studies are required in this field.
Hepatitis C has a complicated and little understood relationship with the immune system. It has been shown that HCV persists in approximately 80% of cases (van der Poel et al, 1991a) despite an apparently strong immune response. This mechanism of persistence appears to reside in the virus' ability to mutate rapidly under immune pressure and to exist simultaneously as a series of related, but immunologically distinct variants, any one of which can become the predominant strain when a coexistent strain comes under immune pressure (Alter, 1995). Among HCV isolates the average mutation rate is $10^{-3}$ to $10^{-4}$ base substitutions/genome site/year (Ogata et al, 1991) and although mutations occur throughout the HCV genome, most occur in a short, hypervariable region of the E2/NS1 domain (Weiner et al, 1991), implying that this region contains key target epitopes which are under constant immune attack. Other immune escape mechanisms include the formation of defective virus particles which direct the immune system away from the replicative virus particles (Huang et al, 1988). HCV could also downregulate its replication in order to escape immune eradication and remain in the liver in a quiescent state, or continue replication in other, extrahepatic, sites (Shimizu et al, 1992).

An in vitro neutralisation study (Shimizu et al, in press) directly confirmed the in vivo studies of Farci et al (1992) and concluded that neutralising antibodies to HCV do develop but that they are highly strain specific and are ineffective against emerging strains and that persistent infections can occur despite the presence of neutralising antibodies. In support are cross-challenge studies in chimpanzees (Farci et al, 1992) showing that an animal infected with one strain can be reinfected with a different strain. These studies also showed that a chimpanzee after appearing to recover from one episode of hepatitis could be reinfected with exactly the same strain of HCV, therefore, in chimpanzees, there appears to be no protective immunity against heterologous or homologous rechallenge.
The role of T-cell responses in hepatitis C infection is highly complex and it is unknown why hepatitis C can persist in the face of a cytotoxic T lymphocyte (CTL) response. Rehermann et al (1995) found that chronically infected patients displayed a polyclonal and multispecific CTL response to HCV which was 4-12 times lower than their CTL response to the influenza virus. This low level of CTL response may be one reason why hepatitis C can continue to persist. Several studies have found that cytotoxic T lymphocytes may play an important role in the pathogenesis of hepatocyte injury in patients with chronic active hepatitis C (Onji et al, 1992; Freni et al, 1991). Napoli et al (1995) found that progressive tissue damage in HCV infection was associated with the upregulation of intrahepatic Th1 cytokines, such as IFNγ and IL-2. Their results suggested a role for cytokine mediated tissue injury which was not related to viral load. They hypothesised that the continual immune pressure (Th1) on HCV drives mutation which causes further Th1 production and, therefore, creates a continual cycle of tissue damage.

Considering the obstacles detailed above, the prospects for a vaccine seem remote. Preliminary studies (Ralston et al, 1993) have used E1 and E2 glycoprotein complexes expressed by recombinant vaccinia viruses as a vaccine to immunise seven chimpanzees. Five out of the seven appeared to be protected from an homologous rechallenge which infected all of the controls. However, the challenging dose was low titre and was given when the antibody response to the original challenge was at its peak. A new approach to vaccination is that of gene transfer by the injection of plasmids containing parts of the viral genome resulting in the induction of cytotoxic T-cell and neutralising antibody responses. This approach has been used successfully for heterologous protection against influenza (Ulmer et al, 1993) and is being considered as a possibility for HCV immunisation (Lagging et al, 1995).
1.3.2 Diagnosis

The first HCV clone isolated resulted in the production of the first HCV protein which was designated 5-1-1, and this was extended to form the c 100-3 antigen which served as the basis of the first generation anti-HCV tests with an enzyme-linked immunosorbent assay (ELISA). The main problem with this original assay was that antibodies to c 100-3 often did not appear until 12 to 26 weeks after infection. The second generation ELISA had two other epitopes derived from the core protein and the NS3 protein designated c22-3 and c33c, respectively. Antibodies to these antigens generally appeared earlier than anti-c100-3 with the majority of HCV-infected individuals being detected within 15 weeks. Third generation ELISA tests, which have been introduced in Europe, incorporate an antigen in the NS5 region and omit the c100-3 antigen. Unfortunately, all these ELISA tests have a problem with false positive results. This can be associated with aged sera, hyperglobulinemia, rheumatoid factor-positive sera and sera from individuals recently vaccinated against influenza, but is often unexplained.

Due to the nonspecificity of the ELISA tests it is necessary to confirm the results with a supplemental assay. The only licensed confirmatory assay is the recombinant immunoblot assay (RIBA) where HCV antigens are applied separately on nitrocellulose strips and, after incubation with the patient’s serum, the anti-HCV antibody recognition patterns are visualised using anti-gamma globulin and substrate. A positive result requires reactivity with at least two protein positions and reactivity at only one protein position is reported as indeterminate. The third generation RIBA used in Europe involves the use of synthetic peptides from the core and NS4 regions and recombinant antigens from the NS3 and NS5 regions.

In view of the limitations of the detection of antibodies to HCV the most sensitive way to detect HCV is the amplification of HCV RNA by the use of the polymerase
chain reaction (PCR). PCR studies have shown that HCV RNA can be detected very early after infection with animal studies detecting HCV RNA within days of exposure and almost always within two weeks (Farci et al, 1992). In humans HCV RNA becomes detectable in the serum within one to two weeks of exposure, and so it precedes the increase in serum alanine aminotransferases (ALT). Quantitative PCR has shown that the highest levels of HCV RNA are early in the course of infection and usually coincide with first significant rise in ALT levels (Lau et al, 1993). In the majority (approximately 80%) of infected individuals HCV RNA persists, often with fluctuating levels generally associated with fluctuating ALT elevations, but sometimes even in the absence of biochemical abnormalities. The implications of this observation are that individuals may be most infectious before clinical signs appear, that chronic HCV infection may be more common than ALT results alone indicate and that there may be a small group of asymptomatic carriers with normal ALT levels and minimal damage in the liver. The increasing importance of HCV nucleic acid detection for diagnosis requires standardisation of the techniques involved and this may be more readily achieved by the introduction of a commercially available HCV PCR assay using a single enzyme for reverse transcription and PCR (Tilston et al, 1994).

Quantitation of HCV RNA is important as it may provide an insight into the progression of disease and it may be a prognostic marker of treatment (Lau et al, 1993). Quantitation by standard PCR is unreliable as the amount of the PCR product increases exponentially with each cycle of amplification. Therefore, any differences that affect the efficiency of amplification can dramatically alter product yield (Syvanen et al, 1988; Gilliland et al, 1990). To circumvent this problem, competitive PCR has been used for accurate quantitation of RNA (Andre and Hahlbrock, 1989; Gilliland et al, 1990; Lundeberg et al, 1991). Competitive PCR involves the establishment of calibration of an external standard which is identical to the target sequence (Rappolee et al, 1988) or comparison with a co-amplified, unrelated
'reporter' RNA (Chelly et al, 1988). However, this method of quantitation is expensive, time-consuming and of limited application when analysing large numbers of samples (Kaneko et al, 1992). These problems have resulted in a new approach, the branched DNA (bDNA) signal amplification, being developed (Urdea et al, 1990; Urdea et al, 1991). A comparative study (Bresters et al, 1994) show that a semi-quantitative cDNA PCR assay correlates strongly with the quantitative bDNA assay. The bDNA assay is easy to perform and less contamination-prone as compared to the cDNA PCR assay. However, it is expensive and lacks sensitivity in patients with low viral titres in that, at present, it cannot detect viral titres of less than $3.5 \times 10^5$ genome equivalents/ml. This study suggested testing first with the bDNA assay and then testing all negative samples with a competitive PCR assay.

Clinical assessment of hepatitis C infection usually involves serum aminotransferase measurements. Chronic HCV is characterised by raised or fluctuating ALT levels or long term marginal increases in ALTs. In cross-sectional studies, 60% of HCV infected individuals have HCV viraemia and normal ALT values but most (80%) of these have evidence of chronic persistent, and sometimes chronic active, hepatitis with or without cirrhosis (Esteban et al, 1991). Therefore, a normal ALT result in patients with HCV infection is of limited prognostic value.

1.3.3 The course of HCV infection

Acute hepatitis C is best documented in the transfusion setting where it is a generally benign event with 70% to 80% of cases being asymptomatic. Community-acquired cases, where individuals had to be sufficiently ill to seek help from their medical practitioner, showed a higher incidence of symptomatic hepatitis illness. These cases confirm that hepatitis C can present as an acute hepatitis which is indistinguishable from hepatitis A or B. Using the data available from transfusion studies it can be estimated that no more than 25% of hepatitis C infections result in an acute illness.
Documented fulminant hepatitis C is extremely rare. Farci et al (1994) have studied three cases and found that the fulminant nature of the infection was probably related either to a high viral burden or to a unique host response. In HCV infection the mechanisms of liver cell destruction are still largely unknown. The virus may be directly cytopathic, in which case the viral load would seem to be the critical determinant in fulminant hepatitis. Alternatively, there may an important immune-mediated component via either cytotoxic T cells or immune complex formation. It has been shown that acute HCV infection of liver grafts in immunocompromised patients can result in subfulminant hepatitis (Pereira et al, 1991) and this indicates that HCV is directly cytopathic rather than the liver injury occurring as a result of an immune response to the virus. Both these hypotheses probably have an important role to play in HCV-related liver injury.

The major consequences of HCV infection relate to the establishment of chronic infection. HCV infection persists in approximately 80% of cases but the majority of these patients are asymptomatic or only mildly symptomatic. Serum aminotransferases decline from the values seen during the acute phase of the infection but will usually remain abnormal. The ALT levels fluctuate over time and some individuals have normal ALT levels for prolonged periods of time while remaining HCV RNA positive. Initially, it was thought that non-A, non-B hepatitis represented little more than a non-specific transaminitis, but evidence began to show that the vast majority of individuals had chronic persistent hepatitis (CPH), chronic active hepatitis (CAH) or cirrhosis. HCV appears to be a very indolent infection with the natural history of the disease taking place over many decades. A study by Kiyosawa et al (1982) showed that the mean time to clinical presentation of chronic hepatitis was 14 years whereas clinical cirrhosis and hepatocellular carcinoma were not evident for 18 and 23 years respectively after transfusion.
The severity of chronic HCV infection can be assessed by studying the proportion of liver transplants performed due to HCV. Although there are many variables which favour these cases for transplantation, surveys have shown that many HCV infected patients develop end-stage liver disease and survive only by virtue of transplantation. Another way to assess the severity of HCV infection is to evaluate asymptomatic individuals. One study of anti-HCV positive donors (Esteban et al, 1991) showed that 8% had minimal changes, 23% had CPH, 60% had CAH and 9% had active cirrhosis. This study also found that there was a significant correlation between ALT values and histologic activity. A similar study (Conry-Cantilena et al, 1992) looked at 31 asymptomatic anti-HCV positive donors and found that biopsy samples had more severe lesions with increasing ALT values.

The final main complication of HCV infection is its association with hepatocellular carcinoma (HCC). Almost all HCV related HCC occurs with the presence of cirrhosis and it is currently unclear if the virus is directly oncogenic or if it induces cirrhosis and the mitotic events that accompany attempts at rapid regeneration in the chronically damaged liver.

The main question about chronic HCV infection is why do some individuals, even those with cirrhosis, survive well for long periods while others progress to a fatal outcome in as little as 10 years. Rapid, progressive disease may be aggravated by concurrent HBV or HIV infections and concomitant alcohol abuse. A study in Italy (Bellentani et al, in press) showed that among a population of 6,917 individuals alcohol was the leading cause of liver disease, but the combination of HCV and alcohol resulted in a ten-fold increase in cirrhosis and a six-fold increase in HCC compared with alcoholics with no evidence of any viral hepatitis. Other reasons for rapid progression of disease may include more virulent strains of HCV, higher levels of virus in the liver, different immune responses or genetic susceptibilities or the presence of cofactors which enhance the severity of HCV infection.
1.3.4 Epidemiology and Transmission

Hepatitis C has a worldwide distribution with relatively high prevalences in Japan, the southern part of the USA, the Mediterranean countries of Europe, Africa and the Middle East, where 0.5-1.5% of blood donors are anti-HCV positive. In Northern Europe, the northern part of the USA and Canada prevalences are between 0.1-0.5% (van der Poel et al, 1994).

Epidemiologic studies (McOmith et al, 1994) have found that HCV types 1, 2 and 3 account for the vast majority of infections in Europe and Australia, although types 2 and 3 were not found in the Eastern European country of Hungary where the majority of infected blood donors were type 1 (see Figure 1.6). Southern European donors were also found to be predominately infected with type 1. North American donors were found to be infected with types 1 and 2 with a small number of type 3 infections. Donors from Japan, China and Taiwan were infected with only types 1 and 2, and donors from Hong Kong had type 6 as well as types 1 and 2. South Africa is the only region so far with evidence of type 5 infections, and it also has a small percentage of types 1 to 4. Type 1 predominates in Turkey, whereas Middle Eastern countries such as Saudi Arabia, Iraq, Yemen and Kuwait have mainly type 4. Type 4 infections were also found to predominate in Egypt. In Western countries (types 1, 2 and 3) there appears to be little subtype diversity, whereas in countries such as Egypt (type 4) there is a large subtype diversity which may suggest that type 4 has a longer evolutionary history than types 1 to 3.

The main route of transmission of hepatitis C is parenteral and most HCV-infected individuals are either intravenous drug users or recipients of blood products that have not been screened for anti-HCV (van der Poel et al, 1991b). Blood donor screening has virtually eliminated post-transfusion hepatitis C and sterilisation of blood clotting factors has also eliminated the risk to haemophilic patients. Other
Figure 1.6: Worldwide hepatitis C virus genotype distribution
parenteral risks include ear-piercing, tattoos and needle-stick injuries which account for 3-10% of HCV-infected individuals. Haemodialysis patients have also been at risk of infection from the dialysis procedure and steps to eliminate this have been taken in the UK including using patient-specific equipment.

Although parenteral transmission is the main route of transmission for HCV, there are also many cases of community acquired infection with no demonstrable parenteral exposure (Alter MJ et al, 1990). The true risk of sexual transmission of HCV is unknown but studies so far have shown it to be low. In high risk populations such as homosexuals (Nelson et al, 1991) and female prostitutes and their male clients (Lissen et al, 1993) HCV infection was found in 4% after controlling for coexistent intravenous drug use. This prevalence is above the blood donor population (0.3-1%) but below that of other viruses such as hepatitis B and HIV (60-80%) in the homosexual and prostitute populations. It has also been shown in the USA that the anti-HCV prevalence is five fold higher in the general population than in the highly selected blood donor population and, therefore, not significantly different from the high risk populations. Two other studies (Bresters et al, 1993; Gordon et al, 1992) have reviewed the incidence of anti-HCV antibodies in partners of 92 HCV positive individuals and found no evidence of HCV transmission. However, in Japan a similar study with 154 index cases (Akahane et al, 1994) found that 16% of spouses were also infected with the same genotype of HCV. These infections were associated with longer duration of marriage so these couples may have been exposed to the same risks decades ago. Eyster et al (1991) studied 235 haemophiliacs and their sexual partners. Among the 170 female partners of HIV and HCV positive haemophiliacs 12% were HIV positive and 4% were HCV positive. In contrast, of the 30 females whose partners were HCV positive but HIV negative none were HCV positive. From these studies, and the many others which have been carried out, it is likely that HCV is either not sexually transmitted or is transmitted at a low efficiency except where co-infection with HIV, or other immunodeficiency,
exists as the viral load may be higher. Guidelines from the Centres for Disease Control (CDC) for prevention of sexual transmission of HCV include the use of barrier contraception for those with multiple partners. Those infected individuals within a monogamous relationship should have their partners tested and allowed to make their own decision regarding the use of barrier contraception after being advised of the uncertainties of sexual transmission of HCV.

The role of vertical transmission in the spread of hepatitis C is also little understood. The vast majority of infants born to anti-HCV positive mothers are themselves anti-HCV positive, but at this early stage it is unknown if this is a sign of true infection or merely the passive transfer of maternal antibody. The determination of neonatal transmission is now entirely based on the use of RT-PCR to detect the virus. Most studies (Wejstal et al, 1992; Lam et al, 1993; Reesinck et al, 1990) show that the risk of vertical transmission is between 0-5%, but one study (Giovannini et al, 1990) showed that 44% of infants born to anti-HCV positive mothers became infected. However, in this particular study all the HCV positive infants were also HIV positive, indicating, as with sexual transmission, that there may be a greater risk of HCV transmission when HIV is also present. The results from the many published studies are conflicting, but it seems likely that the risk of vertical transmission is low except in cases where the maternal viral load is likely to be high. There are currently no specific CDC recommendations regarding vertical transmission and there are no recommendations against pregnancy in anti-HCV positive women.

There is some evidence that household transmission is involved in infecting some HCV positive individuals. Unfortunately, the studies carried out so far have been too small, the methods too diverse and the follow-up not good enough to draw any definite conclusions about the risks of household transmission. Extensive medical histories are required in order to eliminate intravenous drug use or other parenteral risks from the possible routes of transmission. However, in one study (Conry-
Cantilena et al, 1992) 44% of individuals interviewed admitted to intravenous drug use with shared needles despite having denied this on earlier occasions. In order to minimise the risk of household transmission the CDC recommends that razors and toothbrushes should not be shared.

Healthcare workers are also at risk of contracting hepatitis C (Alter MJ et al, 1982; Kiyosawa et al, 1989) mainly by needlestick injuries (Vaglia et al, 1990). There have also been cases of transmission from surgeon to patient (Alter MJ, 1994) and transmission via the human bite (Dusheiko et al, 1990). In conclusion the epidemiology and transmission of this virus remains little understood, therefore, further large-scale studies are required.

1.3.5 Therapy

The first treatment for non-A, non-B hepatitis was tested in 1986 (Hoofnagle et al, 1986) and was a member of the interferon family. Interferons comprise three families of proteins - alpha, beta and gamma (Rubin et al, 1994). Lymphocytes, macrophages and fibroblasts normally produce interferons in response to viral antigenic stimulation. Interferons can induce cytokine gene expression and enhance the function of natural killer cells, cytotoxic T cells and macrophages. Interferons stimulate target cells by binding to specific cell surface receptors rather than directly inactivating the virus particles. The antiviral effect of the interferons is due to the modulation of different steps of the viral replication cycle (Baron et al, 1991), including the activation of endoribonucleases that degrade viral RNA (Peters et al, 1989). Interferon also induces the synthesis of a double stranded RNA-dependent protein kinase that interferes with the elongation step in viral protein synthesis. Interferon also has immunomodulatory actions including increasing HLA class I expression.
The typical HCV treatment protocol for interferon is three mega units three times a week for six months. Many variations on this protocol have been tried including increasing the dose and the length of time of treatment. Generally, increasing the dose does not seem to improve the overall outcome of treatment and can result in intolerable side effects. Prolonged administration may be beneficial, and a treatment course for one year and a possible low dose (1 MU) maintenance therapy may improve response rates. Initial studies showed that approximately 50% of treated patients had a response (defined as a normalisation or a near normalisation of ALT) but when therapy was withdrawn only 20% of patients had a sustained response. Although this relatively low rate of biochemical response is discouraging, it far exceeds the spontaneous clearance rate of chronic HCV infection.

The use of interferon does have adverse effects which increase with dose. These include fatigue, malaise, transient minor flu-like symptoms such as fever, myalgia, arthralgia and headache and often follow the injections for several hours. The symptoms can usually be alleviated by acetaminophen or nonsteroidal anti-inflammatory drugs and may be minimised if the patient administers the interferon prior to going to bed. Other, less common effects include diarrhoea, anorexia, mild alopecia, anxiety or increased irritability. Laboratory abnormalities include usually reversible mild thrombocytopenia or leukopenia. Mild asymptomatic thyroid function test abnormalities may also be seen, but clinically significant thyroid dysfunction is rare.

Interferon has a low response rate, therefore, many studies have assessed the variables which could influence this response. Preliminary evidence has shown that histological stage, viral load, HCV genotype and age affect the response to therapy. Dusheiko et al (1994b) studied 610 patients with chronic hepatitis C and found that those who had either serotypes 2 or 3, were non-cirrhotics and were younger than 40 years old responded better to therapy with interferon. It is now apparent,
however, that the best candidate for interferon response is a low viral load (less than $3 \times 10^5$ genome equivalents/ml), low ALT levels and generally less severe histologic lesions. The consensus of opinion is that those patients with compensated liver disease who have sustained biochemical evidence of chronic hepatitis and have chronic active hepatitis or active cirrhosis on liver biopsy should be treated. Most also agree that patients with inactive cirrhosis or decompensated liver disease should not be treated, but controversy still exists as to whether to treat the milder cases (Alter HJ, 1995). Haemophilic patients present an even more complicated problem as their individual cases must be assessed without the results of liver histology for their HCV infection as any invasive procedure for haemophilic patients carries a risk of severe bleeding complications. Therefore, the decision to treat a haemophilic patient usually relies only on the genotyping and quantification data.

Interferon is the main treatment for hepatitis C at present, but another drug has been tested in some studies. Ribavirin is a synthetic nucleoside analogue (1-β-D-ribafuranosyl-1, 2, 4-triazole-3-carboxamide) resembling guanosine and inosine. It appears to interfere with the expression of messenger RNA and to inhibit viral protein synthesis. It is not significantly incorporated into host cell RNA or DNA (Committee on Infectious Diseases, 1993). Single use of this drug for chronic hepatitis C infection has had unsatisfactory results. It appears that while the use of ribavirin can reduce the ALT levels, it has no effect on the serum HCV RNA. Recently a number of studies have assessed the use of a combination therapy of alpha-interferon and ribavirin. Lai et al (1994) looked at 60 patients randomised into three groups: A-interferon plus ribavirin; B-interferon only and C-no drugs. Approximately 75% of group A showed a sustained ALT response one year after treatment was withdrawn compared with 14% of group B and 10% of group C. Histological findings also showed an improvement in those patients who had a response to therapy but HCV RNA was not tested. Therefore, the combination of
interferon plus ribavirin appears promising but further large-scale studies are required.

There is a third promising therapeutic concept currently under consideration. This is the use of antisense oligonucleotides. The hepatitis C virus RNA is considered the 'sense' strand since it contains the information needed to produce the protein product (Samara et al, 1993). An oligonucleotide that has the complementary base sequence to the RNA is called the antisense strand. This antisense strand is not found naturally, but it can be easily synthesised using automated machines. If a specific antisense oligonucleotide is introduced to the HCV RNA, it will bind by complementary base pairing and so prevent translation. Four studies (Yoo et al, 1994; Wakita et al, 1994; Alt et al, 1994; Yagi et al, 1994) have looked at the possibility of directing antisense oligonucleotides at the highly conserved 5' UTR in animal models. They all concluded from their results that this form of therapy could be used for the treatment of chronic hepatitis C. The main problems with the antisense approach include the cost of development and eventual treatment as oligonucleotide production is highly expensive, and the difficulty of directing the oligonucleotides to the target in sufficient amounts.

It has been estimated that between 300 and 500 million people in the world are infected with the hepatitis C virus. In the UK, 3122 haemophilic patients are living who have received unsterilised clotting factor concentrates between 1977 (when records began) and 1985 and, therefore, must have been exposed to hepatitis C. Since its characterisation in 1989 our knowledge of this virus has vastly increased, but, as outlined above, there is still an immense amount of work required especially in the fields of virus persistence, vaccine development, therapy and disease progression.
CHAPTER 2

OBJECTIVES

2.1 The molecular biology of HCV prior to 1992

The early published studies which looked at the molecular biology of the hepatitis C virus were mainly concerned with the heterogeneity of the virus sequences. Arima et al (1989) cloned serum RNA associated with hepatitis C infection and found that different clones reacted with different sera, so suggesting the presence of heterogeneity in Japanese hepatitis C. Later studies (Kato et al, 1990b; Hosoda et al, 1991) used PCR and direct sequencing to show viral heterogeneity. These studies also suggested that direct detection of viral RNA by PCR may be a useful indicator in the study of hepatitis C infection. Many publications followed with the discovery of novel viral sequences (Kato et al, 1990a; Choo et al, 1991; Takamizawa et al, 1991; Okamoto et al, 1991; Chan et al, 1992), and once variable genomic sequences had been described several papers were published which attempted to group the more closely related variants into types. Okamoto et al (1992b) designed a method for distinguishing between four different types by the use of type-specific primers in the core region of the genome. It is only since 1992 that Simmonds et al (1993a) used phylogenetic analysis to distinguish between the six major genotypes, and to design the classification system that is most widely used at present.

With identification of a heterologous population of HCV sequences came the possibility of assessing clinical conditions in relation to the HCV sequences present in the patients. Pozzato et al (1991) used ‘Japanese-type’ HCV PCR primers and primers regarded as universal HCV primers to distinguish two types of HCV clones. Ten patient samples amplified with the Japanese primers and nine patients amplified
with the universal primers (Japanese-type negative). Six patients with each type were treated with 6MU of interferon three times a week for one month and then 3MU three times a week for nine months. They found that detection of Japanese-type HCV RNA in serum seemed to be associated with the presence of more serious liver disease and a poor response to interferon therapy. Therefore, during the period prior to 1992, the majority of published work in the field of the molecular biology of HCV concentrated on studying the heterogeneity of the viral genome and the classification of the different viral groups into genotypes.

2.2 HCV and haemophilia prior to 1992

After the isolation and identification of the main causative agent of non-A, non-B hepatitis (hepatitis C) in 1989 and prior to the commencement of this project in 1992 there had already been many papers published on the subject of hepatitis C infection. Included in the topics of discussion was the prevalence of hepatitis C amongst various geographical groups. The majority of these studies looked at blood donors, for example, an Italian study (Sirchia et al, 1990) found an anti-HCV seroprevalence amongst Italian blood donors of 0.87%, and a study from the Tokyo area (Watanabe et al, 1990) found a prevalence amongst blood donors of 1.14%. These, and the many other prevalence studies, were carried out by the use of the first available ELISA test system.

Although the majority of studies concentrated on seroprevalences amongst blood donors, some studies did assess the prevalence of HCV in haemophilic patients. Makris et al (1990) found that the prevalence of anti-HCV was associated with exposure to clotting factor concentrates; 76% of patients who required over 10,000 units of concentrate annually were anti-HCV positive, 46% were positive who required less concentrate and 0% were positive who had never received treatment.
Allain et al (1991) also found that all haemophilic patients exposed to non-heat treated products were anti-HCV positive.

The introduction of this ELISA test for hepatitis C caused much correspondence concerning the screening of blood and blood products for transfusion and for fractionation to derivatives such as albumin, immunoglobulin and clotting factor concentrates. Those who were reluctant to introduce screening included the US Food and Drug Administration (FDA) (Finlayson and Tankersley, 1990) who were concerned that removing anti-HCV positive units from the plasma pools may remove neutralising antibodies to HCV and, hence, reduce the safety of immunoglobulin prepared from the plasma, and they also considered that the procedure ‘seems unlikely to improve the safety of other plasma derivatives’. Other concerns about the introduction of anti-HCV testing were raised by the National Blood Transfusion Service, London (Barbara, 1991; Contreras et al, 1991), including the cost of donor screening and the lack of specificity of the early tests which may have precluded anti-HCV negative donors with false positive results from donating. However, many others urged the elimination of HCV from clotting factor concentrates by screening and other means (Makris et al, 1990; Pasi et al, 1990).

The main concern at this time seemed to be the lack of specificity of the first ELISA tests. False positive results were found in patients with paraproteinaemia (Boudart et al, 1990) and in patients with rheumatoid arthritis (Theilmann et al, 1990) as well as unexplained false positive anti-HCV tests. It was generally agreed that a reliable, independent confirmatory anti-HCV assay was urgently needed (Colombo et al, 1990; Skidmore, 1990a) and the RIBA test was soon introduced.

The treatment of chronic non-A, non-B hepatitis with recombinant alpha interferon was being reported as early as 1986 (Hoofnagle et al, 1986). One of the first studies to assess treatment with interferon in HCV infected haemophilic patients (Lee et al,
1989) looked at a patient with non-A, non-B hepatitis (patient 1) and a second patient infected with NANBH, HBV, HDV and HIV (patient 2). Patient 1 showed a normalisation of AST levels at an interferon dose of 5 million units (MU) three times a week which was gradually reduced to 1.5MU three times a week. However, when therapy was withdrawn the AST levels rose dramatically so treatment was continued long-term on a maintenance dose of 1.5MU three times a week. Patient 2 also showed a reduction of his AST levels and a resolution of his HIV-related thrombocytopenia.

Two other studies looked at the treatment of HCV in haemophilic patients with interferon. Makris et al (1991) studied eighteen patients, ten received 3MU three times a week for one year and eight patients received no treatment. Of the treated patients, four normalised their ALT levels and the histologic scores of the treated group were significantly improved compared to the control group. The second study (Bresters et al, 1992) treated eight haemophilic patients with 5MU daily for two weeks, 2.5MU daily for four weeks and 1.5MU three times a week for 18 weeks. The results of this study demonstrated that one patient had a normalisation of his ALT levels and HCV RNA was no longer detectable in three patients.

Prior to 1992, detection of HCV RNA was only beginning to be used as a diagnostic tool, and HCV genotyping and quantitation of HCV RNA had not appeared as yet in any of the published studies assessing HCV infection. Therefore, the molecular biology of the hepatitis C virus in haemophilic patients was little studied at this time. One study (Garson et al, 1990) assessed the viraemia patterns in five haemophilic patients. They found that three temporal patterns of viraemia were observed: transient viraemia in acute resolving HCV; viraemia lasting for several years in chronic HCV and intermittent viraemia in chronic HCV. Another study (Simmonds et al, 1990) used HCV quantification (by limiting dilution PCR) and sequencing in blood products, haemophiliacs and drug users. The quantification of positive
samples showed levels of HCV RNA between $10^4$ and $10^6$ copies/ml. Phylogenetic analysis of the viral sequences showed two distinct groups: one was associated with the drug users and the other with haemophiliacs infected with Scottish factor VIII concentrates. Both were distinct from sequences found in commercial factor VIII. Therefore, although much work had already been done in assessing the hepatitis C virus in haemophilic patients, very little of the molecular biology of the virus in these patients had been studied.

2.3 The haemophilic population at the Royal Free Hospital

There were 255 anti-HCV positive haemophilic patients registered at the Haemophilia Centre at the Royal Free Hospital in 1993 (Telfer et al, 1994). Comprehensive clinical records for each patient have been kept at the centre since they were first registered. Patients with severe haemophilia were seen six monthly and those with mild or moderate haemophilia were seen annually. From 1985 onwards anti-HIV positive patients were seen every three months. Assessment at these review visits included medical history, review of home and in-patient treatment records, physical examination, a standard panel of blood tests and, since 1979, a serum sample was taken for storage at -40°C.

Telfer et al (1994) assessed the clinical records of all the anti-HCV positive patients and found that the median age on August 1st, 1993 in surviving patients was 32.1 years and in deceased patients was 40.8 years. 185 (72.5%) of the patients had haemophilia A, 42 (16.5%) had haemophilia B, 19 (7.5%) had Von Willebrand’s Disease, seven (0.25%) were haemophilia A carriers, one (0.5%) had hypofibrinogenaemia and one (0.5%) had factor XI deficiency. 164 (64%) patients had severe haemophilia, 30 (12%) had moderate haemophilia and 61 (24%) had mild haemophilia. The median annual concentrate usage among the patients was 28,000 units. Seropositivity testing showed that 103 (40%) patients were anti-HIV positive.
and six (2%) patients were hepatitis B surface antigen (HBsAg) positive. Transaminase levels had been tested for each patient at every visit, and analysis of these results showed that 42 (16%) patients had normal levels, 96 (38%) had intermittently abnormal levels and 117 (46%) had persistently abnormal levels. Out of the 255 patients 48 (19%) were deceased. The causes of death were AIDS without hepatic decompensation in 25 (52%), AIDS and hepatic decompensation in six (13%), hepatic decompensation without AIDS in four (8%), other causes in 11 (23%) and there was insufficient information to define the cause of death in two patients. They also found, by analysis of this data, that the risk of progression to liver failure was 1.7% at ten years after exposure to concentrate, 10.8% at twenty years and 21.4% if HIV was also present.

2.4 Study aims

The haemophiliac patients at our centre have been treated with large quantities of unsterilised multi-donor pool clotting factor concentrates. Therefore, all of these patients have been exposed to hepatitis C virus and the majority of them have become infected. Haemophilic patients infected with hepatitis C virus are a complex group as they must have been exposed to multiple HCV quasispecies. Many of the patients have also been infected with other viruses, such as HIV and HBV, which further complicates the course of their HCV infection. However, due to the long term care they have received for their haemophilia, this group of patients have comprehensive clinical data available as well as a large bank of stored sera which dates back to 1979 for most patients. This has enabled a series of short and, more importantly, long term studies to be carried out into molecular diversity of HCV in haemophilic patients who are likely to be multiply infected.

Initially, a study which assessed whether the pattern of transaminase results and HCV serological tests correlated with the pattern of HCV viraemia in haemophilic
patients was performed, and whether conclusions can thus be drawn concerning the likelihood of future liver disease based on these tests.

The second aim was to assess how the hepatitis C genotype, determined by restriction length polymorphism (RFLP) analysis, in 25 anti-HCV positive haemophilic patients influenced the response to treatment with alpha interferon; and also to determine if RFLP methods are suitable to study the HCV genotypes in patients who are likely to have multiple infections.

The aim of the third study, which assessed five patients from the above study in more detail, was to analyse the changes in viral load and the composition of the HCV quasispecies in haemophilic patients receiving therapy with alpha interferon using the four major methods available for HCV genotyping.

At present there is a lack of data relating sequence evolution over extensive time periods (i.e. more than 10 years) and HCV load in HCV positive patients. Due to the large amount of sera available over a number of years for our haemophilic patients, I undertook HCV quantitation and cDNA sequence analysis of the highly conserved 5′ UTR and in an NS4 CTL-epitope in an HIV positive haemophilic patient and in an HIV negative haemophilic patient over 12 and 14 years, respectively. The aim of this final study was to provide an insight into the pathogenesis of HCV and allow the relative effects of declining T-helper functions on HCV replication and diversity to be assessed.
CHAPTER 3

METHODS

3.1 Routine Testing

3.1.1 Liver function tests

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using a Hitachi 747 analyser by the Department of Chemical Pathology at the Royal Free Hospital.

AST is widely distributed in the body tissues and is mainly found in the liver and heart. It has two isoenzyme forms: cytoplasmic and mitochondrial AST. Plasma AST is elevated in hepatocellular destruction as in viral hepatitis, toxic hepatitis, shock liver, alcoholic hepatitis; myocardial infarction, in vivo haemolysis, pulmonary infarction and post hepatic obstructive biliary tract disease. AST acts on oxoglutarate and aspartate to yield glutamate and oxaloacetate. The latter reacts with NADH in the presence of malate dehydrogenase to produce malate and NAD. The decrease in absorbance of NADH is monitored at 340nm and is proportional to AST activity (Bergmeyer et al, 1986a).

ALT is present in the cytoplasm of most cells, the highest concentration being in the liver. Increased plasma ALT is caused by viral/toxic/alcoholic hepatitis, shock liver, infectious mononucleosis and polymyositis. A low plasma ALT is associated with end stage liver disease, renal haemodialysis and renal insufficiency. ALT acts on oxoglutarate and alanine to yield glutamate and pyruvate. The latter reacts with NADH in the presence of LD to produce lactate and NAD. The decrease in
absorbance of NADH is monitored at 340nm and is proportional to the ALT activity (Bergmeyer et al, 1986b).

3.1.2 Virology

Hepatitis C virus antibody was measured using the third generation Ortho™ HCV 3.0 ELISA test system (Ortho Diagnostics, UK). The assay procedure is a three-stage test carried out in a microwell coated with a combination of recombinant hepatitis C virus antigen (c22-3 [core], c200 [non-structural region 3] and NS5 [non-structural region 5]).

In the first stage, a diluted test specimen is incubated in the test well for a specified length of time. If antibody reactive to any of the three antigens is present in the specimen, antigen-antibody complexes will be formed on the microwell surface. In the subsequent washing step, unbound serum or plasma proteins will be removed.

In the second stage, anti-human murine monoclonal antibody conjugated to horseradish peroxidase is added to the microwell. The conjugate binds specifically to the human IgG portion of the antigen-antibody complexes. If antigen-antibody complexes are not present, the unbound conjugate will be removed by subsequent washing.

In the third stage, an enzyme detection system composed of o-phenylenediamine (OPD) and hydrogen peroxide is added to the well. If bound conjugate is present, the OPD will be oxidised, resulting in a coloured end-product. The reaction is stopped by the addition of sulphuric acid. The resulting form of OPD has an orange colour which is measured with a microwell reader (photometer) designed to measure light absorbance in a microwell.
These results were confirmed by the use of the third generation Chiron™ RIBA™ HCV 3.0 SIA (Strip Immunoblot Assay) (Ortho Diagnostics, UK). This is a three stage test which utilises individual recombinant antigens (c33c and NS5 - derived from putative non-structural regions of the genome) and synthetic peptides (c100p and 5-1-1p - from putative non-structural regions - and c22p from core viral protein) immobilised as individual bands onto test strips.

In the first stage, the specimen is diluted and incubated with the strip. Antibodies specific to HCV, if present, will bind to the corresponding antigen and/or peptide bands on the strip. Unbound serum or plasma components are removed by aspiration and washing.

In the second stage, the strip is incubated in the presence of a peroxidase-labelled goat anti-human IgG conjugate. The conjugate binds to the human IgG portion of the antigen-antibody complexes. Unbound conjugate was removed by decantation and subsequent wash steps.

In the third stage, a colorimetric enzyme detection system composed of hydrogen peroxide and 4-chloro-1-naphthol is added. If bound conjugate is present, the enzymic reaction will produce an insoluble blue-black coloured reaction product at each specific HCV antigen, peptide or control band. The reaction is stopped by removal of the reactants by decantation and final wash steps. Anti-HCV reactivity in a specimen is determined by comparing the intensity of each band to the human IgG internal control bands. A sample is negative if all bands are negative; indeterminate if one band is positive and positive if two or more bands are positive. Both tests were carried out by the Department of Virology at the Royal Free Hospital.

Antibodies to HIV1 and HIV2 in serum were detected using the Genelavia™ Mixt enzyme immunoassay kit (Sanofi Diagnostics Pasteur, France) by the Department of
Virology at the Royal Free Hospital. This is a three stage test using microplate wells coated with purified antigens - GP160 recombinant protein and peptides mimicking the immunodominant epitopes of the HIV1 and HIV2 envelope glycoproteins. The samples are added to these wells and any antibodies present will bind to the antigens. The wells are washed and peroxidase labelled anti-human IgG and IgM antibodies are added. They bind in turn to the IgG and IgM captured on the plate. After removal of unbound conjugate the presence of the enzyme is shown by incubation with the substrate (OPD). The reaction is stopped by the addition of sulphuric acid and the absorbances are read using a spectrophotometer at 492/620nm.

3.1.3 Immunology

The CD4 absolute count was carried out by using the ORTHO CytoronAbsolute lymphocyte count by the Department of Clinical Immunology at the Royal Free Hospital. Samples were prepared by a whole blood stain and erythrocyte lysis procedure, and were stained with Ortho Trio panel (OTP) reagents (Ortho Diagnostic Systems, Raritan, NJ). The samples were passed through the flow cytometer and the data was analysed using Ortho ImmunoCount Software (Mercolino et al, 1995).
3.2 General Research Methods

3.2.1 Electrophoresis

3.2.1.1 Agarose gel electrophoresis (AGE)

Agarose gels were cast by melting 1g of Bio-Rad High Strength Analytical Grade Ultra Pure DNA Grade Agarose (Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547) in 50ml of 1 x Tris-borate (TBE) buffer (5 x buffer contains 54g Tris base, 27.5g boric acid and 20ml 0.5M EDTA (pH 8.0) made up to one litre with deionised water) until a clear, transparent solution was achieved. 3.5μl of 10mg/ml ethidium bromide was added (this allows detection of DNA as ethidium bromide intercalates between stacked base pairs, and glows orange when exposed to UV light) and the gels were allowed to set in a mold containing a comb to form wells for loading the samples. 5μl of each polymerase chain reaction (PCR) sample was loaded with 5μl of 6 x loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 40% (w/v) sucrose in water - stored at 4°C). The gels were run at 100 volts and the DNA, which was negatively charged at neutral pH, migrated towards the anode. The DNA was visualised using a UV transilluminator. These 2% gels were used for the detection of PCR fragments of 100 - 300 base pairs in size.

3.2.1.2 Polyacrylamide gel electrophoresis (PAGE)

For resolving restriction products a Bio-Rad PROTEAN® II xi Cell was used as PAGE had superior separating capabilities for the small DNA fragments. The gel plates were assembled using 1mm spacers and a 12% polyacrylamide gel was poured. The gel contained 10.5ml 40% Acrylamide/Bis-acrylamide (19:1), 8.75ml 1.5M Tris-HCl (pH 8.8), 350μl 10% (w/v) SDS, 15.3ml deionised water, 200μl
ammonium persulphate (APS) and 20µl TEMED. The running buffer used (5 x Running Buffer, pH 8.3) contained 45g Tris base, 216g glycine and 15g SDS and was made up to three litres with deionised water. 300ml of this stock was diluted with 1,200ml deionised water for one electrophoresis run. 15µl of each PCR product was loaded with 5µl of loading buffer (see section 3.2.1.1) and run for four hours at 150 volts. The gel plates were separated and the gel was immersed in a 10% solution of ethidium bromide and water for 15 minutes, and then visualised using the UV transilluminator.

3.2.1.3 DNA sequencing urea gels

The sequencing plates were both washed and the smaller plate was siliconised on one side with Sigmacote™ (Sigma Chemical Company, Poole, Dorset BH17 7NH). The 6% polyacrylamide gel was prepared using 63g urea, 24ml 40% Acrylamide/Bis-acrylamide (19:1), 15ml 10 x TBE and 58ml deionised water. After addition of the setting agents (400µl APS and 70µl TEMED) the gel was poured between the plates using wedge spacers (0.4mm - 1.2mm) and two 24 well sharkstooth combs. The gel was preheated to 50°C using 1 x TBE as a running buffer (see section 3.2.1.1). 5µl of each sequencing sample was loaded in the order G, A, T, C, and the gel was run for six hours at 70 watts using a Model S2 Sequencing Gel Electrophoresis Apparatus (Life Technologies Inc., Gaithersburg, MD 20898) and a high voltage powerpack (Bio-Rad). The plates were separated after running and the gel was transferred to two layers of 3MM Chromatography Paper (Whatman®), covered with clingfilm and dried using a Slab Gel Dryer (Bio-Rad) at 80°C for four hours. The dry gel was placed in an autoradiography cassette for two days with X-ray film (Genetic Research Instrumentation Ltd, Dunmow, Essex CM6 3LD).
3.2.2 Hepatitis C virus RNA extraction

HCV RNA was extracted from 100μl of serum by addition of 20μl 3M sodium acetate pH 5.2, 2μl of 1ug/μl yeast tRNA and 100μl 4M guanidinium thiocyanate. Samples were vortexed and the proteins were removed by the addition of 400μl of phenol/chloroform. Samples were vortexed and incubated on ice for 15 minutes. Samples were centrifuged for 20 minutes at 13,000 rpm in a microfuge. 120μl of the aqueous layer of the centrifuged samples was added to 120μl of RNA binding salt (RNaid™ kit, BIO 101 Inc., P.O. Box 2284, La Jolla, CA 92038-2284) and vortexed. The RNA was purified using 5μl of a silica matrix (RNaid kit), vortexed and left at room temperature for 5 minutes. Samples were centrifuged at 13,000 rpm for 1 minute and the supernatant was discarded. The RNA was washed twice using 400μl of RNase free wash solution (RNaid kit) and the pellets were resuspended by vortexing. Following the second wash the supernatant was discarded and the pellet was resuspended in 30μl of diethyl pyrocarbonate (DEPC) treated water (RNaid kit).

3.2.3 Hepatitis C virus RNA reverse transcription reaction

The extraction product from section 3.2.2 was heated to 70°C for 5 minutes immediately prior to reverse transcription. The HCV RNA was reverse transcribed to cDNA in a volume of 25μl containing 10μl of template, 25 pmoles of random hexamers (Pharmacia), 2.5μl of 0.5mM dNTPs (Boehringer Mannheim UK), 250 units of Moloney-Murine Leukaemia Virus (M-MLV) reverse transcriptase (Gibco BRL) and 5μl of 5 x First Strand Buffer (250mM Tris-HCl pH 8.3, 375mM KCl,
15mM MgCl₂). The reaction was incubated for one hour at 37°C. The cDNA product was heated to above 90°C for five minutes prior to amplification by the polymerase chain reaction (PCR).

3.2.4 Hepatitis C virus polymerase chain reaction

The HCV cDNA from section 3.2.3 was amplified using two rounds of PCR (nested). The reaction mixture for the first round PCR contained 5μl template, 16μl 1.25mM dNTPs, 50 pmols of each primer (R and D Systems, UK), 5 units of Taq polymerase (Bioline UK), 3μl of 50mM MgCl₂ (Bioline, UK) and 10μl of 10 x Taq buffer (160mM (NH₄)₂SO₄, 670mM Tris HCl (pH 8.8 at 25°C), 0.1% Tween-20) in a final volume of 100μl. The second round PCR mixture contained 2μl of the first round product, 8μl 1.25mM dNTPs, 50 pmoles of each primer, 5 units of Taq polymerase, 1.5μl of 50mM MgCl₂ and 5μl of Taq buffer in a final volume of 50μl.

3.2.4.1 5' untranslated region (5'UTR) primers and PCR conditions

The sequence of the outer primers were 5’ CCCAACACTACTCGGCTA 3’ and 5’ ATGGGGGCGACACTCCACCATGAAT CACTCCCC 3’, and the inner primers were 5’ AGTCTTGCGGGGGCACGCCAAATC 3’ and 5’ GAGGAACTACTGTTTCACGCAGAAAG 3’. The first round PCR conditions were 5 cycles at 94°C for 2 minutes, 50°C for 2 minutes and 72°C for 3 minutes followed by 30 cycles at 94°C for 1.5 minutes, 60°C for 1.5 minutes and 72°C for 2 minutes, with a final extension of 72°C for 5 minutes. The second round PCR conditions were 30 cycles at 94°C for 1 minute and 24 seconds, 40°C for 1 minute and 72°C for 1 minute with a final extension of 72°C for 5 minutes. The PCR
product (251 bases) was visualised on a 2% agarose gel using ethidium bromide (see section 3.2.1.1).

3.2.4.2 Non-structural region 4 (NS4) primers and PCR conditions

Due to the heterogeneity of the NS4 region the following primers were used in various combinations in order to achieve amplification of the maximum number of samples. The most successful combination was:  

$$\text{5'}\ TTAACCGGTCCCTCGGTGGAGGGCACCT\ CGCCGCTCATGA\ 3'$$  
and  

$$\text{5'}\ AAGTGGGCGAGCCCTTTGAGGCCCTTCTGGGCGAAG\ GCACATGT\ 3'$$  
for the first round and  

$$\text{5'}\ GGAATTTTCATCAGCGGGGATA\ C\ 3'$$  
and  

$$\text{5'}\ CAGAATGTCCACAAGCACCT\ 3'$$  
for the second round. Other first round primers used which resulted in positive NS4 PCR results were  

$$\text{5'}\ TTGACCCAGGT\ CCTCGGTGGAGGGCATTTCCGCGCTC\ 3'$$,  
$$\text{5'}\ GCCTTTCTGGGCGAAG\ CATATGT\ 3'$$,  
$$\text{5'}\ GAGGGGAGCTCACGC\ CT\ 3'$$,  
$$\text{5'}\ ACATTGT\ G\ AAAACACATGT\ 3'$$,  
$$\text{5'}\ CAATTTCGCGCAACACATGT\ 3'$$  
and  

$$\text{5'}\ GAGGG\ C\ T\ G\ AAAACACATGT\ 3'$$.

The first round PCR conditions were 35 cycles at 94°C for 1 minute, 50°C for 2 minutes and 72°C for 3 minutes with a final extension of 72°C for 7 minutes. The second round conditions were as above except for 30 cycles only. The PCR product (260 bases) was visualised on a 2% agarose gel using ethidium bromide (see section 3.2.1.1). Of the 260 base pair fragment 135 base pairs were sequenced as they contained a 10 amino acid epitope - LLFNILGGWV - which is recognised by CD8+ MHC class I-restricted cytotoxic lymphocytes in vitro and in vivo (Battegay et al, 1995).
3.3 Cloning and sequencing methods

3.3.1 Hepatitis C virus cloning

The HCV PCR product from section 3.2.4 was diluted to produce the amount of PCR product required to ligate with 50ng of the vector using the following formula:

\[
X \text{ng PCR product} = \frac{(Y \text{ bp PCR product})(50 \text{ng vector})}{(\text{size in bp of the vector} = 3932)}
\]

This formula provides an estimate, therefore, most PCR products were diluted 1:50 with deionised water. 1\(\mu\)l of this was ligated overnight at 14°C using 2\(\mu\)l of 25ng/\(\mu\)l pCR™II vector (TA Cloning™ Kit, Invitrogen) (see Table 3.1), 1\(\mu\)l 10 x Ligation Buffer (60mM Tris-HCl pH 7.5, 60mM MgCl₂, 50mM NaCl, 1mg/ml bovine serum albumin, 70mM β-mercaptoethanol, 1mM ATP, 20mM dithiothreitol, 10mM spermidine), 5\(\mu\)l sterile water and 1\(\mu\)l T4 DNA Ligase. The appropriate number of vials of INVαF™ One Shot™ competent cells (TA Cloning™ Kit) were placed on ice and 2\(\mu\)l of 0.5M β-mercaptoethanol was added and mixed gently by tapping. 1\(\mu\)l of the ligation reaction was added and mixed gently by tapping and then incubated on ice for 30 minutes. The cells were heat shocked for 30 seconds in a 42°C water bath and then incubated on ice for 2 minutes. 450\(\mu\)l of SOC medium (2% Tryptone, 0.5% Yeast Extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 10mM MgSO₄, 20mM glucose) (TA Cloning™ Kit) was added to each vial, and vials were shaken at 37°C for one hour at 225 rpm. LB plates (10g Bacto-tryptone™, 5g Bacto-yeast™ extract, 10g NaCl and made up to one litre with deionised water. Prior to autoclaving 15g/l Bacto-agar was added. This was allowed to cool to 55°C then 50µg/ml ampicillin was added.) were coated with 25\(\mu\)l of 40mg/ml X-Galactosidase
Table 3.1: Features of the pCR II vector

<table>
<thead>
<tr>
<th>Feature</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>lac promoter</td>
<td>For bacterial expression of the lac Zalpha fragment for alpha-complementation (blue-white screening)</td>
</tr>
<tr>
<td>lac Zalpha fragment</td>
<td>Encodes the first 146 amino acids of beta-galactosidase. Complementation in trans gives active beta-galactosidase for blue-white screening</td>
</tr>
<tr>
<td>Kanamycin resistance gene</td>
<td>Selection and maintenance in <em>E. coli</em></td>
</tr>
<tr>
<td>Ampicillin resistance gene</td>
<td>Selection and maintenance in <em>E. coli</em></td>
</tr>
<tr>
<td>ColEI origin</td>
<td>Replication, maintenance, and high copy number in <em>E. coli</em></td>
</tr>
<tr>
<td>T7 and Sp7 promoters and priming sites</td>
<td><em>In vivo</em> or <em>in vitro</em> transcription of sense and antisense RNA Sequencing of insert</td>
</tr>
<tr>
<td>M13 Forward (-20 and -40) and M13 Reverse priming sites</td>
<td>Sequencing of insert</td>
</tr>
<tr>
<td>f1 origin</td>
<td>Rescue of sense strand for mutagenesis and single strand sequencing</td>
</tr>
</tbody>
</table>
(diluted with dimethylformamide). 100μl of each transformation was plated out and incubated overnight at 37°C. The plates were moved to 4°C to allow colour development (white colonies contained insert, blue colonies did not).

3.3.2 Purification of pCR™II containing HCV PCR inserts

Five white colonies were selected from each transformation (see section 3.3.1), grown up overnight at 37°C in 5mls of LB medium (see section 3.3.1, but do not include agar) with 50μg/ml ampicillin, and purified using the QIAprep Spin Plasmid Kit (Qiagen GmbH, Hilden, Germany). 2mls of the overnight cultures were centrifuged at 13,000 rpm for two minutes. The supernatant was discarded and the pellet was resuspended in 250μl of resuspension buffer P1 (100μg/ml RNase A, 50mM Tris/HCl, 10mM EDTA pH 8.0). 250μl of lysis buffer P2 (200mM NaOH, 1% SDS) was added and the samples were inverted to mix and incubated at room temperature for five minutes. 350μl of chilled buffer N3 (containing guanidine hydrochloride) was added, and the samples mixed immediately by gentle inversion. The samples were incubated on ice for five minutes. The samples were centrifuged at 13,000 rpm for ten minutes. A QIAprep-spin column was placed in a 2ml microcentrifuge tube and the supernatant from the samples was added to the column. These were centrifuged at 13,000 rpm for one minute, and the flowthrough fraction was drained from the tube. 0.5ml of buffer PB was added to the column for the first wash, and centrifuged for one minute. The column was washed once with 0.75ml of buffer PE and centrifuged for one minute. The column was centrifuged for a further minute to remove residual wash buffer. The column was placed in a clean 1.5ml microcentrifuge tube and the DNA was eluted by addition of 100μl water and centrifuged for 30 seconds. The DNA was stored at -20°C until used.
3.3.3 Denaturation of pCR™II containing HCV PCR inserts

The samples from section 3.3.2 were denatured prior to sequencing by incubating 20μl of the purified DNA at 37°C for 30 minutes with 4μl of 2M NaOH, 4μl of 2mM EDTA and 12μl of distilled water. 4μl of 3M NaAc pH 5.2 and 140μl of ice-cold absolute alcohol were added and the samples were incubated at -70°C for 15 minutes. The samples were centrifuged at 13,000 rpm for 5 minutes and the supernatant was discarded. The pellets were washed in 140μl of 70% ethanol, centrifuged for 5 minutes at 13,000 rpm and the supernatant was discarded. The pellets were dried in an 80°C oven prior to resuspension in 7μl of distilled water.

3.3.4 DNA sequencing of plasmids containing HCV inserts

The DNA denatured in section 3.3.3 was sequenced using the Sequenase version 2.0 Sequencing Kit (United States Biochemical Corporation, Cleveland, Ohio). To the resuspended DNA was added 2μl of Sequenase reaction buffer and 1μl of M13(-40) forward sequencing primer (5' GTTTTCCCAGTCACGAC 3'), and the mixture was annealed by heating for 2 minutes at 65°C, then cooled slowly to less than 35°C over 15-30 minutes. While cooling, four tubes were labelled G, A, T, C and 2.5μl of each termination mixture (ddGTP, ddATP, ddTTP and ddCTP) was added and kept at room temperature. The labelling mix was diluted 1:5 with water to the working concentration and kept on ice. To the ice-cold annealing mixture was added 1μl of 0.1M DTT, 2μl of diluted labelling mix (1:5 with water), 0.5μl of [35S] dATP (Amersham International, UK) and 2μl of diluted Sequenase Polymerase (1:7 with Sequenase dilution buffer). The labelling reactions were mixed well and left at room temperature for 2 minutes. During this time the termination mixes were heated to
37°C. 3.5μl of the labelling reaction was added to each termination tube (G, A, T and C), mixed and incubated for 5 minutes at 37°C. The reactions were stopped by adding 4μl of Stop Solution. The samples were heated to 72°C for 2 minutes and then loaded onto a 6% polyacrylamide wedge gel (see section 3.2.1.3).

3.3.5 Computer analysis of hepatitis C virus sequence data

The DNA sequences were analysed using the Sequence Analysis Software Package (version 7) by Genetic Computers Inc. from the University of Wisconsin’s Department of Genetics. Multiple sequence alignments were produced using the Clustal software and alignments produced as PHYLIP-compatible files. The following alignment parameters were used: pairwise parameters with a gap penalty of 3, K-tuple 1 and window size 5 and multiple sequence parameters with a gap penalty of 10 and weighted transitions.

Maximum parsimony trees were generated using the DNAPARS program, available within the Phylib 3.5 package. A randomised input order of sequences was used along with ordinary parsimony methods. In order to test the robustness of the trees bootstrapped data for each multiple alignment was produced in SEQBOOT using bootstrap and 100 replicates. The consensus tree was generated using CONSENSE. Unrooted trees were displayed using DRAWTREE and rooted trees were displayed using DRAWGRAM. For rooted trees the appropriate outgroup sequence was used within DNAPARS, and the analysis was categorised as rooted within the CONSENSE program.
3.3.6 Factor concentrate ultracentrifugation

In order to amplify HCV from stored factor VIII concentrates ultracentrifugation was required. 4ml of stored commercial factor VIII (made up to 9ml with sterile phosphate buffered saline) and 9ml of stored NHS factor VIII concentrate were ultracentrifuged at 20,000g in a SW41 (Beckman Instruments) for three hours at 4°C. The supernatant was discarded and the pellet was lysed immediately with 100μl of guanidinium thiocyanate and the extraction proceeded as detailed in section 3.2.2.

3.3.7 Restriction of clones containing pCR™II with HCV NS4 inserts

To distinguish between the plasmids containing HCV NS4 inserts and those containing vector inserts (an artifact of the cloning of the NS4 sequences), 5μl of the purified DNA (see section 3.3.2) was incubated overnight at 37°C with 10 units of NarI and 2μl of Working Carlo's Universal Buffer (88μl 10 x Carlo’s universal buffer - 3mls 1M Tris acetate pH 7.5, 3mls 2M potassium acetate, 1ml 1M magnesium acetate, 1ml 10mg/ml bovine serum albumen - 10μl 300mM spermidine and 2.5μl 400mM DTT) in a final volume of 20μl. The restriction products were run on a 1% agarose gel (see section 3.2.1.1) and banding patterns were as follows (figures in base pairs):

<table>
<thead>
<tr>
<th>NS4 (+ strand insert)</th>
<th>NS4 (- strand insert)</th>
<th>No NS4 insert</th>
</tr>
</thead>
<tbody>
<tr>
<td>248</td>
<td>248</td>
<td>248</td>
</tr>
<tr>
<td>1150</td>
<td>941</td>
<td>3684</td>
</tr>
<tr>
<td>2794</td>
<td>3003</td>
<td></td>
</tr>
</tbody>
</table>
3.4 Genotyping and quantitation methods

3.4.1 Hepatitis C virus restriction fragment length polymorphism (RFLP) analysis

One 5μl aliquot of the 5′UTR PCR product from section 3.2.4 was digested with ScrFI and a second 5μl aliquot was digested with a combination of HaeIII and Rsal. The restrictions took place overnight at 37°C with 10 units of the appropriate enzyme and 2μl of Working Carlo’s Universal Buffer (see section 3.3.7) in a final volume of 20μl. The restriction fragments were separated on a 12% polyacrylamide gel using a BioRad PROTEAN® II xi Cell according to the manufacturer’s instructions (see section 3.2.1.2). The band sizes were determined by comparison with a Phi X174/HinfI digest and DNA Molecular-Marker V (Boehringer Mannheim UK). The hepatitis C genotype was then determined using the cleavage patterns described by McOmish et al, 1993 and shown in Figure 3.1.

3.4.2 Hepatitis C virus serotyping

The serotyping was carried out using an Anti-HCV Serotyping Assay, provided by Murex, Dartford, UK, which detected type-specific antibodies to serotypes 1, 2 and 3 in non-structural region 4 (NS4). The diluted sample was incubated, in the presence of serotype-specific competing peptides, with microwells coated with serotype-specific antigens of HCV. During the course of the first incubation any serotype-specific anti-HCV antibodies in the sample bound to the immobilised antigens. Following washing to remove unbound material, the captured anti-HCV antibodies were incubated with peroxidase-conjugated monoclonal anti-human IgG. During the course of the second incubation an antigen - human antibody - anti-human antibody/enzyme complex was formed in those microwells which contained
Figure 3.1: Possible cleavage patterns for RFLP analysis of HCV 5'UTR (McOmish, 1993)

**HaeII/Rsal**

<table>
<thead>
<tr>
<th>Types</th>
<th>44</th>
<th>58</th>
<th>114</th>
<th>9</th>
<th>26</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>102</td>
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<td>114</td>
</tr>
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<td>2</td>
<td>44</td>
<td>12</td>
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<td>58</td>
<td>56</td>
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<tr>
<td>3</td>
<td>33</td>
<td>23</td>
<td>46</td>
<td>114</td>
<td>9</td>
</tr>
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</table>

**ScrFI**

<table>
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<th>9</th>
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<th>94</th>
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<tr>
<td>1</td>
<td>53</td>
<td>14</td>
<td>49</td>
<td>9</td>
<td>32</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>53</td>
<td>14</td>
<td>1</td>
<td>57</td>
<td>32</td>
<td>94</td>
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</tr>
<tr>
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<td>53</td>
<td>14</td>
<td>1</td>
<td>48</td>
<td>41</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>53</td>
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<td>1</td>
<td>183</td>
<td></td>
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<td>53</td>
<td>14</td>
<td>1</td>
<td>48</td>
<td>135</td>
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</tr>
<tr>
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<td>53</td>
<td>14</td>
<td>1</td>
<td>57</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>61</td>
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</tbody>
</table>
samples with antibodies to a specific serotype of HCV. After removal of excess conjugate, bound enzyme was detected by the addition of a solution containing 3, 3', 5, 5'-tetramethylbenzidine (TMB) and hydrogen peroxide. A purple colour developed in the wells which contained anti-HCV reactive samples. The enzyme reaction was terminated with sulphuric acid which gave an orange colour which was read photometrically. The amount of conjugate bound, and hence colour, related to the concentration of specific antibody in the sample.

3.4.3 Hepatitis C virus type-specific primers

The type-specific primers were previously described by Okamoto et al (1993) and detected type-specific differences in the core region sequence. The primers used are shown in Table 3.2.

3.4.4 Hepatitis C virus quantitation - Chiron assay

The HCV RNA was quantified using the Nucleic Acid Hybridisation Assay for the Detection and Quantitation of Hepatitis C Viral RNA (bDNA signal amplification assay) by the Chiron Corporation, Emeryville, CA, USA. This assay was based on the specific hybridisation of synthetic oligonucleotides to the 5' untranslated region and core genes of HCV RNA, which allows the RNA to be captured onto the surface of a well in a 96 microwell plate. Synthetic branched DNA molecules (bDNA) and multiple copies of an alkaline phosphatase-linked probe were hybridised to the immobilised complex (signal amplification), see Figure 3.3. Detection was achieved by incubating the complex with a chemiluminescent substrate (LUMI-PHOS™, Lumigen, Inc.) and measuring the light emission generated by the bound alkaline phosphatase. The data management system software calculated average relative luminescence counts and %CV (coefficient of variation) for all standards, controls and samples, plotted the standard curve and printed a
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Type-specific oligonucleotides 5'-3'</th>
<th>Band size (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype 1a</td>
<td>ATGTACCCCATGAGGTCGGC CGCGCGACTAGGAAGACTTC CGCGCGACGCGTAAAACTTC</td>
<td>49</td>
</tr>
<tr>
<td>Genotype 1b</td>
<td>GAGCCATCCTGCCCACCCCA</td>
<td>144</td>
</tr>
<tr>
<td>Genotype 2a</td>
<td>CCAAGAGGGACGGGAACCTC</td>
<td>174</td>
</tr>
<tr>
<td>Genotype 2b</td>
<td>ACCCTCGTTTCCGTACAGAG</td>
<td>123</td>
</tr>
<tr>
<td>Genotype 3</td>
<td>GCTGAGCCCAGGACCGGTCT</td>
<td>88</td>
</tr>
</tbody>
</table>

Table 3.2: Oligonucleotides for type-specific primers
Figure 3.3: Chiron bDNA Signal Amplification Assay

1. Degrade RNases and release RNA
2. Release and denature DNA
3. Target Probes
4. Hybridize probes to target and to solid phase
5. Capture probe
6. Microwell surface coated with capture probes
7. Hybridize bDNA (amplifier)
8. Hybridize enzyme labeled probes to amplifier
9. Add Dioxetane substrate and measure chemiluminescence
report automatically. Specimen values that were below the cut-off \(<3.5 \times 10^5\) genome equivalents/ml), above the standard curve limit, with a high %CV or indeterminate were not included. The numbers generated by the luminometer reflected light count emissions from each microwell. The number of light counts measured was directly proportional to the amount of HCV equivalents present in the microwell.

3.4.5 Hepatitis C virus quantitation - Roche assay

This assay was a PCR based system which included a quantitation standard (QS) that was co-amplified with the target to monitor the efficiency of the system. The QS was a synthetic RNA molecule with primer binding sites identical to the HCV target and a unique probe sequence specific to the QS molecule. The amplification target was a 244 base pair region of the 5' UTR of the HCV genome.

100µl of serum or plasma was extracted, together with the QS, with guanidine thiocyanate and the RNA was recovered by isopropanol precipitation. HCV target RNA and QS RNA were reverse transcribed and amplified in a single tube reaction using the enzyme rTth polymerase. The products were then denatured, transferred to a microwell detection plate, and serially diluted from row A (containing undiluted amplicon) to 1:5, 1:25, 1:125 and 1:625 in rows B-E where they were hybridised to HCV specific microwells, and serially diluted from row F (containing undiluted amplicon) to 1:5 and 1:25 where they were hybridised to QS specific microwells. The plate was then incubated for one hour at 37°C before the addition of 100µl streptavidin horseradish peroxidase to each well and a further incubation for 15 minutes at 37°C. The plate was washed and 100µl of Working Substrate was added to each well. The colour was then allowed to develop for 10 minutes in the dark at room temperature.
The highest dilutions that give an absorbance at 450nm between 0.5 and 2.0 for both the HCV specific microwells and the QS specific microwells were then used to calculate the number of HCV RNA copies/ml in the sample.
CHAPTER 4

A Study of the Patterns of Viraemia in Haemophilic Patients
who have been Exposed to Unsterilised
Clotting Factor Concentrates

4.1 Introduction

All haemophilic patients exposed to unsterilised large donor pool clotting factor concentrates must have been exposed to the hepatitis C virus (Allain et al, 1991). To date, Garson et al (1990) have reported the patterns of viraemia in five anti-HCV positive patients. This study was designed to look at the patterns of viraemia and clinical data of larger numbers of haemophilic patients (n = 53) for a more in-depth assessment of this subject. The HCV RNA was extracted, reverse transcribed and amplified by PCR as detailed in Chapter 3. The PCR assay used had a sensitivity of approximately 1000 genomes per ml as determined using serial dilutions of an HCV RNA standard of known concentration.

AST values were used in this longitudinal study as ALT measurements were not introduced until 1991. The AST normal range was 5-15 IU/l prior to June 1981 and 5-40 IU/l after this date.

4.2 Patient Details

The clinical records of all living haemophilic patients registered at the Royal Free Hospital Haemophilia Centre were reviewed between January 1979 and January 1993. Age was regarded as that at the time of the last HCV RNA assay and concentrate usage was calculated as the average annual consumption over the
Table 4.1: Patient Data

<table>
<thead>
<tr>
<th>Group (number)</th>
<th>Age (years)</th>
<th>HIV Status % +ve</th>
<th>Treatment usage over 5 years (units/annum)</th>
<th>Duration of exposure (years)</th>
<th>Type of haemophilia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (anti-HCV -ve) (n = 6)</td>
<td>Mean 40 Range 10 to 68</td>
<td>0%</td>
<td>Mean 8660 Range 315 to 16,368</td>
<td>Mean 15 Range 8 to 21</td>
<td>2 A Mild, 2 B Mild, 1 B Sev, 1 Carr Mild</td>
</tr>
<tr>
<td>2 (normal transaminases) (n = 18)</td>
<td>Mean 31 Range 12 to 57</td>
<td>32%</td>
<td>Mean 19,175 Range 100 to 68,000</td>
<td>Mean 14 Range 9 to 25</td>
<td>1 A Mild, 2 A Mod, 6 A Sev, 1 B Mod, 4 B Sev, 2 Carr Mild, 1 VWD Mild, 2 VWD Mod</td>
</tr>
<tr>
<td>3 (intermittently abnormal transaminases) (n = 17)</td>
<td>Mean 39 Range 17 to 79</td>
<td>29%</td>
<td>Mean 21,614 Range 500 to 63,000</td>
<td>Mean 16 Range 6 to 23</td>
<td>1 A Mild, 3 A Mod, 8 A Sev, 1 B Mild, 2 B Sev, 2 VWD Mild</td>
</tr>
<tr>
<td>4 (persistently abnormal transaminases) (n = 12)</td>
<td>Mean 43 Range 29 to 64</td>
<td>17%</td>
<td>Mean 23,545 Range 500 to 84,000</td>
<td>Mean 16 Range 12 to 20</td>
<td>2 A Mild, 5 A Sev, 2 B Mild, 3 B Sev</td>
</tr>
</tbody>
</table>
previous five years. The clinical information on the patients included in this study is shown in Table 4.1.

4.3 Results

Four patterns of viraemia were identified for in-depth study with particular emphasis on those patients with normal transaminases. The four patterns were: patients with normal transaminases who were anti-HCV negative (Group 1); patients with normal transaminases who were anti-HCV positive (Group 2); patients with intermittently abnormal transaminases who were anti-HCV positive (Group 3) and patients with persistently abnormal transaminases who were anti-HCV positive (Group 4) (see Figures 4.1 - 4.26). 255 patients were identified who had received blood product therapy. There was insufficient information or lack of stored serum for some patients, therefore, Group 1 contained six patients; Group 2 contained 18 patients; Group 3 contained 17 patients and Group 4 contained 12 patients.

Patients with normal transaminases (Groups 1 and 2) were also studied longitudinally. Their patterns of viraemia were assessed by PCR amplification of five samples from each patient which were selected at random from a period between 1979 and 1993, depending on their clinic visits, with at least one year in between each sample. An example of the PCR amplicons derived from these samples is shown in Figure 4.27.

The clinical features of the 53 patients involved in this study are documented in Table 4.1. There was no significant difference in age, diagnosis, severity of haemophilia, concentrate usage or anti-HIV serology between the groups.

The serological and HCV PCR results on the most recent serum samples from all groups are shown in Table 4.2.
Figure 4.1: Longitudinal AST, EIA and PCR results for Patient 1, Group 1

Figure 4.2: Longitudinal AST, EIA and PCR results for Patient 2, Group 1

Figure 4.3: Longitudinal AST, EIA and PCR results for Patient 3, Group 1

Figure 4.4: Longitudinal AST, EIA and PCR results for Patient 4, Group 1

AST values for patient 1 (IU/\text{L})

AST upper limit of normal (40 IU/\text{L})
Figure 4.5: Longitudinal AST, EIA and PCR results for Patient 5, Group 1

Figure 4.6: Longitudinal AST, EIA and PCR results for Patient 6, Group 1

Figure 4.7: Longitudinal AST, EIA and PCR results for Patient 1, Group 2

Figure 4.8: Longitudinal AST, EIA and PCR results for Patient 2, Group 2
Figure 4.9: Longitudinal AST, EIA and PCR results for Patient 3, Group 2

- AST values for patient 3
- AST upper limit of normal (40 IU/L)

PCR

EIA

Time (months)

Figure 4.10: Longitudinal AST, EIA and PCR results for Patient 4, Group 2

- AST values for patient 4
- AST upper limit of normal (40 IU/L)

PCR

EIA

Time (months)

Figure 4.11: Longitudinal AST, EIA and PCR results for Patient 5, Group 2

- AST values for patient 5
- AST upper limit of normal (40 IU/L)

PCR

EIA

Time (months)

Figure 4.12: Longitudinal AST, EIA and PCR results for Patient 6, Group 2

- AST values for patient 6
- AST upper limit of normal (40 IU/L)

PCR

EIA

Time (months)
Figure 4.13: Longitudinal AST, EIA and PCR results for Patient 7, Group 2

Figure 4.14: Longitudinal AST, EIA and PCR results for Patient 8, Group 2

Figure 4.15: Longitudinal AST, EIA and PCR results for Patient 9, Group 2

Figure 4.16: Longitudinal AST, EIA and PCR results for Patient 10, Group 2
Figure 4.17: Longitudinal AST, EIA and PCR results for Patient 11, Group 2

Figure 4.18: Longitudinal AST, EIA and PCR results for Patient 12, Group 2

Figure 4.19: Longitudinal AST, EIA and PCR results for Patient 13, Group 2

Figure 4.20: Longitudinal AST, EIA and PCR results for Patient 14, Group 2
Figure 4.21: Longitudinal AST, EIA and PCR results for Patient 15, Group 2

Figure 4.22: Longitudinal AST, EIA and PCR results for Patient 16, Group 2

Figure 4.23: Longitudinal AST, EIA and PCR results for Patient 17, Group 2

Figure 4.24: Longitudinal AST, EIA and PCR results for Patient 18, Group 2
Figure 4.25: Example of longitudinal AST, EIA and PCR results for Group 3

- AST values (IU/l)
- AST upper limit of normal (40 IU/l)
- EIA
- PCR -/+
Figure 4.26: Example of longitudinal AST, EIA and PCR results for Group 4

- AST values (IU/l)
- AST upper limit of normal (40 IU/l)
- PCR
- EIA

Time (months)
Figure 4.27: An example of the 5'UTR PCR amplicons

Lane 1: Extraction negative control
Lanes 2-11: Samples
Lane 12: Positive control
Lanes 13, 14 and 15: Reverse transcription, first and second round PCR negative controls, respectively
Lane 16: DNA Molecular-Marker V
Table 4.2: ELISA, RIBA II and PCR results for all groups

<table>
<thead>
<tr>
<th>Group</th>
<th>ELISA OD (Mean)</th>
<th>RIBA II (% +ve)</th>
<th>c100-3 (% +ve)</th>
<th>c33 (% +ve)</th>
<th>c22 (% +ve)</th>
<th>PCR +ve (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0988</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>n=6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.277</td>
<td>78%</td>
<td>55%</td>
<td>77%</td>
<td>100%</td>
<td>3 (17%)</td>
</tr>
<tr>
<td>n=18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.334</td>
<td>89%</td>
<td>53%</td>
<td>90%</td>
<td>100%</td>
<td>9 (53%)</td>
</tr>
<tr>
<td>n=17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2.75</td>
<td>100%</td>
<td>89%</td>
<td>100%</td>
<td>94%</td>
<td>9 (78%)</td>
</tr>
<tr>
<td>n=12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The proportion of patients who were PCR positive in each increasing group was significant, p=0.0006 (Logistic regression)

Key
ND = Not Done as ELISA was negative
All of the anti-HCV negative patients (Group 1) were negative by the HCV ELISA assay and, therefore, it was not necessary to carry out RIBA II tests on these samples. The majority of the other patients in Groups 2, 3 and 4 were ELISA and RIBA II positive, but 22% of patients in Group 2 (normal transaminases, anti-HCV positive) were RIBA II indeterminate, and there was a significant trend in the proportion RIBA II positive with increasing transaminase abnormality. All patients positive for the HCV ELISA assay were positive for antibody to c22 and there was a trend in the proportion positive for antibody to c33 with transaminase abnormality. Antibody to c100-3 was detected in approximately 50% of those with normal and intermittently abnormal transaminase levels, but in more than 80% of those with persistently abnormal transaminase levels.

HCV RNA was not detected in any of the anti-HCV negative patients; in a minority (17%) of those with normal transaminase levels and was detected in over 70% of those with persistently abnormal transaminase levels. There was a significant trend in the proportion of HCV RNA positive patients with increasing transaminase abnormality. None of the six patients with RIBA II indeterminate serology were HCV RNA positive, compared with 24/40 patients (60%) who were RIBA II positive (p=0.01, Fisher’s Exact test), and this difference may be associated with the absence of antibody to c33 in the RIBA II indeterminate patients. The transaminase levels were raised in 5/17 patients (29%) with intermittently abnormal transaminase levels at the time when serum HCV RNA was assayed. All five patients were HCV RNA positive, compared with 4/12 patients (33%) from the same group who had normal transaminase levels at the time of testing for HCV RNA (p=0.06, Fisher’s Exact test).

The patterns of viraemia over a number of years was studied in the six patients with normal transaminase levels and who were anti-HCV negative (Group 1) (see Table
Table 4.3: Longitudinal PCR analysis of anti-HCV negative patients with normal transaminases (Group 1)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Diagnosis</th>
<th>HIV</th>
<th>Treatment usage (units/annum)</th>
<th>PCR 1</th>
<th>PCR 2</th>
<th>PCR 3</th>
<th>PCR 4</th>
<th>PCR 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41</td>
<td>B Mild</td>
<td>-</td>
<td>5,710</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>Carrier Mild</td>
<td>-</td>
<td>15,706</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>B Severe</td>
<td>NK</td>
<td>equ</td>
<td>equ</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td>A Mild</td>
<td>-</td>
<td>16,368</td>
<td>equ</td>
<td>-</td>
<td>equ</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>35</td>
<td>A Mild</td>
<td>-</td>
<td>315</td>
<td>-</td>
<td>-</td>
<td>equ</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>6</td>
<td>33</td>
<td>B Mild</td>
<td>-</td>
<td>5,200</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Key
NK = Not Known as this patient was seen at another centre
N/A = Not Available as the patients did not attend the centre at this time
4.3), and in the 18 patients with normal transaminase levels who were anti-HCV positive (Group 2) (see Table 4.4). Each sample was tested in duplicate and patients were classified as PCR negative if both results were negative, PCR positive if both results were positive and PCR equivocal if one result was positive and the other negative. Group 1 showed two patterns of viraemia over time. 50% of patients showed a pattern of persistent HCV RNA negativity and 50% show a pattern of intermittent positivity. The major pattern of viraemia shown by group 2 was that of intermittent PCR positivity with 11/18 patients (61%) showing at least one PCR equivocal result. 2/18 patients (11%) showed a pattern of persistent PCR positivity and 5/18 patients (28%) showed a pattern of persistent PCR negativity.

4.4 Discussion

The results in this study demonstrate that a relationship does exist between the detection of HCV viraemia and the elevation of transaminase levels. The figures rose from 0% HCV PCR positive patients who were anti-HCV negative and had persistently normal transaminases to 78% of patients who had persistently abnormal transaminases. These data show that transaminase results can reflect the amount of detectable, circulating virus and that high circulating viral titres indicate higher rates of HCV replication and, therefore, higher levels of liver cell damage. Not all patients with intermittently or persistently abnormal transaminases were HCV PCR positive and this is probably due to a naturally fluctuating viraemia.

Five patients (see Figures 4.7, 4.9, 4.11, 4.12 and 4.16) had initially raised transaminase levels which became, and remained, normal. However, their ELISA results, which were carried out sometime after their transaminitis, were initially negative and then became positive. This could be due to a reinfection, or a mutation of the original strain, which elicits a much stronger immune response. Other
Table 4.4: Longitudinal PCR analysis of anti-HCV positive patients with normal transaminases (Group 2)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Diagnosis</th>
<th>HIV</th>
<th>Treatment usage (units/annum)</th>
<th>PCR 1</th>
<th>PCR 2</th>
<th>PCR 3</th>
<th>PCR 4</th>
<th>PCR 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14</td>
<td>B Severe</td>
<td>-</td>
<td>3,765</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>VWD Mild</td>
<td>-</td>
<td>561</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>A Severe</td>
<td>+</td>
<td>37,500</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>33</td>
<td>Carrier Mild</td>
<td>+</td>
<td>240</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>A Severe</td>
<td>+</td>
<td>30,500</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>B Moderate</td>
<td>-</td>
<td>1,615</td>
<td>-</td>
<td>-</td>
<td>equ</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>B Severe</td>
<td>-</td>
<td>&gt;20,000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>equ</td>
</tr>
<tr>
<td>8</td>
<td>22</td>
<td>Carrier Mild</td>
<td>-</td>
<td>4,500</td>
<td>equ</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>47</td>
<td>B Severe</td>
<td>-</td>
<td>50,000</td>
<td>-</td>
<td>-</td>
<td>equ</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>14</td>
<td>A Moderate</td>
<td>-</td>
<td>2,850</td>
<td>-</td>
<td>-</td>
<td>equ</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>28</td>
<td>A Mild</td>
<td>-</td>
<td>12,974</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>equ</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>22</td>
<td>A Severe</td>
<td>+</td>
<td>&gt;20,000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>equ</td>
</tr>
<tr>
<td>13</td>
<td>38</td>
<td>B Severe</td>
<td>-</td>
<td>26,500</td>
<td>-</td>
<td>equ</td>
<td>-</td>
<td>-</td>
<td>equ</td>
</tr>
<tr>
<td>14</td>
<td>32</td>
<td>A Severe</td>
<td>-</td>
<td>42,000</td>
<td>equ</td>
<td>-</td>
<td>equ</td>
<td>-</td>
<td>equ</td>
</tr>
<tr>
<td>15</td>
<td>43</td>
<td>VWD Mild</td>
<td>-</td>
<td>4,430</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>equ</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>57</td>
<td>VWD Moderate</td>
<td>-</td>
<td>10</td>
<td>+</td>
<td>+</td>
<td>equ</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>43</td>
<td>A Severe</td>
<td>+</td>
<td>19,704</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>25</td>
<td>A Severe</td>
<td>+</td>
<td>68,000</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Key

equ = equivalent result. One amplification gave a negative result, the other gave a positive result.
possibilities could be that the transaminase peak was not HCV related or that the initial ELISA results are false negatives.

Five patients were anti-HCV positive, had normal transaminases and were persistently HCV PCR negative over time. These patients have been exposed to hepatitis C virus but appear to have developed an acute hepatitic illness as opposed to the more common chronic HCV infection. In this group of 255 haemophilic patients 2% appear to have had an acute HCV infection. These data support other studies which have concluded that acute hepatitis C only occurs in a small percentage of HCV infections (Alter HJ, 1995).

Nine out of the 18 patients with normal transaminases had at least one equivocal HCV PCR result, and four out of 18 of these patients had at least four positive HCV PCR results in the longitudinal analysis. Thus 72% of the patients in this study had persistently normal transaminase levels despite ongoing viral replication and the implications of this for the liver, and other sites of replication, may be similar to those who have abnormal transaminase levels.

Two patients were anti-HCV negative, had normal transaminases and were persistently negative for HCV PCR. These patients have been exposed to unsterilised clotting factor concentrates but have probably not developed chronic HCV infection. This may be due to low treatment usage as both patients have mild haemophilia. Another explanation may be low viral titres, or an absense of HCV, in the concentrates with which they were treated.

Three out of six patients who were anti-HCV negative and have persistently normal transaminases have at least one equivocal HCV PCR result. None of these patients had any other epidemiological evidence of HCV infection. Therefore, it seems likely that these equivocal PCR results are an artefact of the laboratory test. For the
purpose of this study, these equivocal results will be considered as HCV RNA negative for Group 1 patients. Thus, this data shows that of the 255 patients studied at our centre 98% show some evidence of infection with hepatitis C virus.

This study shows four main patterns of viraemia which are closely related to the transaminase pattern. Only 9% of patients exposed to unsterilised clotting factor concentrates at this centre had normal transaminase levels, and only 2% were HCV PCR negative, suggesting clearance of the virus or that an infection was not established. These data suggests that in only 2% of our patients can minimal liver damage be assumed without the evidence of liver biopsy. Therefore, in haemophilic patients, where liver biopsy is not advised, HCV viraemia and transaminase levels are the only methods of gaining information on the progression of hepatitis C virus infection.
CHAPTER 5

Hepatitis C Genotypes in Haemophilic Patients treated
with Alpha Interferon

5.1 Introduction

The majority of studies which have assessed HCV genotypes and response to interferon have investigated blood donors and intravenous drug users (Dusheiko et al, 1994a; Chemello et al, 1994). This study was designed to assess how the HCV genotype in 25 haemophilic patients affected their response to alpha interferon. The HCV RNA was extracted from serum, reverse transcribed, amplified by PCR and genotyped by RFLP analysis as detailed in Chapter 3.

5.2 Patient Details

Nineteen haemophilic patients had been entered in a pilot study using alpha interferon involving six months of treatment and six months of observation (Telfer et al, 1994). Six additional patients were also receiving alpha interferon but were not involved with the pilot study (see Table 5.1 for patient details). All patients had alanine aminotransferase (ALT) values of at least 1.5 times the upper limit of normal (40 IU/l) for at least one year prior to the start of treatment. Patients were seen monthly for clinical assessment and samples were taken for full blood count, serum electrolytes, bilirubin, total protein, albumin, ALT, prothrombin time and CD4 positive T-cell counts. A serum sample was stored at -40°C for later analysis.

Recombinant alpha interferon (Roferon, Roche) was administered thrice weekly at an initial dose of three mega units. The dose was increased to six mega units at three
Table 5.1: Patient Data

<table>
<thead>
<tr>
<th>Patient</th>
<th>Haemophilia Diagnosis</th>
<th>HIV Antibody</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B Sev</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>A Sev</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>B Mild</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>A Sev</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>A Sev</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>A Sev</td>
<td>Pos</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>A Mild</td>
<td>Pos</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>A Sev</td>
<td>Pos</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>A Sev</td>
<td>Pos</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>A Mild</td>
<td>Pos</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>A Mod</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>A</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>A</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Carr Mild</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>A</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>A Sev</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>A</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>B Sev</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>B Sev</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>A</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>A Sev</td>
<td>Pos</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>A Sev</td>
<td>Pos</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>A Sev</td>
<td>Pos</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>A Sev</td>
<td>Pos</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>A</td>
<td>Pos</td>
<td></td>
</tr>
</tbody>
</table>

All patients were HBsAG negative

No history of: psychiatric disease, renal problems, neurological disease, autoimmune disease, iv drug abuse, alcohol abuse

No evidence of hepatic decompensation
months if the ALT had failed to normalise and if side effects were tolerable. Complete biochemical response was defined as a normalisation of ALT levels for at least the last two months of treatment. Partial response was defined as at least a 50% improvement in the ALT abnormality to below 1.5 times the upper limit of normal. An HCV RNA response was defined as a transition from PCR positive at week 0 to PCR negative at months three and six of treatment. Ten (40%) patients were HIV antibody positive and the remaining fifteen (60%) patients were HIV antibody negative. Serum samples were genotyped six months pre-treatment, at week 0 and during the six months post-treatment. The week 0 and six months post-treatment samples were tested in duplicate.

A 12% polyacrylamide gel showing the ScrFl and HaeIII/RsaI digests of the 5’UTR is shown in Figure 5.1.

5.3 Results

Table 5.2 and Figure 5.2 show the hepatitis C virus genotypes of these haemophilic patients as determined by RFLP analysis.

Prior to treatment with alpha interferon 3/25 (12%) patients showed a change in hepatitis C genotype. One patient changed from 3 to 1+3, the second from 1 to 1+3 and the third from 1+3 to 3. Post-treatment, 10/25 (40%) patients showed a change. Four patients changed from 1 to 1+3, two changed from 3 to 1+3, two changed from 1+3 to 1, one changed from 2 to 1+2 and one changed from 1+3 to 3. The difference between the number of patients who showed a change in genotype pre- and post-treatment with alpha interferon was significant at the p<0.05 level (Fisher’s Exact Test).
Table 5.2: RFLP Results

<table>
<thead>
<tr>
<th>Patient</th>
<th>Clinical Response</th>
<th>6 months pre-IFN</th>
<th>Week 0</th>
<th>12 months post-IFN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>CR+PCR</td>
<td>N/A</td>
<td>1</td>
<td>1+3[1]=1+3</td>
</tr>
<tr>
<td>7</td>
<td>PR</td>
<td>1</td>
<td>1[1]=1</td>
<td>1[1]=1</td>
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<tr>
<td>8</td>
<td>PR</td>
<td>1</td>
<td>1[1]=1</td>
<td>1[1]=1</td>
</tr>
<tr>
<td>9</td>
<td>PR</td>
<td>1</td>
<td>1[1]=1</td>
<td>1+3[1]=1+3</td>
</tr>
<tr>
<td>10</td>
<td>PR</td>
<td>1</td>
<td>1[1]=1</td>
<td>1+3[1]=1+3</td>
</tr>
<tr>
<td>11</td>
<td>PR</td>
<td>1+2</td>
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<tr>
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<td>1[1]=1</td>
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<tr>
<td>13</td>
<td>PCR</td>
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<td>1[1]=1</td>
<td>1[1]=1</td>
</tr>
<tr>
<td>14</td>
<td>NR</td>
<td>1</td>
<td>1</td>
<td>1[1]=1</td>
</tr>
<tr>
<td>15</td>
<td>NR</td>
<td>1+3</td>
<td>3[1+3]=1+3</td>
<td>1[1]=1</td>
</tr>
<tr>
<td>16</td>
<td>NR</td>
<td>1</td>
<td>1[1]=1</td>
<td>1[1]=1</td>
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<tr>
<td>17</td>
<td>NR</td>
<td>1</td>
<td>1[3]=1+3</td>
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<tr>
<td>18</td>
<td>NR</td>
<td>1</td>
<td>1[1]=1</td>
<td>3[1]=1+3</td>
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<tr>
<td>19</td>
<td>NR</td>
<td>1</td>
<td>1[1]=1</td>
<td>1[1]=1</td>
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<td>20</td>
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<td>1[1]=1</td>
<td>1[1]=1</td>
</tr>
<tr>
<td>24</td>
<td>NR</td>
<td>1</td>
<td>1[1]=1</td>
<td>1[1]=1</td>
</tr>
</tbody>
</table>

Key
NR=No Response
PR=Partial ALT Response
CR=Complete ALT Response
PCR=PCR negative during treatment
N/A=Not Available

Notes
Week 0 and 6 months post-IFN:
1st test[2nd test]=combined result
Samples from these dates with only one result were PCR negative the second time
Figure 5.1a: The restriction patterns of HCV genotypes 1 to 3 using ScrFI

Lane 1: Genotype 2
Lane 2: Genotype 3
Lane 3: Genotype 1
Lane 4: PhiX174/Hinfl DNA marker
Lane 5: DNA Molecular Marker V
Lanes 6-12: Genotype 1
Figure 5.1b: The restriction patterns of HCV genotypes 1 to 3 using HaeIII/Rsal

Lanes 1 - 3: Genotype 1
Lane 4: Genotypes 1 and 2
Lane 5: Genotype 1
Lanes 6 & 7: Genotype 3
Lane 8: Molecular Marker V
Lane 9: PhiX174/HinfI DNA marker
Lane 10: Genotype 3
Lanes 11 - 14: Genotype 1
Lanes 15 & 16: Genotype 3
Figure 5.2: HCV distribution pre- and post-treatment

- Type 1
- Type 2
- Type 3
- Type 1+2
- Type 1+3

Legend:
- ■ 6 months pre-IFN
- ● Week 0
- □ 6 months post-IFN

% of patients vs. Time:
- 0
- 10
- 20
- 30
- 40
- 50
- 60
- 70

Time:
- Week 0
- 6 months post-IFN
- 6 months pre-IFN
Analysis of the HCV types found in samples from week 0 and six months post-treatment showed that of those ten patients who showed a difference in genotype, five (50%) also had a complete ALT response to alpha interferon (a normalisation of ALT levels) whereas 2/15 (13%) patients who did not show a change of type had a complete ALT response. Therefore, definite trends were seen in the number of patients who showed a change of genotype and who also had a complete ALT response to alpha interferon.

Eight out of ten (80%) anti-HIV positive patients in this study were infected with HCV type 1, 1/10 (10%) had type 3 and 1/10 (10%) had both types 1 and 3. HIV status was found not to influence the rate of change of the dominant genotype pre- or post-treatment (not significant at the p<0.05 level, Fisher's Exact Test).

Seven patients showed a complete ALT response (normalisation of ALT levels) to alpha interferon. Of these, four patients (57%) were either type 2 or 3, two patients (29%) showed mixed types of 1 and 2 and 1 and 3 and only one patient (14%) had type 1 alone.

5.4 Discussion

There are various methods of detecting hepatitis C virus genotypes, including serotyping by detection of specific antibodies (Chemello et al, 1994; Chan et al, 1992; Simmonds et al, 1993b), type-specific primers (Okamoto et al, 1992; Okamoto et al, 1993), DNA sequencing (McOmish et al, 1993; Preston et al, 1994) and genotyping using RFLP analysis (McOmish et al, 1993). The main difficulty with haemophilic patients is the presence of multiple HCV types (Simmonds et al, 1993b). Genotyping by RFLP analysis was chosen for this study as a restriction enzyme analysis of a PCR product would detect the dominant type(s) present. This
would enable an assessment to be made regarding treatment as it has been shown that genotypes 2 and 3 are more likely to respond to interferon than genotype 1 (Dusheiko et al, 1994a; Chemello et al, 1994). Problems associated with the alternative methods for typing in haemophilic patients include detecting type-specific antibodies from previous infections and the inaccuracy of type-specific primers due to the heterogeneity of HCV sequences. DNA sequencing provides more precise information than RFLP analysis but this method also requires amplification by PCR and is more labour intensive.

There are a number of different classifications for hepatitis C virus genotypes (Takamizawa et al, 1991; Mori et al, 1992; Okamoto et al, 1992; Houghton et al, 1991; Cha et al, 1992). A classification based on phylogenetic analysis seems to be becoming the most comprehensive and widely used (Chan et al, 1992).

Current evidence suggests that genotype 1 is least likely to respond to alpha interferon therapy (Dusheiko et al, 1994a; Chemello et al, 1994). This study has shown similar results: of the seven patients who showed a complete ALT response to treatment the majority had either types 2, 3 or a mixed infection and only one patient had type 1. However, although seven patients showed a complete ALT response, only three of these had reductions in HCV RNA below the cut off of the PCR assay (1000 genomes/ml). However, when treatment was withdrawn, the ALT levels rose and HCV RNA became detectable in all patients.

Comparison of the pre-treatment and week 0 results showed that there was a change in genotype in three out of the 25 patients. Comparison of the week 0 and post-treatment results showed that the proportion of patients who had a change of genotype had increased to ten out of 25. These patients are infected with multiple strains of HCV and, therefore, it seems more likely that the changes in genotype are due to a change in dominance rather than a mutation caused by alpha interferon. The
difference in the changes pre- and post-treatment was shown to be significant, therefore, alpha interferon seems to be associated with a higher rate of change of the predominant HCV genotype in haemophilic patients. The pattern of change is quite variable, for example, in some patients type 1 levels were reduced by alpha interferon resulting in type 3 levels increasing, and in others type 2 levels were reduced resulting in type 1 levels increasing. This could indicate that the predominant strain at any given point in time is the one that responds and other strains then repopulate the quasispecies. The clinical significance of these observations are not understood as yet.

Analysis of the week 0 and post-treatment results showed that half of the patients who had a change of genotype after treatment also responded clinically to alpha interferon. The association between these factors shows definite trends and it may be that alpha interferon reduced the concentration of the dominant HCV genotype sufficiently for transaminase levels to improve. The data generated from these studies also suggests that after alpha interferon was withdrawn other viral types were allowed the opportunity to emerge and so began to circulate at detectable concentrations. The mechanisms which favour the emergence of HCV genotypes and interferon response require further study.

The effect of specific HCV viral genotypes on the progression of liver disease is unclear. Therefore, it is difficult to interpret the significance of these results. It could be speculated that treatment with alpha interferon may be associated with the appearance of a more resistant genotype (type 1) (Dusheiko et al, 1994a; Chemello et al, 1994) in non-responders with mixed genotype infections (1+2 or 1+3) where genotypes 2 or 3 may be eradicated. It may also be possible that the appearance of a new dominant genotype could cause further liver inflammation and, therefore, accelerate the progression of liver disease in non-responders. Interferon may be
detrimental to progression of liver disease in the long term. Only histological data and further genotyping will provide more information on this issue.

In a previous study of the patients described in this chapter it has been shown that the haemophilic patients studied have been infected with HCV for a median of 15 years (range 3.5 - 28) (Telfer et al, 1994). Therefore, until clotting factor concentrates were sterilised in 1985 these patients will have received many infusions of unsterilised clotting factor concentrates. Thus, a high prevalence of mixed genotype infections would have been expected if re-infection does occur. These results show a lower than expected prevalence of mixed infections, so this method may be insensitive to lower concentrations of other major genotypes if one type is present at a much higher concentration. The week 0 and the six months post-treatment samples were tested in duplicate. A small percentage of samples showed a different genotype to that shown on the first test. This is probably due to the fact that these haemophilic patients are multiply infected and the PCR has preferentially amplified a different type. Further study is required to assess the proportion of genotypes comprising the quasispecies circulating in an individual at a given point in time. Therefore, RFLP analysis gives valuable information on the dominant type of hepatitis C virus in these patients, so giving an indication of the result of treatment, but it is not advisable to test samples just once and it does not provide information on all the types an individual may be infected with. Type-specific primers may be the most ideal method for studying hepatitis C genotypes in multiply infected patients. However, this method still requires some development to overcome difficulties probably due to the heterogeneity of HCV sequences found in haemophilic patients.
CHAPTER 6

Longitudinal Genotype Analysis and Quantitation of Hepatitis C Virus in Haemophilic Patients Receiving Alpha Interferon Therapy

6.1 Introduction

The study described in Chapter 5 showed, by the use of RFLP analysis, that haemophilic patients are infected with multiple HCV genotypes. It also demonstrated that RFLP analysis was perhaps not ideal for studying mixed infections. For this reason, a study was designed to compare the four most important, currently available, methods for HCV genotyping. It has been concluded that branched DNA quantitation of the virus is also an indicator of interferon response (Cooksley et al, 1994), therefore, the newly devised method by the Chiron Corporation was also used in this study.

HCV genotyping in haemophilic patients is highly complex due to the large amounts of unsterilised concentrates which they have received. Therefore, only five patients were assessed as a large number of analyses were required for each sample. The HCV RNA was extracted, reverse transcribed, amplified by PCR, restricted, cloned and sequenced as described in Chapter 3. The computer analysis, serotyping and quantitation were also carried out as detailed in Chapter 3.

6.2 Patient Details

Five haemophilic patients were selected at random from the 25 patients described in Chapter 5 who had received alpha interferon for six months at a dose of three mega
Table 6.1: Patient Data

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Type of haemophilia</th>
<th>HIV Antibody</th>
<th>Year of first exposure to concentrate</th>
<th>Mean usage/annum (units)</th>
<th>Origin of concentrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22</td>
<td>A Severe</td>
<td>Pos</td>
<td>1977</td>
<td>90,000</td>
<td>US + UK</td>
</tr>
<tr>
<td>2</td>
<td>34</td>
<td>B Severe</td>
<td>Neg</td>
<td>Mid 1970s</td>
<td>32,000</td>
<td>UK</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>A Severe</td>
<td>Neg</td>
<td>1978</td>
<td>47,000</td>
<td>UK</td>
</tr>
<tr>
<td>4</td>
<td>59</td>
<td>A Moderate</td>
<td>Neg</td>
<td>1978</td>
<td>5,000</td>
<td>US + UK</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>A Severe</td>
<td>Neg</td>
<td>1976</td>
<td>12,000</td>
<td>US + UK</td>
</tr>
</tbody>
</table>
units three times a week (Telfer et al, 1995). The clinical details of these patients are given in Table 6.1. Complete biochemical response to alpha interferon was defined as a normalisation of alanine aminotransferase (ALT) levels; partial response was defined as at least a 50% improvement in the ALT levels and a virological response was defined as PCR negative for HCV RNA at month 12 (cut off = 1000 genomes/ml).

A 3% agarose gel shows the banding pattern produced by the type-specific primers in Figure 6.1. The autoradiograph in Figure 6.2 shows the entire 251 base pair region of the 5'UTR which was sequenced in this study.

6.3 Results

The five haemophilic patients analysed in this study received large amounts of concentrates per annum for at least seven years prior to the introduction of sterilisation procedures in 1985 as shown in Table 6.1, therefore, it would be expected that these patients would be multiply infected.

The quantity of HCV RNA in the plasma of these patients at initiation of therapy with alpha interferon ranged from $8.03 \times 10^5$ genomes per ml to $8.56 \times 10^5$ genomes per ml of plasma (see Table 6.2). Four out of the five patients showed a reduction in the HCV RNA levels to below that detectable by the Chiron assay ($<3.5 \times 10^5$ genomes per ml) after the six months treatment with alpha interferon, but HCV RNA remained detectable by PCR in all patients at this time.

Among the five patients studied all had multiple HCV genotypes and the four different methods of detecting these genotypes did not always provide consistent results (see Table 6.2). In all patients analysed, more complex genotype results were obtained using the serotyping assay or type-specific primers when compared to the
Figure 6.1: HCV genotypes as determined by type-specific primers

Lane 1: 1b
Lane 2: 1b and 2b
Lane 3: Negative
Lanes 4, 6: 1a, 1b and 2b
Lane 5: 1a, 1b, 2b and 3
Lanes 7, 8, 12: 3
Lane 9: 1a
Lane 10: 1a, 1b, 2a and 3
Lane 11: 1a, 1b and 3
Lanes 13, 14: Negative controls
Lane 15: DNA Molecular Marker V
### Table 6.2: HCV Quantitation and Genotyping

<table>
<thead>
<tr>
<th>Patient samples</th>
<th>Clinical Response</th>
<th>Quantitation (genome equiv/ml x 100,000)</th>
<th>RFLP analysis</th>
<th>Sequencing (5 clones/sample)</th>
<th>Serotype</th>
<th>Type-specific primers</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Complete ALT</td>
<td>8.028</td>
<td>1</td>
<td>11111</td>
<td>1+2+3</td>
<td>3</td>
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<tr>
<td>Month 0</td>
<td>PCR Response</td>
<td>&lt;3.5</td>
<td>3</td>
<td>33333</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Month 6</td>
<td></td>
<td>&lt;3.5</td>
<td>Neg</td>
<td>Neg</td>
<td>3</td>
<td>Neg</td>
</tr>
<tr>
<td>Month 12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Complete ALT</td>
<td>15.85</td>
<td>3</td>
<td>33333</td>
<td>1+3</td>
<td>3</td>
</tr>
<tr>
<td>Month 0</td>
<td>PCR Response</td>
<td>&lt;3.5</td>
<td>1+3</td>
<td>11133</td>
<td>1+3</td>
<td>Neg</td>
</tr>
<tr>
<td>Month 6</td>
<td></td>
<td>&lt;3.5</td>
<td>Neg</td>
<td>Neg</td>
<td>3</td>
<td>Neg</td>
</tr>
<tr>
<td>Month 12</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Complete ALT</td>
<td>35.12</td>
<td>2</td>
<td>22222</td>
<td>2</td>
<td>1b</td>
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<tr>
<td>Month 0</td>
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<td>&lt;3.5</td>
<td>1</td>
<td>11111</td>
<td>2</td>
<td>1b+2b</td>
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<tr>
<td>Month 6</td>
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<td>&lt;3.5</td>
<td>2</td>
<td>22222</td>
<td>2</td>
<td>Neg</td>
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<td>Month 12</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Partial ALT</td>
<td>85.65</td>
<td>1+2</td>
<td>11111</td>
<td>1</td>
<td>1a+1b+2b</td>
</tr>
<tr>
<td>Month 0</td>
<td>PCR Response</td>
<td>&lt;3.5</td>
<td>1</td>
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<td>1</td>
<td>1a+1b+2b+3</td>
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<td>Month 6</td>
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<td>51.62</td>
<td>1+2</td>
<td>11222</td>
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<td>1a+1b+2b</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>5</td>
<td>No Response</td>
<td>28.82</td>
<td>3</td>
<td>13333</td>
<td>1</td>
<td>1a+1b</td>
</tr>
<tr>
<td>Month 0</td>
<td></td>
<td></td>
<td>1</td>
<td>11113</td>
<td>1</td>
<td>1a+1b+2a+3</td>
</tr>
<tr>
<td>Month 6</td>
<td></td>
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<td>1</td>
<td>11111</td>
<td>1</td>
<td>1a+1b+3</td>
</tr>
<tr>
<td>Month 12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Figure 6.2: Sequence of a 251 base pair region of the 5'UTR
DNA sequence analysis. Some examples of these inconsistencies include the genotype results for patient one which showed a mixed infection by the serotype assay at month 0 but singular infections by all other methods. The type-specific primers were negative for patient two at month 6 but all other methods gave a genotype result. The RFLP analysis and type-specific primers for patient four, and the sequencing and type-specific primers for patient five all show mixed infections but the serotyping assays show single infections for both these patients. The sequence analysis in patient four only detected type 1 at month 0, whereas the RFLP analysis detected both types 1 and 2. However, the sequence analysis in patient five demonstrated types 1 and 3 at month 0 (type 3 only by RFLP analysis) and types 1 and 3 at month 6 (type 1 only by RFLP analysis).

Three of the five patients showed a complete biochemical response to alpha interferon which persisted six months after therapy was withdrawn. In these three patients the sequence analysis correlated with the results of the RFLP analysis showing a change of genotype after initiation of therapy. During the six months after withdrawal of alpha interferon, patients one and two did not possess sufficient HCV RNA for PCR amplification, whilst patient three returned to pre-interferon type 2 by both RFLP analysis and DNA sequencing. However, the type 2 sequences circulating at month 12 contained point mutations which illustrated their unique molecular identity from those present at month 0. All three patients had low levels of virus circulating at month 0 and after therapy all levels were reduced to below the cut-off value (3.5 x 10^5 genomes/ml for the Chiron assay) and remained below this at month 12.

Patient four had a partial biochemical response to therapy. He had a high viral load (8.56x10^6 genomes per ml) at month 0 which was reduced to below detectable levels at month 6. This coincided with a disappearance of type 2 from the circulating
Figure 6.3: Unrooted phylogenetic tree of 65 5'UTR sequences from patients 1 to 5

KEY
1-5: Patients 1 to 5
0: Month 0
6: Month 6
12: Month 12
A, B, C: Prototype sequences for genotypes 1, 2 and 3, respectively
population of genotypes. At month 12 the viral load had increased to $5.16 \times 10^6$ genomes per ml and type 2 was detectable once more.

Patient five did not have any biochemical response to alpha interferon, however, a change of genotype was shown after therapy by RFLP analysis and sequencing. The quantitation results showed a low viral load in this patient at month 0. This increased slightly after therapy at month 6, and decreased to below detectable levels after the six months of observation at month 12.

In order to fully investigate the sequence evolution of HCV quasispecies in these patients, DNA parsimony methods were used with bootstrap resampling. Prototype sequences of genotypes 1, 2 and 3 (A, B and C respectively in Figure 6.1) were included in this analysis. The unrooted maximum parsimony tree of the 65 5' UTR sequences is shown in Figure 6.3. All sequences cluster according to the three genotypes identified by RFLP and DNA sequence analysis. However, there are certain species such as the three sequences present in patient four at month 12 which are genotype 2 in character but cluster in an independent branch possibly indicating their closer phylogenetic relationship with the genotype 1 sequences present in this patient at months 0 and 6. This may be indicative of recombination events.

6.4 Discussion

Evaluation of the four different genotyping techniques in the longitudinal analysis of patients pre- and post-treatment with interferon showed that the methods produced identical results in 5% of cases. The RFLP analysis did not detect mixed genotypes for the majority of serum samples tested. It seems likely, therefore, that genotypes circulate at differing concentrations within individual patients and hence this distribution is reflected in the PCR amplicons.
The DNA cloning and sequencing assessed type-specific differences in the 5’ UTR. This region is highly conserved and, therefore, much more easily amplified in patients who are likely to have a highly heterogeneous population of HCV sequences. Type-specific differences in this region have also been shown to be representative of the whole genome (Simmonds et al, 1993), therefore, it is probable that sequencing in more variable regions, such as the envelope regions or NS5, would have provided little more information on the genotypes present. Five clones were selected from each sample for sequencing as only a maximum of two species were present in each sample (if more species than this had been present then more clones would have been sequenced). This analysis, which is generally considered the gold standard for genotype analysis, also often failed to detect multiple genotypes despite the sequencing of five different clones in each sample. This was probably due to the fact that RT-PCR amplification of each sample was required before DNA sequencing could be carried out and, as for the RFLP analysis, only the dominant genotypes were sampled in the final amplicons.

The serotype analysis is likely to be confused by past as well as ongoing infection as shown by the two patients who had a sustained response to alpha interferon (patients 1 and 2, Table 6.3). This may be a potential problem when investigating the genotypes circulating at a particular time point. For example, it may be that in haemophilic patients, who are likely to have mixed infections, the serotyping assay will be of less use than in non-haemophilic populations. It is interesting to note that many of the samples analysed produced values that were close to the cut-off for the assay, which, if reduced, would have yielded an improved correlation with the sequence data. Patient two had serotype 1 just below cut-off; patient four had serotypes 2 and 3 just below cut-off for all samples and patient five had serotypes 2 and 3 just below cut-off at month 0.
The most heterogeneous identification of HCV genotypes in individual patients was
detected using the type-specific primers. It is likely that such analysis is more
sensitive to genotypes circulating at lower levels, although infections with multiple
genotypes were only identified in three out of the five patients under investigation.

Therefore, as the RFLP analysis provided the most important information on the
dominant genotypes present in patients who are likely to be infected with multiple
HCV genotypes it is probably the most useful and convenient of the four currently
available methods for genotyping hepatitis C in infected haemophilic patients.

The RFLP analysis showed that treatment with alpha interferon appeared to cause a
change of genotype in all five patients. As the PCR probably preferentially amplifies
the dominant genotypes present it seems likely that treatment with alpha interferon
reduces the concentration of the dominant circulating genotypes below detectable
levels and allows those circulating at lower levels to increase their rates of replication
and become detectable.

This study shows that the three patients who had the best clinical response to
interferon (patients one, two and three) were the youngest (< 40 years) and those
who had low levels of HCV RNA. However, one patient (patient five) who did not
respond to interferon treatment also had a low viral load. This patient showed a
reduction of HCV RNA at month 12 after the six months of observation which may
be due to the natural fluctuations of viraemia. The genotyping results cannot give
any definite conclusions as to the response to interferon as the three patients who
had the best response had predominantly types 1 (patient one), 2 (patient three) and
3 (patient two) (by RFLP analysis) at the start of therapy, the patient with a partial
response had types 1 and 2 and the non-responder had type 3. Therefore, it seems
probable that those patients with mixed genotype infections have more complex
responses to treatment than patients who are singularly infected.
At present, data on the changes of genotypes in haemophilic patients not receiving therapy is limited, but changes appear to be relatively infrequent (Jarvis et al, 1994). Hence, the data presented here shows that to fully understand strain evolution and the benefits of therapy in haemophilic patients (who cannot easily undergo liver biopsy due to the increased risk of bleeding), the majority of whom are likely to be multiply infected, frequent sampling is required at multiple genetic loci together with viral load measurements.
CHAPTER 7

Long term evolution of two regions of the hepatitis C virus genome: comparison between an HIV positive and an HIV negative patient

7.1 Introduction

This study assessed the HCV sequence evolution in the highly conserved 5'UTR and in an NS4 epitope in an HIV positive haemophilic patient and in an HIV negative haemophilic patient from their date of infection to 1994. Both patients had mild haemophilia and, therefore, have received very little unsterilised clotting factor concentrate. Due to the large amount of clinical data available on both these patients, the aim of this study was also to compare the clinical condition of the patients with the molecular biology of their hepatitis C viruses, including sequence data, quantitation of the virus and HCV genotype.

7.2 Methods

The HCV RNA extraction, reverse transcription, PCR of the 5'UTR and NS4, restriction analysis, quantitation, sequencing and sequence analysis were carried out as detailed in Chapter 3. Figures 7.1 and 7.2 show mutations in the 5'UTR from clones within the same year and in different years, respectively. The region of NS4 which was sequenced in this study is shown in Figure 7.3 and the NS4 T-cell epitope is shown in Figure 7.4. Figures 7.5 and 7.6 show mutations in NS4 from clones within the same year and in different years, respectively.
Figure 7.1: Sequences showing a mutation in two 5'UTR clones from the same year

Mutation shown is from C to T
Figure 7.2: Sequences of 5'UTR clones showing mutations in different years (1989 and 1990)

Mutation is from T to C
Figure 7.3: Sequences showing the NS4 region studied and a mutation in different years (1987 and 1988)

Mutation is from G to A
This is a 10 amino acid epitope (LLFNILGGWV) which is recognised by CD8+ MHC class I-restricted cytotoxic lymphocytes in vitro and in vivo (Wentworth et al, 1994)
Figure 7.5: Sequences showing two NS4 clones from the same year demonstrating two mutations.

Mutations are from A to G and G to A.
Prior to the discovery of the NS4 primers, various other attempts were made to amplify other regions. The primers which did not produce a PCR product are shown in Table 7.1. The first region to be attempted was E2/NS1 as this contains a hypervariable region and would have been very useful to study longitudinally. Primers designed by alignment of eight E2/NS1 sequences from GeneBank were used and were found not to amplify sufficient numbers of samples. The sensitivity of the PCR is 1000 genome equivalents/ml, therefore, at least 100 sequences identical to the primer sequences must be present in 100μl of serum in order for amplification of the template to occur. Haemophilic patients are normally infected with multiple viral sequences (Devereux et al, 1995; Jarvis et al, 1994) and so would be less likely than transfusion patients or intravenous drug users to have enough identical sequences present necessary for amplification.

Amplification of other regions such as NS5 were attempted (as this region is reasonably variable and is used for genotyping (Simmonds et al, 1993)), and NS3 (as this region is also variable among different isolates). The primers used for these regions are shown in Table 7.1. The NS5 primers have been published previously whilst the NS3 and NS4 primers were designed by aligning eight sequences from the appropriate region from the GeneBank Database and using the consensus sequence, or variations of it, as appropriate.

7.3 Patient details

Patient 1

This patient had mild haemophilia A and was anti-HIV positive (see Table 7.2). During 1982 he received two batches of commercial factor VIII concentrate manufactured by Immuno in the US. Prior to this date he had received whole blood and cryoprecipitate, but his ALT values remained normal until one week after his
Table 7.1: Primers used initially for longitudinal study

<table>
<thead>
<tr>
<th>Region of HCV Genome</th>
<th>First Round 5' - 3'</th>
<th>Second Round 5' - 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2/NS1</td>
<td>CACGAATTCCGGGGCTGGGAGTGAAGCAAT ATAAAGCTTACCCGCATGGCATGGGATAT</td>
<td>GGTAAGCTTATGGCATGGGATATGATGATGAT CTGGAATTCAAGCAATATACCCGGACCACA</td>
</tr>
<tr>
<td>NS3 1</td>
<td>ACCGGAATGACATCAGCAGCATGTCTCGTGAACCAAGTAAAGGA GAGCCCGTCGTCTCTTCTCCGACATGGGAGACCAAGATCAT</td>
<td>GGTCCCTGGTCTACATTTGCTTACAT ACTAGCCTCACAGGCGGGACAA</td>
</tr>
<tr>
<td>NS3 2</td>
<td>GTGCACACCGCGGCCCTAAATA GAGCCCGTCGTCTCTTCTCCGACATGGGAGACCAAGATCAT</td>
<td>TGACATCGCGCTCGTGCTGACCA CCTAACTGCGCGGGACAA</td>
</tr>
<tr>
<td>NS3 3</td>
<td>TCCTAGGGTGCTAATCACCAGCCTACTG CATGGTTGCTCTAGGTTCTCCAGGATA TTTATCCACCTCACGGGTGCACAA</td>
<td>TTTATCCACTCCACGGGTGCACAA CAGATTGTGCTACAAGCTGCCCAA</td>
</tr>
<tr>
<td>NS5</td>
<td>GGCGGAATTCTCTGGTGCTAGCAGCTCCGTGAA TGGGATCCGGTATGATTCCGGCTGCTT</td>
<td>CCACGACTAGATCATCTCCG CTCAACCCTGCATGACAGACAC</td>
</tr>
</tbody>
</table>

* : Used with the same first round primers
first exposure to concentrate. Therefore, it is highly likely that he became infected with HCV after administration of this concentrate. It is also thought that he contracted HIV infection from this same batch of concentrate. This patient also received five different batches of low donor pool NHS factor VIII (manufactured in the UK) in 1984, prior to the introduction of sterilisation procedures in 1985. Both batches of concentrate from Immuno and four batches of NHS concentrate were available for analysis.

This patient suffered from jaundice after receiving concentrate in 1982 and also after the concentrate in 1984. He has had fluctuating symptoms of hepatitis, including malaise, anorexia, diarrhoea and an enlarged liver and spleen throughout the time following infection. However, these symptoms were complicated by the presence of other medical conditions, see Table 7.3.

Patient 2

This patient had mild haemophilia A, was anti-HIV negative and was matched to patient one for age, haemophilia diagnosis, factor VIII usage and duration of HCV infection (see Table 7.2). He was exposed to unsterilised concentrate initially in 1980, and had been treated with whole blood and cryoprecipitate, but he did not develop the symptoms of hepatitis until approximately one month after the factor VIII treatment in 1980. He did not receive any other concentrate until after sterilisation procedures were introduced in 1985. From the clinical notes, this patient suffered from nausea and malaise for five months after infection but has had no clinical symptoms of HCV infection since then, and his liver and spleen have also remained in palpable over this time (see Table 7.3). The concentrate used to treat this patient was not available for analysis.

Both patients were negative for hepatitis A, B and CMV. Neither patient drank more than the recommended weekly allowance of alcohol for men, but patient one did
Table 7.2: Clinical details for patients one and two

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (Years) (to 1994)</th>
<th>Diagnosis</th>
<th>HIV Status</th>
<th>Year of First Exposure to Concentrate</th>
<th>Total Amount of Unsterilised Concentrate Received (units)</th>
<th>Origin of Concentrate</th>
<th>Duration of HCV Infection (to 1994)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>65</td>
<td>Mild Haemophilia A</td>
<td>Positive</td>
<td>1982</td>
<td>Commercial: 42,044</td>
<td>Commercial + UK</td>
<td>12 years</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>UK: 289,995</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>49</td>
<td>Mild Haemophilia A</td>
<td>Negative</td>
<td>1980</td>
<td>6,000</td>
<td>Commercial</td>
<td>14 years</td>
</tr>
</tbody>
</table>
Table 7.3: Longitudinal clinical history for patients one and two

**Patient 1**
Mild haemophilia A (12%)
HIV +ve
Alcohol: Seldom drinks (smokes 15/day)
First exposure to FVIII: 1982
HBsAg -ve
HAAb -ve
CMV -ve
Note: HCV and HIV were probably contracted from same batch of concentrate
1953 Right hip replacement
1964 Right hip replacement removed
1982 Left hip replacement

**1980**
- 23/6/80: AST 13 (N=5-15) (IU/l)
- 8/8/80: AST 8
- 10/11/80: Cryoprecipitate for thumb injury AST 17
- 17/11/80: Cryoprecipitate and metronidazole

**1981**
- 8/10/81: AST 44 (N=5-40)
- 9/10/81: One discharging sinus in right lower abdomen (caused by abscess in 1965). Left hip has deteriorated. Takes brufen

**1982**
RFLP type 1
NS4 PCR positive
Quantitation (Chiron): 3.3x10^6 (genomes/ml)
- 12/9/82: Admitted for left hip replacement
- 17/9/82: GP B Streptococcus isolated from sinus. 500mg penicillin V 4 times/day
- 21/9/82: Operation on left hip. Gentamicin, whole blood and FVIII AST 42
- 22/9/82: Whole blood and FVIII. Eating and drinking AST 29
- 27/9/82: AST 32
- 30/9/82: AST 79
- 4/10/82: AST 89
- 8/10/82: Reduction of FVIII over weekend AST 447
- 11/10/82: AST 810
- 15/10/82: Jaundice, unwell. Eating and drinking AST 1460
- 19/10/82: AST 1140
- 22/10/82: Very unwell
- 26/10/82: AST 465
- 2/11/82: AST 2500
- 9/11/82: AST 483
- 12/11/82: Fluctuating symptoms of malaise and anorexia. Liver tender, 1.5"
- 23/11/82: AST 730
- 30/11/82: AST 35
- 7/12/82: AST 26
- 21/12/82: AST 475

**1983**
RFLP type 1
NS4 PCR positive
Quantitation (Chiron): 1.7x10^6
- 6/1/83: 500mg penicillin. Eating, no abdominal pain AST 304
11/1/83  AST 407
20/1/83  Unwell  AST 323
1/2/83  Unwell  AST 451
28/2/83  Liver 1.5"  AST 94
28/3/83  AST 48
18/4/83  AST 434
4/5/83  AST 31
23/5/83  AST 105
20/6/83  AST 47
18/7/83  AST 146
31/8/83  AST 95
26/9/83  CT scan - enlarged liver  AST 233
31/10/83  Low grade temperature for one month. Unwell for past 4-5 weeks
          Urine dark, loose bowels. Penicillin for past year. Liver 2".
          AST 328
3/11/83  Rectal bleeding-used to be once a year now once every three weeks
9/11/83  Pseudomonas detected
23/11/83  Barium enema
4/12/83  Colonoscopy. Smooth enlarged liver
          Rectal biopsy. No abnormality detected
6/12/83  Colonoscopy. Palpable carcinoma in upper rectum  AST 97
8/12/83  AST 114
11/12/83  Anterior resection and transverse colostomy
          Tumour 2cm
18/12/83  Inhibitors to porcine FVIII. Given low donor pool NHS FVIII
          Lightheaded 12hrs post-treatment  AST 35
20/12/83  Laparotomy - abnormal liver
21/12/83  Closure of colostomy
28/12/83  AST 31
31/12/83  Paracolic collection aspirated - E. coli and faecal type streptococcus isolated

1984
RFLP type 1
NS4 PCR positive
Quantitation (Roche) : 4.4x10^4
4/1/84  AST 26
9/1/84  Whole blood  AST 64
10/1/84  Whole blood and cryoprecipitate  AST 39
11/1/84  Whole blood  AST 32
13/1/84  Cryoprecipitate
16/1/84  Metronidazole and cefotaxime  AST 18
24/1/84  No antibiotics. Cryoprecipitate  AST 26
26/1/84  Bacteraemia. Metronidazole, trimethoprim, sulphmethoxazole,
          septrin
27/1/84  Abdominal fistula flush
28/1/84  Whole blood and cryoprecipitate
30/1/84  Still bleeding and abdominal fistula still draining  AST 379
31/1/84  Vitamin K, ferrous sulphate
1/2/84  Jaundiced  AST 363
2/2/84  Operation - sinus irrigation. FVIII. Jaundiced post-operatively
3/2/84  Whole blood and FFP
4/2/84  500mg IV metronidazole 3 times/day, 200mg IV trimethoprim 2
          times/day. Jaundiced
6/2/84  Jaundiced. Fistula between bowel and abdominal wall  AST 234
7/2/84  FVIII continued. Jaundiced
8/2/84  Liver appears smalll. Weight loss and anorexia due to local sepsis
10/2/84  AST 101
13/2/84  Jaundice improving  AST 68
15/2/84  Pyrexial 38.4°C. Diarrhoea. Wound discharging green pus and blood. Given metronidazole, cotrimoxazole, cefotaxime and netilmicin IV
16/2/84  Change cefotaxime to trimethoprim  AST 59
17/2/84  Whole blood
18/2/84  Whole blood
20/2/84  Stop antibiotics. Start colistin to combat itch caused by liver disease and neomycin orally  AST 53
23/2/84  Diarrhoea  AST 60
28/2/84  Rigors. Discharge continues. 39.7°C. Cefotaxime 2g IV 6 hourly and metronidazole 500mg IV 3 times/day
1/3/84  Whole blood
2/3/84  Whole blood
5/3/84  Right achilles tender. Still bleeding  AST 150
6/3/84  Eating. Antibiotics stopped
8/3/84  Diarrhoea. Given neomycin and colistin
15/3/84  Pyrexial. Feels better. Still has discharge  AST 139
19/3/84  AST 73
21/3/84  Weight loss, malaise, pyrexial  AST 51
23/3/84  K+ supplement. FVIII
25/3/84  39°C. Given trimethoprim 200mg 2 times/day
26/3/84  37.2°C
27/3/84  FVIII  AST 62
3/4/84  AST 69
5/4/84  Continue daily FVIII, antibiotics, iron and vitamins  AST 60
13/4/84  AST 78
22/4/84  Bleeding from bowel. Blood in urine
26/4/84  AST 109
8/5/84  Taken septrim for 10 days. FVIII daily. No antibiotics  AST 224
24/5/84  Stopped FVIII  AST 264
15/6/84  AST 220
30/7/84  AST 97
4/9/84  AST 80
21/9/84  AST 136
20/12/84  No malaise. Eating well  AST 151

1985
RFLP type 1
NS4 PCR positive
Quantitation (Chiron) : 1.7x10^6
22/3/85  Rash on scrotum. Bowels erratic. No jaundice
Two spidernaemias, one on each arm
X-ray : left hip prosthesis satifactory
Right hip gross deformity - pseudoarthrosis

1986
RFLP type 1
NS4 PCR positive
Quantitation (Chiron) : 7.9x10^6
7/3/86  Appetite good  AST 52
14/5/86  Lump in right buttock increasing in size  AST 62
19/5/86  Admitted 4 days ago with intermittent fever. Liver 1” firm. 5x6cm fluctuant tender mass in left lower abdomen (started 12 weeks ago).
CT scan - normal
AST 58
3/6/86  500mg penicillin 2 times/day
11/6/86  IV antibiotics - netilmicin, azlocillin. treat daily with FVIII

121
12/6/86  IV netilmicyn 200mg 2 times/day and ciprofloxacin orally
           AST 55   CD4 0.95 (x 10^9/l)
23/6/86  Vibamycin given for cold
24/6/86  Incision and drainage of abscess. No organisms found. Given
           gentamycin. Wound left open AST 75
11/9/86  Liver 2”, not tender AST 80
19/9/86  CT scan - liver and spleen enlarged with no deposits and no ascites
2/10/86  AST 69

1987
RFLP type 1
NS4 PCR positive
Quantitation (Chiron) : 4.1x10^6
29/1/87  CD4 0.93
19/2/87  1 week ago dark urine for a few days. Liver 2.5”, not tender
           AST 154
14/5/87  Liver 2.5” AST 72
5/11/87  Occasional diarrhoea. Liver 2.5”. No masses AST 113
12/87  Hip bleed

1988
RFLP type 1
NS4 PCR positive
Quantitation (Chiron): 3.4x10^6
17/3/88  CD4 0.72
15/9/88  AST 75   CD4 0.72
18/10/88  AZT trial started (placebo) AST 70   CD4 0.73
15/11/88  Mouth bleed. AST 68   CD4 0.94
13/12/88  AST 71

1989
RFLP type 1
NS4 PCR positive
Quantitation (Chiron) : 3.9x10^6
10/1/89  Malaise, anorexia, abdominal discomfort, dark urine
           Liver 1.5”, spleen 1”. No ascites AST 56   CD4 0.52
7/2/89  AST 56
3/3/89  AST 40   CD4 0.52
25/4/89  Liver 0.5-1”, spleen 0.5-1” AST 93   CD4 0.47
23/5/89  AST 106
20/6/89  AST 55   CD4 0.47
20/7/89  Paracetamol for headaches. Liver 0.5” AST 55   CD4 0.43
19/9/89  Been on flucloxacillin for 6 weeks (stopped 7/89).
17/10/89  Liver 0.5” AST 74   CD4 0.51
12/12/89  Flu. Liver 0.5” AST 85   CD4 0.345
**1990**
RFLP type 1
NS4 PCR positive
Quantitation (Chiron): \(6.4 \times 10^6\)

<table>
<thead>
<tr>
<th>Date</th>
<th>AST</th>
<th>CD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>30/1/90</td>
<td>81</td>
<td>0.435</td>
</tr>
<tr>
<td>13/3/90</td>
<td>Not eating well. Liver 2&quot;</td>
<td>88</td>
</tr>
<tr>
<td>29/3/90</td>
<td>Ciprofloxacin started</td>
<td></td>
</tr>
<tr>
<td>3/4/90</td>
<td>Ciprofloxacin stopped, ampicillin started</td>
<td></td>
</tr>
<tr>
<td>24/4/90</td>
<td>Appetite poor. Liver 1&quot;</td>
<td>85</td>
</tr>
<tr>
<td>5/6/90</td>
<td>Liver 1&quot;, spleen 1&quot;</td>
<td>112</td>
</tr>
<tr>
<td>17/7/90</td>
<td>Anorexic. Drinks 3-4 glasses milk/day. Liver 1.5&quot;</td>
<td>103</td>
</tr>
<tr>
<td>2/10/90</td>
<td>Leg bleed, fevers and night sweats</td>
<td></td>
</tr>
<tr>
<td>18/10/90</td>
<td>Liver 2&quot;</td>
<td>191</td>
</tr>
<tr>
<td>22/11/90</td>
<td>Ciprofloxacin for 7 days for right groin sinus discharge, pseudomonas found</td>
<td></td>
</tr>
<tr>
<td>29/11/90</td>
<td>Candidiasis in right groin</td>
<td></td>
</tr>
</tbody>
</table>

**1991**
RFLP type 1
NS4 PCR positive
Quantitation (Chiron): \(9.9 \times 10^6\)

<table>
<thead>
<tr>
<th>Date</th>
<th>AST</th>
<th>CD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>8/1/91</td>
<td>Liver 1.5&quot;, spleen 1&quot;</td>
<td>135</td>
</tr>
<tr>
<td>22/4/91</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>1/7/91</td>
<td>2 weeks of ciprofloxacin</td>
<td>0.45</td>
</tr>
<tr>
<td>16/9/91</td>
<td>Liver 0.5&quot;</td>
<td>0.2</td>
</tr>
<tr>
<td>5/11/91</td>
<td>AST 64</td>
<td>0.39</td>
</tr>
<tr>
<td>9/12/91</td>
<td>Liver 1&quot;</td>
<td>63</td>
</tr>
</tbody>
</table>

**1992**
RFLP type 1
NS4 PCR positive
Quantitation (Chiron): \(1.3 \times 10^7\)

<table>
<thead>
<tr>
<th>Date</th>
<th>AST</th>
<th>CD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>13/1/92</td>
<td>60</td>
<td>0.44</td>
</tr>
<tr>
<td>10/2/92</td>
<td>87</td>
<td>0.33</td>
</tr>
<tr>
<td>2/3/92</td>
<td>Liver 2.5&quot;</td>
<td>84</td>
</tr>
<tr>
<td>30/3/92</td>
<td>73</td>
<td>0.29</td>
</tr>
<tr>
<td>27/4/92</td>
<td>Interferon started 3MU x 3/week</td>
<td>66</td>
</tr>
<tr>
<td>28/5/92</td>
<td>Paracetamol for headaches post-interferon. Chronic irregular bowel movements. Appetite normal</td>
<td>50</td>
</tr>
<tr>
<td>22/6/92</td>
<td>44</td>
<td>0.30</td>
</tr>
<tr>
<td>26/6/92</td>
<td>Diarrhoea for past few days. Lower abdominal pain. Interferon stopped</td>
<td></td>
</tr>
<tr>
<td>13/7/92</td>
<td>Re-started interferon 3MU x 3/week</td>
<td>45</td>
</tr>
<tr>
<td>10/8/92</td>
<td>47</td>
<td>0.30</td>
</tr>
<tr>
<td>8/9/92</td>
<td>Interferon reduced to 2MU x 3/week for 1 week</td>
<td>50</td>
</tr>
<tr>
<td>5/10/92</td>
<td>Interferon stopped</td>
<td>37</td>
</tr>
<tr>
<td>2/11/92</td>
<td>42</td>
<td>0.17</td>
</tr>
<tr>
<td>9/11/92</td>
<td>Swab from sinuses grew pseudomonas. Ciprofloxacin for past 10 days</td>
<td></td>
</tr>
<tr>
<td>17/11/92</td>
<td>Started AZT 400mg daily, septrin 480mg daily, fluconazole 150mg weekly, feros sulphate 200mg daily</td>
<td>45</td>
</tr>
<tr>
<td>30/11/92</td>
<td>Feels weak. Started smoking again 2/52 ago. HBV Ab -ve (did not rise on booster)</td>
<td>35</td>
</tr>
<tr>
<td>14/12/92</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>
**1993**

RFLP type 1
NS4 PCR positive
Quantitation (Chiron) : $2.2 \times 10^7$

<table>
<thead>
<tr>
<th>Date</th>
<th>AST 1993</th>
<th>CD4 0.19</th>
</tr>
</thead>
<tbody>
<tr>
<td>19/1/93</td>
<td>AST 197</td>
<td>CD4 0.19</td>
</tr>
<tr>
<td>15/2/93</td>
<td>AST 195</td>
<td>CD4 0.16</td>
</tr>
<tr>
<td>13/4/93</td>
<td>AST 116</td>
<td>CD4 0.17</td>
</tr>
<tr>
<td>4/5/93</td>
<td>Chest pain over past 5 days</td>
<td>AST 105 CD4 0.26</td>
</tr>
<tr>
<td>16/5/93</td>
<td>Chest pain improved. Episode of breathlessness and faintness</td>
<td></td>
</tr>
<tr>
<td>26/5/93</td>
<td>Delta trial week 0. Increase AZT to 200mg 3 times/day</td>
<td>AST 82 CD4 0.18</td>
</tr>
<tr>
<td>8/6/93</td>
<td>Constipation, anorectic, tired, lower abdominal pain</td>
<td>AST 115</td>
</tr>
<tr>
<td>22/6/93</td>
<td>Severe mouth ulcers for 10 days. Skin rash for 13 days. Sweating, no fevers. Stop trial drug/placebo. Acyclovir 400mg 5 times/day. Stop AZT.</td>
<td>AST 83 CD4 0.17</td>
</tr>
<tr>
<td>5/7/93</td>
<td>AZT re-started 200mg TDS. Ulcers and rash better</td>
<td></td>
</tr>
<tr>
<td>26/7/93</td>
<td>AST 56 CD4 0.16</td>
<td></td>
</tr>
<tr>
<td>22/8/93</td>
<td>Stopped AZT 8 days ago. Start AZT at 100mg 3 times/day</td>
<td></td>
</tr>
<tr>
<td>20/9/93</td>
<td>AZT 300mg/24hrs. Does not take for 2 days or nausea builds up. Anorectic</td>
<td>AST 64 CD4 0.08</td>
</tr>
<tr>
<td>17/10/93</td>
<td>Takes AZT Wed-Sat</td>
<td>AST 65 CD4 0.05</td>
</tr>
<tr>
<td>18/10/93</td>
<td>HAV Ab +ve (had hepatitis A at age 20)</td>
<td></td>
</tr>
<tr>
<td>2/11/93</td>
<td>Itching for 4 days. In contact with measles 1 week ago</td>
<td></td>
</tr>
<tr>
<td>9/11/93</td>
<td>Rash settled</td>
<td>AST 59 CD4 0.06</td>
</tr>
<tr>
<td>13/12/93</td>
<td>Significant aortic stenosis.</td>
<td>AST 56 CD4 0.06</td>
</tr>
</tbody>
</table>

**1994**

RFLP type 1
NS4 PCR positive
Quantitation (Chiron) : $1.6 \times 10^7$

<table>
<thead>
<tr>
<th>Date</th>
<th>AST 58</th>
<th>CD4 0.06</th>
</tr>
</thead>
<tbody>
<tr>
<td>10/1/94</td>
<td>AST 58</td>
<td>CD4 0.04</td>
</tr>
<tr>
<td>7/2/94</td>
<td>Chest infection for 1 week. Septrin 2 times/day for 5 days</td>
<td></td>
</tr>
<tr>
<td>11/2/94</td>
<td>AST 50</td>
<td>CD4 0.05</td>
</tr>
<tr>
<td>7/3/94</td>
<td>Spontaneous bleed in right forearm</td>
<td>AST 49 CD4 0.06</td>
</tr>
<tr>
<td>5/4/94</td>
<td>Rash for 5 days. Chest infection for 1 week (septrin had been stopped). Septrin re-started</td>
<td>AST 60 CD4 0.03</td>
</tr>
<tr>
<td>9/5/94</td>
<td>Rash improved, chest infection improving</td>
<td>AST 60 CD4 0.03</td>
</tr>
<tr>
<td>7/6/94</td>
<td>Start rifabutin prophlaxis</td>
<td>AST 63 CD4 0.02</td>
</tr>
<tr>
<td>4/7/94</td>
<td>Ast 52</td>
<td>CD4 0.02</td>
</tr>
<tr>
<td>8/8/94</td>
<td>Stopped AZT 2 weeks ago</td>
<td></td>
</tr>
<tr>
<td>6/9/94</td>
<td>Fluconazole 150mg weekly. Rifabutin 300mg once daily. Septrin 960mg 3 times/week. Ferrous sulphate 200mg once daily.</td>
<td>AST 62 CD4 0.01</td>
</tr>
<tr>
<td>3/10/94</td>
<td>Sinus still discharging. Follicular lesions on skin. Intermittent pruritis. Replace rifabutin with clorithromycin 300mg once daily. Restarted AZT : 100mg 2 times/week, 100mg3 times/week, 100mg once daily</td>
<td>AST 57 CD4 0.01</td>
</tr>
</tbody>
</table>
**Patient 2**
Mild haemophilia A (14%)
HIV -ve
Alcohol : 4-12 units per week
First exposure to FVII : 1980
HBsAg -ve
HAAb -ve
CMV -ve
Note : Neither liver or spleen have been palpable at any time

**1980**
RFLP type 1
NS4 PCR negative (insufficient serum for extensive testing-ultracentrifugation required)
Quantitation (Roche) : Negative
24/8/80 Right ankle bleed. FVIII daily for one week
29/8/80 Operation. Treated twice daily for 48hrs post-operatively and then daily for 5 days
8/9/80 Loss of appetite, nausea, vomiting, epigastric pain
15/10/80 AST 123 Bilirubin 23
8/12/80 AST 216

**1981**
No sample available
10/7/81 Nausea and malaise persisted until Jan 1981
1/81 AST 117
7/81 AST 165

**1982**
Did not visit centre

**1983**
RFLP type 1
NS4 PCR negative
Quantitation (Roche) : $1.4 \times 10^4$
7,8,9/8/83 Cryoprecipitate for nose bleeds

**1984**
RFLP type 1
NS4 PCR positive
Quantitation (Chiron) : $1.2 \times 10^6$
26/6/84 AST 25

**1985**
RFLP type 1
NS4 PCR negative
Quantitation (Chiron) : $2.1 \times 10^6$
8/1/85 AST 65
23/10/85 AST 54 CD4 0.96

**1986**
RFLP type 1
NS4 PCR positive
Quantitation (Chiron) : $1.2 \times 10^6$
12/5/86 AST 53
6/11/86 AST 46 CD4 1.29
1987
RFLP type 1
NS4 PCR positive
Quantitation (Chiron) : $1.6 \times 10^6$
24/11/87 AST 64
27/7/87 AST 47

1988
RFLP type 1
NS4 PCR positive
Quantitation (Chiron) : $4.1 \times 10^6$
28/11/88 AST 39 CD4 1.12

1989
RFLP type 1
NS4 PCR positive
Quantitation (Chiron) : $7.4 \times 10^5$
16/11/89 AST 49 CD4 1.104

1990
RFLP type 1
NS4 PCR positive
Quantitation (Chiron) : $1.6 \times 10^6$
2/1/90 FVIII for fingernail injury AST 50
23/11/90 Alpha-fetoprotein normal AST 48

1991
RFLP type 1
NS4 PCR positive
Quantitation (Chiron) : $2.2 \times 10^6$
8/5/91 CD4 0.88

1992
RFLP type 1
NS4 PCR positive
Quantitation (Chiron) : $6.3 \times 10^6$
28/4/92 6 units of alcohol per day AST 55
12/5/92 AST 48
27/5/92 AST 46
12/6/92 AST 70
13/7/92 Ribavirin started AST 55
27/7/92 AST 59
10/8/92 AST 55
8/9/92 AST 55
6/10/92 Mild nausea AST 46
7/11/92 AST 60

1993
RFLP type 1
NS4 PCR positive
Quantitation (Chiron) : $6.8 \times 10^6$
11/1/93 Ribavirin trail ended. AST 61
11/3/93 AST 64
14/9/93 AST 83
1994
RFLP type 1
NS4 PCR positive
Quantitation (Chiron) : $1.6 \times 10^7$
26/1/94 Right sided chest pain for 3 days, now improving. Feels well, diet good
18/10/94 General health good. No recurrence of chest pain AST 80
CD4 1.10
smoke approximately 15 cigarettes per day. One sample for each year from 1982 to 1994 was analysed for patient one, and from 1980 to 1994 for patient two.

7.4 Results

The HCV RNA quantitation for both patients over time shows a similar pattern of gradually increasing viral load (see Figure 7.6). Patient one shows generally higher levels of virus than patient two, but this difference was not significant (Mann-Whitney U-test). Of the three batches of concentrate tested only one of the commercial concentrates gave an HCV RNA quantitation result. This was $1.0 \times 10^4$ genomes/ml, which is a factor of ten lower than most of both patients’ results.

Figures 7.7 and 7.8 show the AST and HCV RNA quantitation results over 12 and 14 years of patients one and two, respectively. Both patients had persistently abnormal ASTs, although patient one had consistently higher AST levels at most time points. Both patients possessed infection with HCV genotype 1 throughout the study period (patients’ samples were genotyped every year from the date of infection to 1994). Both the commercial concentrates were also genotype 1, whereas one NHS concentrate was genotype 3 and the remaining three NHS batches were HCV PCR negative.

The viral load in serum for patient one remained below $5 \times 10^6$ genomes/ml from infection to 1990 with the exception of one sample in 1986, even though his AST levels were greatly elevated for the first three years after infection. After 1990, his viral load has remained consistently above $1 \times 10^7$ genomes/ml, whereas his AST levels have returned to near normal for most of this time.

Patient two had a viral load of $5 \times 10^6$ genomes/ml for the majority of the study period, until 1994 when it rose to above $1.5 \times 10^7$ genomes/ml. Fluctuations in his
Figure 7.6: HCV RNA Quantitation for Patients 1 and 2

- **Patient 1 HIV +ve**
- **Patient 2 HIV -ve**
- **Chiron assay cut-off**

**HCV RNA Equival./ml x 1000**

**Time (years)**

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Figure 7.7: Patient 1 AST Values and HCV RNA Quantitation

HCV Genotype 1

- AST values
- AST upper limit of normal (40 IU/l)
- HCV RNA equs/ml

Time (months)
Figure 7.8: Patient 2 AST Values and HCV RNA Quantitation

AST values:
- HCV Genotype 1

- AST upper limit of normal (40 IU/l)
- HCV RNA equiv/ml
AST levels follow a similar pattern to his viral load, although his AST levels are not significantly elevated during the follow-up period of 14 years.

The CD4 counts for patient one (HIV positive) are shown in Figure 7.9. This shows that a decline in the CD4 count coincides with an increase in HCV viral load. However, Figure 7.10 shows the CD4 counts for patient two (HIV negative), and this demonstrates that the HCV viral load can increase even when not accompanied by a decrease in CD4 levels.

7.4.1 Phylogenetic analysis of the 5'UTR and NS4 sequences

The unrooted and rooted phylogenetic trees of 140 5'UTR sequences for both patients and the concentrates are shown in Figures 7.11 and 7.12, respectively. The rooted tree was constructed with patient one sequences only and by using the concentrate sequence most closely related to patient one’s sequences as the root (this sequence was derived from the commercial batch of concentrate which most likely infected patient one). Both these trees demonstrate that all the sequences segregate together and the genotype 3 sequences (from the NHS concentrate) forming a separate group within the trees.

Figures 7.13 and 7.14 show the unrooted and rooted phylogenetic trees of 125 NS4 sequences for patients one and two, respectively (patient two’s samples from 1980, 1983 and 1985 did not amplify using the NS4 primers) and the concentrates (not including the NHS concentrate as there was not enough sample remaining to allow amplification by PCR). The rooted tree was constructed using the same concentrate sequence as above and patient one sequences only. The unrooted tree shows that the NS4 sequences segregate into three separate groups - one for each patient and one for the concentrate.
Figure 7.9: Patient 1 CD4 Counts and HCV RNA Quantitation
Figure 7.10: Patient 2 CD4 Counts and HCV RNA Quantitation

- **CD4 Absolute x 10**: Solid line with square markers.
- **CD4 Lower Limit of Normal 0.4 x 10^5**: Dotted line with square markers.
- **HCV RNA Equs/ml**: Dotted line with circle markers.

The graph shows the changes in CD4 counts and HCV RNA levels over time (in years). The CD4 counts are indicated by the solid line with square markers, the CD4 lower limit of normal by the dotted line with square markers, and the HCV RNA levels by the dotted line with circle markers. The y-axis represents CD4 absolute counts x 10^9, and the x-axis represents time in years.
Figure 7.11: Unrooted phylogenetic tree of 140 5'UTR sequences for Patients 1 and 2.

Key:
- C: concentrate
- a, b: commercial
- c: NHS
- 1: Patient 1
- 2: Patient 2
- 80-94: Sample year
Figure 7.12: Rooted phylogenetic tree of 5'UTR sequences for Patient 1 and concentrate
Figure 7.13: Unrooted phylogenetic tree of 125 NS4 sequences for Patients 1 and 2
Figure 7.14: Rooted phylogenetic tree of NS4 sequences for Patient 1 and concentrate
Figure 7.15: Unrooted phylogenetic tree of combined 5' UTR and NS4 sequences for Patients 1 and 2
Figure 7.16: Rooted phylogenetic tree of combined 5'UTR and NS4 sequences for Patient 1 and concentrate.
In order to assess whether the 5'UTR sequences affected the segregation of the NS4 sequences, using a spacer of AAAAA, the 5'UTR sequence of clone 1 was artificially joined to the NS4 sequence of clone 1 and the resulting 386 base pair sequences were used to construct an unrooted and a rooted phylogenetic tree (patient one and concentrate only - rooted to the same sequence as above), see Figures 7.15 and 7.16, respectively. The unrooted tree shows a similar pattern to the NS4 only tree (Figure 7.13), that is, the sequences segregate into patient specific groups. The rooted tree shows four of the five clones from 1982 (the year of infection for patient one) segregating in a group away from the concentrate sequence used to root the tree. This tree also shows all 1994 clones segregating in a separate group from all the other patient one sequences.

The NS4 sequences for both patients and the concentrates were translated and an unrooted phylogenetic tree was constructed using these peptide sequences, see Figure 7.17. This tree shows that the concentrate peptide sequences cluster with the patient one sequences more closely than to the NS4 sequences of patient one, and is consistent with the likely source of infection.

The mean genetic distances for the 5'UTR and NS4 clones obtained from the same year are shown in Figures 7.18 and 7.19 for patients one and two, respectively. In patient one, diversity has remained fairly constant over time until year 8 (1989) at which point an inflexion occurred in the NS4 sequences, although the 5'UTR sequences remained relatively homogeneous until 1993/4. At this point 5'UTR sequence variation increased markedly over previous levels. In patient two, the mean genetic distances have been more variable over time and since 1989 have shown an oscillatory pattern in both the 5'UTR and NS4 with the NS4 being more prominent, that is, more divergent.
Figure 7.17: Unrooted phylogenetic tree of NS4 peptide sequences for Patients 1 and 2

Patient 1 and concentrate

Patient 2
Figure 7.18: Mean evolutionary distances for 5 clones at each time point for Patient 1 (HIV positive)

Distance of 1 = Homogenous population of sequences
Figure 7.19: Mean evolutionary distances for 5 clones at each time point for Patient 2 (HIV negative)

Distance of 1 = Homogenous population of sequences
7.5 Discussion

At present there is a lack of data relating sequence evolution over extensive time periods (i.e. > 10 years) and HCV load in HCV positive patients. This study was designed to redress this issue. In the two patients intensively investigated, hepatitis C viral load has relentlessly increased in both patients from the date of infection to 1994. By performing such an analysis in an HIV/HCV co-infected patient and in an age and concentrate usage matched HIV negative patient with HCV infection, we have shown that the influence of HIV results in a consistently higher HCV load at any given time point over a number of years. However, at any given time point there was no significant difference between the total quantity of virus present in each patient. This suggests that total hepatitis C viral loads over time can be as high for HIV negative patients as for HIV positive patients and is host-dependent.

Genotypic analysis and the clinical data has provided an insight into the reinfection of HCV infected individuals. Multiple infection with different HCV genotypes has been documented in haemophilic patients (Devereux et al, 1995; Jarvis et al, 1994) but whether these reflect simultaneous co-infection or sequential re-infection has not been investigated. Patient one was treated with two batches of commercial clotting factor concentrate and five batches of low donor pool NHS clotting factor concentrate before sterilisation procedures were introduced in 1985. Three of the seven batches were PCR positive for the hepatitis C virus, two of these were the commercial concentrates and one was an NHS concentrate. However, patient one only became infected due to treatment with one of the commercial batches. Two reasons could account for this occurrence. Firstly, only the infecting concentrate had a high enough viral load to initiate an infection or secondly, the previous infection protected the individual from re-infection with a new HCV strain. Only one of the commercial batches had a high enough viral load to be quantitated using the Roche assay (cut-off 100 genomes/ml (Young et al, 1993)). Patient one received 1.52 litres
of this concentrate and was, therefore, exposed to approximately 15 million hepatitis C viral genomes. It seems highly likely that he received his HCV infection from this particular batch.

The results of the phylogenetic analysis of the 5'UTR was consistent with data showing a low level of divergence within genotypes (Simmonds et al, 1993a). Thus, the 5'UTR nucleotide sequences from both the patients and the concentrate were clustered together. In contrast, the NS4 phylogenetic analysis of the nucleotide and peptides sequences showed a patient specific clustering with the concentrates also forming a separate group. This suggests that, while the highly conserved 5'UTR population changes little over time in both patients, the NS4 sequences - containing a T-cell and possibly a B-cell epitope - have adapted to the immune environment presented in each individual.

It has been hypothesised that subsequent mutants are more likely to escape the immune system than the originating strain, resulting in a chronic HCV infection (Weiner A. et al, 2nd International Meeting on Hepatitis C and Related Viruses, 1994). The phylogenetic trees of the 5'UTR and NS4 nucleotide and peptide sequences have not identified an identical sequence in the infecting concentrate to the sequences in patient 1. Therefore, the data demonstrates that HCV has quickly mutated away from the infecting strain, in a similar manner to HIV (Nowak M.A. et al, 1995), possibly to escape the immune response.

It has been shown that mutation is considerably reduced in agammaglobulinaemics (Thomas H.C. et al, 2nd International Meeting on Hepatitis C and Related Viruses, 1994). The mean genetic distances and the phylogenetic trees show that HCV mutation has continued in the HIV positive patient even though his CD4 count is now virtually zero. This could be due to other elements of his immune system.
functioning as normal, and new CD4 cells performing their function before being destroyed.

This study has given some insight into the evolution of the hepatitis C virus in two patients over a number of years. Somewhat reminiscent of HIV (Ho et al, 1995; Wei et al, 1995), HCV appears to be a dynamic, host-dependent infection. Hence, it will be important to assess many different patients using viral load and sequence analysis of different regions of the viral genome before correlations between specific HCV strains and their pathogenic potential can be made.
CHAPTER 8

GENERAL DISCUSSION

The hepatitis C virus was first characterised only six years ago (Choo et al, 1989) and much progress has since been made in the development of diagnostic and research techniques used to study the virus. There are currently thought to be hundreds of millions of hepatitis C virus carriers world-wide and haemophilic patients have one of the highest prevalences of the virus (Makris et al, 1990). This is due to the fact that patients with haemophilia have been exposed to thousands of donors when they received unsterilised clotting factor concentrates prior to the introduction of sterilisation procedures in 1985 (Lee et al, 1985). Haemophilia patients have been exposed to multiple hepatitis C virus genotypes, as well as other viruses such as HIV (Hanley et al, 1993; Eyster et al, 1993; Telfer et al, 1994), and this makes them a complicated group to study. However, due to their comprehensive haemophilia care they also have detailed clinical records and an availability of stored sera from 1979 to the present at the Royal Free Hospital. Since no other group of hepatitis C virus infected patients is likely to have such information over a long period of time, this thesis exploited these factors to study hepatitis C virus infection in haemophilic patients in more detail.

Prior to the start of this thesis there was little published work on the relationship between transaminase levels, HCV serological tests and HCV viraemia in haemophilic patients. For example, Garson et al (1990) had investigated these parameters in five haemophilic patients. The aim of my initial studies was to assess the relationships between the above parameters in a larger number of patients with an emphasis on those with normal transaminase levels. In these studies the patterns of hepatitis C viraemia in four groups of haemophilic patients were assessed. The patients were
grouped according to transaminase abnormality and HCV seropositivity, ranging from persistently abnormal transaminase levels and anti-HCV positive to persistently normal transaminase levels and anti-HCV negative.

The results of my investigations demonstrated that there was a significant relationship between transaminase levels and HCV viraemia. Of the four groups with varying transaminase patterns, the patients who had persistently abnormal transaminases were more likely to be HCV PCR positive. Thus, high circulating levels of virus is indicative of high levels of replication and higher levels of cell damage which are manifested as higher transaminase values. These results also indicate that most liver damage is probably due to long term chronic viral replication rather than to immunopathological mechanisms. These conclusions are supported by Hagiwara et al (1993) who found that the replicative level of hepatitis C virus was higher in advanced liver disease and that an elevation of viral replication may play an important role in liver injury and progression of liver disease. Naito et al (1994) also found that most HCV infected patients with persistently normal transaminase levels had inflammatory changes in the portal tracts, with the severity depending on the viral load. Finally, Petrelli et al (1994) found that detectable HCV RNA was associated with liver damage, even in the absence of clinical or biochemical signs of overt liver disease.

The serological tests did not correlate with HCV viraemia as well as the transaminase levels. Indeed the mean ELISA optical densities were similar for patients with normal, intermittently abnormal and persistently abnormal transaminase levels. The level of antibody did not vary much between patients because the viral load necessary to produce an antibody response may be less than that needed to cause sufficient liver damage to raise transaminase levels above normal. If this is the case then ELISA optical densities would not be related to either the level of transaminase abnormality or HCV viraemia. Although ELISA optical densities can provide an answer as to whether HCV antibodies are present or not, they cannot give information on the quantity of
antibody present. This is due to the fact that there is only a limited amount of antigen present on the plate so limiting the amount of antibody that can be bound. Therefore, ELISA optical densities do not appear to be particularly useful for monitoring progression of HCV infection. It would be interesting to perform end-point dilution of sera to attempt to determine whether quantitative antibody levels correlate better with viral load and disease progression.

In contrast to the ELISA results, the overall percentage of positive RIBA II results increased with higher levels of viraemia. This suggests that if there is a high amount of antigen present then antibody production will be increased resulting in an increased probability of a positive RIBA II result. Whilst this conclusion is true overall, not all individual recombinant antigens followed this pattern. A possible explanation for this discrepancy could be HCV sequence variability affecting the sensitivity of the test.

Haemophilic patients do not routinely undergo liver biopsy due to the increased risk of bleeding. Therefore, any information on the amount of liver damage can only be gained from the results of tests which assess transaminase levels and HCV viraemia. Interestingly, in other groups of patients, such as blood donors, a clear relationship between raised transaminase levels and increasing liver damage has not been demonstrated (Hardiman et al, 1993; McMahon et al, 1994). However, it may be possible that patients with repeatedly detectable HCV viraemia and persistently raised transaminase levels will have more advanced liver damage than patients who do not have detectable HCV viraemia and normal transaminase levels, but this cannot be assumed without the data from a liver biopsy.

Six major HCV genotypes have so far been described with genotypes 1 to 3 found in the indigenous UK population (Simmonds et al, 1993). Many studies have investigated the incidence of HCV genotypes in various populations (Ohno et al, 1995; Wu et al, 1994; McOmish et al, 1994; Dusheiko et al, 1994b) and the
relationship between HCV genotype and the response to therapy with interferon
(Matsumoto et al, 1994; Mita et al, 1994; Dusheiko et al, 1994b). However, prior to
the start of this thesis no one had assessed the effect of therapy on the HCV genotype
in any group of infected patients. The experiments described in Chapter 5 assessed the
hepatitis C virus genotypes in haemophilic patients pre- and post-treatment with
interferon and showed that genotypes 2 and 3 responded better to interferon treatment
than genotype 1. In this study seven patients had a complete transaminase response
(transaminase levels returned to normal during treatment with interferon) and six of
these had either genotypes 2, 3 or a mixed infection.

All the patients analysed in Chapter 5 have been exposed to large amounts of
unsterilised clotting factor concentrates and, therefore, would be expected to have
mixed genotype infections. The RFLP analysis used in Chapter 5 did not detect mixed
infections in many of the samples tested. Therefore, RFLP analysis may be insensitive
to lower concentrations of other genotypes if one is present at a much higher
concentration. From my results I would conclude that RFLP analysis gives valuable
information on the dominant genotype but it does not provide information on all
genotypes present in an individual.

To my knowledge the results presented in Chapter 5 were the first to show that
treatment with interferon influences the dominant genotype present in an individual.
One explanation of these results is that during treatment with interferon the dominant
genotype responds, therefore, allowing other strains to repopulate the quasispecies.
This effect could influence the clinical outcome in an individual if the original
dominant strain is replaced by a more virulent strain.

Prior to the start of the study described in Chapter 6 there was no published work on
the effectiveness of each of the four methods of genotyping in haemophilic patients.
Chapter 6 assessed the viral load and the four main methods of detecting hepatitis C
genotypes in haemophilic patients who were receiving treatment with interferon. The results showed that interferon reduced the viral load in four out of the five patients to below detectable levels. Viral load remained below detectable levels in three of these four patients after treatment with interferon was withdrawn.

The four methods of HCV genotyping gave identical results for only a small number of the samples tested but they did show that haemophilic patients are infected with multiple HCV genotypes. Consistent with the results of Chapter 5 the RFLP analysis only detected the dominant genotypes present in the sample. DNA sequencing following PCR amplification is confounded by the same problem unless large numbers of clones are analysed. Serotyping detects past as well as present infections and this may produce a complex genotype pattern in patients who are likely to have mixed genotype infections. Finally, the results indicated that although type specific primers were more sensitive to genotypes present at lower levels they gave the most discordant results with the other methods. Therefore, it was concluded that RFLP analysis is the most suitable technique available at present for studying genotypes in patients who are likely to have mixed infections.

More recent data (Preston et al, 1995) has also found differences between HCV serotype results and HCV genotypes in haemophilic patients. This explanation of these findings was that haemophilic patients have been reinfected with different HCV types which may differ in replication rates and in their capacity to induce HCV antibody formation. These results of Preston et al (1995) were in contrast to the situation in HCV infected blood donors where there was a close agreement between HCV serotypes and HCV genotypes (Preston et al, 1995).

At present, there is a lack of published sequence evolution information and viral load data in hepatitis C virus infected patients over extensive time periods (more than ten years). The final chapter of this thesis has assessed one HIV positive HCV infected
patient and one HIV negative HCV infected patient over 12 and 14 years, respectively. Viral load measurements showed that HCV levels increased in both the patients irrespective of their HIV status over time. This shows that in these two patients the HCV viral load was independent of the CD4 count. However, even though in 1994 their HCV viral loads were very similar, the viral load of the HIV positive patient had reached $10^7$ genomes/ml approximately three years before that of the HIV negative patient. This suggests that there was a higher rate of replication in the HIV positive patient. The HIV positive patient also had consistently higher transaminase levels than the HIV negative patient. Unfortunately, no liver histology was available for either of these patients, but it could be surmised that a high viral load does not necessarily mean that a high level of liver damage is occurring and that host specific factors must play a role in the pathogenesis of HCV infection.

The HIV positive patient received batches of unsterilised clotting factor concentrate containing genotypes 1 and 3. However, genotype analysis over the 12 years of the study showed only evidence of infection with genotype 1. This may be due to the primary infection preventing reinfection or an insufficient viral load present in concentrates following the one that transmitted infection to introduce a superinfection.

The sequence evolution information demonstrated that the 5'UTR changed little over time, whereas the NS4 (containing a T-cell epitope) had adapted over time to the immune environment present in each individual. The sequence data could not identify an infecting sequence in the concentrate in either the 5'UTR or NS4 regions. This indicates that HCV has mutated rapidly in the host following initial infection possibly to escape the immune response. Weiner et al (1995) showed that HCV can escape from the immune response. Using a chimpanzee model CTLs directed against a conserved epitope in NS3 persisted for at least two years after infection. However, these CTLs did not recognise the HCV quasispecies present in the plasma of this animal at week 16 post-infection or at any later time points. Taniguchi et al (1993)
also found that the hypervariable domain of the HCV envelope region appears to be structurally flexible and antigenically variable, providing the virus with a way to escape from host immunity. Thus, HCV is probably similar to HIV which has also been shown to mutate to escape the host’s immune response (Nowak et al., 1995).

This data in Chapter 7 also showed that, despite a CD4 count of zero at the end of the study, the HCV sequences in the HIV positive patient continued to diversify. This may be due to other elements of the immune system functioning as normal (CD8 levels remained normal in both patients over the study period except from November 1988 to March 1989 in the HIV positive patient when his levels were slightly raised above normal) and/or sufficient numbers of CD4 cells performing their function before being destroyed.

This thesis has begun to provide an understanding of hepatitis C infection in haemophilic patients, but much further work is required. Studies are required to assess whether a relationship exists between the extent of liver damage and the patterns of transaminase levels and HCV viraemia. This will allow the level of liver damage to be assessed for haemophilic patients in whom liver biopsies are contraindicated.

The majority of haemophilic patients have mixed genotype infections. There is no ideal method for genotyping mixed infections available at present. Therefore, frequent sampling is necessary, together with viral load measurements, in order to fully understand the HCV quasispecies and the benefits of therapy in haemophilic patients.

HCV is a dynamic, host-dependent infection. Therefore, studies are required to assess many different patients, using viral load and sequence analysis of different regions of the viral genome, before correlations between specific HCV strains and their pathogenic potential can be made. The true nature of the dynamics of HCV infection
may be revealed when better antiviral agents become available as seen with HIV (Wei et al, 1995; Ho et al, 1995).

In conclusion, therefore, this thesis has presented a number of studies carried out on a cohort of HCV infected haemophilic patients. Many of the results presented can be generalised to other groups of patients who have contracted their HCV via other routes of transmission but do not have the clinical data or stored sera available for analysis. It is hoped that the work in this field will continue to provide answers to important clinical questions in the future.
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