UNIVERSITY OF SOUTHAMPTON

Viral Persistence in Hepatitis C Virus Infection

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Correction sheet

This is the final version of the thesis. It contains the corrections suggested by the examiners following the original submission of my thesis within the text of the document.

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ABSTRACT

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Hepatitis C virus (HCV) results in chronic infection in the majority of patients. The reason for viral persistence remains unknown. The aims of the thesis are firstly to study the natural history of HCV in order to establish what proportion of patients fail to clear HCV; secondly, to determine factors that might predict risk of developing chronic infection and rate of disease progression; thirdly, to determine underlying host and viral mechanisms that result in viral persistence.

Three different groups were studied: patients attending the Oxford hepatitis C clinic, patients identified as having been infected by contaminated blood by the Oxford blood centre and antibody deficient patients in the United Kingdom who were infected by a single HCV isolate from contaminated immunoglobulin. Each study group had different strengths: the first group represented a broad cross section of infected patients, in the second group the exact duration of infection was known and the third prospective study provided insight into the role of the immune system in determining disease outcome. In each study group, approximately 85% of patients failed to clear HCV spontaneously. Host factors appeared to be more important than viral factors in determining viral persistence; male sex and older age at infection being associated with viral persistence and worse disease outcome. Antibody deficiency did not increase the risk of developing chronic infection but significantly increased the rate of disease progression in chronically infected patients.

Different immune responses were seen in patients who cleared virus compared to those who developed chronic infection. In patients who cleared the virus, $CD8^+$ T lymphocyte responses to HCV were found to be multi-specific compared to absent or oligospecific responses in patients who failed to eradicate HCV. In addition the T cell responses were phenotypically different with gamma interferon production mirroring cytotoxicity in patients with viral clearance but not in chronically infected patients. Finally, "escape mutation" by which the virus persists as a result of selection of HCV quasispecies containing mutations within immune epitopes does not appear to be the predominant mechanism by which the virus persists.

List of Contents

Chapters

List of Figures and Tables

List of Figures and Tables (continued)

Chapter 4: Outcome of HCV in hypogammaglogammaglobulinaemic patients

Chapter 5: $CD8^+$ lymphocyte responses in acute self limiting HCV

List of Figures and Tables (continued)

Chapter 6: Immune selection and genetic sequence variation in HCV

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Definitions, abbreviations used

ALT	Alanine transaminase
CHC	Chronic hepatitis C
CTL	Cytotoxic T Lymphocyte
EIA	Enzyme immuno-assays
HCV	Hepatitis C Virus
HLA	Human leukocyte antigen
HVR	Hypervariable region
MHC	Major histocompatability complex
PCR	Polymerase chain reaction
RIBA	Recombinant immuno-blot assay

CHAPTER 1: INTRODUCTION
HEPATITIS C VIRUS

Hepatitis C virus (HCV) was first identified in 1989[1] and is now known to be one of the commonest causes of chronic liver disease[2]. The prevalence of the virus is high[3], with an estimated 170 million people infected worldwide and physicians are therefore seeing an increasing number of infected patients both in specialist and non-specialist settings. Hepatologists have seen a change and sharp expansion in their already busy workload due to the escalating referral of HCV infected patients. The virus is not cleared by the majority of patients thus resulting in chronic infection[4-6]. Chronic infection can lead to chronic hepatitis, cirrhosis and eventually hepatocellular carcinoma[7]. Liver failure due to HCV related cirrhosis has now become the commonest indication for liver transplantation in the United States[8] and Europe. It is therefore recognised that HCV infection represents a major health care problem and as a result is the subject of much active research.

Many questions concerning HCV infection remain unanswered. One fundamental question is why some patients appear to clear HCV, whilst in the majority of patients exposure to the virus results in chronic infection. A second question is why the virus causes serious liver disease, morbidity and mortality in some patients but not others. Answers to these questions not only will further our understanding of the virus and the consequences of infection for the patient but will also lead to the discovery of new therapies.

In this thesis the natural history of HCV disease has been studied in different populations in order to establish what factors are involved in determining viral persistence. The thesis then investigates molecular and immunological mechanisms that may contribute to viral persistence. In this introduction, I have discussed the current knowledge of the virology, epidemiology, natural history, and immunology of hepatitis C, on which background the thesis is based. Finally I have reviewed the mechanisms leading to chronicity in other viral infections in order to apply this knowledge to HCV viral persistence.

1.1 VIROLOGY

Background

Before 1975, only two hepatitis viruses were recognised: hepatitis A virus (infectious hepatitis virus) and hepatitis B virus (serum hepatitis virus). Diagnostic tests for hepatitis B were first developed in 1964 and for hepatitis A in 1973. Following the advent of diagnostic testing, serum from patients with non-B hepatitis acquired after transfusion were tested for hepatitis A; none of the cases was found to been caused by hepatitis A[9]. Thus these cases were termed non-A, non-B hepatitis. At that time, non-A, non-B hepatitis accounted for as many as two thirds of transfusion associated hepatitis cases. With the advent of recombinant DNA technology, Hepatitis C virus was first cloned and characterised in 1989[10] and diagnostic tests were subsequently developed[11]. The molecular biological techniques that allowed the identification of HCV were a major breakthrough as until that point the agent responsible for causing hepatitis had not been visualised, grown in culture or immunologically defined. Antibody testing of patients previously labeled as having non-A, non-B hepatitis has revealed that greater than 90% are positive for hepatitis C[12].

Despite the relatively recent identification of HCV much had been learned about the virus from the research into non-A, non-B hepatitis. The disease was transmitted to chimpanzees in 1978, thereby establishing that it was caused by a transmissible agent. By subjecting the innoculum to various treatments before attempting to infect chimpanzees, it was established that non-A, non-B hepatitis virus contained essential lipids (and was therefore enveloped) and had a diameter of approximately 30 to 60nm[13;14].

The identification of HCV has been shown it to be an RNA virus related to viruses of the family *Flaviviridae*, which includes other human pathogens such as dengue fever and yellow fever as well as agents that cause disease in animals such as hog cholera virus and bovine viral diarrhoea virus. HCV has now been specified in a separate genus, known as *Hepacivirus*, in this family *Flaviviridae*[15]. The other genera within the *Flaviviridae* family are the *Pestivirus* and the *Flavivirus*, whose viruses share some sequence homology with HCV[16]. The recently described hepatitis G virus[17] is also related, although evidence now suggests that this virus is not a hepatitis virus.

The viral genome

The genome of HCV is a single stranded linear RNA of positive sense and is approximately 10,000 nucleotides in length[18]. It has a single open reading frame that codes for both structural and non-structural proteins with an untranslated region at either end. At the 5' end of the HCV genome, the untranslated region is between 329-341 base pairs in length and forms a stem loop structure that appears to function as an internal ribosomal entry site[19]. Its nucleotide sequence is highly conserved with a 92% homology among different HCV types, therefore making it an ideal target for diagnostic testing of viral nucleic acid by polymerase chain reaction. The 3' untranslated region is shorter and is also highly conserved, although its function is not certain[20].

Immediately downstream of the 5' non-coding region is a single large open reading frame of 9379-9481 nucleotides, which encodes for a large polyprotein precursor of approximately 3000 amino acids. The polyprotein is cotranslationally or posttranslationally cleaved to make up to 10 polypeptides by a combination of host and viral proteases. The first proteins encoded for are the structural proteins, followed by the non-structural proteins.

The first structural protein is a nuclear capsid core protein, which is relatively well conserved amongst HCV isolates. Two envelope proteins follow this, designated E1 and E2, which coat the virus and a small putative protein of undermined function (P7). The E1 and E2 proteins appear to form heterodimers on the surface of the virus.

At least six non-structural proteins (designated NS 2 - 5) are positioned in the 3' portion of the open reading frame. These non-structural proteins seem to be critical for viral replication and include protease, helicase, and RNA polymerase enzymes and regulatory peptides. The functions of the protein encoded by the NS2 region remain unclear. The NS3 region encodes a viral helicase and a protease. NS4 functions as a serine protease co factor involved in the cleavage between NS4B and NS5A. It also facilitates the protease activity of the amino terminal quarter of the product of gene NS3 and, in addition, regulates the phosphorylation of the product of gene 5A[21;22]. NS5A is a serine phosphoprotein whose function has not yet been determined, while NS5B has both RNA dependent RNA polymerase and terminal transferase activity.

Viral Heterogeneity

A striking feature of the HCV genome is its sequence heterogeneity and in this respect, it resembles the human immunodeficiency virus. At the protein level the heterogeneity of HCV has previously been shown not to be uniform across the genome but differs between the regions. The most conserved regions of the genome are the 5' and 3' untranslated region. The most conserved regions of the open reading frame are the putative core and NS3 regions and antigens from these regions are used in anti-HCV antibody assays.

In contrast, the most heterogeneous portions of the genome are the genes encoding the envelope proteins. The 5' end of the E2 gene is the most heterogeneous region of all and has been named the first hypervariable region (HVR1)[23;24] and seems to mutate rapidly. The HVR1 consists of approximately 90 nucleotides (30 aminoacids). A few strains have a second hypervariable region (HVR2) just 3' of the first hypervariable region; hypervariable region 2 appears to be limited to strains of genotype 1 B[24].

These observations are further evaluated in this thesis by studying the relationship between genetic variation with the host immune response (see Chapter 6).

Genotypes

In view of the considerable variation between different HCV isolates, attempts have been made to classify the HCV into different genotypes. Typing can be performed in several different ways either serologically with specific peptide ELISA (serotyping), or by analysis of PCR products by direct sequencing, use of type specific primers, restriction fragment length polymorphisms, or with sequence specific DNA probes (genotyping). These genotypes may also be sub divided into sub types. Genotypes and subtypes species are defined according to the percentage nucleotide diversity. Most patients are infected with a single genotype. However in addition, nucleotide variability may be present in viruses circulating within an individual, referred to as quasispecies. These quasispecies will be of the same genotype but will have nucleotide variation between their genomes.

Evolutionary phylogenectic analysis of nucleotide sequences have shown that isolates of HCV cluster into six broad genotypes, whereas sub-types represent clusters with similar sequences within each genotype[25]. Genotypes have been of considerable epidemiological interest and their distribution varies considerably in different parts of the

world. The clinical significance of genotypes remains to be determined. Some genotypes such as 1a, 1b, and 4 seen to be less responsive to interferon therapy[26], but no clear association with disease outcome or severity have been demonstrated [27]despite initial suggestions that infection with genotype 1b is more likely to lead to cirrhosis or hepatocellular carcinoma[28]. There is now evidence that genotype 1 has been present for a longer time in the population and therefore these initial observations may have been subject to lead time bias with duration of infection not genotype being the reason for the increased incidence of cirrhosis.

1.2 EPIDEMIOLOGY

Infection with HCV occurs worldwide, with the prevalence in most developed countries ranging between 0.1 and 2%[29;30]. Significantly higher rates of infection are seen in Eastern Europe and Africa[31], with Egypt having the highest prevalence rate reaching approximately 15% of the general population[32]. Studies have looked at the prevalence of anti-HCV in the blood donating population. The prevalence in blood donors is 0.5% in United States[33] and 0.1% in the United Kingdom[29]. However this prevalence probably does not reflect the prevalence in the general population as donors with risk factors for hepatitis are advised not to give blood. Therefore the prevalence in the general population would be expected to be higher than the blood donor group. Indeed in the *Third and National Health and Nutrition Survey* conducted between 1988 to 1994 in United States, 1.8% of a representative sample of the civilian non-institutionalised population tested positive for anti-HCV[34]. This latter data has allowed the Centers for Disease Control to estimate that nearly four million people are infected with HCV in the USA. They have estimated that approximately 10,000 deaths occur in the US from HCV and that the number of deaths from HCV are expected to triple over the next two decades, eventually becoming responsible for greater mortality than AIDS.

The main route for transmission of the virus appears to be via blood, with intravenous drug use and blood transfusion being the best recognised paths of infection. Since the advent of screening tests, HCV has been shown to be widespread in the blood doning population and HCV has been shown to account for 90% of previous transfusion transmitted hepatitis[35]. Patients with haemophilia who were heavily transfused and treated with pooled factor VIII concentrates have a prevalence rate of anti-HCV exceeding 90%[36].

Since 1991 all blood donations in the UK are now screened for antibodies to hepatitis C and therefore the risk of blood transfusion passing on virus has fallen significantly. In addition, the viral inactivation of clotting factor concentrates has virtually eliminated these blood products as the source of HCV infection. It is now estimated that only 1 in 103,000 of blood units will transmit the virus[37]. The reason for continuing transmission is that that donors are only tested for antibodies to HCV, which take 1 to 2 months to develop and therefore there is still a window where the donor if acutely infected with HCV, will be infective but not be identified by antibody testing.

Introduction of PCR testing for HCV RNA would probably reduce the risk of transfusion related HCV further.

The commonest route of transmission now seen is intravenous drug use[38]. This had been recognised in the earlier studies of non-A, non-B hepatitis where case control studies of patients had shown a strong association between acquiring disease and a history of injection drug use in the last 6 months[39]. Approximately 50% of new cases of hepatitis C are now a result from injecting illegal drugs. HCV infection has been shown to be acquired rapidly so that within 6 to 12 months up to 80% of users are infected[39]. Interesting work from the United States has shown however that there is a decline in the number of acute cases of HCV among drug users in the last five years. The reason for this decline is unknown but may be related to HIV awareness and needle/syringe exchange. Coupled with the fall of HCV in transfusion recipients, this has resulted in a fall of new cases of HCV reported in the United States with an estimated peak of in the mid-1980's of 180,000 cases per year to 28,000 in 1995[38]. In the United Kingdom, similar prevalence rates of HCV amongst drug users are found.

Healthcare Workers are at slightly increased risk of HCV infection compared with the general population. Studies have reported an average anti-HCV rate of 1% among hospital based health care workers and in one study a history of accidental needle stick exposure was independently associated with anti-HCV positivity[40]. A recent review of follow-up studies of health care workers who sustained percutaneous exposures to blood from anti-HCV positive patients found that the incidence of anti-HCV seroconversion averaged 1.8% (range 0 - 7)[41]. There has been one case of the transmission of HCV from blood splash to the conjunctiva[42]. There have been two case reports of the virus having been spread from surgeons to patient. However at present as the risk of transmission appears to be low, there is no policy with regards HCV infected health care professionals working or performing exposure- prone procedures.

Sexual transmission is uncommon. The consensus view is that the overall risk in the order of 5%. It can be difficult to ascertain whether the virus has been passed on sexually as partners may share in other high risk activities such as intravenous drug use. In a study of the sexual partners of 85 anti-HCV asymptomatic blood donors 9 (11%) were anti-HCV positive[43]. However all of these nine had independent parenteral risk factors for acquiring HCV. Large studies of the partners of HCV infected haemophiliacs has shown that the majority are not infected[44;45]. Sequence analysis of the virus can help

to determine whether the same isolate of the virus has been passed from one patient to another. The consensus view is not to recommend a change in sexual practice among stable monogamous couples, although partners of anti-HCV positive individuals should be tested at least once for evidence of HCV infection. Couples should be counselled that there is an increased risk of transmission for sexual intercourse during menstruation. HCV patients not in a stable relationship are recommended to use barrier contraception not only to reduce the risk of HCV infection but also the risk of being infected with other sexually transmissible diseases.

Vertical transmission is also uncommon with an incidence of less than 5%[46 47]in early series. A more recent study from Italy found a 6 % transmission rate. This risk is much greater if the mother is co infected with HIV[46;47]. There is no evidence to advise HCV infected mothers against breast feeding[48].

It seems that HCV may also have spread iatrogenically through the use of non-disposable needles and syringes and the practice of traditional healing techniques involving puncture of the skin. For example, in Egypt HCV may have been spread up contaminated needles used to administer treatment for schistosomiasis, which is prevalent in the area.

Finally there appears to be still a large proportion of patients in whom we can identify no risk factors. This group ranges from 1-30% in different series. The difference may be related to the inability to elicit a history of past drug use from the patients. Other possible but unproven routes of transmission are other percutaneous exposures such as tattooing, acupuncture, non-professional ear piercing and body piercing. Further work is needed to establish these and other possible routes of transmission in this group of patients.

1.3 Natural History of Hepatitis C Virus Infection

Acute Infection

Acute HCV infection is usually mild and often goes unnoticed, making prospective follow up of patients difficult. The majority of patients are asymptomatic with less than 25% of patients developing jaundice. Where prospective data is available, patient follow up shows that most fail to clear the virus, with greater than 80% of individuals developing chronic infection[49].

Further evidence that only a minority of patients clear HCV comes from cross sectional studies of HCV patients, comparing HCV antibody and HCV-RNA results. There are no antigen tests for HCV and therefore reverse transcriptase polymerase chain reaction (RT-PCR) detection of viral RNA is used to establish whether the virus is circulating in the blood. The test can pick up the virus down to a sensitivity of 100 copies /ml of blood. Approximately 20% of patients with antibodies against HCV have persistently undetectable HCV RNA in their serum[50].

A positive antibody test in the presence of a negative PCR test for HCV-RNA can be interpreted in a number of ways. One explanation is that the antibody result is a false positive, but this is unlikely with the advent of newer generation ELISA and confirmatory tests with RIBA, which give >99% sensitivity and specificity[51;52]. A second explanation is that the virus is present in blood but below the level of the detection of the PCR test. A third is that the virus may be not be present in the blood but continues to exist in the liver or other sites. The final explanation is that the positive antibody test indicates previous exposure to HCV but the inability to detect HCV-RNA indicates that these patients have cleared the virus.

Follow up of antibody positive and HCV-RNA negative patients have shown that their risk of developing serious liver disease is significantly lower than patients with detectable HCV-RNA. Thus in terms of clinical evaluation, whether these patients have truly cleared HCV or not is perhaps academic. For practical purposes these patients can be considered "healed".

This cross sectional analysis looking at antibody positive, HCV-RNA negative individuals, may underestimate the proportion of patients who have cleared virus as there may be patients who have been infected, cleared the virus but either have never developed an antibody response or have developed and then lost their antibody response

by the time they are tested. These patients cannot be identified on a cross-sectional basis, as they have no HCV antibodies to indicate past infection. One possible way to identify such patients is to look for alternative markers of infection in high risk populations, such as proliferative or cytotoxic T cell responses to HCV rather than antibody responses.

Chronic infection

The natural history of chronic infection remains ill defined. In the absence of prospective cohort studies, it is unclear whether chronic HCV infection will inevitably sequentially progress through advancing histological stages of chronic hepatitis, cirrhosis and culminate in hepatocellular carcinoma. If disease progression is inevitable the differing spectrum of disease seen in cross sectional studies implies that the rate of progression must be variable. It is important to identify those factors that influence this rate of progression. An alternative hypothesis is that only a proportion of infected persons will develop progressive disease and that attention should therefore focus on establishing as early as possible who is likely to show advancing disease and on attempting to define factors that may be responsible for such progression. These contradictory views can be resolved only by conducting appropriate long term studies of the natural history of disease.

There are a number of problems when designing studies to look at the natural history of HCV. The first, as discussed above, is to establish the time of the acute infection. Without establishing the timing of the acute infection, one is unable to measure the duration of infection nor can one study the disease prospectively. The second major problem is that the disease progresses slowly and decades of follow-up are required to judge the true effect of HCV on morbidity and mortality. The third problem is to assemble a properly managed control group of individuals without HCV and follow them for the same period of time. The final problem in the 1990's, is the impact of treatment, which will modify the natural history.

There are a number of different methods that have been employed for looking at the natural history of HCV. One method is to perform retrospective studies in patients with already established chronic liver disease, subsequently tested as HCV positive. The majority of the literature is based on such studies. This approach excludes subjects - possibly the bulk of cases - that do not reach clinical awareness. This will tend to bias the outcome data in the direction of the more severe liver disease. A second and more ideal

method of studying the natural history of chronic HCV requires screening at risk groups, such as transfusion recipients, prospectively in order to establish the onset of acute infection and then following them up for at least two to three decades. An alternative method to the above is to perform a retrospective analysis of a prospective study. This is possible if a common source of the HCV infection can be traced (for example blood or immunoglobulin transfusion) and then the outcome of the patients in the present day evaluated. Ideally the onset of infection should be in the distant past and serum samples have been saved from the outbreak, which can be tested for HCV. Not surprisingly this is a rare event.

The following text reviews the current literature on the natural history of HCV, categorizing the studies as defined above. Much of the literature is based on studying the natural history of disease in patients previously labelled as having non-A, non-B hepatitis, the vast majority of whom are now known to be infected with HCV.

1. Patients with established chronic liver disease

There are a number of studies of the natural history of HCV in infected individuals with known chronic hepatitis[35;53-55]. These have shown that cirrhosis developed in between 8% to 46% during a mean follow up period of 4 to 11 years. Hepatocellular carcinoma (HCC) was found in 11% to 19% during the same follow up period. Liver related mortality was increased in one study[35] and not reported in the others. However as HCC was common in the two studies from Japan[53;54], liver mortality rates must also be presumed to have been high in these patients. This high rate of serious liver disease was also found by Tong et al from the United States[35]. The study looked at 131 HCV positive patients referred to their tertiary care centre. The patients represent a selected group as they were referred because they all had evidence of liver disease (abnormal serum liver function tests, established chronic liver disease, or the presence of a liver mass). Liver biopsies were performed in 101 of the patients. The mean age of the patients at transfusion was 35 years and at the time of initial outpatient evaluation, 57 years. At the initial assessment, 67.2% complained of fatigue, 67.9% had hepatomegaly, and on liver biopsy, 20.6% had chronic hepatitis, 22.9% had chronic active hepatitis, 51.1 % had cirrhosis, and 5.3% had HCC. During a follow-up period that averaged 3.9 years, an additional 5.3% developed HCC and 15.3% died from liver disease.

This study by Tong also provided useful information on the rate of progression of the disease once chronic hepatitis is established. In the retrospective analysis, the investigators estimated that after transfusion, chronic hepatitis was identified 13.7 ± 10.9 years later; chronic active hepatitis 18.4 ± 11.2 years later; cirrhosis, 20.6 ± 10.1 years later; and HCC, 28.3 ± 11.5 years later. Similar data was reported by Kiyosawa et al in Japan who studied 231 patients with chronic non-A, non-B hepatitis[56]. Based on the blood transfusions recipients in their study, where the time of infection was known, the mean intervals between transfusion and detection of chronic hepatitis was 10 years, 21.2 years for cirrhosis and 29 years for HCC. In four patients, the intervals exceeded 40 years, and in three even 50 years.

These studies show that follow up of patients with already established chronic hepatitis C will reveal a high rate of progression to cirrhosis and HCC. However these studies also show that the rate of progression was extremely slow with serious liver disease beginning to evolve mainly between two and three decades after initial infection. However, all of the studies furnished numerator but not denominator information. Patients with sub-clinical, asymptomatic disease would not have been identified for these studies, because the studies focused on patients with already identified end stage or near end stage liver disease. It is now known that less than 25% of patients will develop symptoms with acute HCV infection. Indeed the screening of at risk populations and the blood doning populations has revealed a significant proportion of asymptomatic carriers of the virus. Therefore one must be careful in extrapolating these results to the entire HCV infected population.

Nonetheless, these studies do show that serious liver disease develops in a proportion of persons with chronic HCV infection. This is clearly apparent to those who work in the field of liver transplantation, where hepatitis C is reported to be the most common basis for liver transplantation. However again the denominator remains unknown; what proportion of all HCV infected persons will develop end stage liver disease remains to be established. The majority of infected individuals may for example remain healthy with undetected HCV infection and therefore never require transplantation.

2. Prospective studies

There are number of studies from the United States and Europe which have followed up patients with transfusion associated non-A, non-B hepatitis[12;57-60]. These studies have the advantage that they begin with apparent onset of acute disease. However they were not designed with the express intent of undertaking long time follow up evaluations and hence the follow up was relatively short with a mean duration ranging from 8 to 14 years. Another fault with these studies was that they lacked control groups. However there is consistency between the results of the studies.

Clinical symptoms were noted in approximately 10% of the study subjects. However these symptoms were not all defined and varied between patients. The results of liver biopsy showed cirrhosis in 8 to 24%. In two of the studies, HCC was detected in 0.7% and 1.3% of them, respectively. Mortality from liver disease reported to range from 1.6% - 6.0%. Thus these limited studies show that there is an unequivocal but relatively modest frequency of morbidity and mortality in the first decade of infection.

3. Retrospective - Prospective studies

In order to define the onset of infection, these studies must have a clearly defined outbreak and ideally involve a large number of individuals. Thus far, only two situations have met these requirements, namely that of exposure to HCV contaminated lots of immunoglobulins and exposure to blood transfusions in specifically designed monitored studies, both occurring approximately 20 years earlier.

The best study of immunoglobulin transmitted HCV comes from Ireland[61]. They identified 417 of 53,178 recipients of anti-D immunoglobulins in the early 1970's to be anti-HCV positive. At the time of the report, 232 had been assessed 17 years after inoculation. Their mean age at that time was 44.9 years. Mild fatigue was reported by 26.5%. Physical examination demonstrated that none had signs of liver disease. ALT values were abnormal in 72.4%. Liver biopsy revealed mild chronic hepatitis in 55%, mild to moderate disease in 38.1% and severe hepatitis in 6.8%. Only 2.4 % cent of the biopsies showed cirrhosis. The study thus shows only a small proportion of serious liver disease after 17 years in this cohort of healthy women infected at a young age.

A similar study has also been reported from Germany[62]. One hundred and sixty women out of 2,533 women who had received anti-D immunoglobulin between 1978 and 1979 were found to be anti-HCV positive. Eighty-six of these 160 patients had evidence

of chronic hepatitis whilst the others appeared to have recovered. The study did not however report on the clinical outcome of the patients and follow up is still relatively short. However the study highlights the slowly progressive nature of HCV infection.

The largest studies of blood transfusion transmitted hepatitis have come from the United States. Between 1968 and 1980, there were five separate studies exploring the incidence of transfusion associated hepatitis[63-67]. Many of the patients had undergone transfusion at the time of cardiac surgery. The criteria to define hepatitis was similar in all these studies and involved screening of blood recipients with serum liver function tests at frequent intervals to detect biochemical evidence of hepatitis. Thus it is probable that all cases whether symptomatic or asymptomatic would have been detected. Indeed, more than two thirds of patients were totally asymptomatic during the acute episode. Hepatitis developed in 8% to 18% of recipients in the various studies, 90% of which could not be attributed to either hepatitis A or B or any other cause and was therefore labelled non-A, non-B hepatitis at the time. Fifteen years later the original investigators decided to conduct a long term follow-up study to include all cases from each of the 5 studies with an original diagnosis of non-A, non-B hepatitis (568 cases), as well as a carefully matched transfusion control group (984 controls) from the same studies who had not developed hepatitis[68]. The aim of their study was to establish whether there was any difference in overall or liver related mortality or morbidity between the groups.

Their data revealed that there was no difference in all cause mortality after an average follow-up period of 18 years (51% cases versus 51% controls). The survival curves of the cases and controls overlapped almost completely. Most deaths in both groups had occurred by five to six years after transfusion, suggesting that they might have been the consequence of the original disease for which the transfusions had been administered.

Liver related mortality was infrequent, as derived from death certificate analysis, but was significantly higher at 3.2% among the cases compared with 1.5% of controls (P=0.033). The majority (71%) of the patients who had died of liver disease whether cases or controls, were identified to be heavy drinkers. Stored samples from the original studies were screened for anti-HCV using the second generation enzyme immunoassay and then mortality was compared between the HCV positive cases and the controls but again life table analysis for mortality continued to show no difference (51% versus 54%).

Among the 287 cases and 492 controls who were alive at the time of tracing, 205 cases (71%) and 335 controls (68%), respectively, could be traced, interviewed, and bled. In addition, a subgroup of 146 person's among the 205 living cases had serum available both from the time of the initial infection and the time of follow-up evaluation, permitting paired analysis of biochemical and serological indices. Of the 205 living cases, approximately one third were symptomatic with abnormal biochemistry; one third were asymptomatic but had biochemical evidence of chronic hepatitis; one third were asymptomatic and had HCV viral markers without biochemical abnormalities. Approximately a third of the living cases have been assessed with liver biopsy of which one third showed histological cirrhosis (10% of the total living cases). Patients were much more likely to be symptomatic with abnormal physical signs if they had cirrhosis on their liver biopsy as opposed to just chronic hepatitis (70% versus 5%). Therefore after 18 to 20 years there is evidence of increased liver related morbidity and mortality although at this time point the increase is small. However it is probable that those patients with cirrhosis will be expected to have an increased morbidity and mortality over time. In addition the patients with biopsy changes of chronic hepatitis may progress to cirrhosis although this is unknown and will only be answered by further follow up.

This non-concurrent prospective study has many desirable features. Firstly that the patients were identified by repeat biochemical monitoring in the acute phase and thus would have identified the majority of new cases. Secondly, the database is large. Thirdly, it was able to pick a matched control group. Fourthly, the patients were followed up by the same investigators and there were stored samples that could be re-tested. Finally, at the time of this study no treatment was available and therefore no intervention took place.

The study however has a number of problems. Firstly, the study defined chronic hepatitis as two or more raised ALT levels. However it is now well established that histological evidence of hepatitis can be seen in the presence of normal ALT. Liver biopsies were only recommended to patients with an abnormal ALT value and indeed only a proportion of patients with abnormal ALT values were biopsied. The second and most important problem with the study is that the patients are relatively old, meaning that many of them have died of other causes. Many of the patients had severe concomitant illness such as heart disease and indeed the mortality is very high at 50%. The study therefore does not tell us about young patients who are infected with HCV. In addition

this study only relates to HCV transmitted by blood transfusion which may not reflect the course of HCV transmitted by other routes. Finally and probably the main drawback of the study is that the follow up is still too short at 20 years.

Thus we can see from all of the above literature that HCV infection is associated with significant liver disease, morbidity and mortality. However it still remains unclear what proportion of patients will develop the serious complications of infection and over what time period. As the natural history of HCV is extremely long follow-up for 50 years is probably required to get an accurate assessment of the effects of hepatitis C on mortality. Such long follow-up is difficult to achieve and therefore a final and helpful method of studying the natural history is to look at surrogate markers of disease progression rather than mortality. As HCV causes disease through the development of cirrhosis, liver fibrosis is probably the best surrogate marker to assess disease progression. The best study to date looking at the natural history of liver fibrosis progression in patients with chronic HCV comes from France[27]. This collaborative French study recruited 2225 patients from across France, who had been involved in earlier studies of HCV. All the patients had a liver biopsy sample compatible with chronic hepatitis C. The biopsies were scored for the grade of disease activity (i.e. inflammation) and for the stage of fibrosis. None of the patients had had any treatment prior to the biopsy sample. The authors assessed nine factors on fibrosis progression: age at biopsy, estimated duration of infection, sex, age at infection, alcohol consumption, HCV genotype, HCV viraemia, cause of infection, and histological activity grade. They defined fibrosis progression per year as the ratio between fibrosis stage in Metavir units (scored from 1 to 4, where 4 represents cirrhosis)[66] and the duration of infection.

They found that the median rate of fibrosis progression per year was 0.133 fibrosis units (95% CL 0.125 - 0.143), which was similar to the estimates from previous studies (0.146- 0.154). Three independent factors were associated with an increased rate of fibrosis progression: age at infection older than 40 years, daily alcohol consumption of 50 grams or more, and male sex. There was no association between fibrosis progression and HCV genotype. The median estimated duration of infection for progression to cirrhosis was 30 years (28 - 32), ranging from 13 years in man infected after the age of 40 to 42 years in women who did not drink alcohol and were infected before the age of 40. Without treatment, 377 (33%) patients had an expected median time to cirrhosis of less than 20 years, and 356 (31%) will never progress to cirrhosis or will not progress for

at least 50 years. Their conclusions were that host factors of ageing, alcohol consumption, and male sex have a stronger association with fibrosis progression than virological factors in HCV infection.

The main limitation of their study was the fact that it was a cross sectional observational study aimed at estimating longitudinal parameters. The second problem with the study was that duration of infection had to be estimated in the majority of patients. This is estimated by using the day of the first transfusion or of the first intravenous drug use. Duration of infection was known in only 52% of the overall population. However when the estimated rates of progression were compared with the rate of progression in patients in whom the duration of infection was known the results were very similar. In addition the estimates of the rate of progression were also similar to those calculated in the seventy patients who had two biopsies at different time points.

The rate of fibrosis progression was not normally distributed with an asymmetrical distribution. This finding suggests the presence of at least three populations: rapid fibrosers, intermediate fibrosers, and slow fibrosers. Therefore the expression of a mean rate of fibrosis progression per year and of a mean expected time to cirrhosis does not mean that the progression to cirrhosis is universal and inevitable. These findings could explain conflicting findings on the incidence of cirrhosis observed in different cohorts such as the low instance of cirrhosis observed in the Irish anti-D study, which included women infected before the age of 40 years.

This thesis studies the natural history of HCV infection in the Oxford population. The thesis studies factors that might determine viral persistence or clearance as well as factors related to progression of liver disease, as measured by fibrosis. The methods of studying the natural history of HCV as outlined above have been employed. Firstly in Chapter 2, a cross sectional study of the outcome of HCV in our entire clinic population is reported. Our patients are derived not only from patients presenting with liver disease or abnormal liver function tests but also from asymptomatic individuals identified as a result of testing of high risk groups and blood donors. Therefore unlike many of the above studies that have looked at patients presenting with liver disease, this study group represents a broad based cross section of HCV infected patients.

In Chapter 3, a retrospective prospective follow-up of blood transfusion recipients is reported. These patients have been identified by tracing all the recipients of

blood donors who have been identified as being anti-HCV positive, after the introduction of blood donor screening. A very thorough review of the blood transfusion records, which have been on a computer database, and review of the patient's case records has insured that no cases have been missed. The advantage of this study is that the timing of the onset of infection is known.

Finally in Chapter 4, a prospective follow-up study of HCV infection following the outbreak of HCV in antibody deficient patients following transfusion with contaminated immunoglobulin is reported. This study not only enables prospective follow up of all the patients exposed to this single HCV isolate but also gives insight into the relationship of the host immune system with HCV infection.

1.4 HEPATITIS C VIRUS AND THE HOST IMMUNE RESPONSE

A better understanding of the interaction between the host immune response and HCV will help elucidate why some patients appear to clear the virus whilst the majority do not. In addition it may help to explain why a proportion of chronically infected patients progress to serious liver disease whilst others do not. Histological examination of liver biopsies from patients with chronic HCV infection reveals a spectrum of disease. Inflammation is usually present but can range from minimal changes to severe portal and lobular changes with interface hepatitis. The inflammatory infiltrate includes T and B lymphocytes and neutrophils. From our knowledge of other viral infections, T lymphocyte responses are likely to be important in viral clearance. However T lymphocyte responses to viruses may have two opposing effects. On one hand, they may be critical for protection, either directly through $CD8^+$ T killer cells or indirectly through $CD4^+$ T cells, which help B lymphocytes to produce neutralising antibody and can support priming of $CD8^+$ T cells specific for virus infected cells. On the other hand, T cells may be harmful, mainly through $CD8^+$ T cells, which damage infected tissues in an attempt to clear the virus. In chronic HCV infection, it is unclear to what extent the immune response to HCV is protective in trying to limit HCV replication, and to what extent the immune response is the cause of the progressive liver damage.

A number of observations provide circumstantial evidence that cell mediated immune responses limit virus replication and contribute to immunopathogenic mechanisms of disease. Firstly, high levels of HCV replication occur in the absence of obvious liver damage during the first few days to weeks after infection[69;70]. Morphological studies indicate that $CD8^+$ T lymphocytes are in the immediate proximity of infected hepatocytes and increased ALT activity correlates with the number of intrahepatic $CD8^+$ T cells[71]. Recent descriptions of HCV specific CTL in the liver and peripheral blood of humans[72-78] and chimpanzees[79] provide further evidence for involvement of host immune responses in the pathogenic process. On the other hand accelerated liver disease seen in the immunocompromised patient, including liver transplant patients[80], patients coinfected with HIV[81;82] and patients with primary immune deficiency[83] indicate the importance of the host immune response in controlling disease.

Previous work on the interaction between HCV and the immune system can be divided into studies of $CD4^+$ T cell responses and $CD8^+$ T cell responses. One further

line of evidence that the immune response is important in determining the clinical outcome of chronic HCV infection comes from studies of the association between clinical course and HLA type. These studies are discussed below.

MHC Associations

The human leukocyte antigen is a crucial genetic factor that initiates or regulates immune response by presenting foreign or self-antigens to T lymphocytes. $CD4^+$ and $CD8^+$ T cells recognise antigens as peptides bound to the major histocompatibility complex class II and class I molecules, respectively, on the surface of an antigen presenting cell (APC). Generally, peptides generated by proteolytic degradation of antigens such as viral particles or viral proteins internalised by APC bind to MHC class II molecules, whereas peptides generated by proteolytic degradation of antigens that are synthesised de novo within APC, as is the case in virus infected cells bind to MHC class I molecules.

HLA polymorphisms have been studied to see whether any are associated with the onset or progression of liver disease in chronic HCV. Different studies have compared HLA types between anti-HCV positive patients and matched healthy controls; between anti-HCV positive, HCV-RNA positive patients and anti-HCV, HCV-RNA negative patients; and between chronically infected patients with persistently normal biochemical liver function tests and abnormal liver function tests.

In one study HLA class I and class II alleles were studied in 130 HCV infected patients along with matched healthy controls[84]. Of the infected patients, thirty-three had persistently normal ALT values and 97 had abnormal ALT with evidence of chronic liver disease. The frequencies of DRB1*0405 and DQB1*0401 were higher in HCV infected patients than in unaffected controls. Among HCV infected patients, the frequencies of B54, DRB1*0405 and DQB1*0401 were significantly higher in patients with abnormal liver function tests than those with persistently normal ALT values, whereas DRB1*1302, DRB1*1101 and DQB1*0604 were more frequently found in carriers with persistently normal ALT values. They showed that extended haplotypes including class I B54 were closely associated with the progression of liver injury whereas extended haplotypes including class II DRB1*1302 -DQB1*0604 were associated with low hepatitis activity in chronic HCV. However other studies have found different associations with one showing a decrease in the frequency of DQB1*05 and another

DQB1* 0302 and DQA1*03 in patients with chronic HCV, compared to healthy controls.

Interesting work, looking at class II genotypes in patients who are antibody positive for HCV but persistently HCV RNA negative was conducted at King's College Hospital[85]. They studied 36 patients who had persistently HCV RNA negative results by PCR and 134 matched healthy controls. They found that DQB1*05 and DQB1*0301 were found at significantly higher frequencies in patients compared with controls and DRB1*1501 and DQB1*0602 were significantly decreased. The increased prevalence in HCV RNA negative patients of the 2 alleles (DQB1*05 and DQB1*0301) has previously been shown to be present at a reduced frequency in patients with persistent HCV infection[86]. This suggests that these alleles may be associated with more efficient viral clearance. In contrast, the novel negative association with the other two alleles may suggest that these patients have poor viral clearance and are more susceptible to chronic viral infection. These findings support the hypothesis that an HLA class II restricted immune response is important in determining the outcome of HCV infection.

$CD4^+$ Responses

Preliminary work in acute infection has suggested that a vigorous qualitative response by HCV specific $CD4^+$ T cells is associated with viral clearance in the minority of patients who eliminate the virus[87]. $CD8^+$ T cell responses in these individuals have not yet been studied. In the majority of patients who develop chronic infection $CD4^+$ T cell responses appears to be weak.

The first study of $CD4^+$ responses to hepatitis C virus in different clinical courses of infection was published in Gastroenterology in 1993[88]. The proliferative responses of peripheral blood mononuclear cells were assessed using six recombinant proteins of HCV. The responses were compared between 41 patients with chronic HCV, 11 patients whose chronic hepatitis successfully treated with interferon alfa and 11 healthy HCV individuals. The definition of healthy carriers were patients who were asymptomatic with normal liver function tests. They found that 65% of HCV antibody positive individuals had $CD4^+$ T cell responses to viral proteins. All viral proteins were immunogenic for T cells with NS4 being the most immunogenic. There was a significant correlation between the presence of $CD4^+$ T cell responses to core and a benign course of infection in healthy sero-positives. However, the study definition of healthy controls is questionable as 6 of 9

of the individuals tested were actually viraemic by PCR testing (2 were not tested). In addition only three of these "healthy" patients were biopsied; it is now well recognised that histological disease can be seen in the presence of normal liver function tests. Nonetheless they concluded that $CD4^+$ T cell responses to core although not coinciding with viral clearance, is associated with a more benign course of infection and may be required to maintain humoral and cellular responses protective against the disease.

Ferrari and colleagues studied the $CD4^+$ T cell responses between patients with chronic infection and patients who had apparently cleared the virus (antibody positive patients but persistently HCV RNA negative with normal liver function tests for one year)[89]. They tested peripheral blood mononuclear cell proliferative responses to core, envelope, non-structural 4 and non-structural 5 proteins. Each of the antigens tested was able to induce significant although variable levels of proliferation. Significant proliferative responses to core, non-structural 4 and non-structural 5 antigens were more frequently detected in subjects who were able to eradicate infection than in patients with chronic HCV, although the difference was not statistically significant. They found no difference between the two groups of patients with respect to the response to the putative envelope antigens.

Cramp and colleagues performed a similar study at Kings[90]. They compared patients who were antibody positive but persistently HCV RNA negative without evidence of liver disease with patients with chronic infection who were persistently HCV RNA positive with abnormal ALT and biopsy proven chronic hepatitis. They found that $CD4^+$ proliferative lymphocytes responses to the HCV protein was detected in 14/26 RNA negative patients compared to only 2/14 of the RNA positive patients at a stimulation index of greater than 3. Twelve out of 26 patients of the RNA negative patients responded to more than 1 protein compared to none of the 14 RNA positive patients. Thus they concluded that a strong and multi-specific $CD4^+$ lymphocyte response to HCV proteins is associated with viral clearance. Of particular interest is the maintenance of the $CD4^+$ lymphocyte response for many years after initial exposure to HCV raising the possibility of continuing immunostimulation by viral proteins from hidden reservoirs of infection.

Further studies by Cramp and colleagues have shown that HCV specific T helper cell responses were more common and multispecific against HCV proteins in patients with the DQB1*0301 allele than in patients without this allele. They also showed that

patients with this allele had a stronger quantitative response to the core protein. This finding suggests that the previously described association of viral clearance with this allele may be mediated by promoting more effective antiviral T cell responses.

$CD8^+$ Responses

While the $CD4^+$ T cell response certainly plays a role in viral persistance and immunopathology, its involvement at the effector cell level is unclear since it is targeted primarily at internalised extra cellular antigens in the context of HLA class II molecules, and because multiple, often antagonistic, functions have been attributed to the different subsets of $CD4^+$ T helper cells. HLA class I restricted cytotoxic T lymphocytes (CTL) on the other hand, are widely acknowledged as a major effector mechanism in most viral infections. CTL mediated lysis of virus infected cells is thought to lead to clearance of the virus or if incomplete, to viral persistence and chronic tissue injury. Virus specific CTL have been demonstrated in many viral infections, including hepatitis B virus, and from these parallels with other viruses probably have an important role in HCV.

$CD8^+$ CTL will recognise a complex consisting of a class I MHC molecule, beta-2 microglobulin, and antigenic peptide on the surface of a cell. This complex is usually formed when peptides are released from newly synthesised viral proteins which are transported from the cytoplasm to the endoplasmic reticulum by ATP dependent peptide transporters (Tap 1 and Tap 2) where they encounter class I MHC molecules and are then presented on the surface of infected cells. This has not been clearly demonstrated in hepatocytes as a cell culture system of HCV has not been established. It is of note however that under normal conditions hepatocytes have low or undetectable amounts of class I MHC molecules but in chronic hepatitis infection, the expression of class I MHC genes are increased.

Mapping of $CD8^+$ T cell epitopes

Studies of $CD8^+$ T cell responses to HCV have been undertaken in humans[72;74], mice[78] and chimpanzees[79]. Intrahepatic and peripheral blood CTL have been isolated leading to the identification of a number of HCV epitopes through a combination of HLA binding motif scanning, HLA assembly assays and epitope mapping using synthetic peptides.

The method of HLA binding motif scanning is based on work that has characterised the common peptide binding motifs of HLA class I molecules. Peptides that bind to class I molecules consist of epitopes consist of 8 to 11 residues (usually 9 or 10). HLA A2.1 is often studied as it is of the highest frequency in most populations. A2 restricted epitopes tend to have a leucine, isoleucine, methionine or valine residue at position 2 and a leucine or other residue with an aliphatic hydrocarbon side-chain at the C-terminus. The binding motifs of class one alleles such as A2.1, B7 have thus been published. The HCV viral genome can be scanned for probable epitopes, based on these described peptide sequences, and this approach can be used to detects HCV specific CTL epitopes. These epitopes are scattered throughout the genome and restricted by a limited number of MHC molecules.

Battegay and colleagues used these techniques to show the presence of 5 HLA A2.1 restricted epitopes recognised by CTL of patients with chronic HCV infection, 3 in core, 1 in NS4 and 1 in NS5. They were unable to demonstrate CTL in 2 patients who had cleared the virus[75].

Chisari and colleagues described 9 novel A2 restricted epitopes[76]. They did this by stimulating peripheral blood mononuclear cells from a group of HLA A2 positive patients with chronic HCV with a panel of 130 HCV derived peptides containing the HLA A2 binding motif. Effector cells were tested for the capacity to lyse HLA A2 matched target cells that were either sensitised with peptide or infected with a vaccinia virus construct containing HCV sequences. They derived 9 immunogenic peptides in HCV, three of which were derived from the core protein, 3 from the non-structural 3 domain, 2 from NS4 and one from NS5. In their study however, unexpectedly they also managed to find peptide specific CTL responses in sero negative individuals, suggesting in vitro activation of naive CTL precursors. However the precursor frequency was 10-100 fold higher in infected patients compared to controls and the responses were greatly diminished by removal of CD45 RO (memory) T cells.

Shirai showed that CTL responses in transgenic mice expressing human HLA A2.1 predicted the same epitopes recognised by human A2.1 restricted CTLs[77]. They found that a highly conserved HCV core peptide was the most immunogenic. The results suggest that in spite of species differences, the T cell repertoire is plastic enough to allow a similar response when the same class one MHC molecule is presenting the peptide.

Peripheral versus Intrahepatic CTL

In contrast to these above studies Koziel and colleagues were unable to detect HCV specific CTL activity when using CTL derived from peripheral blood[73]. However $CD8^+$ lymphocytes derived from the liver and bulk expanded were shown to recognise and lyse target cells sensitised with synthetic HCV peptide. They identified four distinct class I restricted epitopes including two epitopes from the core protein. In a further study Koziel showed that the intrahepatic CTL response to HCV is broadly directed and that as many as five different epitopes may be targeted in a single individual with chronic HCV[74]. In another study CTL activity was detected in unprimed bulk expanded $CD8^+$ cells derived from the liver in 16 of the 35 patients, but not in peripheral circulation suggesting a tissue specific localisation with HCV specific CTL[91]. One hypothesis is that CTL populations are present at low frequency in peripheral blood because of sequestration in the liver.

A summary of the CTL epitopes that have been described to date is shown in **table 1.1**.

Table 1.1: Location and MHC restriction of HCV epitopes recognised by CTL derived from Humans

HCV REGION	AA RESIDUES	SEQUENCE	Liver (L) or peripheral blood (P) derived CTL	HLA Restriction	REF
Core	2-10	STNPKPQKK	L	A11	[73]
Core	35-44	YLLPRRGPRL	P	A2	[75;76]
Core	41-49	GPRLGVRAT	L & P	B7	[73]
Core	88-97	NEGCGWAGWL	P	B44	[92]
Core	131-140	ADLMGYIPLV	P	A2	[75;76;78]
Core	178-187	LLALLSCLTV	P	A2	[75-77]
E1	220-227	ILHTPGCV	P	A2	[77]
E1	233-242	GNASRCWVAM	L	B35	[72]
E1	363-371	SMVGNWAKV	P	A2	[77]
E2	401-411	SLLAPGAKQNV	P	A2	[77]
E2	460-469	RPLTDFDQGW	L	B53	[74]
E2	489-496	YPPKPCGI	L	B51	[73]
E2	569-578	CVIGGAGNNT	L	B50	[73]
E2	621-628	TINYTIFK	L	A11	[74]
NS2	826-838	LMAMTLSPYYKRY	L	A29	[72]
NS2	838-845	YISWCLWW	L	A23	[74]
NS3	1073-1081	CINGVCWTV	L & P	A2	[74;76]
NS3	1169-1177	LLCPAGHAV	P	A2	[76]
NS3	1287-1296	TGAPVTYSTY	P	A2	[76]
NS3	1395-1403	HSKKKCDEL	L	B8	[74]
NS3	1406-1415	KLVALGINAV	P	A2	[76]
NS4	1789-1797	SLMAFTAAV	P	A2	[76]
NS4	1807-1816	LLFNILGGWV	P	A2	[75;76]
NS5	2252-2260	ILDSFDPLV	P	A2	[76]
NS5	2588-2596	RVCEKMALY	L	A3	[93]
NS5	2727-2735	GLQDCTMLV	P	A2	[75]

$CD8^+$ T cell Function

The functional role and clinical relevance of these CTL is unclear. The reason why CTL responses can be elicited from some patients and not others remains to be determined. For example in one representative human study, 9 out of 15 subjects lacked cytolytic activity when intrahepatic $CD8^+$ T cells were expanded in vitro. One explanation is that there may be epitope variation in the infecting virions. In Koziels study, as in most studies, CTL were tested against targets either as peptides or vaccinia viruses expressing a single genotype 1a strain rather than patient derived viral sequences. Koziel in another study of intrahepatic CTLs responses were able to show that the liver derived CTL recognised different strains of HCV in a strain specific way[74]. Therefore if CTL recognition is dependent on HCV genotype, patients infected with genotypes other than 1a, may not respond to assays using 1a derived peptides or vaccinia. In addition even if the CTL assays are matched for HCV genotype, the infecting HCV isolate in the individual patient may still vary from the prototype sequence from which peptides used in the CTL assays are derived.

Work has tried to establish the relationship between the strength of the CTL response, liver disease severity, and viral load. Chisari used CTL precursor frequency analysis to quantitate the peripheral blood CTL response[94]. He showed that patients chronically infected with HCV were less strongly sensitised to a panel of well defined HCV epitopes than they were to an epitope within the Flu matrix protein. However they did not find that CTL correlated with disease activity or viral load in the majority of patients on a cross sectional bases although it did increase in three patients concomitant with sharp increases in serum ALT. They did not find that interferon therapy enhanced CTL precursor frequency. They hypothesised that CTL may contribute to ongoing liver disease while being quantitatively inadequate to destroy all the infected hepatocytes there by facilitating HCV persistence and contributing to chronic liver disease.

Lau in his study of intrahepatic CTL showed that compared with patients without detectable HCV specific CTL activity, those exhibiting CTL activity had lower levels of viraemia and more active disease, as reflected by a higher histological activity index and serum ALT levels with non-specific stimulation[91]. This would support the hypothesis that CTL both control viraemia but are also responsible for tissue damage.

In this thesis the role and function of CTL in determining acute self-limited infection as opposed to chronic infection has been studied and is described in Chapter 5.

Cytolysis/ IFN-gamma Production

Cytolysis may not be the only action of $CD8^+$ T cells in chronic HCV infection. In chronic hepatitis B virus infection viral replication is controlled by interferon gamma producing $CD8^+$ T cells that do not exhibit cytotoxicity[95]. Recent studies in HCV and HBV drawing upon this work have revealed differences between liver derived and peripheral blood T cell responses that have suggested that non-cytolytic, interferon, producing $CD8^+$ T cells may play a critical role in the control of HCV infection or may be responsible for mediating hepatocellular damage[96].

Analysis of cytokine production of $CD8^+$ cell lines has shown that they can produce interferon gamma, tumour necrosis factors alfa and beta, interleukin 8 and interleukin 10. These cytokines may have potent immunoregulatory activities and could be involved in manipulation of the inflammatory response seen in the liver. Differences in interferon gamma production and cytolytic activity of T cells in patients clearing the virus and in patients with chronic infection are described in chapter 5.

1.5 MECHANISMS OF VIRAL PERSISTENCE

The mechanism by which HCV persists and results in chronic infection in the majority of infected individuals is unknown. By analogy with other chronic viral infections a number of strategies may be invoked. Viruses have evolved numerous strategies for avoiding T cell responses including integration into the host genome as employed by retroviruses, the production of proteins that interfere with antigen presentation by inhibiting the TAP1/TAP2 transporter[97], down regulating MHC class 1 transcription (adenovirus and HSV) or interfering with cytokine production (EBV, pox viruses and myxoma virus). Some viruses produce cytokine analogues that interfere with CTL generation.

The most attractive of strategy, in view of the error rate of HCV RNA polymerase, is "escape mutation", whereby mutations arising within the HCV genome introduce protein variation that enables the virus to evade the host's immune response. In other chronic virus infections, such as Hepatitis B virus (HBV), murine lymphocytic choriomeningitis virus[98] and HIV[99], mutations have been found within immune epitopes. It is thought, though not conclusively proven, that these mutations are selected by the host's immune response and that they play a role in viral persistence and disease severity. Mutations within immune epitopes may lead to immune escape through disruption of antigen processing or antigen presentation. Similarly epitope mutation may destroy T cell recognition and activation, or antibody binding. In addition altered peptide ligands containing minor modifications of index peptide have been shown to antagonise both $CD4^+$ and $CD8^+$ T cell responses to the original peptides. CTL antagonism has been described for HIV[100] and HBV and may play a significant part in the failure of new clearance in patients chronically infected with these viruses. Escape mutations in MHC class I restricted epitopes may be of particular importance to the survival of HCV as, unlike retroviruses, it is unable to integrate into the host genome to avoid the CTL response.

Studies of HCV infection in chimpanzees have reported limited evidence of mutation within $CD8^+$T cell epitopes associated with viral persistence. Weiner and colleagues demonstrated that CTL derived from the liver of an experimentally infected chimpanzee could be detected against a conserved epitope in the HCV non-structural 3 protein for at least two years after infection[101]. However these CTLs did not recognise

the HCV quasispecies present in the plasma of this animal at week 16 post infection or at later time points. Escape from the CTL response was facilitated by an aspartic acid to glutamic acid substitution at amino acid position 1449 in all HCV genome that were sequenced. This study supports the concept that CTL responses can select for variant viruses with an enhanced ability to persist in a host. No similar studies have yet been reported in humans.

Escape from antibody recognition has been postulated as the basis for variability within the HVR[102]. Mutation within the variable region of E2 has been shown to be followed by sequential antibody responses. This hypothesis has been further investigated previously by comparing mutation rates in the HVR in immunocompetent patients with those in antibody deficient patients[103]. Although lower rates of mutation in the HVR were reported in hypogammaglobulinaemic patients the lack of evidence for neutralising HCV antibodies in chimpanzees or humans casts doubt on these observations.

The hypothesis examined in this thesis is that variation in mutation rate across the genome is the result of selection pressure exerted by the host on HCV. Secondly that selection by $CD8^+$ T-cells leads to immune escape due to a mutation within $CD8^+$ T cell epitopes encoded by HCV. Thirdly, that mutation in B cell epitopes leads to escape from immune control from antibodies. Experiments to test these hypotheses are described in Chapter 6.

CHAPTER 2:
NATURAL HISTORY OF HCV:
THE OXFORD HEPATITIS C CLINIC

2.1 SUMMARY

Within the Oxford region, referral of HCV antibody positive individuals for specialist review has been encouraged. These cases have included asymptomatic individuals identified through HCV screening, as well as patients with established liver disease. A database recording the clinical details of the patients was set up with the aim of identifying factors that might determine viral persistence or viral clearance and in addition factors that might determine disease progression.

Patients were categorised into patients with chronic HCV infection, as determined by positive testing for HCV RNA by PCR and patients who had cleared HCV, who had persistently negative testing for HCV RNA by PCR. Patient's gender, age at infection, duration of infection, route of transmission, alcohol consumption and co-morbid history were compared between HCV RNA positive and negative patients. In chronically infected patients the effect of these variables on liver histology was studied.

Patients who appear to clear HCV are more likely to the female and have acquired hepatitis C at a younger age. These factors are also significantly related to slower disease progression. In addition alcohol consumption is positively related to increased rate of progression. Liver inflammation correlates significantly with liver fibrosis.

Conclusions: Host and environmental factors are important in determining viral persistence or clearance and disease progression.

2.2 BACKGROUND

Since the discovery of hepatitis C virus in 1989[10], the number of infected individuals that have been detected has steadily risen. There are a number of different routes by which patients have been commonly identified. Firstly, the testing of patients, previously diagnosed as having non-A, non-B hepatitis, has revealed that 90% of these patients have antibodies to hepatitis C[35]. Secondly, anti-HCV testing is now a routinely performed "liver screening test" in patients with suspected liver disease or abnormal serum liver biochemistry in order to establish the aetiology of their condition. Thirdly, increasing public awareness of the disease has led to individuals with a history of high risk behaviour coming forward to request testing. In addition patients who request HIV testing, are now encouraged to also have testing for hepatitis B and C. Finally and one of the commonest routes of identification is the screening of blood donations, which was introduced in 1991. This has resulted in the identification of many, often asymptomatic, blood donors. The prevalence of anti-HCV in United Kingdom blood doning population has been found to range from 0.01-0.02%[29].

The increasing awareness of the complications of HCV, namely chronic hepatitis, cirrhosis, hepatocellular carcinoma and liver failure, has led to these patients being referred for specialist assessment. In the Oxford region the increasing number of referred patients to the local Hepatologist, Dr Chapman, led to the realisation for the need for an additional clinic a week dedicated to hepatitis C. This clinic was set up in 1993 and has been managed by myself since 1995. Since the start of the clinic, the number of new patient referrals has increased from 2 patients a month to 2-3 patients a week. The total number of patients attending this clinic continues to rise. There were 100 patients in 1995 and 297 at beginning of 1998.

Unlike previous studies of patients with established liver disease, our clinic contains many patients who are asymptomatic and have only been identified as a result of HCV screening. Previous studies of HCV that have focused on patients with clinically detectable disease have biased results towards a more severe outcome. The majority of patients attending the Oxford HCV clinic have been identified through either the screening of blood donors or testing of individuals with a history either past or present intravenous drug use; the minority of patients have presented with symptoms or signs of

liver disease. The cross section of patients seen in our clinic allows a representative assessment of the natural history of HCV.

Patients referred to the Oxford HCV clinic have been followed up regularly. Clinical assessment has been made at least once a year, in patients with mild asymptomatic disease, up to once a month in patients receiving treatment or with severe liver disease. In addition to recording symptoms and clinical examination, serum liver biochemistry has been performed and recorded on each visit and HCV-RNA status has been tested at least once a year and more often in patients undergoing treatment. PCR testing has revealed a group of untreated patients who are persistently HCV RNA negative despite having antibodies to HCV. These patients are thought to have spontaneously cleared virus.

Patients have now have been followed up prospectively in the clinic for a mean duration of 3 years (range 1 month to 6 years). The estimated duration of infection in these patients ranges from 0 to 40 years. In view of the long natural history of HCV this length of follow up is still too short to assess the effects of HCV on mortality. However it has been our policy to recommend liver biopsy to all patients who are HCV RNA positive unless there is a contraindication to biopsy. From the biopsy we have been able to judge both the degree of inflammation and in particular the degree of fibrosis and use this as those surrogate marker of disease progression.

A computer database of the patients was set up in 1995. The aim of the database was to accurately record the demographics, clinical findings and outcome of HCV infected patients in our Oxford clinic in order to determine what factors might determine viral clearance or viral persistence. The second aim was to determine in chronically infected patients what factors influenced disease progression.

1.3 METHODS

Patients

Individuals within the Oxford region found to be HCV antibody positive were referred to the specialist HCV clinic. Close liaison with General Practitioners, other departments including the Blood Transfusion service and Sexually Transmitted Diseases unit enabled high referral rates (for example any blood donor testing positive for HCV received a letter from the Blood Transfusion service recommending that their GP referred them to the clinic). Patient details were recorded in the case notes and subsequently recorded onto a computer database. Information recorded included the patients' demographic details, the reason they were tested, their risk factors for HCV and the estimated duration of their infection. In addition a history of previous excess alcohol intake and average weekly alcohol consumption at the time of referral was recorded. At the initial consultation all patients were tested for HCV RNA by PCR.

All patients who were HCV RNA positive were recommended to have a liver biopsy to obtain an accurate assessment of the effects of HCV on the liver. This included patients with normal liver function tests as we have previously demonstrated the poor correlation of aminotransferases with liver histology. In all cases the liver biopsy was performed before the patient received treatment. The results of the liver biopsy were recorded on the database using the scoring system proposed by Ishak and colleagues[104].

Outcome measures

The effect of predictive variables on two outcome measures was assessed. The outcome measures studied were firstly the patients HCV RNA status and secondly, in chronically infected patients, liver histology.

1. HCV RNA

HCV RNA was detected by PCR of the 5'non-coding region. Patients were classified into patients who had a positive PCR or negative PCR test. A positive result was confirmed by repeat testing. Patients were only classified as PCR negative if the result was confirmed by repeat testing, on at least three separate occasions at least one month apart.

2. Liver Histology

Two independent pathologists using the Ishak classification scored the liver biopsies. (Each biopsy was scored by both pathologists and where there were differences in score, the score averaged). This new scoring system aims to improve on previous scoring systems by separating grade and stage of liver disease. Grading is used to describe the intensity of necroinflammatory activity in chronic hepatitis. Staging, on the other hand, is a measure of fibrous and architectural alteration, i.e. structural progression of the disease; these features are currently believed to be the consequence of the necroinflammatory process although this remains controversial. By scoring grade and stage numerically the histological features can provide a semi-quantitative assessment. The scoring system is shown in **table 2.1**.

Table 2.1: Modified histological activity index proposed by Ishak et al[104]

Grade (necroinflammatory score):	
A. Periportal or periseptal interface hepatitis (piecemeal necrosis)	0-4
B. Confluent necrosis	0-6
C. Focal lytic necrosis, apoptosis and focal inflammation	0-4
D. Portal inflammation	0-4
Total	18
Fibrosis:	
No fibrosis	0
Fibrous expansion of some portal areas	1
Fibrous expansion of most portal areas	2
Fibrous expansion of most portal areas with occasional portal to portal bridging	3
Fibrous expansion of portal areas with marked bridging	4
Marked bridging with occasional nodules	5
Cirrhosis	6

For the purposes of analysis the scores were categorised for grade into three categories representing mild (score 0-4), moderate (score 4-8) and severe inflammation (>8).

In order to compare rate of fibrosis progression between patients a value for fibrosis rate was obtained by dividing the fibrosis score by the estimated number of years of infection. For example a patient with a fibrosis score of 3 after 10 years of infection would have a fibrosis rate score of 0.3 fibrosis units/year. Another way of depicting this

score is to estimate the predicted time for a patient to reach cirrhosis. A patient with a fibrosis rate score of 0.3 might be predicted to develop cirrhosis after 20 years (0.3 x 20 years = Fibrosis score of 6). It must however be recognised that the histological score for fibrosis represent a descriptive pattern of the liver histology rather than a true linear scale and therefore the obtained score for fibrosis rate is not a true value but simply enables a method of comparison between patients. In the analysis of the results patients were categorised into patients with low fibrosis rates (<0.15), moderate fibrosis rates (0.15-0.3) and high fibrosis rates (>0.3). Or in other words patients predicted to develop or who had developed cirrhosis after >40, 20 – 40 or <20 years respectively.

Predictive Variables

The following variables and their relationship with the two outcome measures were assessed: patient gender, duration of infection, age at the time of infection, route of transmission, past history of excess alcohol intake, present alcohol consumption, co-morbidity. For analysis patients were divided into patients infected before or after the age of 40. Alcohol consumption was divided into patients who drank less than or more than 20 units per week. In a subgroup where the viral genotype was known this was also assessed.

Statistical Analysis

Statistical analysis was performed using both univariate and multivariate analysis (SPSS for windows release 11.0.0 (copyright SPSS inc.)),

to study not only the impact of risk factors on stage but also fibrosis rates and determine the interdependance of these risk factors.

2.4 RESULTS

Antibody Testing

By the beginning of 1998, 297 patients had been seen in the clinic. All patients were HCV antibody positive by first and second generation ELISA tests. Antibody testing was confirmed with a positive confirmatory RIBA test in 276 patients. Twenty one out of 297 patients had indeterminate RIBA tests. Ten patients with indeterminate RIBA status had risk factors for HCV (8 intravenous drug use, 2 blood transfusion). All these patients were HCV RNA negative and the indeterminate result may in these patients reflect weakening antibody responses as a result of viral clearance. 11 patients, with indeterminate RIBA testing had no risk factors for HCV and were also HCV RNA negative: their antibody tests may represent false positive results. Analysis of results was done excluding patients with RIBA indeterminate antibody responses.

HCV RNA

Of the 276 antibody positive patients who initially attended clinic, 251 returned for repeat confirmatory PCR testing. Two hundred and thirteen patients tested positive for HCV RNA by PCR and this was confirmed by a minimum of two tests. Thirty-eight patients were persistently HCV RNA negative. All HCV RNA negative patients were tested on at least three occasions, 1 month apart. Thirty-two out of 38 (84%) of the HCV RNA negative patients have been tested at least five times. The mean duration follow up of HCV RNA negative patients is 2 years (range 6 months - 5 years) and all patients have been persistently HCV RNA negative during this time.

Factors that determine viral persistence

Of the variables that were correlated with the end point of viral clearance or persistence, female gender was found to be significantly associated with viral clearance (see **table 2.2**)(Chi-test $p< 0.001$). The ratio of female to male patients in HCV RNA negative patients was 23:15 (61% female), compared to 87:126 (41% female) in HCV RNA positive patients. There was a trend for the average age of HCV RNA negative patients at the time of infection to be lower than HCV RNA positive patients (24.8 versus 26.5 years),although this did not reach statistical significance. There was no

difference in route of transmission, viral genotype or co-morbid medical history between patients who clear the virus and those that go on to persistent infection.

Table 2.2: Comparison of HCV RNA negative and positive patients

PREDICTIVE VARIABLES	HCV RNA NEGATIVE (TOTAL 38)	HCV RNA POSITIVE (TOTAL 213)
Gender: F:M (% Female)	23:15 (41%)	87:126 (61%)
Age at infection: Mean (range) Number of observations	24.8(16-61) (33/38)	26.5(15-65) (175/213)
Route of transmission: No. (%) Blood transfusion	11 (29%)	54 (25%)
IVDU	21 (55%)	121 (57%)
Sexual	0	2 (1%)
Other	6 (16%)	36 (17%)
Alcohol consumption: units/week: Mean (range)	9.0	9.9
Past history of heavy drinking: No. (%)	10 (26%)	61 (29%)
Significant co-morbidity; No. (%)	1 (3%)	6 (3%)

Histological Scoring

Of the 213 chronically infected patients, 131 had been biopsied at the time of the analysis. Reasons for not being biopsied were failure to attend either further follow up in clinic or to attend for the biopsy (51 patients), patient refusal (14 patients), contraindication to biopsy (5 patients). Twelve patients are still awaiting biopsy.

The scores obtained by the two independent pathologists were not significantly different with identical scores obtained in 92% of biopsies, thus validating the reproducibility of the scoring system. The results of liver histology are shown in **table 2.3** showing the number of patients in each fibrosis category and the number of patients with mild, moderate or severe inflammation.

Table 2.3: The results of liver histology in 131 chronic HCV carriers

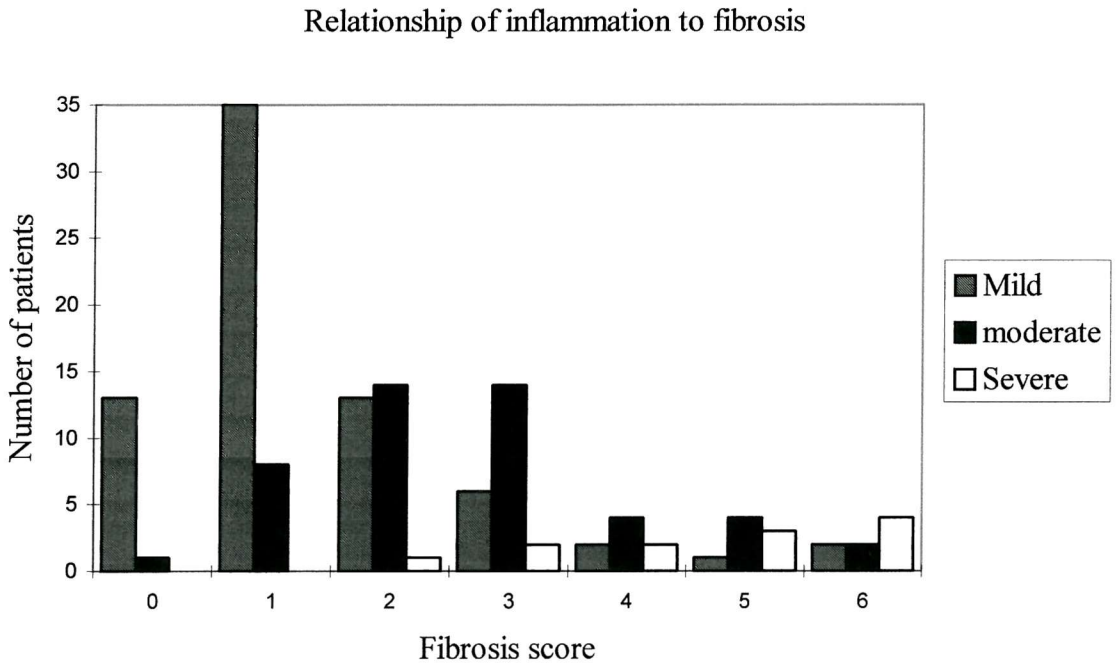
Fibrosis score	**Patient number (%). Total 131**
0	14 (11%)
1	43 (33%)
2	28 (21%)
3	22 (17%)
4	8 (6%)
5	8 (6%)
6	8 (6%)
Histological grade	
Mild	72 (55%)
Moderate	47 (36%)
Severe	12 (9%)

The relationship of histological grade and fibrosis were compared and this is shown in **table 2.4** and **figure 2.1**.

Table 2.4: Histological inflammatory scores associated with each fibrosis grade

STAGE	GRADE 0-4	GRADE 5-8	GRADE >8
0	13	1	0
1	35	8	0
2	13	14	1
3	6	14	2
4	2	4	2
5	1	4	3
6	2	2	4

Figure 2.1:



One can see from these charts that milder inflammation is more commonly associated with less fibrosis although still can be seen in patients with more severe fibrosis and cirrhosis.

In view of the relative small number of patients with moderate and severe inflammation and with moderate and severe fibrosis we grouped these categories together to perform statistical analyis as shown in the table below.

		Fibrosis (no. of patients)		
		Mild	Mod/Severe	Total
Inflammation	Mild	61	11	72
	Mod/severe	24	35	59
	Total	85	46	131

The association of severity of inflammation with severity of fibrosis was highly significant using chi-square tests as detailedd below:

	χ^2Value	df	significance
Pearson chi-square test	24.054	1	p<0.001
Likelihood ratio	24.5	1	p<0.001
Fishers exact test	23.85	1	p<0.001

Relationship of Histological Grade and Stage with predictive variables

The predictive variables were compared with the different histological grades and stages and are shown in **table 2.5** and **table 2.6**. The level of fibrosis correlates as one might predict with duration of infection. One can also see a pattern of less fibrosis in females, patients infected at an earlier age and in patients with no previous history of excessalcohol consumption. This pattern is however better illustrated when the predictive variables are compared with fibrosis rate rather than simply with fibrosis (see below).

Table 2.5: Relationship of predictive variables with Histological Grade

PREDICTIVE VARIABLES	GRADE 0-4 (N=72)	GRADE 5-8 (N=47)	GRADE >8 (N=12)
Gender: F:M (% female)	40:32 (56%)	19:28 (40%)	2:10 (17%)
Duration of infection: Mean (range)	12.4(2-29)	11.4(1-24)	12.4(3-39)
Age at infection: Mean (range)	25.5(16-61)	27.5(17-61)	33.7(17-48)
Route of transmission: No. (%)			
Blood transfusion	24 (33%)	17 (36%)	6 (50%)
IVDU	42 (58%)	21 (45%)	2 (17%)
Sexual	0	2 (4%)	0
Other	6 (8%)	7 (15%)	4 (33%)
Normal LFT: No. (%)	39 (54%)	7 (15%)	2 (17%)
Alcohol units/week: Mean (range)	8.2	13.9	16.1
Past history of heavy drinking. No(%)	20 (28%)	22 (47%)	7 (58%)
Significant co-morbidity; No. (%)	1 (1%)	3 (6%)	2 (16%)

Table 2.6: Relationship of predictive variables with Histological Stage (Fibrosis)

Predictive Variables/ Stage	0 (n=14)	1 (n=43)	2 (n=28)	3 (n=22)	4 (n=8)	5 (n=8)	6 (n=8)
Gender: F:M (% female)	8:6 (57)	27:16(63)	11:17(39)	9:13(41)	2:6(25)	2:6(25)	2:6(25)
Duration of infection: Mean (range)	9.3 (1-17)	11.8 (3-20)	11.4 (5-24)	11.2 (4-32)	14.6 (7-32)	17.1 (9-42)	20.4 (11-41)
Age at infection: Mean (range)	21.8 (16-29)	25.3 (15-54)	26.3 (15-61)	28.7 (17-55)	34.9 (18-56)	29.9 (21-49)	24.9 (20-61)
Route of transmission: No. (%)							
Blood transfusion	3 (21)	14 (33)	11 (39)	10 (43)	4 (50)	5 (63)	2 (25)
IVDU	7 (33)	27 (63)	15 (54)	10 (43)	4 (50)	3 (37)	5 (63)
Sexual	0	1	1	0	0	0	0
Other	4				0	0	1
Normal LFT: No. (%)	11 (79)	16 (37)	11 (39)	5 (23)	3 (34)	1 (13)	1 (13)
Alcohol: units/week: Mean (range)	8.5	8.7	7.8	17	10.75	13.1	18.6
Past history of heavy drinking: No. (%)	1 (7)	11 (26)	6 (21)	11 (50)	4 (50)	3 (34)	6 (75)
Significant co-morbidity; No. (%)	0	0	1 (4)	2 (9)	2 (25)	0	1 (13)

Rate of Fibrosis

Rate of fibrosis was estimated by dividing the score for fibrosis by the estimated number of years patients had been infected with HCV. In order to compare the effect of different variables on fibrosis rate patients were divided into slow, moderate and fast progressers – see **table 2.7**.

Table 2.7: Relationship of predictive variables with fibrosis rate

	FIBROSIS RATE		
Predictive Variables	0–0.15 Slow (n=58)	0.15–0.3 Moderate (n=38)	>0.3 Fast (n=34)
Gender: F:M (% female)	37:21(64)	16:22(42)	8:27(23)
Age at infection: Mean (range)	22.4 (15-54)	27.9 (16-61)	32 (18-61)
Route of transmission: No. (%)			
Blood transfusion	15 (26%)	13 (34%)	21 (60%)
IVDU	37 (64%)	22 (58%)	12 (34%)
Sexual	1 (2%)	1 (3%)	0
Other	5 (10%)	2 (5%)	2 (6%)
Normal LFT: No. (%)	26 (45%)	14 (37%)	8 (23%)
Alcohol : units/week: Mean (range)	8.7	7.9	17.7
Past history of heavy drinking: No. (%)	14 (24)	9 (24)	19 (54)
Significant co-morbidity; No. (%)	1 (2)	3 (8)	2 (6)

Rate of fibrosis was also compared in different patient groups - see **table 2.8**. Rate of fibrosis was higher in male patients, patients infected after the age of 40 and in patients with higher alcohol consumption.

Table 2.8: Fibrosis Rate in different patient groups

PREDICTIVE VARIABLES		FIBROSIS RATE MEAN (Standard Deviation)
Female		0.15 (0.13)
Male		0.33 (0.28)
Age at infection: < 40 years		0.23 (0.20)
Age at infection: > 40 years		0.45 (0.29)
Route of transmission:	Blood transfusion	0.32 (0.17)
	IVDU	0.19 (0.27)
Alcohol consumption: <20 units/week		0.22 (0.22)
Alcohol consumption: >20 units/week		0.33 (0.29)
No Past history of heavy drinking		0.21 (0.25)
Past history of heavy drinking		0.33 (0.23)
Genotype (No. of patients genotype available)	1 (19)	0.27
	2 (8)	0.24
	3 (9)	0.21
	4 (3)	0.26

Genotype data was available for 39 out of 131 patients biopsied. No difference in fibrosis rates was seen between patients infected with different genotypes.

Statistical Analysis

As previously in view of realtively small numbers, we combined moderate and severe fibrosis and compared this with mild fibrosis. Similarly we compared combined moderate and severe fibrosis rate with mild fibrosis rate. For the purpose of analysis we catergorised the predictive variables as below:

mild inflammation vs moderate/severe inflammation,

previous heavy alcohol vs no previous history of heavy alcohol

Female vs male

Age: <40 years versus > 40 years

Age at infection: >25 years versus <25 years

Fisk factors: Blodd transfuion versus IVDU

Duration of infection: <15 versus >15 years

Multivariate analysis

Mild versus moderate/severe fibosis

Variables	Significance P value	Odds ratio (B)	95% Confidence for B	
			upper	lower
Inflammation	<0.001	7.49	2.70	20.81
alcohol	0.005	5.33	1.65	17.24
sex	0.39			
age	0.17			
Age at infection	0.13			
Risk factor	0.25			
Duration of infection	0.60			

Slow versus moderate/fast fibrosis

Variables	significance	Odds ratio (B)	95% Confidence for B	
			upper	lower
Inflammation	0.005	4.42	1.56	12.52
alcohol	0.056	3.05	0.971	9.55
sex	0.004	0.22	0.08	0.63
age	0.17			
Age at infection	<0.001	8.24	2.27	29.95
Risk factor	0.20			
Duration of infection	0.67			

We can see from the above a highly significant relationship between inflammation and alcohol on the degree of fibrosis . When one looks at fibrosis rate, inflammation, male sex, older age at infection and a past history of alcohol excess are all significantly associated with fast fibrosis rate.

2.5 DISCUSSION

In our clinic population 38 out of 251 (15%) of patients with antibodies to HCV have persistently negative tests for HCV RNA by PCR. All these patients were tested on numerous occasions to confirm the result and therefore a negative test is unlikely to be the result of a false negative result. The fact that HCV RNA is undetectable in the serum by PCR may reflect that these patients have cleared the virus. Another explanation is that HCV is still present but below the detection rate of the test or alternatively that the HCV is presiding in other sites but not in blood. Nonetheless this group of HCV RNA negative individuals do represent a phenotypically different subgroup to patients who are PCR positive in that follow up of HCV RNA negative patients has shown that morbidity and mortality related to liver disease in this group is extremely low.

Female sex is associated with ability to clear HCV. In addition there is a trend for patients infected at a younger age to be more likely to be HCV RNA negative. This suggests that host factors play a role in determining viral persistence. The role of the viral factors in determining viral persistence is harder to evaluate. The viral genotype of HCV RNA negative patients cannot be tested (if the viral RNA cannot be amplified genotyping cannot be performed). However the fact that the route of transmission namely intravenous drug use versus blood transfusion, which results in differing doses of infecting virus, is not different in HCV RNA negative and positive patients, suggests that viral load at the time of infection is not a factor that influences viral persistence or clearance.

In order to study predictors of disease progression we looked at the effect of the same variables that we had studied in determining chronic infection on liver histology in HCV RNA positive patients. Male gender, and history of heavy alcohol intakewere associated with increased levels of fibrosis. Male gender, age at infection and history of previous heavy alcohol intake were significantly associated with faster fibrosis rates. In the subgroup where viral genotyping was available, no difference in fibrosis rates could be demonstrated between genotypes. One limitation of the study is the fact that the duration of infection is estimated in many patients. However a previous study has shown that the vast majority of intravenous drug users become infected within the first six months of drug usage[38]. Thus using the year of first intravenous drug use will give a

reasonable estimation to calculate duration of infection. For the patients infected from blood transfusion the exact date of infection was known.

One possible explanation as to why age at infection is a risk factor for disease progression is that the host defence mechanisms against HCV is weaker in older patients. The correlation of alcohol consumption and disease progression is not surprising. However there is some evidence to suggest that alcohol does not merely have an additive affect on disease progression but might have may accelerate fibrosis formation. Thus advice on keeping alcohol consumption to a minimum is essential. The mechanism by which female sex protects against HCV remains unknown.

We found that the degree of inflammation was higher in patients with increased fibrosis scores. This finding was statistically significant and is the first time this has been shown. It is tempting to speculate that more inflammation results in more fibrosis. However only a longitudinal study of paired liver biopsies will determine whether liver inflammation in the first biopsy will predict development of fibrosis in the second biopsy. In Oxford approximately 50 patients have now undergone two or more liver biopsies although this number is presently too small to make meaningful analysis.

It is also of note from our study that biochemical liver function tests are poor markers of histological liver disease. Although there is a trend for normal liver function tests to be associated with the milder spectrum of liver histology, we have again demonstrated that normal liver function test can be found in patients with high levels of inflammation (17%), fibrosis (13%) and fibrosis rates (23%).

In conclusions a wide spectrum of disease has been seen in our clinic population. Fifteen per cent of patients have spontaneously cleared the virus. In chronically infected patients both the spectrum of disease and rate of disease progression is extremely variable. The majority of patients have evidence of relatively mild histological changes on liver histology although the length of follow up is still comparably short. We have identified host factors that appear to be important in not only determining viral persistence or clearance but also disease progression.

CHAPTER 3:
INCIDENCE AND OUTCOME OF HCV IN BLOOD TRANSFUSION RECIPIENTS AT THE OXFORD BLOOD CENTRE

3.1 SUMMARY

The national HCV Lookback was designed to trace recipients of infected blood products which might have transmitted HCV before antibody screening was available; to offer specialist care and to study the natural history of disease in a population where the time of infection is known. All donors subsequently discovered to be HCV antibody positive were entered into the lookback. Recipients of blood products derived from these donors were traced and tested for antibodies to HCV and HCV RNA by PCR. Within the Oxford region infected recipients were referred to the Oxford HCV clinic and liver biopsies were performed on patients with detectable HCV RNA. Virus genotype was performed on donor recipient linked pairs.

399 potentially infectious blood components issued from the Oxford Centre (from 95 donors) were traced to 104 living recipients. 49 recipients showed no sign of infection, although in only three patience was it certain that they had received infected blood. 11 recipients were HCV antibody positive but were HCV RNA negative by PCR whilst 44 were both antibody and HCV RNA positive. The 14 with signs of recovery had a mean age of 27 years when transfused, compared with a mean age of 46 years in the 44 with chronic infection. The majority of patients were asymptomatic with mild histological changes at liver biopsies following their transfusions 4 - 13 years earlier, although there was one case of cirrhosis with hepatoma. Virus genotyping in donor recipient pairs showed complete concordance. No correlation was observed between viral genotype and disease severity.

Conclusions: HCV transported by blood transfusion led to chronic infection in 44 of 58 patients who had definitely received infected blood (76%). Younger patients were more likely to clear virus. Viral genotype had no influence on outcome. Continued follow up of these patients in the specialist Oxford HCV clinic and additional results from the rest of the UK blood centres will provide further information about the natural history of HCV.

3.2 BACKGROUND

Following the establishment of donor screening for hepatitis B surface antigen in the 1970's, it became apparent that other agents could cause post-transfusion hepatitis. Following the identification of Hepatitis C virus in 1989and the development of tests to detect antibody to HCV it was found that that 90% of post-transfusion non A, non B hepatitis was caused by this newly discovered virus. Early clinical studies of hepatitis C were based on patients with detectable disease, and therefore the overall risk of morbidity and mortality which included asymptomatic individuals was unknown. The introduction of blood donor screening for anti-HCV by the UK National Blood Service (NBS) in September 1991 led to the identification of many asymptomatic virus carriers.

There were a number of reasons that led the National Blood Service to decide to conduct a study to identify recipients of blood transfusion infected with HCV before screening was available. Firstly, a retrospective pilot study indicated that substantial numbers of infected recipients would be identified if a nation wide study was carried out. This study was carried out in Scotland by Ayob and colleagues after the first six months of donor screening[105]. Nine living recipients from 15 blood donors with chronic HCV were tested and all had signs of persisting infection. Secondly, previous studies of patients with non-A, non-B hepatitis had shown that patients with chronic infection are at risk of developing cirrhosis of liver and hepatocellular carcinoma. Finally, there was increasing evidence of the benefits of antiviral therapy with interferon alpha for the treatment of HCV infection.

The National Lookback programme was carried out by order of the Chief Medical Officeron the recommendation of the Department of Health Advisory Committee for the Microbiological Safety of Blood and Tissues for Transplantation(MSBT). The programme was directed and co-ordinated by the Medical Director of the National Blood Authority (NBA) and started in all NBS blood centres in April 1995. The main aims of the look-back were (1) to enable referral of HCV infected patients to specialist care and (2) to study a large number of people infected by blood transfusion from a known source at a known time, and thus to discover more about the natural history of hepatitis C.

In the Oxford region the tracing of recipients and the clinical follow-up of patients was co-ordinated by Dr Angela Dike from the blood transfusion service and

myself in the Oxford HCV clinic. The results of the Oxford Blood centre are reported in this chapter.

3.3 METHODS

Blood donor screening

Donor testing was carried out according to NBS Guidelines 1993 using second and third generation enzyme immuno-assays (EIA) for hepatitis C antibody (anti-HCV), supplemented by recombinant immuno-blot assay (RIBA II/RIBA III) for all EIA positive samples.

In May 1995, at the beginning of the national HCV look back study, MSBT gave instructions for the combination of laboratory test results to be used to identify the blood donors whose previous donations were to be considered as potentially infectious for HCV. Donors were entered into the study if they had shown detectable anti-HCV in two or more EIA tests, using tests kits from different manufacturers, together with a positive RIBA result. A year later the MSBT advised that blood donors whose sera was reactive in two or more EIA's but RIBA indeterminate (a strong reactive band with only the NS4 (c33c) or core (c22) region on the immuno-blot strip) should also be considered as potentially infectious and should be included in the study. The reason for this was that several studies have shown that a small portion of patients with indeterminate RIBA status were HCV RNA positive (tests for HCV RNA were not routinely available in the early years of anti- HCV screening blood donors).

HCV look-back

In March 1995 a standard set of questionnaire forms was issued by the NBA to every NBS blood centre, together with guidelines for counselling the recipients of potentially infectious blood components. Data to be gathered included whether transfusion of the blood component was documented in the hospital notes, and whether the recipient had other known risks for hepatitis C. Tests to be done on serum samples, in designated virology laboratories, were as follows: two different EIA tests for anti-HCV, and a RIBA or similar supplementary test for the reactive samples. EIA negative samples were to be tested by a reverse transcriptase polymerase chain reaction (PCR) to exclude any rare cases which might be viraemic in the absence of detectable antibody. At Oxford, in addition to these minimum requirements, PCR tests were carried out on all the recipients with detectable anti- HCV, and on as many as possible of the linked donors, in many cases using samples which had been stored by the Oxford Public Health

Laboratory after finding them anti-HCV positive. HCV RNA was detected using an in house reverse transcriptase PCR. Briefly, HCV RNA was extracted from 100 microlitres of serum using the method of Boom et al[106], and a nested PCR was carried out using primers to the conserved 5'non- coding region of the genome. Reverse transcriptase was incorporated in to the first round PCR mix. Following amplification, specific products were identified with reference to a molecular weight marker by agarose gel electrophoresis, stained with ethidium bromide and examined by ultraviolet transillumination. In addition a blood sample was to be sent for liver function tests.

Identification of the hospitals or other institutions to which blood had been issued from potentially infectious donors from previous donations was available from 1983 on the Oxford blood centre computer database. Tracing of blood transfusion recipients was done with the co-operation of Haematology departments in the ten District Hospitals, two Royal Air Force Hospitals, and in five private Hospitals served by the Oxford blood centre. Blood sampling of the traced patients was undertaken by hospital haematologists or other consultants and by family practitioners.

All blood transfusion recipients with evidence of hepatitis C were offered referral to the Gastroenterology department of the John Radcliffe Hospital where they were seen by me in the hepatitis C clinic. Repeat testing for HCV RNA was performed in clinic along with liver function tests. Patients who were HCV RNA positive were recommended to be further evaluated by liver biopsy regardless of liver function tests. Patients who were HCV RNA negative were seen at regular intervals and had repeat PCR testing.

Where HCV RNA was detected in both donor and recipient linked pairs, HCV genotypes were determined at Central Public Health Laboratory in Colindale (CPHL) using a PCR-restriction fragment length polymorhism procedure. RNA was extracted from serum samples and cDNA was synthesised. A 174 base-pair fragment of the 5' non-coding region of the hepatitis C virus genome, between base-pairs 142 and 316 was amplified by nested PCR. Primer sequences were taken from Lin et al[107]. After PCR, aliquots of each product were digested with the restriction enzymes ScrfI, MvaI, HinfI and BstUI . For each sample, ScrfI and MvaI digests were pooled, as were HinfI and BstUI digests. The pooled digests were then electrophoresed through a polyacrylamide gel, prepared from MDETM gel solution (FMC Bioproducts), at 160-170V for 3 HR. Gels were stained using SYBR-green I (FMC Bioproducts). The patterns were then

analysed to distinguish between at least nine different HCV sub types (1a, 1b, 2a/c, 2b, 3a, 3b, 4, 5,and 6). This method is an adaptation of the one described by Pohjanpelto et al (1995)[108].

3.4 RESULTS

1) Donor identification (figure 3.1)

Seventy anti-HCV positive donors with previous donations on record were identified in Oxford and 7 more who had moved were identified in other NBS blood centres and reported to Oxford, giving a total of 77 anti-HCV positive donors for the look-back. In addition, 14 anti-HCV indeterminate donors with previous donations were entered into the look-back together with 4 more reported to Oxford from other centres (adding 18). Thus in total 95 anti-HCV positive donors were identified who had previously given blood.

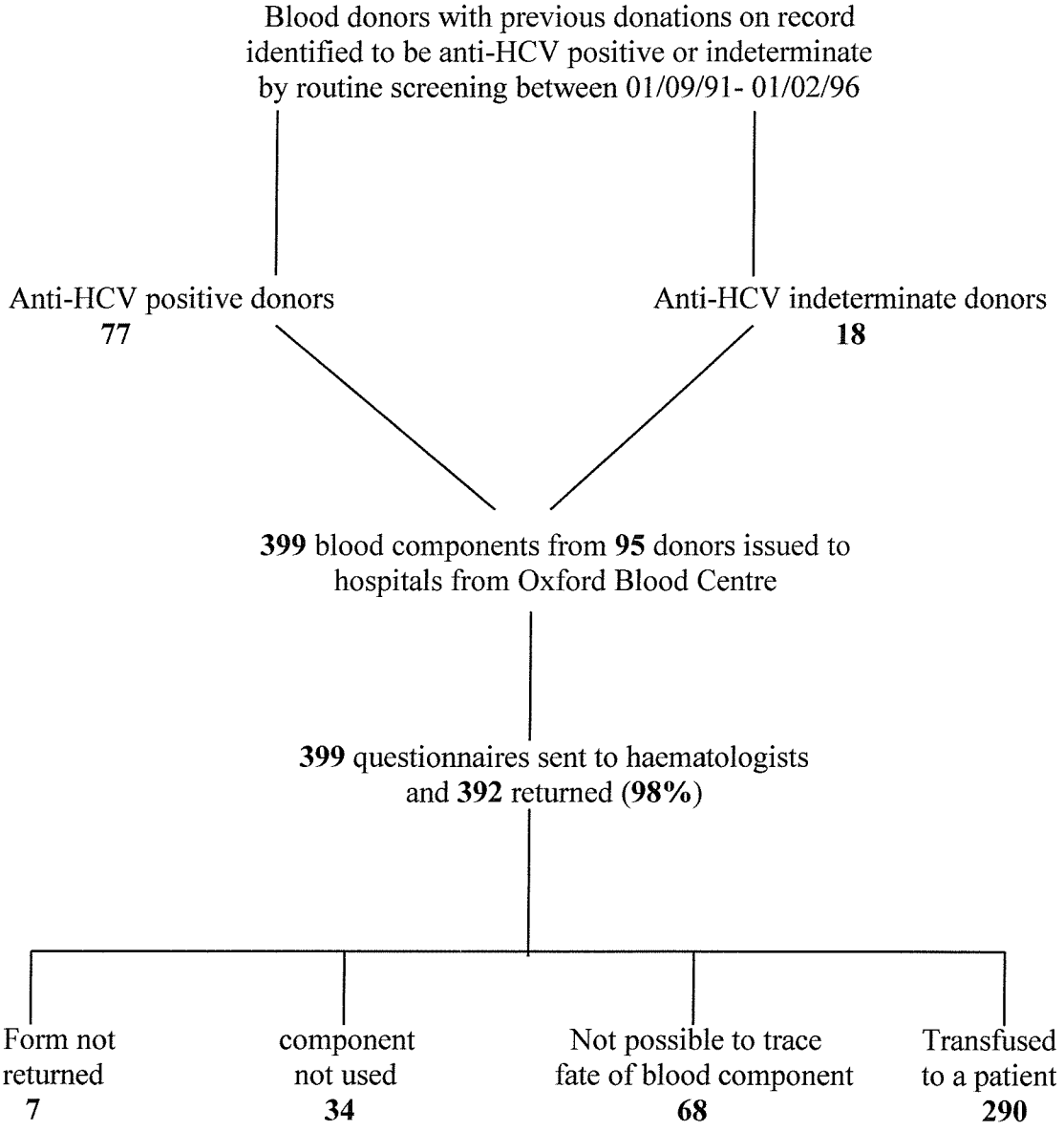
2) Lookback Results

Three hundred and ninety nine potentially infectious blood components from the 95 identified donors had been issued for clinical use and questionnaires relating to each of these blood components were sent to the hospital haematologists, (45 of these were sent to hospitals via four other NBS blood centres which had received blood components from Oxford). The questionnaires had been designed to ascertain the identity of the recipients, and then to trace hospital notes, and to find out whether the recipients might be alive and traceable. Three hundred and ninety two of the 399 questionnaires were returned. These showed that 34 blood components had not been transfused. Sixty-eight (19%) of the remaining 358 components had not been traced for the following reasons: 37 were unrecorded in the hospital transfusion laboratories; 22 were noted in the hospital transfusion laboratories but had insufficient patient details recorded to allow identification of the hospital records; 6 cases had adequate hospital blood bank records but no traceable hospital records; 3 patients had adequate hospital blood bank records and hospital records but were untraceable by the Family Health Service Authorities.

Two hundred and ninety two recipients were identified of whom 177 (61%) had died. Of the remaining 113 alive and traceable patients, there were nine who were either very old or terminally ill or mentally incapable of understanding the reasons for blood testing and therefore were considered unsuitable to be approached by their doctors. This left 104 recipients on whom the following results are based. They were linked to 43 of the 77 anti- HCV positive blood donors and to 11 of the 18 anti-HCV indeterminate donors. No living recipients were traced to the other 41 potentially infectious donors.

Figure 3.1:

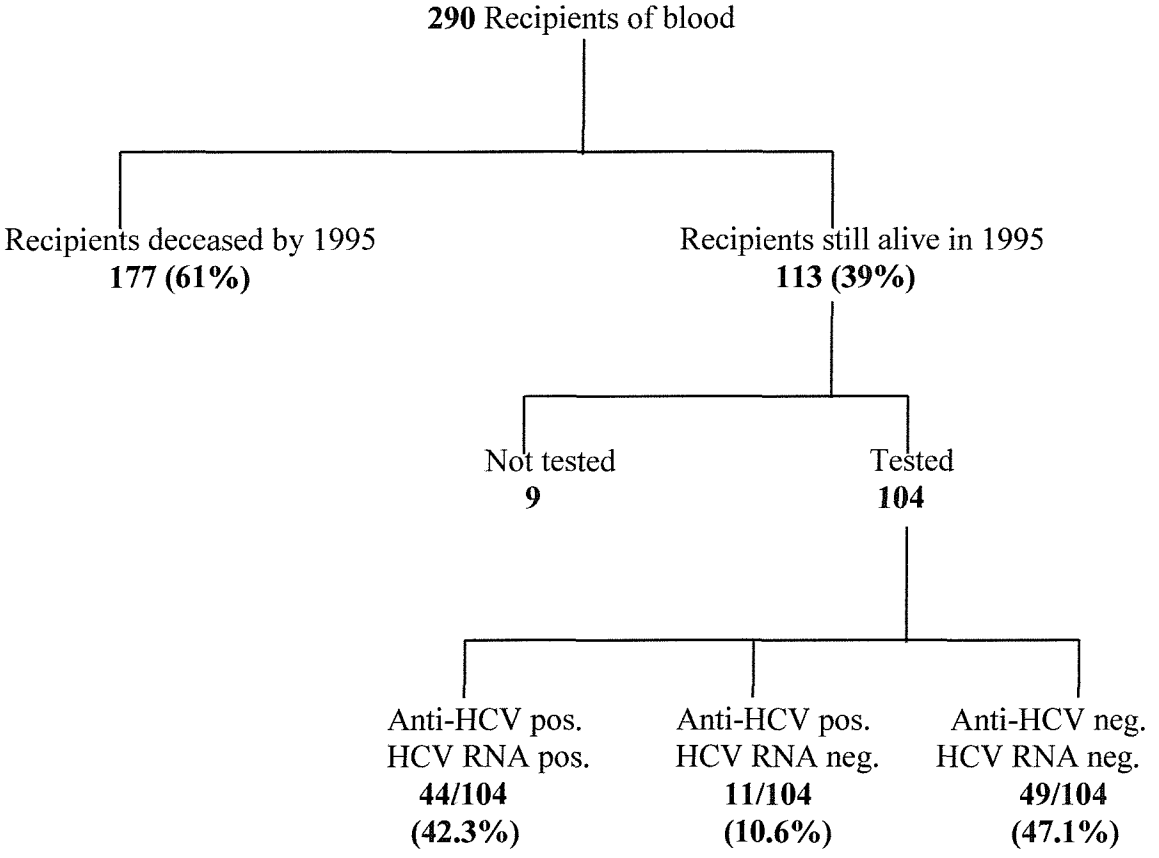
Tracing of blood components from donors to recipients



Recipients were divided into three main categories; (a) anti-HCV and HCV RNA both negative; (b) anti-HCV positive and HCV RNA negative; (c) anti-HCV and HCV RNA both positive (see **figure 3.2**).

Figure 3.2:

Outcome of recipients of blood transfusion from donors subsequently found to be anti-HCV positive



A). Anti-HCV and HCV RNA both negative ***(see table 3.1)***

Forty nine recipients (47%) were shown to be anti-HCV negative and had no detectable serum HCV RNA on PCR. These recipients were transfused between 1983-1993, and were tested in 1995 and 1996. They could be sub-divided into three categories according to the status of the 28 implicated donors as follows:

i) Ten recipients were transfused from 7 donors all of whom were documented as having acquired hepatitis C infections some time after these traced donations: three regular blood donors who were anti-HCV negative between 1991-1993 showed seroconversion between 1994-1996; three other donors acquired HCV at later dates from blood transfusions they themselves received, and were discovered as "look back " recipients; and one donor admitted injecting himself with drugs for a short period after the donation under investigation. Later recipients of two of the sero-converted donors were infected (see below), but the other five donors had no later recipients available for testing.

ii) Twenty nine recipients with no evidence of infection were transfused from 13 donors, 11 of whom had indeterminate RIBA status as defined above, and two of whom had positive anti-HCV results by all tests. However, all these donors were HCV RNA negative by PCR on several occasions, and none of them were linked to any other infected recipients.

iii) Ten recipients with no evidence of infection received blood from 7 donors who were all anti-HCV and HCV RNA positive when screened after 1991. Three of these seven donors were almost certainly HCV RNA positive at the time of their earlier donation to these recipients for the following reasons: one of the 7 donors (Donor-ID 9) were linked to two other infected recipients transfused both before and after the dates of this traced donation; another donor (Donor-ID 18) had transmitted hepatitis C to a second recipient of the same donation; a third donor (Donor-ID 57) had transmitted HCV six months earlier to another recipient. Since in the three recipients (ID numbers 49,52,57) there was clear evidence in the hospital notes that the transfusions had been given, this suggests that they had either resisted or recovered from infection, retaining no detectable antibody; they were one female aged nine, one female aged 33 and one male aged 18 when transfused in 1984, 1990 and 1986, respectively. Further details are presented in **table 3.1** and the prospective donors can also be seen in **tables 3.2** and **3.3** linked to infected recipients.

Table 3.1: 49 Recipients who were anti-HCV negative and HCV RNA negative in 1995-1996 following blood transfusions received 1983-1992

Recip-ient ID	Sex	M/Yr Trans-fused	Age Trans-fused	Trans-fusion confirmed	Don-or ID	Donor anti-HCV	Donor HCV RNA	Donors infection date
105	M	Oct-83	31	Yes	7	Pos	Pos	NK
57	M	Jun-86	18	Yes	9	Pos	Pos	NK
17	M	Aug-84	27	Yes	10	Pos	Pos	1989
41	F	Feb-86	25	No	10	"	"	"
81	M	Nov-87	64	Yes	11	Pos	Pos	1988
9	F	Jun-85	53	No	11	"	"	"
75	F	Feb-85	61	Yes	13	Pos	Pos	1989
90	F	Jun-91	35	Yes	14	Pos	Pos	1983
111	F	May-86	75	Yes	14	"	"	"
53	F	Jun-87	62	Yes	15	Pos	Pos	1990
52	F	Mar-90	33	Yes	18	Pos	Pos	1978
116	M	Jun-91	27	No	25	Pos	Pos	NK
4	M	Jul-91	73	Yes	30	Ind	Neg	1984
86	M	Jan-88	55	Yes	30	"	"	"
68	M	Nov-85	18 months	No	30	"	"	"
67	F	Oct-83	54	Yes	50	Pos	Pos	NK
46	F	Oct-89	40	Yes	54	Pos	Pos	1985
2	F	Jan-83	25	No	57	Pos	Pos	1965
49	F	Jan-84	9	Yes	57	"	"	"
33	M	Feb-90	28	Yes	66	Ind	Neg	NK
59	F	Feb-89	24	Yes	66	"	"	"
21	M	Aug-91	1	No	66	"	"	"
14	M	Feb-90	2 months	No	66	"	"	"
55	M	Mar-90	62	No	66	"	"	"
29	M	Aug-90	12	Yes	66	"	"	"
54	M	Sep-86	54	Yes	76	Ind	Neg	NK
94	M	Mar-87	2 months	Yes	77	Ind	Neg	NK
5	M	Oct-89	43	No	77	"	"	"
79	M	Mar-89	5 days	Yes	77	"	"	"
3	F	May-91	59	No	77	"	"	"
23	M	Mar-85	12 days	Yes	77	"	"	"
38	M	Apr-85	73	Yes	77	"	"	"
24	F	Sep-89	9	No	77	"	"	"
45	F	Mar-87	10	No	77	"	"	"
22	F	Jul-90	75	Yes	78	Ind	Neg	NK
50	M	Jan-86	54	Yes	79	Ind	Neg	NK
108	F	Feb-91	78	Yes	79	"	"	"
16	M	Oct-83	30	Yes	80	Ind	Neg	NK
104	M	Feb-83	64	No	84	Pos	Neg	NK
51	M	Jun-89	77	Yes	84	"	"	"

Table 3.1: 49 Recipients who were anti-HCV negative and HCV RNA negative in 1995-1996 following blood transfusions received 1983-1992 (continued)

Recip-ient ID	Sex	M/Yr Trans-fused	Age Trans-fused	Trans-fusion confirmed	Don-or ID	Donor anti-HCV	Donor HCV RNA	Donors infection date
101	M	Jan-92	54	Yes	94	Pos	Pos	1992
36	M	Mar-93	70	Yes	95	Pos	Pos	1993
92	F	Oct-92	53	Yes	95	"	"	"
87	M	Feb-93	14	Yes	129	Pos	Pos	1994
112	M	Feb-89	11 days	Yes	133	Ind	NT	NK
39	M	Jul-88	58	Yes	135	Ind	NT	NK
31	M	Feb-88	44	No	137	Ind	NT	NK
8	M	Sep-90	78	Yes	145	Pos	Neg	NK
91	M	Oct-86	45	Yes	146	Ind	Neg	NK

B). Anti-HCV positive and HCV RNA negative ***(see table 3.2)***

Eleven recipients, found to have anti-HCV only, were transfused from 9 donors who had evidence of chronic HCV infection. These recipients have been seen in the Oxford HCV clinic and have been retested several times over a period of 6-18 months, showing consistently normal liver transaminases and negative PCR results. None have symptoms or signs of liver disease. No liver biopsies have been done in this group of patients, but they will be continued to be followed up in order to confirm absence of infection. Seven out of the 9 donors for this group are linked to other recipients who were found to be HCV RNA positive, as can be seen from **table 3.3**.

Table 3.2: 11 Recipients who were anti-HCV positive but HCV RNA negative in 1995-1996 following blood transfusions received between 1987-1991

Recipient ID	Sex	M/Yr Transfused	Age Transfused	Donor ID	Donor anti-HCV	Donor HCV-RNA	Genotype of donor
82	F	Dec-90	23	16	Pos	Pos	1a
93	M	Aug-91	3 days	18	Pos	Pos	3a
56	F	Feb-90	2 days	18	"	"	"
27	F	Aug-89	55	53	Pos	NT	2b
11	F	Oct-87	39	64	Pos	Pos	5
89	M	Mar-90	7	65	Pos	Pos	1a
80	F	Aug-91	62	72	Pos	Pos	NT
115	F	Jun-91	29	114	Pos	Pos	1a
95	M	Jul-86	30	128	Pos	Pos	1b
65	F	Oct-87	30	128	"	"	"
110	F	Dec-90	25	144	Pos	Pos	2b

C). Anti-HCV and HCV RNA both positive ***(see table 3.3)***

Forty four recipients in this category were transfused from 29 donors. This includes 42 recipients transfused with blood from 27 donors identified as sero positive, and 2 recipients transfused in 1992 and 1993 from 2 blood donors (Donor-ID 94 and 95) whose blood had given clearly negative results in routine HCV screening tests. A subsequent donation from each of the two donors was positive for anti-HCV and HCV RNA, showing that these donors were almost certainly viraemic in 1992 and 1993, but had donated blood during the period before appearance of detectable anti-HCV. Genotyping in both donor-recipient pairs showed matching virus types.

HCV genotype in 25 donors showed variety genotypes (6 with type 1a, 5 with 1b, 3 with 2b, 10 with 3a and 1 with type 5). Thirty five recipient samples were tested and all the HCV genotypes seen in the donors were found in the recipients. There were 8 with type 1a, 5 with 1b, 3 with 2b, 18 with 3a and one with type 5. Thirty three donor recipient pairs showed matching HCV genotypes, providing supporting evidence of transfusion- transmitted infection. There were no genotype mismatches in the donor-recipient pairs.

Of the 44 antibody and RNA positive patients, 28 have been investigated in the Oxford HCV clinic and 4 are being seen by liver specialists in other hospitals. 11 recipients have not been referred as they are elderly and/or have other significant medical history. None of these unreferred patients have symptoms suggestive of liver disease. One recipient, a 23 year old asymptomatic female has not yet agreed to a referral.

Most of the referred patients were asymptomatic, but four complained of lethargy, one of nausea, and one had symptoms and signs consistent with cirrhosis (ascites and encephalopathy). This male patient who was aged 63, when transfused in 1989, developed hepatocellular carcinoma and died in 1997. In this group of patients transaminase results were normal in 21/44 (48%), abnormal in 17/44 (39%), and fluctuating between the normal and above normal range in 6/44 (13%). Liver biopsy was recommended to all patients with evidence of continuing viraemia (PCR positive on two or more occasions). Twenty three of the 28 patients being investigated in the Oxford clinic have been biopsied. Five patients have not been biopsied; one patient became PCR negative when retested in the clinic, one patient declined a liver biopsy, two patients liver biopsy was contra-indicated due to abnormal blood coagulation and one patients is still awaiting biopsy.

Liver biopsy results in 23 patients were classified by the system proposed by Ishak, giving numerical scores for the degree of liver inflammation (0-18) and the degree of fibrosis (0-6). Seven recipient, all female, with a mean age of 33 years when transfused 4 - 12 years earlier, showed no liver fibrosis. Sixteen recipients, 8 male and eight female, showed mild to moderate the fibrosis. Mean age of these 16, together with the man with HCC, was 46 years when transfused 5 - 13 years earlier. The clinical findings and liver biopsy results are summarised in **table 3.3**.

Table 3.3: 44 Recipients with chronic HCV (HCV RNA positive)

Recip-ient ID	Sex	M/Yr transfused	Age Trans-fused	LFT	Symp-toms	Histological inflammation	Inflam matory score	Fibrosis	Fibrosis score	Don-or ID	Genotype Donor	Recipient
63	F	Apr-84	36	Normal	None	mild	2	None	0	8	1a	1a
99	M	May-87	55	Abnormal	None	mild	2	mild	1	8	1a	
32	M	May-88	61	Normal	None	moderate	4	moderate	3	8	1a	1a
100	F	Jun-84	54	Normal	None	mild	2	mild	1	9	1b	1b
84	F	Dec-90	34	Abnormal	None	moderate	5	mild	1	9	1b	1b
83	F	Jun-91	36	Abnormal	Lethargy	mild	2	moderate	2	16	1a	1a
42	F	Jan-91	26	Normal	None	mild	2	none	0	17	1b	1b
26	F	Sep-91	80	Normal		*No biopsy*				18	3a	3a
69	M	Apr-91	33	Abnormal	None	*Not referred*				18	3a	3a
25	M	Sep-90	40	Abnormal	None	mild	2	mild	1	18	3a	3a
6	M	Jun-89	25	Normal		*Refused biopsy*				19		
12	M	Feb-91	68	Normal	None	*Now PCR neg*				20	3a	3a

Recipient ID	Sex	M/Yr transfused	Age Transfused	LFT	Symptoms	Histological inflammation	Inflammatory score	Fibrosis	Fibrosis score	Donor ID	Genotype Donor	Recipient
62	F	Apr-90	27	Normal	None	minimal	1	none	0	22	3a	3a
34	M	Apr-91	40	Abnormal	Nausea	moderate	6	moderate	2	22	3a	3a
15	F	Dec-90	55	Abnormal	None	*No biopsy (on warfarin)*				24		3a
103	M	May-91	56	Abnormal	Lethargy	moderate	5	moderate	2	24		3a
107	F	Aug-89	27	Fluctuating	None	moderate	4	moderate	2	27	1b	1b
58	F	Jun-91	69	Normal		*Not referred*				31	3a	
13	F	Aug-89	31	Normal	None	minimal	1	none	0	50		
10	M	Apr-89	63	Abnormal	Ascites	No biopsy		**Hepatoma**		51	3a	3a
74	F	May-88	29	Fluctuating	None	mild	2	mild	1	53	2b	2b
1	F	Jun-85	45	Normal		*Unrelated death 1996*				55	3a	3a
47	M	May-88	67	Normal		*Not referred*				55	3a	3a
76	M	Oct-89	76	Abnormal		*Not referred*				56	3a	3a
64	F	Jul-83	54	Normal	None	moderate	4	moderate	2	57	1a	1a

Recipient ID	Sex	M/Yr transfused	Age Transfused	LFT	Symptoms	Histological inflammation	Inflam matory score	Fibrosis	Fibrosis score	Donor ID	Genotype Donor	Recipient
18	M	Feb-87	68	Abnormal		*Not referred*				60	1a	1a
19	F	Sep-87	44	Fluctuating	Lethargy	minimal	1	mild	1	61	3a	3a
98	F	Jul-88	44	Fluctuating	None	mild	2	none	0	61	3a	3a
40	M	Jul-87	47	Normal		*Not referred*				61	3a	3a
73	M	Feb-89	44	Abnormal	None	moderate	5	moderate	2	63		
66	M	Jul-91	83	Abnormal		*Not referred*				64	5	5
7	M	Mar-90	6	Normal	None	No biopsy				65	1a	1a
20	F	Apr-89	18	Normal		No biopsy				65	1a	
43	M	Sep-88	55	Abnormal		*Not referred*				65	1a	1a
114	F	Oct-90	40	Normal	None	No biopsy				65	1a	
35	F	Aug-91	23	Normal	None	moderate	4	None	0	73	3a	3a
97	F	Dec-92	44	Abnormal	None	moderate	4	none	0	94	1a	1a
71	F	Sep-93	82	Normal		*Not referred*				95	2b	2b

Recipient ID	Sex	M/Yr transfused	Age Transfused	LFT	Symptoms	Histological inflammation	Inflammatory score	Fibrosis	Fibrosis score	Donor ID	Genotype Donor	Recipient
88	F	Mar-89	18	Normal		*Not referred*				103	1b	1b
72	F	Feb-87	66	Normal		*Not referred*				103	1b	
113	F	Feb-87	42	Abnormal	Lethargy	mild	2	mild	1	112		
30	M	Sep-90	53	Abnormal		*Not referred*				143	3a	3a
48	M	Jan-89	47	Fluctuating	None	moderate	4	moderate	3	143	3a	3a
102	M	Jun-91	33	Fluctuating	None	moderate	5	mild	1	144	2b	2b

Statistical analysis

Statistical analyis was performed using logistic regression analyis comparing the 44 PCR positive patients with the 11 antibody positive/PCR negative patients combined with the three recipients with no detectable anti-HCV or HCV RNA after seven to 13 years despite of strong evidence of transfusion with infectious blood. These 14 people, four of whom were young children, had a mean age of 27 years when transfused with a sex ratio of four males to 10 females. By comparison, the 44 recipients with chronic hepatitis C infection had mean age at transfusion of 46 years, with 10 recipients over the age of 60 and the sex ratio in this Group was 20 males to 24 females.

Logistic regression analysis indicates that the age difference in the two groups did not quite achieve statistical significance ($p>0.05<0.1$) and that numbers were too small to deduce any influence of sex on the outcome of infection.

3.5 DISCUSSION

The look-back exercise has identified a group of mainly asymptomatic blood transfusion recipients who were infected with hepatitis C at a known time, making them an ideal group of all ages in which to study the natural history of this infection. The pilot study by Ayob et al predicted that it might be possible to find approximately one infected recipient per donor. At Oxford 44 recipients with chronic infection were found and were linked to 43 of the 77 antibody positive donors. Twenty-eight recipients and 22 of the linked donors along with seventy other former donors with HCV infection are now being followed in the Oxford HCV clinic. Unless the are contradictions, patients who are HCV RNA positive are recommended to have a liver biopsy. If there is histological evidence of moderate to severe liver disease these patients are recommended to have anti-viral drugs. For patients with mild liver disease the pros and cons of treatment are weighed up and in all cases patients are encouraged to share in decision making about their management. Our approach in the clinic is similar to that suggested by Foster et al in the BMJ in 1997[109].

Thirty-nine of the 104 tested recipients had probably not received infectious blood, because 13 of the linked donors showed only anti- HCV and no HCV RNA, and 7 more donors were known to have been infected at later dates. Ten recipients, transfused from 7 other donors who were anti- HCV and HCV RNA positive, showed no sign of infection; some may have recovered, and some may not have received infectious blood; for example, the donors might have acquired their infections at later dates, or the transfusions might not have been given since in four cases confirmation of transfusion was not found in the hospital notes. The potential infectivity of donor blood collected between 1983 and August 1991 could not be tested directly as no donor serum samples had been stored during that period. The Oxford study revealed no infected recipients from 11 anti- HCV indeterminate donors, but the numbers are small and accumulated figures from the national study will probably show that a few currently RIBA indeterminate donors did transmit HCV. In most centres, as at Oxford, it will probably not be possible to re- test the relevant blood donations from archives samples. It is now known that some HCV infections resolve spontaneously, and that test results for HCV may become weaker or disappear with time. Therefore an attempt to trace all potentially infected recipients must include donors with currently indeterminate RIBA results as

defined above. The Oxford study showed no recipients with detectable HCV RNA when anti-HCV tests were negative. This would possibly occur only rarely in immunodeficient patients; the combined national results will show if any such cases have been detected.

A recent report on needle stick injuries showed that people who were HCV RNA positive transmitted infection, whereas HCV RNA negative people did not. Likewise in the Oxford study it was shown that 35 HCV RNA positive donors transmitted infection in 55 /58 cases (95%). Forty-four out of 58 (76%) of the infected recipients were persistently HCV RNA positive after intervals of 4 - 13 years. Eleven other recipients were in good health and had indications of recovery from infection since they were repeatedly negative for HCV RNA although anti- HCV positive. There were three recipients with no detectable anti-HCV or HCV RNA after seven to 13 years despite of strong evidence of transfusion with infectious blood. These 14 people, four of whom were young children, had a mean age of 27 years when transfused with a sex ratio of four males to 10 females. By comparison, the 44 recipients with chronic hepatitis C infection had mean age at transfusion of 46 years, with 10 recipients over the age of 60 and the sex ratio in this Group was 20 males to 24 females. Logistic regression analysis indicates that the age difference in the two groups did not quite achieve statistical significance ($p>0.05<0.1$) and that numbers were too small to deduce any influence of sex on the outcome of infection. However the findings accord with the report by Poynard[27] who found that age at infection together with male sex, influenced the outcome more than differences in virus genotypes. In a study as in theirs, liver biopsies in the younger patients showed less fibrosis. Also as found by Poynard, we found a variety of HCV genotypes in the recipients with chronic infection and the same range of genotypes was present in donors linked to recipients who had recovered. This gives no indication of a more severe affect due to a particular virus genotype.

The follow up of these patients infected at a known time point is still relatively short, as HCV is known to cause serious liver damage only after decades of infection. Therefore monitoring of these patients is essential and continues in our Oxford HCV clinic. In addition combination of all the different Blood Service Units from around the nation will make such studies more powerful. Further results from the National HCV look back study will provide valuable information about factors that influence the outcome of HCV infection. Moreover many transfusion recipients and former blood donors with chronic HCV infection will now, as a result of the look Back programme,

receive long term specialist management which will allow earlier diagnosis, counselling. health education and treatment.

In summary, results from the Oxford part of the National HCV look back study showed that 14/58 (24%) of those infected has probably made a full recovery from hepatitis C, but that 44/58 (76%) has become chronic virus carriers and 17 of these had histological evidence of liver disease 4 - 13 years from the date of infection. Our results have shown that the outcome of patients in terms of becoming infected, developing chronic infection or developing liver disease is variable and that host factors play a larger role in determining outcome than viral factors.

CHAPTER 4:
CLINICAL OUTCOME OF HYPOGAMMAGLOBULINAEMIC PATIENTS FOLLOWING AN OUTBREAK OF ACUTE HEPATITIS C

4.1 SUMMARY

In 1994, an outbreak of Hepatitis C virus infection, genotype 1a, occurred in hypogammaglobulinaemic patients in the U.K from one batch of contaminated anti-HCV screened intravenous immunoglobulin. Patients exposed to this batch were identified and prospectively followed up to assess what proportion became infected with HCV, what proportion became infected but spontaneously cleared the virus and what proportion cleared the virus with early treatment with alpha-interferon. In addition clinical outcome data was evaluated at two years follow up and is still ongoing. Data was collected using standardised questionnaires.

Thirty six patients were exposed to the batch; 30 became acutely infected. Infected patients were considered for early treatment with interferon. Five patients with secondary hypogammaglobulinaemia due to lymphoid malignancy were not treated and all have died of their primary malignancy. Of 25 patients with primary hypogammaglobulinaemia, 1 resolved HCV infection before treatment, 17 commenced on treatment within the first year of infection, and 6 declined or treatment was contraindicated. One patient, a child, was treated at a later stage. Thirteen of the initial seventeen treated patients completed therapy and 7 (54%) had a sustained response (normal transaminases, negative serum HCV RNA) at six and twelve months post treatment. All these patients remain HCV RNA negative to the present day.

Liver biopsy was performed in patients not clearing HCV and was abnormal in all. Eight out of 25 patients with primary hypogammaglobulinaemia have died: 3 from decompensated cirrhosis, 2 from pneumonia but had evidence of liver failure and 3 from liver unrelated causes. One further patient developed decompensated cirrhosis but has been successfully transplanted.

Conclusions: HCV can cause rapid severe liver disease in hypogammaglobulinaemic patients. Early treatment with high dose alpha-interferon results in a high clearance of HCV.

4.2 BACKGROUND

In February 1994, Baxter Healthcare Ltd. withdrew Gammagard, an intravenous preparation of human immunoglobulin (IvIg) because of case reports of acute Hepatitis C infection associated with its use[110]. Transmission had occurred despite the introduction of anti-HCV screening of each plasma donation contributing to the large plasma pools (~15,000 donations from ~2,000 donors) from which the product was derived. Since then over 200 hundred suspected cases have been reported world-wide, associated with several different batches of the product which have been shown to contain HCV RNA[111]. Within the UK, only one batch was suspected of transmission[112]. Accurate documentation of batch numbers by both the suppliers and prescribers enabled the identification and subsequent follow-up of all exposed individuals

Experience of HCV in hypogammaglobulinaemic patients infected following infusion of IvIg suggested that the liver disease could be rapidly progressive and responded poorly to alpha-interferon[113-116]. These small studies of alpha-interferon treatment were in patients with chronic infection of undefined length and with unknown or mixed genotypes of HCV. However the UK Gammagard outbreak provides a unique opportunity to study the rate of progression of HCV in hypogammaglobulinaemic patients with known onset of infection from a single source, as well as their response to therapy. We chose therapy in the acute phase with high dose alpha-interferon as greater efficacy had been suggested with higher doses[117;118] and early treatment[119;120].

4.3 METHODS

Patients

Early in 1994, abnormal liver function test results were detected on routine testing among patients in the United Kingdom undergoing intravenous immunoglobulins therapy using the brand Gammagard. At the same time, several cases suspected acute hepatitis C infection were reported after the use of the same product in Sweden and Spain. It became rapidly clear that in the United Kingdom and Spain, all patients developing possible HCV infection had been treated recently with one batch of a product (93F21AB11B - Gammagard). Data were obtained from the manufacturer detailing all centres in the United Kingdom supplied with Gammagard since 1991. This data was used to identify the centres where the implicated batch had been used and thus identify the patients who had received the batch.

Thirty six patients received the implicated batch from 19 local centres in the United Kingdom. Also identified were 12 patients who have received Gammagard over the same time period but had not received this particular batch. These patients came from throughout the UK and were under the supervision of their individual regional immunology centres. Questionnaires were sent to the physicians looking after the 48 patients who had received any of the batches Gammagard at the 19 local centres during the last 14 months. The questionnaire included centre details, individual patient details (indication and history of immunoglobulin therapy, history of previous liver disease, details of current episode, and risk factors for HCV and laboratory results). Any available laboratory data for the year before exposure were collected for each case including liver function tests. To confirm infection serum was tested for HCV RNA by PCR.

Three further questionnaires were sent out over a five year follow up period. The second questionnaire collected information on the initial results of interferon therapy. This questionnaire detailed the clinical details of the patients along with the results of serial liver function tests, PCR tests and liver histology if available. The third questionnaire obtained details on the outcome of patients at 2 years follow up and the final questionnaire, on the outcome and any further treatment of patients at 5 years follow up. All details were recorded on a computer database.

Virology

Patients were tested for antibody responses to HCV along with HCV RNA by reverse transcription polymerase chain reaction for the 5'non- coding region.

In order to establish whether the infection was caused by the same HCV isolate the virus was genotyped and subtyped in a representative number of samples from the different centres. Analysis was performed on a segment (222 base pairs: positions 7975-8196) of the NS-5 gene, which was amplified, sequenced, and analyzed as previously described[121]. The phylogenectic relationship between the nucleotide sequence amplified from the patients along with unrelated type 1a variants from the United States and Europe was compared. Analysis also included sequence of the viral isolate identified in Gammagard batch. Sequence distances were calculated using DNAML program (in PHYLIP inference package, version 3.5, University of Washington, Seattle, WA). Control sequences were obtained from published sources.

Treatment protocol

Alpha-interferon therapy was considered for all patients with proven HCV infection. In view of the previously reported severity of HCV infection amongst patients with primary antibody deficiency and the iatrogenic nature of transmission, the UK Gammagard Users Group considered that it would be unethical to withhold interferon therapy from the patients with primary hypogammaglobulinaemia, although in six treatment was contraindicated or declined. Therapy was recommended at 6 million units of alpha-interferon, three times weekly, subcutaneously for a planned period of 6 months according to a standard protocol; the dose could be reduced by the supervising physician if clinically indicated. Response was measured by both improvement in liver enzyme levels and clearance of circulating HCV RNA detected by RT-PCR. A sustained response was defined as normalisation of liver transaminases and absence of HCV RNA from the serum at both 6 and 12 months post therapy. Treatment and follow-up data was collected centrally by the use of the serial data sheets. The dates of treatment, dosage, duration and side-effect were recorded. Patients with hypogammaglobulinaemia secondary to malignancy were not offered therapy.

4.4 RESULTS

Association of HCV infection with batch numbers of Gammagard

Forty-six out of the 48 initial questionnaires sent were returned. The batch number of Gammagard was known in 27 out of the 46 patients identified as having received Gammagard infusions during the period 1993-1994. Fourteen of these patients were HCV-RNA positive and 13 were HCV-RNA negative. To establish which batch was associated with the transmission of HCV in these cases, univariate analysis was performed on each batch compared with HCV RNA detection. Only one of 17 batches (93F21AB11B - Gammagard) was positively associated with transmission (χ^2 test, 17.1; P< 0.00003 Fisher's Exact test). The relative risk for being HCV-RNA positive and having used the batch was 9.88 (confidence limits, 1.5- 64.92) with an odds ratio of 51.33 (confidence limits, 4.84 - 1315).

Virological investigation

Thirty six UK patients had received treatment with this HCV contaminated batch of IvIg. One patient was already infected with HCV from an earlier gammaglobulin outbreak. This patient has subsequently had a liver transplant but has now died. One patient, a Greek child who had left the country, was not tested. Four patients were documented as having received the batch but tested HCV RNA negative and antibody negative on at least three occasions up to 4 months after the exposure.

Thirty patients were shown to have developed acute infection with HCV. Twenty eight of thirty patients were repeatedly positive for HCV RNA by reverse transcriptase polymerase chain reaction after exposure to the batch. The remaining two patients although they were not documented as having HCV RNA, developed abnormal liver function tests and sero-converted to anti-HCV positive. The liver function tests of one of these two patients subsequently returned to normal and as he has never been found to be positive for HCV RNA, is therefore considered to have resolved the HCV infection acutely. The second was a patient with myeloma. His liver function tests did not return to normal and his negative PCR test may therefore be a false negative result; the high paraprotein level in his serum may have interfered with the assay.

The mean length of time from exposure to the batch and the first positive HCV PCR test was 37.5 days (range 7 to 71 days) with 92.3% of patients being positive on the first test. PCR was either perfomed on stored archive patient sera if available or at the time of identification of the outbreak. Twenty of these viral isolates underwent sequence analysis revealing identical isolates of HCV genotype 1a.

Patient Details

None of the patients had a history of illicit drug use, tattoos, or sexual contact with individuals with hepatitis. Patients can be divided into patients who became infected, and exposed but uninfected patients.

Infected patients: The indication for gammaglobulin therapy can be categorised into two main groups: patients with primary antibody deficiency (16 common variable immunodeficiency, 7 IgG subclass deficiency, 1 X-linked agammaglobulinaemia, 1 drug induced hypogammaglobulinaemia) and patients with immune paresis secondary to malignancy (3 chronic lymphocytic leukaemia (CLL), 2 myeloma). The diagnosis of primary hypogammaglobulinaemia was made using standard criteria[122]. The average age of these 30 patients was 42 with 43% of patients being female.

Exposed but uninfected: Four patients were exposed to the batch but had no findings of HCV viraemia or anti- HCV seroconversion. Due to the small number of exposed but uninfected patients it is difficult to make comparisons with the infected group. The average age of this group was younger at 32 and had a higher proportion of women (50%) but this was not statistically significant. There was no difference in the indication for immunoglobulins (2 CVID and 2 Ig subclass deficiencies), or dose of the implicated batch (see Table 4.2).

Table 4.1: Clinical details of patients receiving Gammagard at the beginning of 1994

Clinical features	Exposed /Uninfected	Exposed /Infected	Unexposed to batch
No. of patients	4	30	12
Male/Female	2/2	17/13	5/7
Age (yr, mean (range)	32 (17-55)	42 (4-75)	36 (22 - 51)
Indication for IV Ig:			
CVID	2	16	10
Ig subclass deficiency	2	6	1
Chronic lymphocytic leukaemia		3	
Myeloma		2	
X-linked gammaglobulinaemia		1	
Drug induced gammaglobulinaemia		1	
Intestinal lymphangiectasia		1	
X-linked hyper IgM syndrome			1

Acute clinical features

Symptoms: Nine patients exposed to the batch developed jaundice. Eighteen patients developed the following symptoms: malaise in 13, dark urine in 3, pruritus in 2, and food intolerance or nausea in 4. All of the patients not exposed to the batch and the 4 patients who were exposed but did not become infected had no symptoms of liver disease.

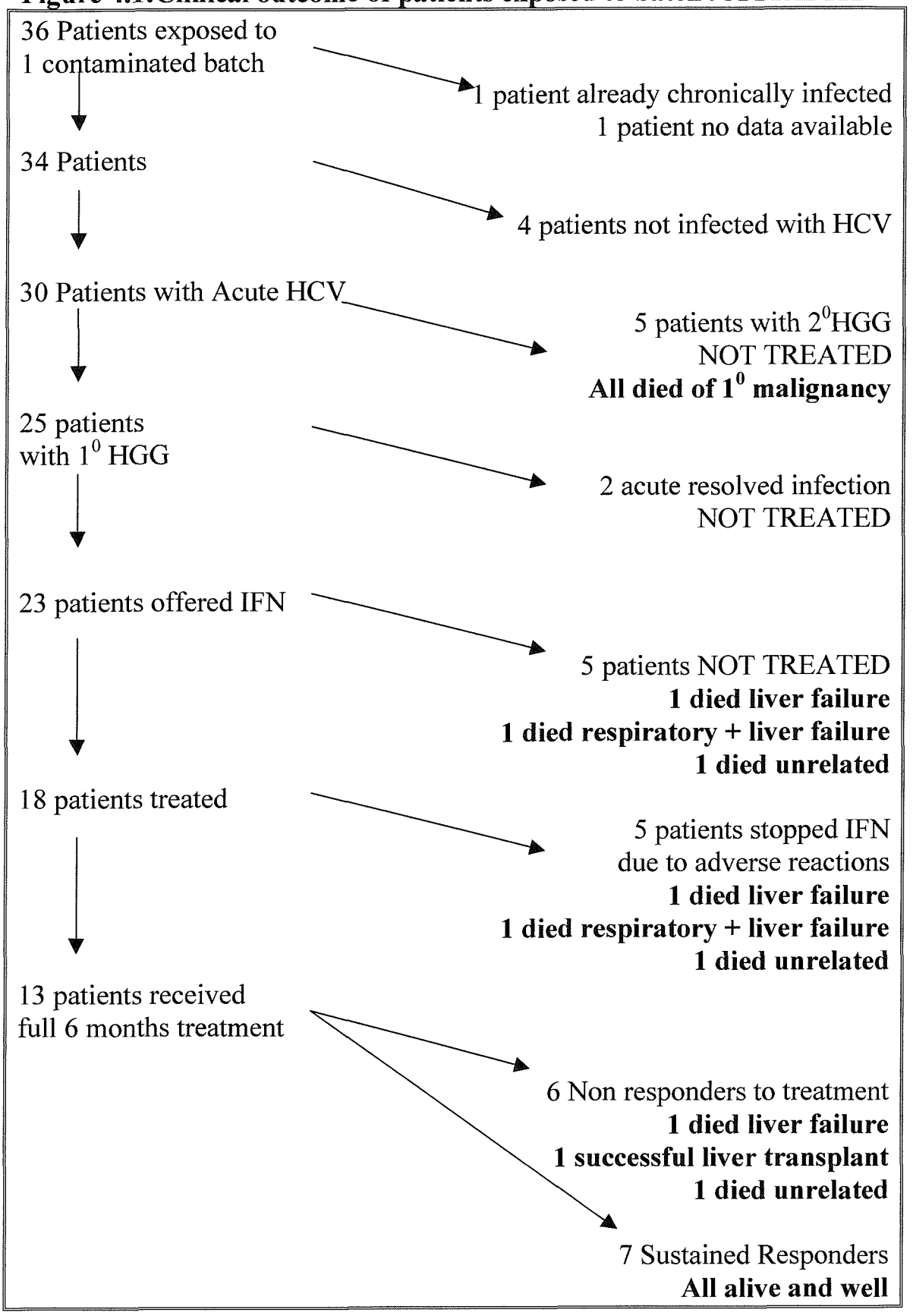
Liver Function Tests: Twenty seven had abnormal liver enzymes within four months after the first exposure to the contaminated batch. The mean time from the individual exposure to the batch to the first documented abnormal liver transaminase level was 44.59 days (range, 4 - 119 days; N=22; with 60% of values abnormal on the first available testing after exposure). None of the 4 patients exposed to the batch but who remained uninfected had any abnormality in the liver function tests. Only one of the 12 patients not exposed to the batch had any documented change in liver function tests results; this change was transient after a chest infection treated with broad spectrum antibiotics.

Clinical follow-up

Thirty patients with acute HCV infection were followed and a summary of their clinical outcome are summarised in **figure 4.1**. The five patients with malignancy (3 CLL, 2 myeloma) were considered inappropriate for therapy with alpha interferon in view of their underlying condition. One patient, as discussed above, resolved the HCV infection acutely.

The remaining 24 patients were considered for therapy with alpha-interferon of which 17 patients started treatment. One patient, a child, was treated at a later stage two years after exposure. Six patients did not receive interferon; 3 declined therapy and therapy was contra-indicated in 3 patients, in view of other severe medical conditions (severe depression, granulomatous liver disease, chronic pulmonary suppuration).

Figure 4.1:Clinical outcome of patients exposed to batch 93F21AB11B



Treatment group ***(see table 4.2)***

Seventeen patients with primary antibody deficiency were commenced on treatment with interferon initially (13 with common variable immunodeficiency, 3 with IgG deficiency and 1 with X-linked agammaglobulinaemia). The mean age of the treated group was 40.4 years, with a male to female ratio of 10:7. The mean time between exposure to the infected batch and commencement of treatment was 4.3 months (range 1-9 months). The majority of patients (14/17) started treatment within 6 months of inoculation. Fourteen patients commenced therapy at full dose and three patients started at reduced dose. Four patients withdrew from treatment before the end of the six months (including all three whom started at a reduced dose). Reasons for withdrawal included severe lethargy (2 patients), epistaxis/thrombocytopaenia (1 patient) and depression (1 patient). Thirteen patients completed therapy with interferon, 11 achieving the full cumulative dose and 2 patients received 80% and 60% of the full dose each (although five patients required a reduction in the dose, three of these patients received longer than 6 months therapy and therefore received the full cumulative dose). The child treated at a later stage, did not tolerate treatment and failed to respond.

Liver enzymes returned quickly (within one month) to pre-treatment levels in 11/13 patients completing treatment with interferon-alpha. Rapid biochemical (within 1 month) and viraemic relapse was seen in 6 patients following cessation of treatment. Liver enzymes have remained normal in 7 patients, all of whom have shown a sustained response with a persistently negative testing for HCV RNA by PCR at six months and 12 months after treatment with alpha-interferon. These patients have now been followed for five years and remain free of HCV by PCR testing with no signs of liver disease. This represents a sustained response rate of 41% of those commenced on interferon or 54% of patients who completed therapy. Clinical responses of two typical cases (one sustained response, one rapid relapse) are shown in **figure 4.2**.

There was no difference between the age of patients who had a sustained response to interferon and patients unresponsive to treatment. We also found no correlation with IgG, IgA or IgM levels with response to treatment.

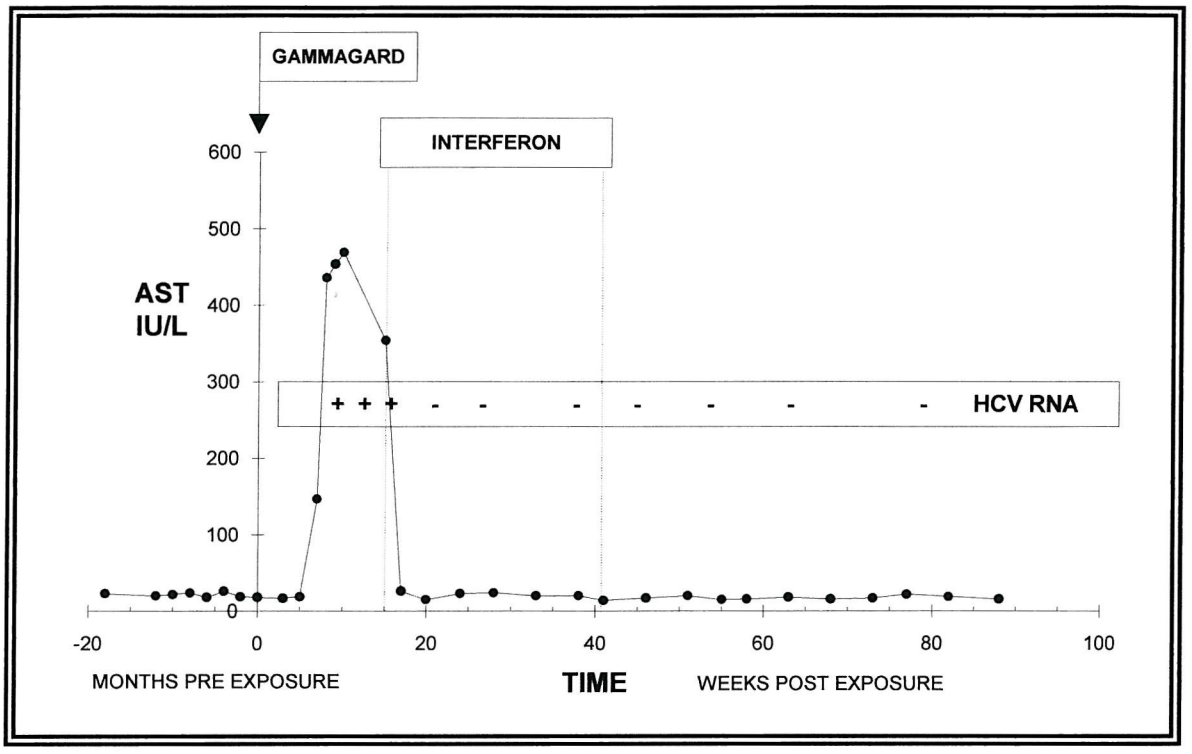
Table 4.2: Clinical details of patients with primary antibody deficiency treated with alpha-interferon

Pt	Disease	Sex	Time to first dose	Initial dose	Dose modifications	Clinical Outcome
1	CVID	M	3 month	Full	none	Sustained response
2	CVID	F	3 month	Full	none	Sustained response
3	CVID	F	2 month	Full	none	Sustained response
4	CVID	M	1 month	Full	none	Sustained response
5	CVID	M	3 month	Full	halved at 3/12* - lethargy	Sustained response
6	IgG def	F	3 month	Full	halved at 2/12* - lethargy	Sustained response
7	CVID	M	9 month	Full	halved at 3/12* - thrombocytopaenia	Sustained response
8	CVID	M	6 month	Full	none	Relapsed. Treated with IFN + Ribavirin - Relapsed
9	CVID	M	4 month	Full	none	Relapsed. 2nd course of IFN given
10	CVID	M	9 month	Full	halved at 4/12 - lethargy	No response. Ribavirin and IFN. Liver transplant. Alive
11	IgG def	M	1 month	Full	halved at 3 months - lethargy*	Relapsed. Treated with Ribavirin + IFN - Relapsed
12	CVID	F	5 month	Full	halved at 3 months - thrombocytopaenia	Failed to respond. Died following Liver transplant
13	IgG def	F	4 month	Full	halved for 1 month - lethargy*	Relapsed following IFN. Died unrelated
14	CVID	M	7 month	Half	stopped at 2/12 - severe lethargy	Died liver failure
15	CVID	F	6 month	Half	stopped at 3/12 - severe lethargy	PCR positive, abnormal LFT. Asymptomatic
16	XLA	M	4 month	Half	stopped at 2 months - depression	PCR positive, abnormal LFT. Died unrelated
17	CVID	F	2 month	Full	stopped at 2 months -thrombocytopaenia	Died respiratory failure Evidence of liver failure
18	CVID	F	29 month	Half	Stopped at 5 months	IFN & amantadine – no response Abn LFT. Asymptomatic

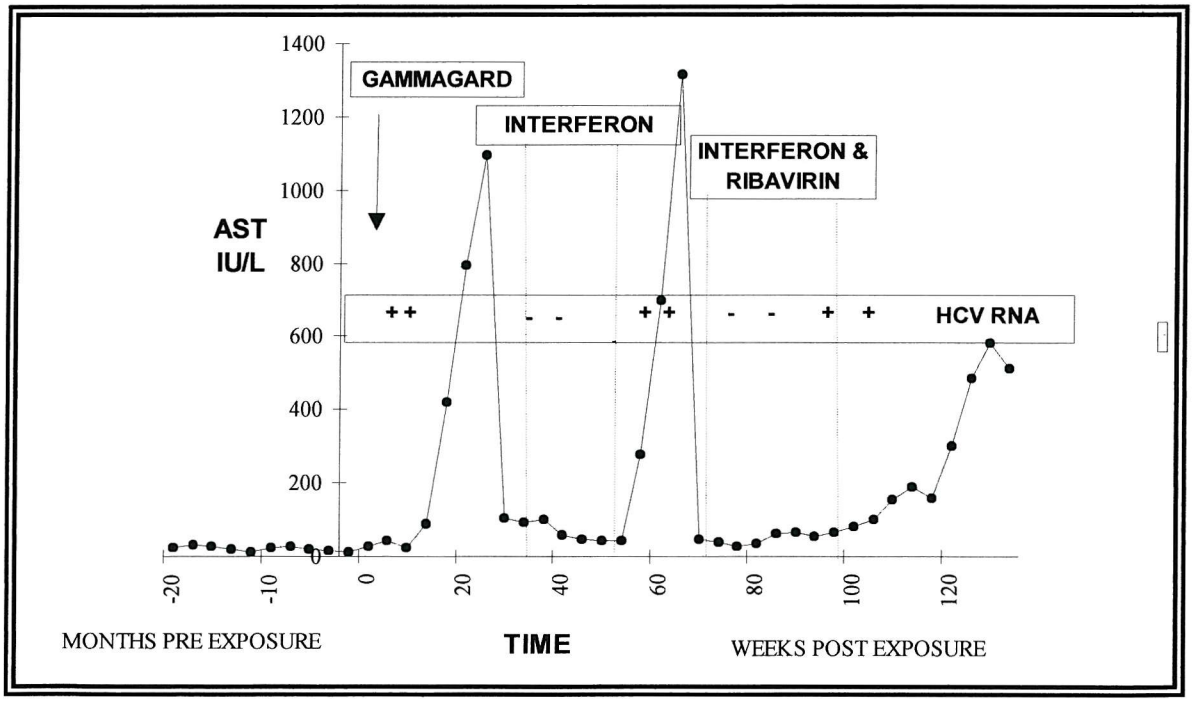
* Continued therapy for longer than 6 months and therefore received full cumulative dose of IFN

Figure 4.2: Clinical response in a patient with A. Sustained response to interferon B. Relapse following interferon

A.



B.



Untreated Group

Twelve patients received no treatment - five with secondary hypogammaglobulinaemia due to lymphoid malignancy and seven with primary hypogammaglobulinaemia.

The five patients with secondary hypogammaglobulinaemia have died of their malignancy within the two year follow-up period, without evidence of hepatitis contributing to their deaths. One of these patients, as discussed above, never became PCR positive but seroconverted within four months of infection and had continuing biochemical evidence of hepatitis.

Of the seven untreated patients with primary hypogammaglobulinaemia, two are HCV PCR negative. One of these patients cleared HCV before treatment could be commenced (considered acute clearance of HCV infection - see before). One other patient, who refused treatment, became HCV PCR negative after 6 months; both patients remain well. One additional patient has died of unrelated causes. Five patients remain HCV PCR positive. In total 2 out of 13 patients not treated with interferon have appeared to clear HCV without interferon treatment.

Disease progression in patients who remained chronically infected with HCV

Sixteen patients became chronically infected with HCV. Five patients did not receive treatment (2 declined therapy and therapy was contra-indicated in 3 patients, in view of other severe medical conditions - severe depression, granulomatous liver disease, chronic pulmonary suppuration). Eleven patients received treatment but did not respond to interferon monotherapy. Following initial treatment, 1 patient was given further interferon, 3 patients were treated with combination therapy of interferon and ribavirin and one patient combination therapy with interferon and amantadine. None of these patients had a sustained response to further therapy.

Within five years, half (8/16) of these chronically infected patients have died: 3 from decompensated cirrhosis, 2 from pneumonia but had evidence of liver failure and 3 from liver unrelated causes. One further patient developed decompensated cirrhosis but has been successfully transplanted. His immediate post-transplant course was uncomplicated. Five months after transplantation the patient developed fibrosing cholestatic hepatitis. Withdrawal of immunosuppression resulted in clearance of the

hepatitis C virus and resolution of the fibrosing cholestatic hepatitis and at five year follow up he is well and back at work

Three of the patients who developed cirrhosis, developed cirrhosis within 2 years - 1 died whilst awaiting liver transplantation, 1 died shortly after liver transplantation and the patient that was successfully transplanted. Two of these patients had additional immune suppression; one was receiving prednisolone for autoimmune haemolytic anaemia and the other was 28/40 weeks pregnant at the time of exposure. A further patient, who died of bronchopneumonia secondary to long-standing bronchiectasis and cor pulmonale, was also suffering from sub-acute liver failure (bilirubin > 500IU/L and prothrombin > 30 seconds). None of these 4 patients received the full cumulative dose of interferon due to significant side effects of treatment (2 discontinued treatment, 2 received reduced dosage). Two patients who were not treated developed decompensated cirrhosis – an elderly woman with a previous diagnosis of granulomatous hepatitis died of decompensated cirrhosis three years after exposure. However a biopsy prior to death showed cirrhosis and a chronic active hepatitis consistent with HCV and no evidence of granuloma. The second was a patient who declined therapy. She died of pneumonia but had clinical evidence of decompensated cirrhosis at time of death 4 years after exposure. This represents a 24% (6/25) rate of progression to end stage liver failure within 5 years amongst the patients with primary immunodeficiency or 38% (6/16) in those that became chronically infected.

Seven patients remain well but chronically infected. Six of these patients have abnormal liver function tests. Only one of these patients has symptoms and signs of liver disease although at least one patient has evidence of progressive fibrosis.

Histology

Patients who achieved a sustained response to treatment were not biopsied. Liver histology was available from 8 of 11 patients who relapsed after full therapy or failed to tolerate alpha-interferon. Three patients, who failed to respond to therapy, were not biopsied – 1 patient died of an unrelated cause, one elderly patient declined liver biopsy and one patient had a failed attempt at liver biopsy. Liver histology was also available in one patient who cleared the virus with treatment and in one patient who was not treated - a total of 16 biopsies from 10 patients. The histological findings are summarised in **table**

4.3, and were abnormal in all. In one patient, liver histology was available from three time points. A first biopsy was taken when he presented with jaundice in 1988 attributed after investigation to be secondary to autoimmune haemolytic anaemia, and was normal. A second biopsy taken 12 months after exposure to HCV during a laproscopic cholecystectomy for cholelithiasis, shows chronic hepatitis with interface hepatitis and a Knodells Histological Activity Index (HAI) score of 14. A final biopsy taken 18 months following exposure to IvIg as part of liver transplant assessment showed cirrhosis. Previous exposure to HCV was conjectured as this patient had received a number of transfusions of packed erythrocytes (prior to the introduction of anti-HCV screening) for haemolytic anaemia. However biochemical evidence of hepatitis developed only after his exposure to the contaminated IvIg batch and the HCV isolate detected was genotype1a and identical in sequence analysis to others from the rest of the outbreak.

Table 4.3: Results of Histology

Pt	Time from exposure to biopsy	Clinical Information at time of biopsy	Description	Knodell score	Ishak score[19]	
					Grade	Stage
5	12 months	PCR –ve after IFN	Minimal Hepatitis No fibrosis	3	3	0
9	13 months	Post IFN. PCR +ve	Chronic hepatitis, Mild fibrosis	3	2	1
9	48 months	On IFN, PCR +ve, Normal LFT	Mild hepatitis No fibrosis	NA		
10	14 months	Post IFN. PCR +ve	Mild chronic active hepatitis	NA		
10	19 months	Post IFN & Ribavirin	Severe chronic active hepatitis Early cirrhosis	NA		
10	28 months	Prior to transplantation	Cirrhosis	NA		
11	12 months	Post IFN. PCR +ve	Chronic active hepatitis Mild fibrosis	10	7	2
11	24 months	on Tx with IFN & Ribavirin	Chronic hepatitis improved from first bx	7	NA	NA
11	29 months	Post IFN & Ribavirin	Severe chronic active hepatitis Severe fibrosis	15	NA	NA
12	14 months	Post IFN. PCR +ve	Chronic active hepatitis Cirrhosis	14	9	5
13	13 months	Post IFN. PCR +ve	Mild chronic hepatitis Mild fibrosis	4	4	1
14	13 months	IFN discontinued. PCR +ve	Chronic hepatitis Severe fibrosis	15	12	4
14	20 months	Transplantation assessment	Cirrhosis	NA		
17	9 months	Post IFN. PCR +ve	Severe chronic hepatitis Moderate fibrosis	11	8	3
18	30 months	Prior to treatment	Moderate inflammation Moderate fibrosis	NA		
19	30 months	Not treated with IFN (granulomatous liver)	Severe chronic active hepatitis. Cirrhosis	12		

DISCUSSION

This epidemiological investigation has proved that the one batch of Gammagard was responsible for this UK outbreak of HCV. No infection was found in the patients treated with 16 different batches of the same products in the United Kingdom during the 14 months before withdrawal. In addition the genotype found in all the patients was identical - genotype 1a. In 20 of the patients sequence analysis of a region in NS5 has shown that nucleotide sequence is almost identical between isolates found in the patients and in the implicated batch. These findings are consistent with all the patients having been infected from a single donor.

Careful record keeping of batch numbers has then allowed identification of all patients exposed to the contaminated batch. This has allowed a unique opportunity to study the natural history of the disease from a known time point of infection. Similar to the blood transfusion data we have again seen that some patients will not develop infection despite definite exposure to a contaminated transfusion. Four patients received similar doses to the 30 patients who became infected but had no evidence of HCV infection as judged by PCR, symptoms and liver function tests. The numbers are small and so it is difficult to see any differences between these patients and patients who went on to develop infection. Although the average age was younger, in the uninfected group this was not statistically significant. It is of particular interest that these patients did not become infected because they had antibody deficiency. Also of note were the two patients who became infected but subsequently became negative for HCV RNA spontaneously without treatment. This is in keeping with current beliefs that approximately 15% of patients will clear the virus, although again this finding is more striking as the patients were antibody deficient.

In 4 out of 25 patients with primary antibody deficiency we have observed rapid progression of HCV infection, with end stage liver disease occurring within a period of 18 months. A further two patients died of liver failure after four years of infection. Such rapid progression to cirrhosis is rarely seen in HCV related liver disease; this outcome is highly unusual when compared with post transfusion HCV in immunocompetent patients[68]. Liver biopsy results also show evidence of rapid progression of fibrosis in a further patient, who has not cleared the virus, with no previous history of liver disease.

Thus overall in our group we have evidence of rapid progression of liver fibrosis in 6 out of 25 patients (24%). Prospective follow-up of the other patients continues and may reveal continuing morbidity and mortality

In contrast to some of the other reports of patients with HCV and hypogammaglobulinaemia, some of the patients have done well. As already mentioned, two patients have spontaneously cleared the virus without any treatment. Of the remaining 10 patients who have not cleared the virus but who have not had rapidly progressive liver disease, two have had liver biopsies showing only stage 1 fibrosis; one of these patients remains well and the other has died of unrelated causes. Two further patients have died of unrelated causes. The remaining six patients are asymptomatic, although have not been assessed by liver biopsy.

Seven patients (54%) treated with interferon for more than 3 months have shown a sustained response with normal liver function tests and negative RT-PCR after cessation of treatment. All of these seven patients have now been followed up for over 5 years since stopping treatment and have remained HCV RNA negative by PCR, with normal liver function tests and no clinical evidence of liver disease. Thus a negative PCR test for HCV at 6 months after therapy has a 100% predictive value of continued sustained response, validating this trial endpoint of treatment. This finding has now also been shown in larger interferon trials in immunocompetent patients. Overall the sustained response to interferon compares favourably to the 20% sustained response rate seen in other studies. This may be due to the prompt treatment and high dose regime that the patients received.

One alternative to acute treatment with alpha-interferon was to wait and treat only when the disease had become chronic as a small proportion of patients will spontaneously clear HCV. Indeed we have found that two patients with antibody deficiency cleared HCV without treatment. However Bjoro et al's experience with similar patients showed very poor efficacy for alpha-interferon in established chronic infection[123]. This may be due to the length of infection prior to treatment, different genotypes or mixed infections. The need for early treatment is highlighted by the suggestion that liver transplantation for end stage liver disease in hypogammaglobulinaemia has a poor outcome[124]. However our results show a high

clearance rate of HCV (7/13 = 54%) when hypogammaglobulinaemic patients receive and tolerate high dose interferon therapy within 9 months of infection.

Previous published reports suggest that the clinical outcome of HCV in primary antibody deficiency is variable. Possible explanations for these differences involve host factors including coexisting morbidity and viral factors. Pre-existing conditions may partly explain the rapid progression as exemplified by two patients in our group. One patient who died of liver failure also suffered from haemolytic anaemia, requiring maintenance corticosteroid therapy, another severely affected patient was pregnant at the time of exposure; both these factors would lead to increased immunosupression. Nonetheless steroid treatment alone or pregnancy has not been previously reported to lead to such rapid progression of HCV liver disease.

Underlying liver disease may also contribute. Patients with CVID are recognised to have an increased risk of liver pathology, but the literature is limited to a few case reports of granulomatous change similar to that seen in sarcoidosis[125]. However the 4 patients who progressed to end-stage liver disease in less than 2 years had no evidence of previous liver abnormality as extensively documented by serial liver function tests performed prior to exposure. The patient that died after three years did have an earlier diagnosis of granulomatous liver disease, however a liver biopsy prior to death was entirely in keeping with HCV infection with no evidence of granulomas.

Viral factors could also aggravate or attenuate the liver disease. There are known difference between viral genotypes in the severity of disease and response to interferon therapy. Our patients were known to be infected with one single genotype 1a. Although genotype 1 infections have been suggested in earlier studies, to be more severe than other genotypes[126], the degree of progression seen in some patients is far greater than could be explained by this factor alone. In addition more recent evidence suggests that genotype is not related to disease progression.

The contribution of other viruses should also be considered. Recently a further flavivirus HGV (HGBV-C), sharing limited homology has been identified[127] and has been suggested to be an other potential co-factor in patients receiving blood products. However more recent evidence suggests that this virus does not contribute to liver disease. This has been confirmed in this cohort with the incidence of HGV infection

being less than 10% and not found in any of the severe cases (Peter Simmonds, personal communication).

All blood products, especially those produced from pooled donations, are potential sources of infection. In order to be aware of such outbreaks and to enable accurate diagnosis and follow-up, it is important to document the products used and their batch numbers together with careful clinical and biochemical monitoring in all recipients. It is also important that patients receive only one product and that the brand is only changed for medical reasons. We believe our study confirms the severe potential nature of HCV infection in some patients with hypogammaglobulinaemia, although it is difficult to predict who will do badly. In the unfortunate event of an HCV outbreak, early diagnosis is important as prompt treatment with high dose interferon benefits patients.

Acknowledgments

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CHAPTER 5:
CD8$^+$ LYMPHOCYTE RESPONSES
IN ACUTE SELF LIMITING HEPATITIS C VIRUS INFECTION

5.1 SUMMARY

Introduction. CD8$^+$ T lymphocyte responses are important in the clearance of many viral infections. One hypothesis, tested in this chapter of the thesis, is that the strength and breadth of the CD8$^+$ T lymphocyte response to HCV determines whether the virus is cleared or persists. The aim of this study was to compare CD8$^+$ T cell responses in patients with limited acute HCV infection with patients with chronic infection.

Methods. We compared the CD8$^+$ T cells responses in three patients with acute self-limited HCV infection with 4 chronically infected patients. All patients expressed HLA A2 and were infected with HCV genotype 1a. CD8$^+$ T cell responses were determined against a panel of 8 peptides representing previously described HCV HLA A2 restricted epitopes using Elispot assays for gamma interferon production and standard chromium release cytotoxicity assays.

Results. CD8$^+$ T cells derived from patients with acute self-limited infection exhibited cytotoxicity against 3-4/8 epitopes after stimulation and IFN-γ production mirrored cytotoxicity. CD8$^+$ T cells derived from patients with chronic infection exhibited cytotoxicity in response to only 1 (2 patients) or none (2 patients) of the epitopes and specific IFN-γ production to 1-2 epitopes. In contrast with acute patients, CD8$^+$ T cells from chronically infected patients with demonstrated peptide specific IFN-γ production in the absence of detectable cytotoxicity.

Conclusions. Vigorous CD8$^+$ T cell responses against a wider repertoire of epitopes is associated with viral clearance. The functional phenotype of CD8$^+$ T lymphocyte in chronic HCV infection, characterised by gamma interferon production but not cytotoxicity may contribute towards a failure to clear HCV.

5.2 BACKGROUND

In the last three chapters, it has been shown that approximately 85% of patients will develop chronic infection with HCV. Host factors appear to be important in determining viral persistence. However the underlying mechanisms that determine whether the virus is cleared or persists remains unknown. The following two chapters explore possible mechanisms of viral persistence. In this chapter differences in the immune responses between patients who have cleared virus and patients who have developed chronic infection have been investigated. In Chapter Six the hypothesis that the virus persists by means of "escape" mutation is explored, by which virus mutation results in loss of host immunological recognition and control.

It is known that T lymphocyte responses to viruses contribute to viral clearance but may also result in host tissue damage. The control and elimination of virus from infected cells is achieved by the direct action of cytolytic $CD8^+$ T lymphocytes and indirectly by $CD4^+$ T cells, which provide help for B lymphocytes that produce neutralising antibody and $CD8^+$ antigen specific T cells. However these activated T cells may damage bystander host cells as well as virally infected cells. At present it remains unclear to what extent the immune response to HCV is protective in trying to limit HCV replication, and to what extent the immune response is the cause of the progressive liver damage.

Preliminary studies of acute infection suggest that a vigorous response by HCV specific $CD4^+$ T cells is associated with viral clearance in the minority of patients who eliminate the virus[90;128-130]. $CD8^+$ T cell responses in these individuals have not yet been studied. In the majority of patients who develop chronic infection $CD4^+$ T cell responses appears to be weak[90].

However much less is known about the role of $CD8^+$ T cells in acute and chronic HCV infection. HLA class I restricted $CD8^+$ T lymphocytes have been shown to play a major role in viral clearance in many virus infections. In Hepatitis B, multispecific, polyclonal $CD8^+$ lymphocyte responses are associated with viral clearance whilst oligospecific, oligoclonal responses are associated with viral persistence[131]. Similarly in chronic viral infections such as HIV vigorous $CD8^+$ T cell responses are associated with control of infection whilst the development of AIDS is associated with loss of $CD8^+$ T

cell recognition of HIV[132](Ref). From these parallels with other viruses it is probable that CD8$^+$ T cells have an important role in HCV.

HCV specific CD8$^+$ T cell responses have been studied in humans[73;74;91;94;133], mice[134] and chimpanzees[135]. In humans, CD8$^+$ lymphocytes have been generated from both the peripheral blood and liver of patients chronically infected with HCV leading to the identification of a number of HCV epitopes through a combination of HLA binding motif scanning, HLA assembly assays and epitope mapping using synthetic peptides. These epitopes are scattered throughout the genome and restricted by a range of MHC molecules. HLA A2.1 restricted epitopes have been the most frequently studied due to the high prevalence of HLA A2 in North American and European populations. No immunodominant epitopes have been identified[136;137] and the role of CD8$^+$ T cells in clearing the virus remains undetermined. Very little is known about CD8$^+$ T cells in acute infection. A consistent finding in chronic disease has been the weakness of the cytotoxic T cell responses when present. Studies of CD8$^+$ T cell cytotoxic activity have reported an inverse correlation with viraemia and a positive correlation with histological disease activity[91]. These data suggest that in chronic hepatitis C cytotoxic CD8$^+$ T cells exert beneficial effects by reducing viraemia and cause harm by causing hepatocyte lysis.

Cellular cytotoxicity is not the only effector action of CD8$^+$ T cells. In HBV infection CD8$^+$ T cells have been described that release gamma interferon and control viral replication and release without causing hepatocyte lysis[129]. Similar responses have been reported in a murine model of HCV infection[138] but the role of these cells has not been studied previously in man.

The hypothesis tested in this chapter was that the strength and breadth of the CD8$^+$ lymphocyte response to HCV determines viral persistence or clearance. Furthermore the hypothesis that in chronic infection CD8$^+$ T cells may recognise antigen but fail to lyse infected cells, resulting in a state of viral control but not elimination, is evaluated. The aims of the study were to compare CD8$^+$ T cell responses in patients who have cleared acute HCV infection with patients who are chronically infected.

5.3 PATIENTS

Two groups of patients were studied: three patients with acute self-limited HCV infection and 4 patients with chronic HCV infection. The patients with acute self-limited HCV infection were anti-HCV positive, indicative of exposure to HCV but HCV-RNA negative by repeated polymerase chain reaction tests. A negative test for HCV RNA is believed to represent either viral clearance or if not clearance then simply immunological control of viral replication. The patients with chronic infection on the other hand had detectable HCV RNA by PCR, indicating continuing viral replication.

The first acute patient (patient A1) had developed an acute hepatitis following a needle stick injury. She was initially antibody negative for HCV at the time of the acute presentation but sero-converted over the subsequent two months, indicating acute infection with HCV. During the acute illness she became jaundiced and had symptoms of lethargy and nausea. Her transaminase levels were greater than 5 times the upper limit of normal and a liver biopsy showed a moderately severe acute hepatitis consistent with a viral hepatitis. However her liver function tests returned to normal by six weeks and have subsequently remained consistently normal. From two months after the initial jaundice she has had persistently negative tests for HCV RNA by PCR. Blood was taken for T cell analysis approximately 6 months after infection.

The second acute patient (patient A2) became anti-HCV positive following sexual exposure with her partner who was known to have chronic HCV. Similar to the first patient she had previously tested negative for antibodies to HCV. The genotype that was identified in both the patient and her partner was genotype 1a. Sequencing of the virus has confirmed that they were infected with the same viral isolate. A decision was made to treat her acutely with interferon-alfa and she became negative for HCV RNA by three months of therapy. Due to side effects of the treatment the patient stopped therapy at this stage but has remained persistently HCV RNA negative since that time point. She has now been followed up for two years since infection. Blood was taken for T cell analysis approximately 8 months after infection.The third patient (patient A3) was identified as part of the national UK lookback program (see chapter 3, patient ID number 115). She contracted HCV from a blood transfusion for a post partum haemorrhage in 1991. The donor of the blood subsequently tested positive for HCV after screening for HCV was introduced in 1991. The viral genotype in the donor was genotype 1a.

However patient A3 although testing anti-HCV positive was persistently RNA negative. She had no symptoms or signs of chronic liver disease and her liver biochemistry was normal. Blood was taken for T cell analysis approximately 6 years after infection.

All patients were HLA typed and were shown to be HLA-A2.1.

Four patients with chronic HCV infection were selected for comparison (patients C1-C4) because they were infected with the same genotype (type 1a) and also expressed HLA A2.1. These patients had detectable HCV RNA in the serum and had abnormal liver function tests. They had been infected for a relatively short period of time (less than 10 years) and had evidence of chronic hepatitis on liver biopsy although this was not severe in any of the cases.

In addition 2 healthy controls, both with HLA A2.1, with no history of HCV infection were studied as negative controls.

5.4 METHODS

5.4.1 Methods: HLA Typing

Class I HLA typing was performed on all patients using sequence specific PCR[139].

5.4.2 Methods: Generation of HCV specific T cells

CD8$^+$ T cells were generated from patients by culturing their peripheral blood mononuclear cells (PBMC) in the presence of HCV A2.1 restricted peptides for 2 weeks. The rationale for culturing the cells was to increase the numbers of circulating HCV specific T cells that are otherwise present at too low levels to perform functional assays. The peptides representing epitopes within both structural and non structural proteins of HCV are shown in **table 5.1** and are based on the genotype 1a sequence. CTL recognition of these 8 epitopes has been previously described in patients chronically infected with HCV. Peptides were synthesised using standard fluorenylmethoxycaronyl techniques and were > 90% pure as determined by HPLC (Research Genectics, Inc (Huntsville, Al, USA and the Multiple Sclerosis Society Peptide laboratory. Oxford, U.K.) All peptides were reconstituted in sterile distilled water containing DMSO.

Table 5.1: HLA-A2 restricted CTL epitopes found in HCV

PEPTIDE NO.	SEQUENCE	REGION	AMINO ACID
1	YLLPRRGPRL	core	35-45
2	ADLMGYIPLV	core	131-140
8	SLLAPGAKQNV	E2	401-411
3	LLCPAGHAV	NS3	1169-1178
4	KLVALGINAV	NS3	1406-1416
5	SLMAFTAAV	NS4	1789-1798
6	LLFNILGGWV	NS4	1807-1817
7	ILDSFDPLV	NS5	2252-2261
9	Flu A2 restricted		

5.4.2 Methods: Generation of HCV specific T cells (continued)

Briefly 40 ml of blood was taken and PBMC obtained by Ficoll-Hypaque density gradient centrifugation. The cells were counted and half the cells were frozen down to be used later for stimulation. The other half was resuspended in R10 solution (RPMI plus 10% fetal calf serum plus penicillin, streptomycin and glutamine) to a concentration of 2 million cells/ml. Eight separate wells were set up with 2 million cells per well to which was added a single HCV A2 peptide at a final concentration of 10 μM. In addition cells were added to a ninth well to which was added an A2 restricted influenza matrix peptide[140], which acted as a positive control. The cells were incubated at 37°C with 5% CO_2.

The cells were stimulated again on day 8 with feeder autologous irradiated PBMC, using the cells that had been stored on day 1 and further peptide. In addition IL-2 was added on days 3 and 8 to a 10% concentration. The cells were washed on day 14 before functional analysis (cytotoxic assays and Elispot assays) was performed.

5.4.3 Methods: Cytotoxic T cell assays

Chromium release assays were used to test the ability of host T cells to lyse target B cells coated with the relevant HCV peptides, representing the HCV A2 restricted epitopes as shown in **table 5.1**. EBV transformed B lymphoblastoid cell lines (B-LCL), which were HLA-A2 restricted, (or mismatched where appropriate for negative controls) were used as targets for these assays. B-LCL were pelleted and resuspended in 100 µl ^{51}Cr for two hours. Cells were then washed 3 times and resuspended in R10 to a final concentration of 5,000 cells per 50 µl.

5,000 target cells were added to each well in a 96 round-bottomed well plate along with the cultured T cells at a target to effector ratio of 1:20 in the presence or absence of peptide (10µM). Assays were performed in duplicate.

The assay was performed by culturing the cells for six hours at 37°C. After this time, 20µl of culture supernatant was harvested and specific chromium release was determined. The percentage lysis was calculated as 100 x [(experimental release - spontaneous release/ (maximum release - spontaneous release)]. Maximum release was determined from supernatant of cells that were lysed by addition of 5 % Triton. Spontaneous release was measured in supernatant from target cells incubated without effector cells, and assays were excluded from analysis if the spontaneous value was >30% of maximum. The percentage specific lysis was calculated by subtracting the percentage lysis seen in the absence of peptide from the percentage lysis seen with peptide added. Significant cytotoxicity was defined as >15% specific lysis. HLA mismatched target LCL were incorporated to confirm that cytotoxicity was both antigen specific and HLA restricted. Specific lysis in response to each HCV peptide was calculated and responses were compared with responses to Flu.

5.4.4 Methods: Elispot Assays (see figure 5.1)

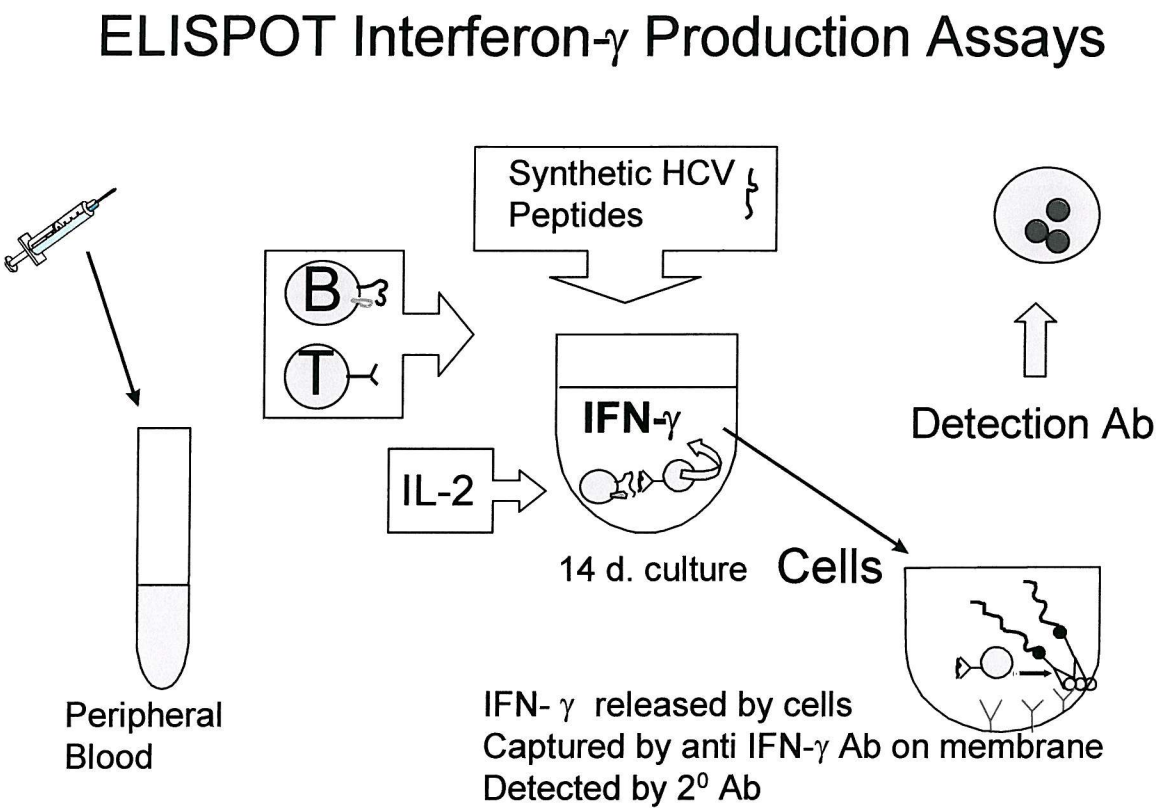
A sterile 96 well plate (Millipore) was used. The bottom of the wells are lined with membrane which was first coated with 100µl of an antibody to interferon gamma (1-D1K) at a concentration of 15 µg/ml. The plate was left for three hours and then the excess antibody was washed off with RPMI. R10 was then added as a blocking solution and the plate incubated for a further one hour.

The T cells that had been cultured in the presence of the relevant 8 HCV peptides or the influenza matrix peptide were washed and then added to 4 separate wells for each of these 9 cultures (total of 36 wells). The number of cells added was approximately 100,000 cells/well. The relevant peptide was then added to two wells at a concentration of 10µM and in two wells no peptide was added as a negative control. The plate was then incubated at 37°C overnight.

The following day the cells and medium were discarded from the wells and the wells were washed 6x with PBS. A detector biotinylated monoclonal antibody was then added to the wells and a further incubation of three hours was performed at room temperature. The excess second antibody was then washed off and 100ml of streptavidin-alkaline phosphatase was applied and left for two hours. This was then washed off and finally 100 ml of substrate (BCIP/NBT fom BioRad, Richmond, USA) was added. This was washed off after approximately 10 minutes with tap water after the plate had developed. The plates were allowed to then dry.

Black dots appear on the membrane, which are then counted. These represent individual cells that have released gamma interferon in response to the peptide. The difference between the wells where peptide has been added was compared to the negative wells. In addition the HCV assays were compared to the well in which the 'Flu peptide had been added. The maximum number of spots in negative wells was 5, while spots in positive wells numbered 20-50. Assays in which the number of spots in test wells was more than twice the number of spots in control wells were considered to be positive.

Figure 5.1: Diagrammatic View of the Elispot Assay



5.5 RESULTS

5.5.1 Results: Cytotoxic Assays (see appendix 1 for raw data)

All patients had a positive response to the flu peptide and there were no differences in the flu response between patients with acute or chronic HCV infection. The patients with acute self limited HCV infection (patients A1-3) showed specific lysis greater than 15% to 3 or 4 of the 8 epitopes studied. However in the patients with chronic infection (patients C1-C4) specific lysis could only be demonstrated against 1 out of 8 epitope in two patients and none out of 8 in 2 patients.

The two healthy control patients with no evidence of any contact with HCV exhibited responses to 'flu but none of the HCV peptides. HCV specificity was confirmed in assays in which peptide was omitted or HLA mismatched LCL were used.

The cytotoxic responses for acute and chronically infected patients are shown in **figure 5.2** and **5.3**. The comparison of the different pattern of response between patients is shown in **table 5.2**.

Figure 5.2: Specific lysis to A2 restricted peptides in patients with acute self limiting infection with HCV

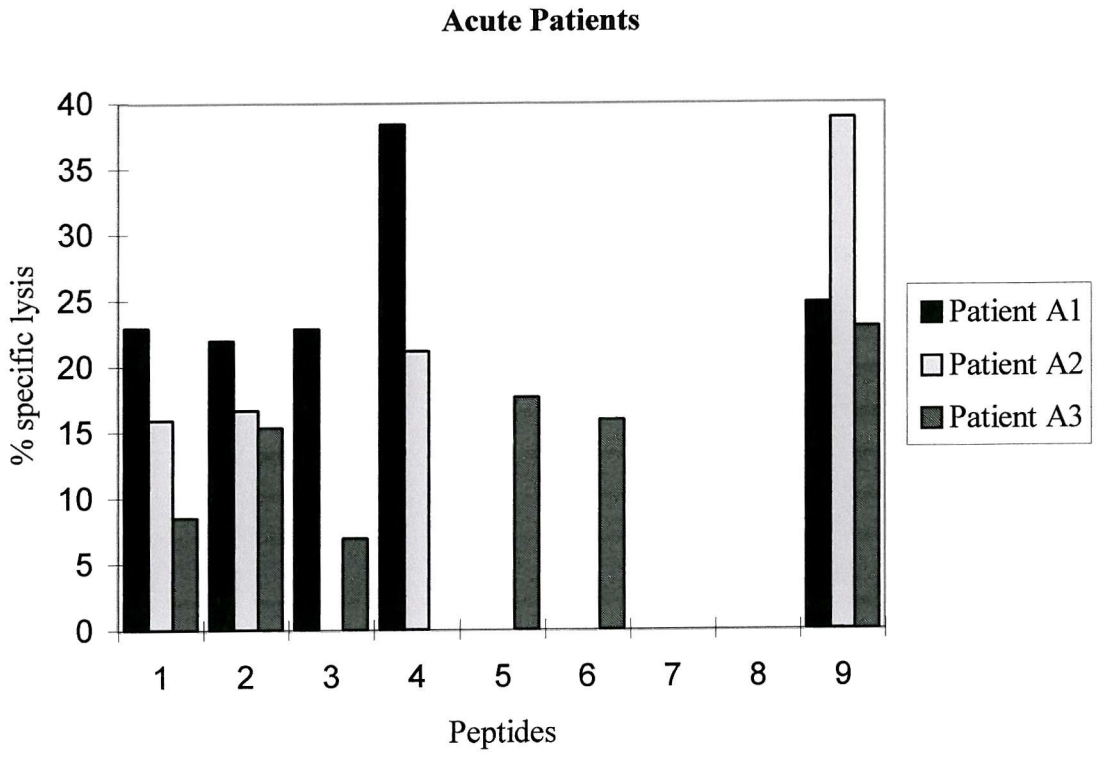


Figure 5.3: Specific lysis to A2 restricted peptides in patients with chronic infection with HCV

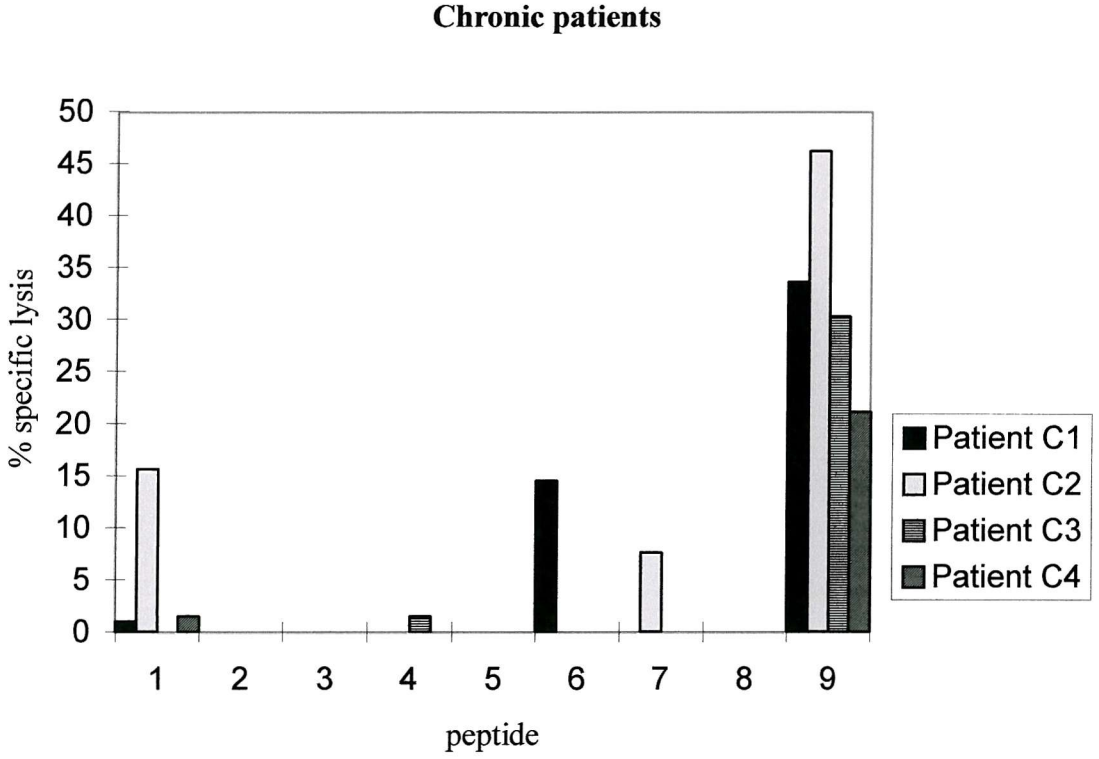


Table 5.2: Positive cytotoxic responses to HCV A2 restricted CTL epitopes and a flu CTL epitope in acute and chronically infected patients

	PEPTIDE								
Patient	1	2	3	4	5	6	7	8	Flu
A1	+	+	+	+					+
A2	+	+		+					+
A3		+			+	+			+
C1						+			+
C2	+								+
C3									+
C4									+

5.5.2 Results: Elispot Assays (see appendix 2 for raw data)

All subjects tested exhibited IFN-γ release in response to flu matrix peptide in a pattern analogous to that seen in the cytotoxicity assays. When tested against the HCV peptides, the 3 patients with acute resolved HCV showed a pattern of IFN-γ release that mirrored that seen in the cytotoxicity assays; IFN-γ release was demonstrated in response to the same 3 or 4 epitopes. Three of the patients with chronic infection exhibited IFN-γ release in response to 2 peptides and one patient responded to only one peptide. Again the two healthy HCV negative control patients responded to 'flu but none of the HCV peptides. The results of the Elispot assays are depicted in **table 5.3**.

Table 5.3: Positive Elispot responses to HCV A2 restricted CTL epitopes and a flu CTL epitope in acute and chronically infected patients

	PEPTIDE								
Patient	1	2	3	4	5	6	7	8	Flu
A1	+	+	+	+					+
A2	+	+		+					+
A3		+			+	+			+
C1		+				+			+
C2	+						+		+
C3				+	+				+
C4	+								+

5.5.3 Results: Comparison of Cytotoxity and IFN-γ Production

For patients with acute resolved HCV infection the cytolytic peptide specific responses exactly matched IFN-γ release (**see table 5.4**). However in the patients with chronic infection, IFN-γ production was seen in the absence of cytotoxicity.

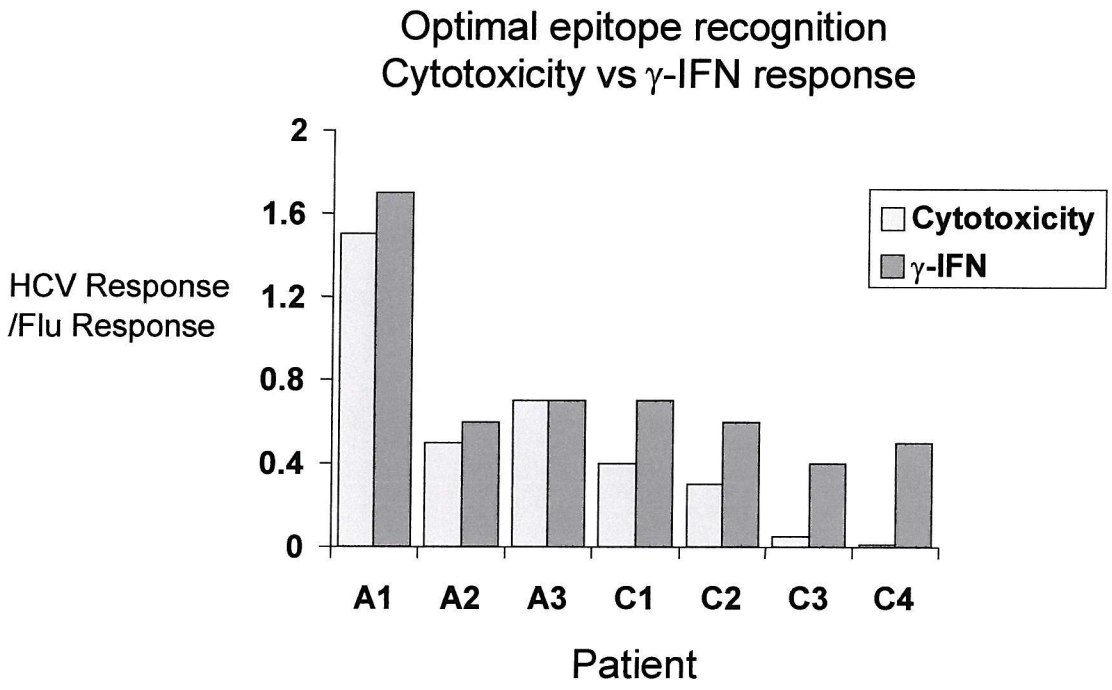
Table 5.4: Comparison of Cytotoxicity and IFN-γ responses to HCV A2 restricted CTL epitopes and a flu CTL epitope in acute and chronically infected patients

	PEPTIDE								
Patient	1	2	3	4	5	6	7	8	Flu
A1	++	++	++	++					++
A2	++	++		++					++
A3		++			++	++			++
C1		+				++			++
C2	++						+		++
C3				+	+				++
C4	+								++

+ = IFN-γ production alone. ++ = IFN-γ and cytotoxic responses

This difference between the CD8+ T cell responses in acute resolved and chronic patients were well illustrated by comparing cytotoxicity and IFN-γ release in response to the optimally recognised peptide for each patient (see **figure 5.4**). The responses were measured by comparing the response to the best recognised HCV epitope relative to the patients response to flu. In acute resolved patients the magnitude of IFN-γ production was matched by the strength of the cytotoxic response. However in patients with chronic infection the strength of the cytotoxic response relevant to 'flu was significantly less than the IFN-γ release relevant to flu.

Figure 5.4: Comparison of cytotoxicity with IFN-γ production for best recognised epitope for each patient



5.6 DISCUSSION

In the present study considerable care was taken to ensure that all subjects were infected with the same HCV genotype. In addition the assays were performed using peptides restricted through the same HLA molecule. These measures eliminate the possibility that a lack of reactivity could be due to a mismatch between the infecting and test antigens. The subjects were HLA matched to reduce the likelihood that differences in response were due to HLA background[84-86;6]. T cell function was analysed using assays of both cytotoxicity and IFN-γ release. This was done to investigate the hypothesis that chronic infection might be due in part to the ability of $CD8^+$ T cells to recognise antigen but fail to lyse infected cells, resulting in a state of viral control but not elimination.

These studies of $CD8^+$ T cell responses to HCV have demonstrated both quantitative and qualitative differences between infected patients who clear the virus and those that do not. Many earlier studies have found that $CD8^+$ T cell responses to HCV are weak or absent in patients with chronic hepatitis C infection. The vast majority of these studies have employed chromium release cytotoxicity assays as the sole evaluation of $CD8^+$ T cell function. These findings support the hypothesis that persistence of HCV is facilitated by an ineffectual CTL response. The present study has again found weak responses in patients with chronic hepatitis C.

In addition we have demonstrated that patients who have cleared acute infection posses $CD8^+$T cells capable of recognising a greater range of epitopes than patients with chronic infection recognise. A detailed quantitative analysis of cytotoxic responses was hampered by the scarcity of responsive patients with chronic hepatitis C. Where analysis was possible lower levels of antigen specific cytotoxicity were seen in the patients with chronic hepatitis C. The Elispot assays allowed us to compare the magnitude of the peptide specific responses in a greater number of patients and again responses were weaker in patients with chronic hepatitis C than in patients with acute resolved hepatitis C. The dichotomy of responses between acute and chronic HCV infection mirrors findings reported for $CD4^+$ T cells[85]. The data for both $CD4^+$ and $CD8^+$ T cells infer that a broader and more diverse T cell response is required for viral clearance in acute HCV infection and suggest that oligospecific and weak T cell responses contribute to the development of chronic infection.

This theory is substantiated by work published this year. Lechner and colleagues also showed a strong and persistent CTL responses in three patients with acute resolving HCV infection[141]. Using both Elispot assays and also human histocompatability leucocyte antigen peptide tetramer assays they also described at the earliest time point after infection that highly activated CTL populations were observed to temporarily fail to secrete IFN-γ, from which they recovered as the viraemia declined. The significance of this "stunned phenotype" is unclear. Takaki and colleagues demonstrated that CD4$^+$ and CD8$^+$ responses in patients who had acute resolved HCV infection persisted up to 18-20 years after infection[142], whereas in many of these patients HCV-specific antibodies were undetectable after this period of time. They also demonstrated using Elispot assays that the IFN-γ phenotype persisted. They were unable to demonstrate CTL activity or IFN-γ production in the majority of their chronically infected patients, although they were working from frozen stored cells rather than freshly extracted cells.

The role of CD8$^+$ T cells in chronic hepatitis C is less clear. Earlier studies have found conflicting results concerning the correlation between cytotolytic activity and viral load and hepatic inflammation[91;94;94]. However this single readout of CD8$^+$ T cell function may have failed to capture the full contribution of these cells to hepatic inflammation and viral control in chronic hepatitis C. Previous studies have reported failure to detect peripheral blood cytotoxic T cell responses in chronic hepatitis C[73]. One explanation for the lack of response in these previous studies that has been postulated is that there may be epitope variation in the infecting virions. In Koziels study, as in most studies, CTL were tested against targets either as peptides or vaccinia viruses expressing a single genotype 1a strain rather than patient derived viral sequences. Koziel in a study of intrahepatic CTLs responses was able to show that the liver derived CTL recognised different strains of HCV in a strain specific way[74]. Therefore if CTL recognition is dependant on HCV genotype, patients infected with genotypes other than 1a, may not respond to assays using 1a derived peptides or vaccinia. Mismatch of genotype may explain why in a previous study no cytolytic responses were seen in patients who had cleared virus, as unlike our study, the infecting genotype had been unknown.

The balance between liver damage and viral control due to CD8$^+$ T cells is very likely to vary between patients with chronic hepatitis C. Antigen specific cytolysis of HCV infected cells may be advantageous in eliminating infection but it may also be harmful to the host through the resultant host cell destruction, by triggering a fibrogenic

response and by releasing virions, thereby spreading infection to neighbouring hepatocytes. By employing assays of gamma interferon release, in addition to cytotoxicity assays, we have been able to examine $CD8^+$ T cell function in CHC in greater detail. The patients with chronic hepatitis C had antigen specific $CD8^+$ T cells that release gamma interferon in the absence of cytotoxicity. This is similar to observations in chronic hepatitis B virus infection[52;143;144], and in an experimental mouse model of HCV infection[138] which lead to the suggestion that IFN-γ releasing, non-cytolytic T cells might be harnessed therapeutically to control infection with little collateral cell damage[138]. The antigen specific $CD8^+$ T cells described in the present study, whilst incapable of eliminating virus are capable of exerting antiviral effects through interferon gamma that may control virus replication, enhance cellular resistance to infection and lysis[52;95;143;143;144]. As a result they may play a significant role in modifying the course of chronic hepatitis C even if they cannot terminate infection. Whether these T cells are entirely beneficial will require further investigation. It is equally possible that persistent gamma interferon release from $CD8^+$ T cells may contribute to fibrogenesis and even to the malaise reported by many patients with chronic hepatitis C infection. It appears that the nature of the $CD8^+$ T cell response in CHC as well as it's breadth and vigour may play a central role in the failure to clear HCV that characterises the infection in almost all cases.

The absence of cytotoxic T cell responses in the patients with chronic hepatitis C limited our ability to investigate the strength of $CD8^+$ T cell responses in chronically infected patients using conventional chromium release assays. However the use of Elispot assays permitted comparison of the vigour of $CD8^+$ T cell responses in acute and chronic hepatitis C infection related to responses to flu as a standard. This revealed that antigen specific responses were stronger in patients who had resolved acute infection compared to those with chronic infection.

The persistence of vigorous $CD8^+$ T cell responses in patients with acute resolved hepatitis C raises the possibility that these T cells may provide protective immunity against HCV. These data may be useful in the search for a vaccine against HCV as they suggest that promotion of an effective $CD8^+$ response may be as important as an effective $CD4^+$ response in the resolution of HCV infection. Further detailed analysis of the nature of $CD8^+$ T cells responses in acute resolved and chronic HCV infection is required.

In summary we have found that 3 patients with acute HCV, exhibited multispecific T cell responses to HCV and that cytotoxicity mirrored IFN-g production. The 4 patients with chronic HCV exhibited oligoclonal or absent cytotoxic T cell responses to HCV and that peptide specific IFN-γ production was seen in the absence of detectable cytotoxicity. Thus, we have shown that multispecific $CD8^+$ T cell responses is associated with viral clearance. The functional phenotype of $CD8^+$ T cells characterised by IFN-γ production but not cytotoxicity may be responsible for failure to clear HCV.

CHAPTER 6:
IMMUNE SELECTION AND GENETIC SEQUENCE VARIATION IN CORE AND ENVELOPE REGIONS OF HEPATITIS C VIRUS

6.1 Summary:

The mechanism by which Hepatitis C Virus causes persistent infection is unknown. One hypothesis is that HCV evades the host immune response through mutation in immune epitopes. The aims of the following experiments were to investigate whether mutations in the HCV genome accumulate over time clustered within cytotoxic T lymphocyte (CTL) epitopes and B cell epitopes and to study the impact of antibody deficiency on mutation rates.

Six patients with chronic HCV were studied: 3 patients with antibody deficiency and 3 patients with normal immunity. Core and envelope regions of the HCV genome, known to contain CTL and humoral epitopes were sequenced at 2 time points, 2 years apart.

The diversity of HCV quasispecies increased with time. The overall mutation rate was higher than previously predicted. The neutral nucleotide mutation rate in core was similar to that observed in envelope, suggesting that the error rate of the HCV RNA polymerase is similar in both regions. However in comparison to the neutral rate of mutation, the coding mutation rate was decreased in core and increased in envelope. No genetic mutation was seen in any of the core CTL epitopes, despite detectable cellular responses. Mutations within a previously described CTL epitope in envelope were detected in all patients but did not correlate with cellular responses to index or mutated peptides. There was no difference in mutation rates between patients with antibody deficiency and normal immunity in cellular or humoral epitopes.

Conclusions: Stability of the HCV genome is selected in core and variation selected in envelope in chronic HCV infection. However we have found no evidence that the selection of protein variation is due to immune escape from pressure exerted by HCV specific CTL or antibody selection. These findings implicate alternative virus host interactions as exerting selection of mutation in HCV.

6.1 BACKGROUND

As shown in earlier chapters, acute HCV infection leads to chronic persistent infection in 85% of patients. In the last chapter differences in T cell response were shown to be associated with viral clearance or persistence. However, by analogy with other chronic viral infections a number of other mechanisms by which chronic HCV infection is maintained may be invoked.

HCV replicates by means of an RNA polymerase encoded within the viral genome[21]. Several different genotypes have been described[25] and within an infected individual several closely related but different quasispecies may be isolated at any one time point[145]. Viral genetic diversity is attributed to replicative errors introduced by the viral RNA polymerase which lacks a proof-reading activity[146]. The distribution of mutations has been reported to be uneven but the basis of this variability is unexplained[145]. A hot-spot for mutation is described within the genome encoding a portion of E2 termed the Hypervariable Region (HVR) which encodes B-cell epitopes[23;102]. It is postulated that genetic variation, translated into protein variability results in the production of HCV quasispecies. Interaction between host and virus selects those quasispecies better adapted to survival.

Thus perhaps the most attractive hypothesis by which HCV persists is "immune escape" whereby mutations arising within the HCV genome enable the virus to evade the host's immune response[99;147-149]. Studies of HCV infection in chimpanzees have reported limited evidence of mutation within $CD8^+$ T cell epitopes associated with viral persistence[101] but only limited studies have yet been reported in humans[133], the results of which are equivocal.

Genetic variability within the HVR has been attributed to the effect of immune selection by antibodies on B cell epitopes leading to immune escape. This hypothesis has been investigated previously by comparing mutation rates in the HCV HVR from immunocompetent patients with those from antibody deficient patients[103;150]. However differences in the duration of infection amongst the patients may have confounded these earlier studies.

The hypothesis tested in this chapter are that variation in mutation rate across the HCV genome is the result of selection pressure exerted by the host immune response upon the virus; that selection by T-cells leads to immune escape through mutation within

$CD8^+$ and $CD4^+$ T cell epitopes encoded in HCV; and that antibody selection results in the emergence of HCV quasispecies mutated in B cell epitopes.

To test these hypotheses sequence variation in two regions of the genome encoding the core and envelope proteins were studied. In order to detect evidence of selection of HCV mutation by the host, the frequency of mutations resulting in amino-acid substitutions with those that are "silent," or "neutral" at the protein level were compared. In order to obtain evidence that HCV mutations are selected by pressure from host immune responses, mutation rates in regions encoding $CD8^+$ and $CD4^+$ T cell epitopes, which are potential immune targets, were compared with flanking regions, which are not; and mutation rates across the region encoding B cell epitopes in patients with normal humoral immunity were contrasted with that observed in patients with antibody deficiency.

6.3 PATIENTS (see table 6.1)

All patients studied were selected as sharing HLA-A2, -B7 or both allotypes, and for being infected with the same HCV genotype (1a) for less than 5 years. Three patients (patients 1 - 3) with hypogammaglobulinaemia infected in January 1994 with an identical HCV isolate (genotype 1a) from the same batch of contaminated immunoglobulin were studied over a two year period (1994-1996). In two patients HCV progressed rapidly to death (see chapter 4). Patient 1, had no pre-existing liver disease, but died of liver failure with cirrhosis two years after infection. Patient 2, with a prior diagnosis of granulomatous hepatitis, died from liver failure 4 years after HCV infection. Liver biopsy prior to death was consistent with chronic HCV infection. Patient 3 is alive and has no symptoms or signs of chronic liver disease. She remains HCV-RNA positive with a fluctuating transaminitis.

At the same time, 3 patients (patients 4-6) with normal immunity were studied and compared with the antibody deficient patients. Criteria for selection were infection with the same HCV genotype (1a), a relatively short duration of infection and HLA compatibility. These patients were identified as part of a national lookback program to identify recipients of infected blood products and had received blood transfusion in 1989, 1990 and 1991 respectively. They remain HCV-RNA positive and have evidence of chronic hepatitis with early fibrosis on liver biopsy (see chapter 3).

Table 6.1: Patient Clinical Characteristics

Pt	Immunity	Age	Sex	Year of Infection	Class 1 HLA type	Outcome
1	CVID	36	M	1994	A1,2 B44,37	Died liver failure 1996
2	CVID	69	F	1994	A2,68 B7,64	Died liver failure 1997
3	CVID	76	F	1994	A2,31 B44,35	Chronic hepatitis - moderate activity
4	Normal	35	F	1990	A1,3 B7,49	Chronic hepatitis - mild activity
5	Normal	42	F	1991	A1,2 B60,55	Chronic hepatitis - mild activity
6	Normal	68	M	1989	A2,31 B18,55	Chronic hepatitis - moderate activity

CVID = Common variable immune deficiency

6.4 METHODS

Two regions of the HCV genome were studied at a minimum of two time points, two years apart in all the patients; the first region of 430 base pairs encodes a fragment of the core protein and the second region of 200 base pairs encodes part of the E2/NS1 protein. In addition HCV RNA isolated from the infecting batch of immunoglobulin was sequenced across the same regions. At each time point, in each patient, 20 clones were sequenced in order to determine not only the commonest sequence at that time point but also the diversity and proportion of quasispecies in each serum isolate. Comparison of isolates at different time points provided information on sequence variability.

The sequenced region of the core gene spanned nucleotides 1-430 (αα 1-144) and contained two HLA-A2 restricted CTL epitopes[76] and one HLA-B7 restricted epitope[151]. The envelope region studied spanned nucleotides 1099-1297 (αα 367-433) contained a putative HLA- A2 restricted CTL epitope[77] as well as the hypervariable region (HVR) (αα 386-412), which contains linear B cell epitopes recognised by neutralising antibodies[152-154]. (See **table 6.2**)

Table 6.2: CTL Epitopes in Core and Envelope Gene

PEPTIDE	AMINO ACID POSITION	PROTEIN	HLA RESTRICTION
YLLPRRGPRL	35-44	Core	A2
ADLMGYIPLV	131-141	Core	A2
DPRRRSRNL	111-120	Core	B7
SLLAPGAKQNV	400-411	Envelope	A2

HCV sequence was obtained as described in detail below.

6.4.1 Methods: RNA extraction

Blood was taken at sequential time points over the two year period. Serum was separated and stored at -70°C. Two methods of RNA extraction were compared:

Method 1:

The first method use a methodology adopted from Boom et al, (Journal of Clinical microbiology 1990). The following regents were prepared: -

RNA extraction buffer was made up by adding 100 grams guanidinium isothiocyanate to 100ml 0.1M Tris-HCL pH 6.4, 22ml 0.2M EDTA pH8.0 and 2.3ml Triton X-100.

Silica suspension was made by adding 60 grams silicon dioxide to 500 ml distilled water in a cylinder; this was left for 24 hours and then 430 ml of supernatant was removed and the pellet resuspended in 500 ml of distilled water. This was then allowed to stand for five hours and again the supernatant was removed. Finally 600µl of concentrated HCL was added and the suspension alliquotted autoclaved and then frozen until ready for use.

100 µl of serum was added to 1 ml of RNA extraction buffer. And 40 µl Silica suspension in a 1.5 ml eppendorf tube. At least one positive control for HCV RNA and at least two negative controls were used during any experiment.

The eppendorfs were then vortexed thoroughly and stood at room temperature for five minutes. The samples were then spun for 10 seconds at 13000 rpm in a microcentifuge. The supernatant was then removed and discarded into 3M NaOH (guanidinium isotiocyanate can liberate cyanide gas in acid pH). 1ml of ethanol was then added to each tube, vortexed to resuspend the silica pellet and then spun again for 10 seconds. A second wash was then performed with acetone, removing the supernatant completely after spinning. The tubes were then air dried for 10 minutes at room temperature and then finally the pellet resuspended in 50µl of sterile distilled water, vortexed and incubated at 55°C for five minutes.

6.4.1 Methods: RNA extraction (continued)

Method 2:

The following reagents were made up:-

RNA Extraction buffer was prepared by adding 9 volumes of TNE buffer (NaCl 0.11M, Tris pH 8.0 55mM, EDTA pH 8.0 1.1mM, 0.55% SDS) to 1 volume of proteinase K(10mg/ml) and 20ml/ml poly A(2mg/ml).

100µl serum was added to 400µl of RNA extraction buffer. Samples were incubated for 10 minutes at 37 °C and then 450µl of phenol was added to each sample and the tubes were shaken for 5 minutes. The samples were then centrifuged at 1300rpm for 5 minutes to separate the layers. The aqueous layer was then transferred to a fresh tube containing 450µl chloroform/iso-amyl alcohol (50:1) and shaken for 2 minutes before being spun again for 5 minutes. After centrifugation 400µl of the aqueous layer was placed in a fresh tube containing 40µl 3M Na-acetate pH 5.2. To this was added 800µl of Ethanol (100%) to precipitate nucleic acids. The samples were frozen overnight at -70°C.

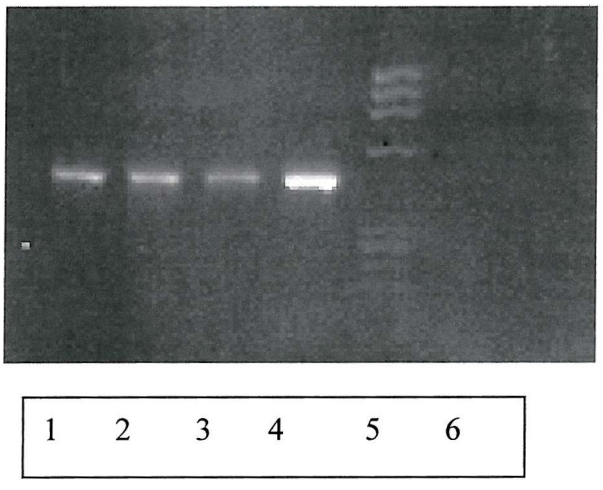
Precipitated nucleic acids were then collected by centrifuging the samples at 1300rpm for 10 minutes at 0°C. The supernatant was discarded and the pellet washed with 600µl of 80% Ethanol. The sample was then centrifuged for 5 minutes and the supernatant then removed. The sample was dried and then re-dissolved in 20ml of nuclease free water.

Comparison of Method 1 and Method 2

These two methods of RNA extraction were compared to ascertain the best method for obtaining HCV RNA. The results of PCR using RNA extracted by these two different methods, using the serum from the same patients taken at the same time point were compared (for method of PCR see next section). It was found that both methods could extract sufficient HCV RNA derived from fresh serum to allow PCR amplification of the 5′ non-coding region. The PCR product of amplification of the 5′ non-coding region were demonstrated clearly on an agarose gel shown in the diagram below (**figure 6.1**).

6.4.1 Methods: RNA extraction (continued)

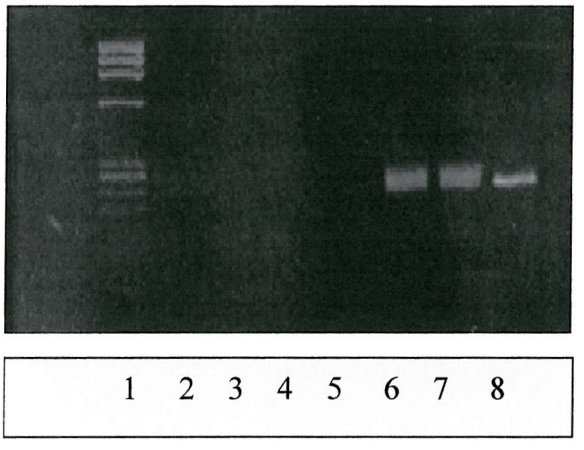
Figure 6.1: Electrophoresis agarose gel of PCR product of 5/ non coding region of HCV comparing RNA extraction method 1 and 2 using fresh serum samples from the same patient



1 = patient 1 method 1, 2 = patient 2 method 1
3 = patient 1 method 2, 4 = patient 2 method 2
5 = ladder, 6 = negative control

However for samples that were older than six months method 1 often extracted insufficient RNA to achieve a positive PCR result. This is shown in **figure 6.2**

Figure 6.2: Electrophoresis agarose gel of PCR product of 5/ non coding region of HCV comparing RNA extraction method 1 and 2 using stored serum samples from the same patient



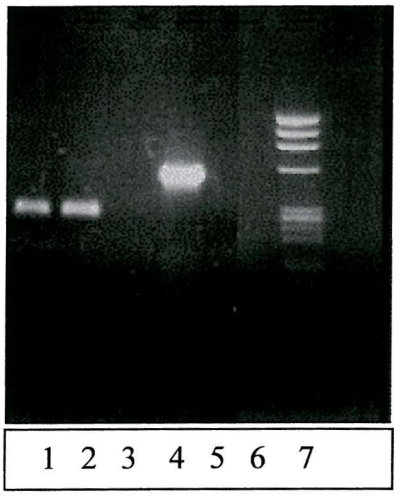
1 = ladder, 2 = negative control,
3 = patient 1 method 1, 4 = patient 2 method 1, 5 = patient 3 method 1,
6 = patient 2 method 2, 7 = patient 2 method 2, 8 = patient 3 method 2

6.4.1 Methods: RNA extraction (continued)

In addition the first method was found to be unreliable for assays for obtaining RNA for amplification of core and envelope. These regions are known to be harder to amplify than the 5'non-coding region. This is demonstrated in **figure 6.3.** In this experiment serum from the same patient at the same time point was subjected to RNA extraction using the two different methods and then subjected to PCR using primers for the non coding region and core region (see next section on PCR). The products were run out on an agarose gel, which shows that although method 1 can result in the detection of HCV RNA encoding the 5'non- coding region, insufficient RNA is extracted to amplify core region. On the other hand method 2 results in detection of both the non coding region and core region of the genome. The PCR reactions following the two methods of RNA extraction were exactly the same showing that the problem lies at the stage of RNA extraction and not at the stage of the PCR reaction.

Having compared these two methods all reactions were performed with method 2.

Figure 6.3: Electrophoresis agarose gel of PCR product of 5/ non coding region, and core region of HCV comparing RNA extraction method 1 and 2, using serum samples from the same patient



1 = patient 1 method 1 non coding region
2 = patient 1 method 2 non coding region
3 = patient 1 method 1 core region
4 = patient 1 method 2 core region
5&6 = negative controls
7 = ladder

6.4.2 Methods: Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)

cDNA synthesis and the first round PCR were performed as a single reaction. Primers were designed to amplify the 5' non-coding, core and envelope regions. The primers were based on the consensus genotype 1a sequence. A standard set of described primers were used to amplify the 5'non- coding region.

Different combination of primers were used to establish the best combination for amplifying the core and envelope regions. In addition the magnesium concentrations and the temperatures of the PCR reactions were adjusted to give the optimal DNA products. Subsequent sequencing of the envelope region revealed sequence variation in the sites where primers that had not produced a PCR product had been located, explaining why these earlier primers probably had not worked. The following text describes the most optimal set of conditions and primers that were established and thus subsequently used for all amplifications.

Core: RNA was transcribed with primer 1 and complementary DNA was amplified in a two-stage nested PCR reaction using in the first round, primer 2 and primer 1 and in the second round, primer 3 and primer 4.

Envelope: RNA was transcribed with primer 5 and complementary DNA was amplified in a two-stage nested PCR reaction using in the first round, primer 6 and primer 5 and in the second round, primer 7 and primer 8 (see **table 6.3**).

Table 6.3: PCR primers

PRIMER	SEQUENCE
1	antisense 5′- GCGAATTCTGGCAGCGCCTCCAAI
2	sense 5′-ACTGCCTGATAGGGTGCTTGCGAGTG
3	sense 5′-GCTCTAGAGGTCTCGTAGACCGTGCACC
4	antisense 5′-GCGAATTCTGGCAGCGCCTCCAAII
5	antisense 5′-GCCCGACCTGTCGGTCGII
6	sense 5′-CACTGGGGAGTCCTGGCGGGC
7	sense 5′-GCTCTAGATGGTGGGGAACTGGG
8	antisense 5′-CGGTGTTGAGGCTATCAT

6.4.2 Methods: RT-PCR (continued)

RT-PCR was carried out in a 50 µl reaction. cDNA synthesis and the first round PCR were performed as a single reaction. The PCR reaction mix contained 20µl of the extracted nucleic acid, 5µl 10x buffer, 3µl 50mM $MgCl_2$, 5µl 10mM dNTP mix, 1µl each of the relevant primers, 1µl Taq polymerase and 1µl reverse transcriptase and 13µl of H_2O. The reaction mix was overlaid with oil.

In the second reaction 2 µl of the first reaction was added to 5µl 10x buffer, 1.5µl 50mM MgCl, 5µl 10mM dNTP mix, 1µl each of the relevant primers, 1µl Taq polymerase and 33.5µl of H_2O. The reaction mix was overlaid with oil.

PCR was performed by cycling as follows for all reactions:- First round: 37°C 15 minutes, 94°C 1 minute x1, followed by 94 °C 30 seconds, 60°C 45 seconds, 72 °C 30 seconds x 35. Second round: 94 °C 30 seconds, 60°C 45 seconds, 72 °C 30 seconds x 35.

Positive controls:

For all reactions a positive control was performed using the serum of a known chronic carrier of HCV. A positive band from this reaction would indicate that both RNA extraction and PCR reaction had been completed successfully.

In addition PCR for the 5'non-coding region was performed along side amplification of core and envelope regions. If no band were seen in the reaction for the 5'non-coding region this would indicate that either the patient was negative for HCV RNA or that there had been failure to extract HCV RNA. However in all cases this reaction produce a positive result.

Negative controls:

At each stage (RNA extraction, first round and second round PCR) a negative control was performed that was then taken on to the next stage. Thus at the RNA extraction stage, water instead of patient serum was used as a negative control and the products of this reaction were then taken forward to both first and second round PCR reactions.

6.4.2 Methods: RT-PCR (continued)

In addition a separate negative control was added at the first round of the PCR reaction, where instead of adding the products of the RNA extraction, water was simply added to the PCR reaction. Likewise a further negative control was performed during the second round PCR reaction. Thus at the end there were three negative control samples. If a band was seen in the first negative control but not the subsequent two negative controls this indicated that there had been contamination at the RNA extraction stage. However if there were bands in the first and second negative controls then contamination would have most likely occurred during the first round PCR reaction (although may have occurred at both RNA extraction and the first round PCR). If bands appeared in all three negative controls then the contamination must have occurred during the second round PCR reaction as well as possibly at earlier stages. If at any stage there was contamination of the negative controls, the reagents used in the implicated contaminated reaction would be discarded and all the reactions repeated.

6.4.3 Methods: Cloning of PCR Products

PCR products for core and envelope were run out on a low melting point 1% agarose and ethidium bromide gel against a molecular weight marker. The relevant DNA bands of the correct molecular size were visualised under ultraviolet light, cut out and cleaned (GeneClean II Kit). This procedure removes residual DNA polymerase and the unwanted side products of the PCR reaction. This results in minimal false positive cloning and the recombinants containing spurious inserts. An estimation of DNA concentration was made against the molecular ladder which has a known DNA concentration. For optimal cloning efficiencies the vector to insert ratio was calculated to be in the range of between 1:5 to 1:10. The estimation of the concentration of the PCR products could be inaccurate and when a high level of false positive clones were observed a range of factor to insert ratio was used to achieve the optimum conditions.

The products were cloned into a vector (pMOS*Blue* T-vector, Amersham). This kit provides an efficient system for direct cloning of PCR products. The methodology eliminates the requirement for additional sequences or restriction sites being incorporated into PCR primers, which when cleaved produce sticky ends. This system exploits the template independent activity of thermostable polymerase which preferably adds a single adenosine nucleotide to the 3'end of double stranded DNA. The amplified DNA can then be ligated into the T-vector which has been specifically constructed to be compatible with PCR products by being thymidine tailed.

Ligation of PCR product into pMOS Blue T-vector

For each PCR product to be cloned the following ligation reaction was made up:

1 µl	10x ligase buffer
0.5µl	100mM DTT
0.5µl	10mM ATP
1.0µl	50ng/µl vector
6.5µl	amplified product
0.5µl	T4 DNA ligase (2-3 weiss units)

The ligation mixture was then integrated at 16 ^{o}C for two hours or overnight.

6.4.3 Methods: Cloning of PCR Products (continued)

Transformation

Competent cells (MOS*blue* competent cells) were thawed on ice. For each transformation one microfuge tubes was pre chilled on ice and to this 20µl of competent cells was added. 1µl of ligation mix was then added directly to the cells and left on ice for 30 minutes. The cells were then heat shocked for 40 seconds in a 42°C water bath and then placed back on ice for two minutes. SOC medium was then added to each tube and the tubes shaken for one hour at 37°C. The cells were then plated on ampicillin and tetracycline agar plates with X-gal (5-bromo-4-chloro-3-indolyl-B-galactoside) and IPTG (isopropyl B-D-thiogalactopyranoside and incubated overnight. The tetracycline ensures that the selectable F' containing Lac Z M15 is maintained and thus eliminates the background of non-white colonies which have lost the F'. The colonies can then be blue-white screened with recombinant colonies appearing white.

6.4.4 Methods: Template Preparation

Twenty four separate white colonies were picked and grown up overnight in separate universal tubes. The plasmid DNA was extracted and purified using Hybaid Recovery plasmid mini prep extraction kit. The kit is designed for rapid isolation and purification of double stranded plasmid DNA from bacterial cultures. The bacterial culture is centrifuged and the supernatant removed and discarded. The cell pellet that is left is re-suspended in 50μl of pre lysis buffer and vortexed. To this is added 100μl of alkaline lysis solution and the suspension is pipetted up and down until the solution is clear and viscous. 75μl of neutralising solution is then added and vortexed. A white precipitate forms which consists of cell membranes, proteins and chromosomal DNA. The vials are centifuged to pellet the white precipitate and the supernatant which contains the plasmid DNA is removed and transferred to a spin filter which is placed on to a micro centifuge vial. To the spin filter is added to 50 microlitres of binding buffer. The tubes are spun and the plasmid DNA will bind to the binding buffer and remain on the filter. The filter is washed and then finally the plasmid DNA is eluted from the spin filter by adding 50μl of sterile water and spinning the tubes. The DNA will collect in the bottom of the vial.

A sample of the DNA can be tested by subjecting it to restriction enzyme digestion and running the DNA on an agarose gel to check for a product the correct size.

6.4.5 Methods: Sequencing

DNA templates were then sequenced by PCR sequencing (Thermosequenase kit, Pharmacia Biotech, Uppsala, Sweden). This technique relies on the synthesis of a new strand of DNA starting at a specific priming site using a primer which is radiolabelled with g-P^{33}-ATP (the relevant sense internal HCV primer for core or envelope), extending with the incorporation of dNTPs and ending with the incorporation of a chain terminating dideoxynucleoside triphosphate. In this kit 7-deaza-dG is substituted for dG, which helps eliminate secondary DNA structure. Cycle sequencing uses repeated cycles of thermal denaturation, annealing and extension/termination using a thermostable DNA polymerase. We found this method resulted in increased signal levels, clearer sequence and also combated crossbanding seen with standard sequencing methods. The following PCR conditions were used: 95 °C 30 seconds, 55 °C 30 seconds, 72 °C 60 seconds x 50. The reaction products were separated in a 6% acrylamide/5.5 mmol/L urea denaturing gel. The gel was dried and autoradiography was performed for 24 hours and then manually read and loaded onto DNA analysis software package that performed sequence alignment and protein sequence analysis.

6.4.6 Methods: Sequence Analysis

The number and diversity of quasispecies was determined by subcloning HCV PCR products and sequencing multiple recombinants at each time point. A minimum of twenty clones from each patient were sequenced at each time point. This allowed accurate identification of the dominant sequence in each sample. For each individual, the most frequently isolated sequence at the first time point was compared with that isolated from the same patient 2 years later. Nucleotide substitutions and the corresponding amino acid sequence were determined. The rate of nucleotide mutations was determined as substitutions per base per year. Six analyses were performed:

1. The rate of mutations per nucleotide per year in each region
2. The ratio of coding: neutral mutations
3. Rate of mutation within CTL epitopes: rate of mutation in flanking regions
4. Rate of mutation within regions containing helper T cell epitopes: rate of mutation in flanking regions
5. Mutation rate across HVR in patients with normal immunity: mutation rate across HVR in patients with antibody deficiency.
6. Ratio of purine to pyrimidine substitutions was calculated in order to detect Taq errors

6.4.7 Methods: HLA typing

Class I HLA typing was performed on all patients using sequence specific PCR[155].

6.4.8 Methods: Antibody assays

Patient sera were tested using commercially available second and third generation ELISA antibody tests for HCV followed by a RIBA-3 test, according to manufactures instructions.

6.4.9 Methods: Assays of $CD8^+$ T cell function

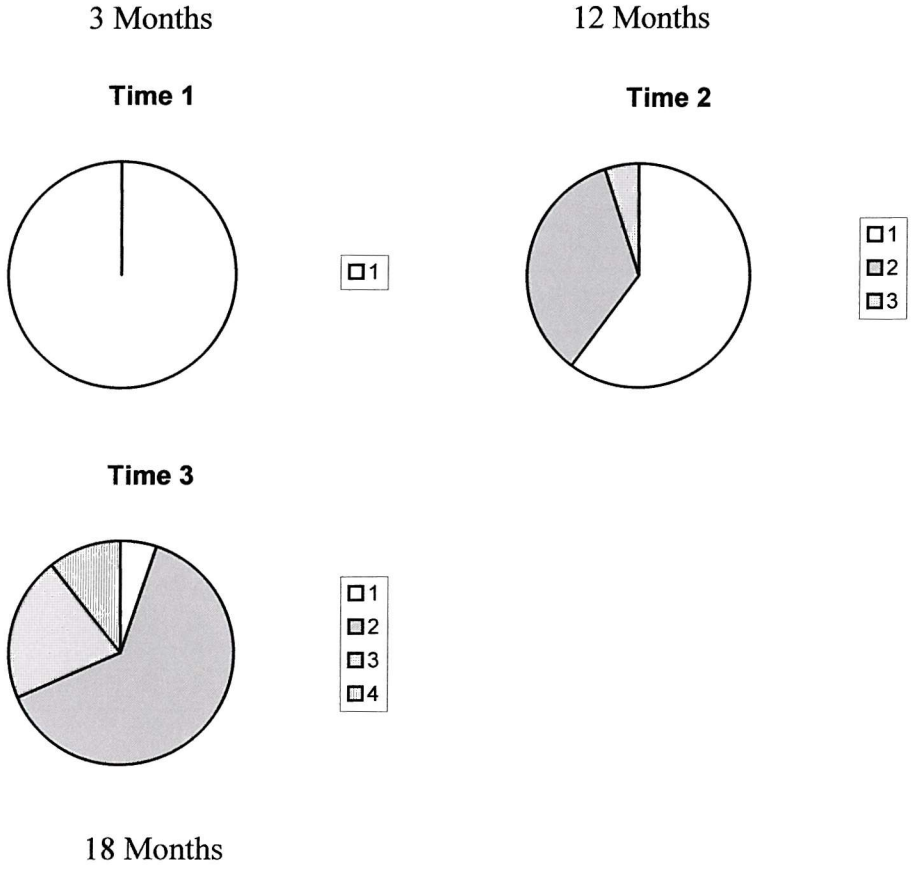
Peripheral blood mononuclear cells were isolated by density centrifugation, cultured in RPMI supplemented with 10% foetal calf serum and stimulated *in vitro* with synthetic peptides representing HLA-A2.1 restricted T cell epitopes. Chromium release cytotoxicity assays were used to test the ability of patients peripherally derived T cells to lyse B cell targets sensitised with HCV peptides representing relevant CTL epitopes. Standard chromium release assays were performed according to established protocols (see chapter 5).

6.5 RESULTS

6.5.1 Results: Comparison of Predominant Sequence at Time 1 and Time 2

Both the diversity of HCV isolates (differences between quasispecies) and their variability (changes over time) were analysed. The diversity of quasispecies in each sample was determined at both nucleotide and deduced protein levels. In all patients the diversity of quasispecies increased over the 2 year observation period. Protein diversity in envelope region exceeded that in core. Variability within the envelope region was greater than that in the core region and increased over time. An example of the increasing diversity of quasispecies based on envelope sequences in 1 patient at different time points are shown in **Figure 6.4**.

Figure 6.4: Evolution of HCV quasispecies in 1 patient showing the relative proportion of each quasispecies at three time points after infection



6.5.1 Results: Comparison of Predominant Sequence at Time 1 and Time 2(continued)

In the entire antibody deficient patients the predominant sequence at the first time point was identical to that of the infecting HCV inocculum. The nucleotide sequence for core is shown in **figure 6.5**, for envelope in **figure 6.6** and derived amino-acid sequence for envelope in **figure 6.7**.

Figure 6.5: Nucleotide changes in Core over 2 years

A. Hypogammaglobulinaemic patients (patients 1-3)

```
1ATGAGCACGAATCCTAAACCTCAAAGAAAAACCAAACGTAACACCAACCGTCGCCCACAGGACGTTAAGTTCCCGGGTGGCGGTCAGATCGTTGGTGGAGTTTACTTGTTGC
2--------a-------------------------------------------------------c-----------------------------------------------
3--------a-------------------------------------------------------c-----------------------------------------------
4--------a-------------------------------------------------------c-----------------------------------------------

1CGCGCAGGGGCCCTAGATTGGGTGTGCGCGCGACGAGGAAGACTTCCGAGCGGTCGCAACCTCGAGGTAGACGTCAGCCTATCCCCAAGGCACGTCGGCCCGAGGGCAGGAC
2-------------------------------C-----------------------------------------------------------t-------------------
3-------------------------------C-----------------------------t-------------------------t-------------------
4-------------------------------------------------------------t-----------------------------------------------

1CTGGGCTCAGCCCGGGTACCCTTGGCCCCTCTATGGCAATGAGGGCTGCGGGTGGGCGGGATGGCTCCTGTCTCCCCGTGGCCTCGGCCTAGCTGGGGCCCCACAGACCCCC
2----------------------------------------------------------------------------------------------------------------
3------------------t-----------------------------------------------------------------------------------------------
4------------------t-----------------------------------------------------------------------------------------------

1GGCGTAGGTCGCGCAATTTGGGTAAGGTCATCGATACCCTTACGTGCCGGCTTCGCCGACCTCATGGGGTACATACCGCTCGTCGGCGCCCCTC
2-------------------------------------c--------------------------------------------------------
3----------------------------------------------------------------------------------------------
4----------------------------------------------------------------------------------------------
```

1= Gammaglobulin sequence, 2= Sequence of Pt 1 time point 2, 3= Sequence of Pt 2 time point 2, 4= Sequence of Pt 3 time point 2
Capital letter = coding change, Small letter = non coding change

Figure 6.5 (continued) B. Patients with normal immunity (patients 4-6)

PATIENT 4

```
T.1ATGAGCACGAATCCTAAACCTCAAAGAAAAACCAAACGTAACACCAACCGTCGCCCACAGGACGTCAAGTTCCCGGGTGGCGGTCAGATCGTTGGTGGAGTTTACTTGTT
T.2------------------------------------------------------------t-----------------------------------------------

T.1GCCGCGCAGGGGCCCTAGATTGGGTGTGCGCGCGACGAGGAAGACTTCCGAGCGGTCACAACCTCGAGGTAGACGTCAGCCTATCCCCAAGGCTCGTCGGCCCGAGGGCA
T.2-----------------------------------------------------g-----------------------------------------------------

T.1GGACCTGGGCTCAGCCCGGGTACCCTTGGCCCCTCTATGGCAATGAGGGCTGCGGGTGGGCGGGATGGCTCCTGTCTCCCCGTGGCCTCGGCCTAGCTGGGGCCCCACAG
T.2-----------------------------------------------a-----------------------------------------------------

T.1ACCCCCGGCGTAGGTCGCGCAATTTGGGTAAGGTCATCGATACCCTTACGTGCCGGCTTCGCCGACCTCATGGGGTACATACCGCTCGTCGGCGCCCCTC
T.2-----------------------------------------------------------------------------------------------------

PATIENT 5

T.1ATGAGCACGAATCCTAAACCTCAAAGAAAAACCAAACGTAACACCAACCGTCGCCCACAGGACGTCAAGTTCCCGGGTGGCGGCCAGATCGTTGGTGGAGTTTACTTGTT
T.2--------a--------------------------------------------------------------------------t-------------------------

T.1GCCGCGCAGGGGCCCTAGATTGGGTGTGCGCGCGACGAGAAAGACTTCCGAGCGGTCGCAACCTCGAGGTAGACGTCAGCCTATCCCCAAGGCTCGTCGGCCCGAGGGCA
T.2-----------------------------------G-----------------------------------------------------------------------

T.1GGACCTGGGCTCAGCCCGGGTACCCTTGGCCCCTCTATGGCAATGAGGGCTGCGGGTGGGCGGGATGGCTCCTGTCTCCCCGTGGCCTCGGCCTAGCTGGGGCCCCACAG
T.2------------------t-----------------------------------------------------------------------------------

T.2ACCCCCGGCGTAGGTCGCGCAATTTGGGTAAGGTCATCGATACCCTTACGTGCCGGCTTCGCCGACCTCATGGGGTACATACCGCTCGTCGGCGCCCCTC
T.2-------------------------------------------------------------------------c-------------------------
```

T.1 = Time point 1, T.2 = Time point 2

Figure 6.5 (continued) B. Patients with normal immunity (patients 4-6)

```
PATIENT 6

T.1ATGAGCACGAATCCTAAACCTCAAAGAAAAACCAAACGTAACACCAACCGTCGCCCACAGGACGTCAAGTTCCCGGGTGGCGGTCAGATCGTTGGTGGAGTTTACTTGTT
T.2--------a-----------------------------------------------------------a-----------------------------------------

T.1GCCGCGCAGGGGCCCTAGATTGGGTGTGCGCGCGACGAGGAAGACTTCCGAGCGGTCGCAACCTCGAGGTAGACGTCAGCCTATCCCCAAGGCTCGTCGGCCCGAGGGCA
T.2---------------------------------a-----------------------------------------------------------------------------

T.1GGACCTGGGCTCAGCCCGGGTACCCTTGGCCCCTCTATGGCAATGAGGGCTGCGGGTGGGCGGGATGGCTCCTGTCTCCCCGTGGCCTCGGCCTAGCTGGGGCCCCACAG
T.2-----------------------------------------------c-----------------------------------------------------------------

T.2ACCCCCGGCGTAGGTCGCGCAATTTGGGTAAGGTCATCGATACCCTTACGTGCCGGCTTCGCCGACCTCATGGGGTACATACCGCTCGTCGGCGCCCCTC
T.2-------------------------------------------------------------------------------------------------
```

T.1 = Time point 1, T.2 = Time point 2

Figure 6.6: Nucleotide changes in Envelope over 2 years

A. Hypogammaglobulinaemic patients (patients 1-3)

```
1.AACTGGGCGAAGGTTCTGGTAGTACTGCTGCTATTTGCCGGCGTCGACGCGGAAACCCGCGTCACCGGGGGAGTTGCCGGCCGCGTTACGTCCGGACTTGCTGGCCTCTTT
2.---------------A-------g-----------c----------------------A------------------------------------------------------
3.---------------A-----------------------------A--------------------------C-A---------C-------------------------
4.-------------------------------------------------G-----A----------a---C-----------C----------T--------------
-

1.GCACCAGGCGCCAAGCAGAACATCCAGCTGATCAACACCAACGGCAGTTGGCACATCAATAGCACGGCCCTCAACTGCAATGATAGC
2.-----------------------------------G-----------------------------------------------------
3.---------------AC-------------G----------------A-T-----------------------------------
4.----G-------C---------------------------------A-T-----------------------------------
```

1= Gammaglobulin sequence, 2= Sequence of Pt 1 time point 2, 3= Sequence of Pt 2 time point 2, 4= Sequence of Pt 3 time point

Figure 6.6: Nucleotide changes in Envelope over 2 years (continued)

B. Patients with normal immunity (patients 4-6)

```
PATIENT 4
T.1AACTGGGCGAAGGTCCTGGTGGTGCTGCTGCTTTTTGCCGGCGTCGACGCGGAAGAACCCCGCCACAGGGGAAGTGCCGGCAGCAATCATCGTGGAACTTGTCGCCTTCT
T.2--------------------------------a--c------------G---------------G-----------T--------------------A-------

T.1ACTAACAGGCCCCCCGCAGAACATCCAGCTGATCAACACCAACGGCAGTTGGCACCTCAATCGCACGGCCCTGAACTGCAATGATAGC
T.2--C-------G---T-------------------------------------A------------------------------

PATIENT 5
T.1AACTGGGCGAAGGTCATGGTAGTGCTGCTCCTGTTTGCCGGCGTCGACGCACAAACCCACGTCACCGGGGGAAGTGCCGGCCACACTGTGTCTGGATTAGTTAGCCTCCT
T.2---------------C-------------g--------------------------T----------a----------C----G----------c------------T-

T.1CACACCAGGCGCCAAGCAGAACATCCAGCTGATCAACACCAACGGCAGTTGGCACCTAAATAGCACGGCCCTGAACTGCAATGATAGC
T.2----------C---G-------G---------------G---------------A------------------------------

PATIENT 6
T.1AACTGGGCGAAGGTCCTGGTAGTGCTGCTCCTGTTTGCCGGCGTCGACGCACAAACCCACGTCACCGGGGGAAGTGCCGGCCACACTGTGTCTGGATTTGTTGGCCTCTT
T.2--------------a-----------------a----------------------------------------G------C-----------T----------------

T.1CGCACCAGGCGCCAAGCAGAACATCCAGCTGATCAACACCAACGGCAGTTGGCACCTCAATAGCACGGCCCTGAACTGCAATGATAGC
T.2-C---T--------------------------------t-----------------------------------------C----
```

T.1= time 1, T.2 = time 2. Capital letter = coding change, Small letter = non coding change

Figure 6.7: Amino acid changes in envelope over 2 years

A. Hypogammaglobulinaemic patients (patients 1-3)

```
                                        HVR
                              *-----------------------------------*
1 . NWAKVLVVLLLFAGVDAETHVTGGSAGHTVSGFVSLLAPGAKQNVQLINTNGSWHLNSTALNCNDS

2 . NWAKVLVVLLLFAGVDAETRVTGGVAGRVTSGLAGLFTPGAKQNIQLINTNGSWHINSTALNCNDS
3 . -----M----------H-----------------------------S----------------
4 . -----M----------N---------AS--A-------------T----V----RL----------
5 . ------------------G-H----A---A---F-----R--Q----------RL----------
```

1= Consensus 1a sequence Choo et al PNAS 1991, 2= Gammaglobulin sequence, 3= Sequence of Pt 1 time point 2, 4= Sequence of Pt 2 time point 2, 5= Sequence of Pt 3 time point 2

B. Patients with normal immunity (patients 4-6)

```
                                        HVR
                              *-----------------------------------*
PATIENT 4
T . 1 NWAKVLVVLLLFAGVDAEEPRHRGSAGSNHRGTCRLLLTGPPQNIQLINTNGSWHLNRTALNCNDS
T . 2 ----------------G-----G---V-------S--P--AL-------------I----------

PATIENT 5
T . 1 NWAKVMVVLLLFAGVDATTHVTGGSAGHTVSGLVSLLTPGAKQNIQLINTNGSWHLNSTALNCNDS
T . 2 -----L-------------Y------A-A-------F---PR--V----S-----I----------

PATIENT 6
T . 1 NWAKVLVVLLLFAGVDATTHVTGGSAGHTVSGFVGLFAPGAKQNIQLINTNGSWHLNSTALNCNDS
T . 2 ----------------------G-A---F------PL----------------------A-
```

T1 = time 1, T2 = time2, HVR = Hypervariable region, Italic = A2 CTL epitope

6.5.1 Results: Comparison of Predominant Sequence at Time 1 and Time 2(continued)

The nucleotide mutation rates are presented in **table 6.4** (core) and **table 6.5** (envelope). Numbers represent the number of mutations observed over 2 years. Mean substitution rates per site per year have been calculated.

Core Region- Nucleotide mutation rate in the core gene was 5.23 $x10^{-3}$/site/year and exceeded the number predicted from previous studies[156]. In the core gene, the frequency of coding changes was at a rate predicted by previous studies in both groups of patients (0.5 $x10^{-3}$/site/year). Assuming random accumulation of nucleotide mutations, the predicted ratio of neutral to coding mutations would be 2.3:1 and that observed was 6.6:1, almost three times greater than predicted.

Table 6.4: Mutations Observed in 430 nucleotides of Core Region over 2 years

	Neutral Changes	Coding Changes	Total changes	Mutation Rate $x10^{-3}$ /site/year
Predicted	1.2	0.48	1.6	1.86
Patient 1	4	1	5	5.81
Patient 2	5	1	6	6.98
Patient 3	4	0	4	4.65
Patient 4	3	0	3	3.49
Patient 5	4	1	5	5.81
Patient 6	4	0	4	4.65

Ratio of Neutral: Coding mutations Observed 8:1; Predicted 2.3:1
(Calculated by Ina et al. 2.15:1)

6.5.1 Results: Comparison of Predominant Sequence at Time 1 and Time 2(continued)

Envelope region- In the envelope gene the opposite was found. The mean observed overall mutation rate was 23.8 $x10^{-3}$/site/year and exceeded that predicted from previous studies. By contrast with the core gene data, the rate of coding mutations in envelope greatly exceeded the rate of neutral mutations. The observed ratio of neutral: coding mutations was 1:4.8, 10 times greater than predicted (2.3:1). The mean observed neutral mutation rate was similar to that predicted and was at the same rate in envelope as core in individual patients.

Table 6.5: Mutations Observed in 200 nucleotides of Envelope Region over 2 years

	Neutral Changes	Coding Changes	Total Changes	Mutation Rate $x10^{-3}$ /site/year
Predicted	0.56	0.23	0.79	1.975
Patient 1	2	3	5	12.5
Patient 2	0	9	9	22.5
Patient 3	1	9	10	25
Patient 4	2	8	10	25
Patient 5	3	10	13	32.5
Patient 6	3	6	10	22.5

Ratio of Neutral: Coding mutations Observed 1:4.18; Predicted 2.3:1
(Calculated by Ina et al. 19.6:1)

Tables 4 and 5 present the predicted and observed numbers of mutations within core (table 4) and envelope (table 5) regions of HCV. The observed and predicted ratios of neutral: coding mutations are presented.

6.5.2. Results: Mutation rates within the HVR in antibody deficient and normal patients.

All patients with hypogammaglobulinaemia had abnormally reduced levels of immunoglobulins A, G and M and lacked detectable antibodies to HCV as determined in second and third generation ELISA tests (Abbott 2 and 3 ELISA) and recombinant immunoblot assay (Ortho RIBA-3). When mutations rates in the HVR were compared between patients with normal immunity and those with antibody deficiency, no significant difference could be detected (**table 6.6**) and there was no difference in the ratio of coding: neutral mutations within the HVR.

Table 6.6: Mutation rate within the hypervariable region: comparison of patients with antibody deficiency and normal immunity

PATIENT	IMMUNITY	NUMBER OF CODING CHANGES IN HVR OVER 2 YEARS	
		Coding	Neutral
1	CVID	1	0
2	CVID	4	0
3	CVID	7	1
4	Normal	7	0
5	Normal	6	2
6	Normal	5	0

6.5.3 Results: Mutation in CTL Epitopes

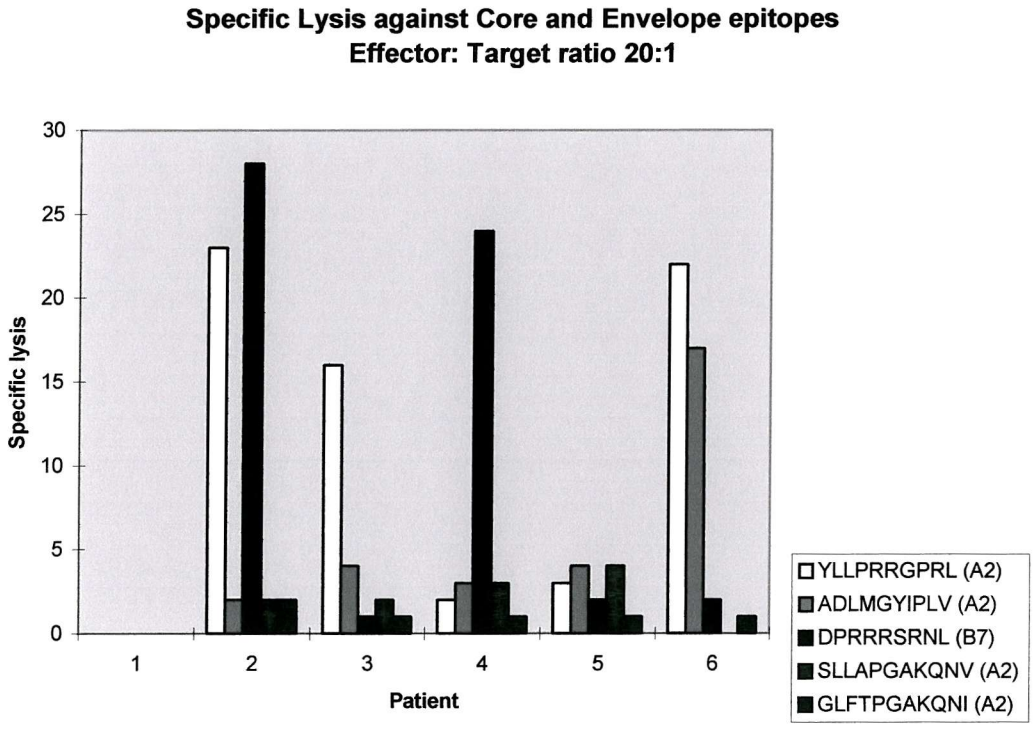
Core Region: No mutations were detected within the HLA-A2.1 epitopes encoded in core in any patient despite the presence of detectable CTL activity against core peptides in patients 2,3,5 and 6 (see **figure 6.8**). Furthermore the mutation rate in regions encoding CTL epitopes did not differ between those patients with or without detectable CTL responses to the epitopes. There was no difference in the mutation rate in patients with normal immunity compared to those with antibody deficiency, who might be expected to rely more heavily on CTL to control viral infection.

Envelope Region: Although mutations were detected in the gene encoding an HLA-A2.1 CTL epitope in the five HLA-A2.1 haplo-identical patients, these were present at the outset and mutation in this region was not increased relative to the flanking regions (see **table 6.7**). In addition there was no evidence of CTL recognition of synthetic peptides translated from either consensus or mutated sequence (see **figure 6.8**).

Table 6.7: Coding mutation rate/aa/2 years in envelope comparing A2 CTL epitope and its flanking region

HLA-A2 patients	Envelope Epitope	Flanking Region	Ratio epitope: flanking
Patient 1	0	0.02	1.0:1
Patient 2	0.03	0.05	0.6:1
Patient 3	0.06	0.05	1.2:1
Patient 5	0.12	0.035	3.4:1
Patient 6	0.06	0.03	2.0:1

Figure 6.8: Cytotoxic responses to CTL epitopes in core and CTL index and mutated epitope in envelope



Patient	A1,2	A2,68	A2,31	A1,3	A1,2	A2,31
HLA	B44,37	B7,64	B44,35	B7,49	B60,55	B18,55

CTL responses were demonstrated against core epitopes in patients 2,3,4 and 6 as demonstrated above. CTL assays were performed approximately half way between the samples for mutational analysis i.e after 1 year from the first sample. The responses in the patients 4 and 6 were more impressive than responses in other patients with chronic infection in Chapter 5. This may have been due to a relatively shorter duration of infection.

6.6 DISCUSSION

We have found evidence of selection of mutation in the envelope gene and conservation in the core gene of HCV over a 2 year period of infection. In the present study neither genetic sequence conservation in core, nor variation in envelope can be attributed to host immune responses. The evidence for these assertions is that the core region remained unchanged despite CTL recognition and mutations in envelope were not clustered within known CTL or B cell epitopes. In addition envelope mutations occurred at the same frequency in patients with normal B cell immunity and antibody deficiency. These findings imply that sequence variation or stability in core and envelope genes of HCV in chronic infection must be attributed to selection pressures other than those exerted by CTL or antibodies.

The present investigation was designed to address many of the problems encountered in earlier studies[103;150;157;158]. We conducted a longitudinal study of virus mutation in individual patients, rather than a cross-sectional study, as this is a more appropriate methodology for observing the natural history of virus-host interactions. Many earlier studies have included patients carrying a variety of HLA types, infected with differing HCV genotypes for varying periods of time. Each of these factors might influence the observed mutation rate of HCV suggesting spurious variation or obscuring true changes. In order to permit valid comparisons between subjects, patients were matched for HLA background, HCV genotype and duration of infection. In studying sequence variation we elected to subclone PCR products and analyse multiple clones at each time point, (rather than directly sequencing amplicons), to provide an accurate picture of the relative representation of quasispecies. In addition studies of CTL function were performed to confirm that the core protein was exposed to host immune selection.

Longitudinal analysis revealed the changing diversity of quasispecies. In all individuals the number of quasispecies increased with time. The significance of this increasing diversity is uncertain but it has been suggested as a means by which HCV might secure an evolutionary advantage by diverting the host immunity into a wide range of inadequate responses.

By performing a longitudinal study we were able to obtain an accurate measure of the mutation rate of HCV. This appears to be higher than that observed in previous

studies which have relied on cross-sectional analyses of sequence diversity between large numbers of isolates[156]. Our findings refute the suggestion from this earlier work that the synonymous mutation rate is reduced in core. The high rate of mutability emphasises HCV's potential to change its proteins when exposed to negative selection and is consistent with recent reports of the high replication rate of HCV[157].

Interestingly whilst neutral mutations were evenly distributed across core and envelope regions there was a marked difference in the frequency of coding mutations between the two regions studied. The even distribution of neutral mutations suggests that the error rate of HCV's RNA polymerase is not significantly template dependent, at least in the two regions studied. Suppression of coding mutations within the core region implies that this viral protein's function is dependent on a high degree of sequence conservation. The core protein binds and encapsulates viral RNA as well as anchoring the envelope protein to its external surface. These functions may be critically affected by point mutations in the core gene which would render core mutants non-viable. Such a loss of function would explain the apparent selection of stability in core. The core protein has also been shown to act in ways that might confer a survival advantage on HCV. It can modulate cellular and viral promoter activities[158-161], bind to host RNA helicase to interfere with host RNA translation, and interact with host[162]. Recent data has suggested that HCV core may mediate an immunosuppressive effect in vivo. In addition, core can directly bind TGF-B receptor and may modify the host response to HCV facilitating persistent infection[163]. Positive selection of these actions that might benefit HCV provides an alternative explanation for the observed sequence stability in core.

In contrast, the selection of coding mutations within the envelope region implies that these mutations provided the quasispecies with survival advantages. It has been suggested that a major determinant of such selection is host immune pressure and that such mutations enable the virus to escape from immune control by the host. Despite using a variety of analyses to search for evidence of immune selection of mutations we could find no evidence of either immune escape from T cell or antibody recognition. Earlier studies have reported lower rates of coding mutation in antibody deficient compared to immunocompetent patients[103;150]. This has been interpreted as evidence that mutation in the HVR is selected by the host antibody response. However a review of the data reveals a lower frequency of both neutral and coding mutations in the antibody

deficient patients when compared to the controls. The reason for the reduced neutral and coding mutation rate amongst immunodeficient patients seen in this earlier study is unclear but may well have been due to the shorter duration of infection compared to the control subjects. In the present study we have endeavoured to match the immunodeficient and immunocompetent patients for duration of infection to avoid this potential bias. Furthermore earlier studies made no attempt to control for HCV genotype but all patients in the present study were selected for genotype 1a infection and we found no difference in the rate of coding mutations between the two groups.

Thus the present study has found clear evidence of selection of coding mutations in the envelope gene of HCV during the course of chronic infection which cannot be attributed to selection of mutants by CTL or antibodies. Alternative mechanisms must be sought to explain the selection of these mutants. In other chronic viral infections viruses have been shown to express proteins that interfere with antigen processing and presentation[164], to down regulate the expression of HLA molecules[165] and to produce proteins that interfere with the action of host cytokines[166-170] and CTL responses[74]. It is possible that mutations within the envelope gene of HCV result in one or some of these actions. These potential interactions between HCV proteins and host immune responses warrant further investigation.

It must be stressed that the present study concentrated on mutations in known immune epitopes in only two regions of the HCV genome. It is possible that mutations within the envelope region accumulated in immune epitopes that have yet to be recognised. However the mutations were randomly distributed throughout envelope and the observed mutations were not clustered within sequences that represent known HLA binding motifs[171]. Nothing is yet known about the immunodominance of $CD8^+$ T cell epitopes in HCV. It is possible that the epitopes encoded in core and envelope are of little significance in the immune response to the whole virus and it is for this reason that we failed to detect a correlation between mutation and persistence. Identification of immunodominant epitopes and a more comprehensive study may resolve this question. However the lack of evidence of immune escape in this study and the detection of the emergence of only one antagonist mutant in an earlier study[133] suggests that immune escape from CTL is not the major mechanism by which HCV ensures persistence in chronic infection.

The finding that CTL epitopes were conserved within the core region despite evidence of CTL recognition suggests that the $CD8^+$ T cell response to core may be ineffective in chronic hepatitis C infection. This raises the possibility that HCV interferes with effector T cell function in some way after T cell activation has occurred. This alternative explanation for HCV persistence has precedence in other chronic viral infections[95] and warrants investigation in chronic hepatitis C. Greater understanding of the reasons why $CD8^+$ T cells directed against core CTL epitopes fail to eliminate HCV infection and why coding mutations in envelope accumulate during persistent HCV infection may provide the basis for the design of immunotherapy and an effective vaccine against HCV.

CHAPTER 7:
SUMMARY AND CONCLUSIONS

The outcome of HCV infection in different individuals is extremely variable. Some patients will spontaneously clear HCV, whilst the vast majority will go on to develop chronic infection. In patients with chronic infection, the rate at which progressive liver disease develops is again variable. Up to 30% of patients will develop cirrhosis of the liver, which may then be complicated by liver failure and hepatocellular carcinoma. On the other hand many patients will remain asymptomatic without cirrhosis despite decades of chronic infection. This wide disparity in clinical outcome of patients infected with HCV must either be a result of viral or host factors or both.

From the first study of the Oxford clinic population, it was found that 15% of patients with antibodies to HCV had persistently negative tests for HCV RNA by PCR. Whether these patients represent patients who have truly cleared HCV still remains controversial. However there is no doubt that these patients have a different clinical course to patients who are HCV RNA positive patients. Follow up of HCV RNA negative patients in the Oxford and other series has shown that morbidity and mortality related to liver disease in this group is extremely low compared with HCV RNA positive individuals.

In this study female gender was shown to influence whether a patient is HCV RNA positive or negative. In addition there was a trend for patients who were infected at a younger age to be more likely to clear virus. This suggests that host factors play a role in determining viral persistence. In addition female gender and younger age of infection was also found to correlate with milder disease progression.

The role of the viral factors in determining viral persistence is harder to evaluate. Viral genotype, viral load or viral sequence variation cannot be assessed in HCV RNA negative patients in such a study. This can only be evaluated prospectively in patients identified in the acute stages of HCV infection who then go onto to clear the virus. As patients with acute HCV are usually asymptomatic, they are rarely identified, making such a study extremely difficult. It has been postulated that viral load at the time of infection may determine disease outcome. If this hypothesis were correct one might expect to see a diiference in the outcome of patients infected from blood transfusion, as viral load associated with blood transfusion is almost certainly greater than that with

infection through contaminated needles with intravenous drug use. In our population we found that that the route of transmission in HCV RNA negative and positive patients was not significantly different. This would suggest that viral load at the time of infection is not a factor that influences viral persistence or clearance.

The reasons for age at infection influencing clinical outcome needs further evaluation but one possible explanation is that the host defence mechanisms against HCV are weaker in older patients. The mechanism by which female sex protects against HCV remains unknown.

In addition to female sex and age at infection, alcohol consumption was shown to correlate with disease progression. This correlation is not surprising as alcohol is well established as a cause of liver disease in its own right. However there is some evidence to suggest that alcohol does not merely have an additive affect on disease progression but might have may even accelerate fibrosis formation. Thus advice on keeping alcohol consumption to a minimum is essential.

An important observation from the liver histology of our chronically infected patients was that fibrosis but not histological grade (degree of inflammation) correlated with duration of infection. Thus clinically relevant progression of chronic hepatitis C is better estimated by fibrosis stage than by the grade of histological activity. The degree of inflammation may help to predict who is likely to progress faster although only further studies with serial liver biopsies will answer this question.

One problem with this initial study of the Oxford HCV clinic is that in many patients the duration of infection was estimated. The second study, involving patients identified from the national look-back programme identified a group of blood transfusion recipients who were infected with hepatitis C at a known time, making them an ideal group of all ages in which to study the natural history of this infection. At Oxford 44 out of 58 (76%) of the infected recipients were persistently HCV RNA positive after intervals of 4 - 13 years. This figure is in keeping with the overall results of the clinic.

Eleven recipients were repeatedly negative for HCV RNA although anti- HCV positive. There were three recipients with no detectable anti-HCV or HCV RNA after seven to 13 years, despite strong evidence of transfusion with infectious blood. These patients were in good health and had no symptoms, signs or biochemical evidence of liver disease. These 14 people, four of whom were young children, had a mean age of 27 years when transfused with a sex ratio of 4 males to 10 females. By comparison, the 44

recipients with chronic hepatitis C infection had mean age at transfusion of 46 years, with 10 recipients over the age of 60 and the sex ratio in this Group was 20 males to 24 females. Logistic regression analysis indicates that the age difference in the two groups did not quite achieve statistical significance ($p>0.05<0.1$) and that numbers were too small to deduce any influence of sex on the outcome of infection.

Thus although not quite reaching statistical significance due to the small number of patients, the findings of the first study of the entire clinic population were borne out. Further results from the National HCV look back study will provide valuable information about factors that influence the outcome of HCV infection. In addition as with the first study, the liver biopsies of patients infected at a younger age showed less fibrosis. Furthermore as the genotypes of the donors was known in this study, the effect of viral genotype on determining viral persistence or clearance could be evaluated. A variety of genotypes were present in donors linked to recipients who had recovered and this did not differ from the HCV genotypes in the recipients with chronic infection. This gives no indication of a more severe effect due to a particular virus genotype, again suggesting that host factors rather than viral factors are more important in determining disease outcome.

The Gammagard outbreak allowed a unique opportunity to study the natural history of HCV prospectively from a known time point of infection and to asses the role of the antibody response in determining disease outcome. The epidemiological investigation proved that the one batch of Gammagard was responsible for this UK outbreak of HCV. In addition the genotype and the nucleotide sequence between isolates found in all the patients and in the implicated batch was identical - genotype 1a. These findings were consistent with all the patients having been infected from a single donor. Thus the effect of the same virus, controlling for viral factors, could be studied in different hosts.

Similar to the blood transfusion data it was established that some patients will not develop infection despite definite exposure to a contaminated transfusion. Four patients received similar doses to the 30 patients who became infected but had no evidence of HCV infection as judged by PCR, symptoms and liver function tests. The numbers are small and so it is difficult to see any differences between these patients and patients who went on to develop infection. Although the average age was younger, in the uninfected group this was not statistically significant. It is of particular interest that these patients

did not become infected because they had antibody deficiency. Two patients out of the 12 untreated patients, who became infected, spontaneously became negative for HCV RNA. Thus in the prospective study we could establish again that approximately 15% of patients will spontaneously clear the virus. This finding is more striking as the patients were antibody deficient.

In 6 out of 25 patients with primary antibody deficiency we have observed rapid progression of HCV infection, with end stage liver disease occurring within a period of 18 months. Such rapid progression to cirrhosis is rarely seen in HCV related liver disease; this outcome is highly unusual when compared with post transfusion HCV in immunocompetent patients. Prospective follow-up of the other patients continues and may reveal continuing morbidity and mortality. Thus although the observed spontaneous clearance rate of HCV was similar to immunocompetent patients, once chronic infection was established progression to serious liver disease was much more rapid than in patients with normal immunity. This suggests that the antibody response is not important in determining viral clearance or persistence but may be important in subsequent control of HCV once infection is established.

In chapter 5 the role of $CD8^+$ T cell responses in determining viral clearance of HCV were studied. $CD8^+$ T cell responses are known to be important in the clearance of many viral infections, although their role in HCV infection remains to be established. In 3 patients with acute HCV, multispecific T cell responses were demonstrated against HCV. In contrast, 4 patients with chronic HCV exhibited oligoclonal or absent cytotoxic T cell responses to HCV. In addition patients with acute self-limiting infection showed peptide specific IFN-γ production which mirrored their cytotoxic responses. In chronic patients IFN-γ production was seen in the absence of detectable cytotoxicity. Thus, there appears to be an association between multispecific $CD8^+$ T cell responses and viral clearance. The functional phenotype of $CD8^+$ T cells characterised by IFN-γ production but not cytotoxicity may be responsible for failure to clear HCV. In addition the continuing production of gamma interferon may fuel the inflammatory process resulting in liver damage. As well as a phenotypic difference in response, a quantitative difference in the $CD8^+$ T cells responses relative to flu was seen, with lower levels of response seen in chronic infection compared to patients with acute infection. From these findings it can be hypothesised that $CD8^+$ T cells may contribute to ongoing liver disease by causing

inflammation but being quantitatively inadequate to destroy all the infected hepatocytes there by facilitating HCV persistence.

In the final chapter, the hypothesis that immune escape is the mechanism by which HCV causes chronic infection, was explored. A careful longitudinal investigation of HCV sequence variation in patients with normal immunity and antibody deficiency was performed and correlated with studies of cellular and humoral immune recognition of B and T cell epitopes.

It was found that the non-coding rate of nucleotide mutation was the same in core and envelope, which suggests that the error rate of the HCV RNA polymerase is the same in these regions. In addition it was found that the rate of non-coding mutation was at a higher rate than previously calculated from cross-sectional studies. In the core region it was found however that the coding rate of mutation was lower than expected relative to the non-coding rate of mutation. In contrast in the envelope region the coding mutation rate was higher than expected relative to the non-coding rate of mutation. This gives evidence for selection of expressed protein variation over and above nucleic acid variation in envelope.

The hypermutation seen in envelope occurred in the absence of demonstrable CTL recognition. Conversely, despite demonstrating CTL recognition to epitopes in core, there was no evidence of the accumulation of mutations in these epitopes over 2 years. Furthermore hypermutation in envelope was seen irrespective of antibody responses. Taken together these findings imply that escape mutation from immune recognition does not play a significant role in maintaining persistent HCV infection in chronic HCV.

It must be stressed that the present study concentrated on mutations in known immune epitopes in only two regions of the HCV genome. It is possible that mutations within the envelope region accumulated in immune epitopes that have yet to be recognised. However the mutations were randomly distributed throughout envelope and the observed mutations were not clustered within sequences that represent known HLA binding motifs. Nothing is yet known about the immunodominance of $CD8^+$ T cell epitopes in HCV. It is possible that the epitopes encoded in core and envelope are of little significance in the immune response to the whole virus and it is for this reason that we failed to detect a correlation between mutation and persistence. Identification of immunodominant epitopes and a more comprehensive study may resolve this question.

However the lack of evidence of immune escape in this study and the detection of the emergence of only one antagonist mutant in an earlier study suggests that immune escape from CTL is not the major mechanism by which HCV ensures persistence in chronic infection. The data relating to the envelope region imply that non immune mediated host/virus interactions are responsible for the selection of hypermutation in the HVR of envelope. Alternative mechanisms must be sought to explain the selection of these mutants.

The finding that CTL epitopes were conserved within the core region despite evidence of CTL recognition suggests that the $CD8^+$ T cell response to core may be ineffective in chronic hepatitis C infection. This raises the possibility that HCV interferes with effector T cell function in some way after T cell activation has occurred. This alternative explanation for HCV persistence has precedence in other chronic viral infections and warrants investigation in chronic hepatitis C. Greater understanding of the reasons why $CD8^+$ T cells directed against core CTL epitopes fail to eliminate HCV infection and why coding mutations in envelope accumulate during persistent HCV infection may provide the basis for the design of immunotherapy and an effective vaccine against HCV.

APPENDIX: 1: RESULTS OF CYTOTOXIC AND ELISPOT ASSAYS

CTL assay and Elispot results Patient A1

	Elispot: No. of reactive cells in each well				Pos-neg	Ratio to Flu	CTL assay 10:01							
Peptide	neg	neg	pos	pos			neg	neg	% lysis	pos	pos	% lysis	Specific Lysis	Ratio to Flu
ADLMGYIPLV	4	2	23	17	17	0.97	642	654	3.48	1856	2008	26.62	23.14	0.94
YLLPRRGPRL	0	3	15	13	12.5	0.71	653	740	4.35	2045	1890	27.26	22.91	0.93
ADLMGYIPLV	2	3	16	19	15	0.86	712	689	4.42	1978	1856	26.35	21.93	0.87
LLCPAGHAV	3	5	21	17	15	0.86	548	624	2.36	1724	1980	25.18	22.82	0.92
KLVALGINAV	12	9	44	39	31	**1.77**	534	546	1.53	2556	2781	39.89	38.36	**1.55**
SLMAFTAAV	2	1	0	4			578	589	2.31	623	645			
LLFNILGGWV	2	1	5	4			623	712	3.83	567	689			
ILDSFDPLV	4	0	2	4			656	567	2.82	589	745			
SLLAPGAKQNV	0	0	3	2			547	618	2.3	489	678	2.32		
FLU	3	7	21	24	17.5		745	709	4.9	1991	2207	29.63	24.73	
						R10	421	413		510	476			
						Triton	6004	5899		6107	6005			

Neg = no peptide added to well
Pos = peptide added to well

CTL assay and Elispot results Patient A2

	Elispot:No. of reactive cells						CTL assay ratio 20:01							
Peptide	neg	neg	pos	pos	pos-neg	ratio to flu	neg	neg	% lysis	pos	pos	% lysis	Sp lysis	Ratio :flu
ADLMGYIPLV	2	5	15	14	11	0.35	912	868	3.1	1345	1332	19.8	16.7	0.43
YLLPRRGPRL	3	1	14	11	10.5	0.33	879	895	3	1263	1367	18.9	15.9	0.41
ADLMGYIPLV	4	0	18	12	13	0.42	887	960	4.3	1371	1366	20.9	16.6	0.43
LLCPAGHAV	3	3	4	3			870	954		956	1005			
KLVALGINAV	4	10	30	22	19	**0.6**	906	868	3	1502	1407	24.1	21.1	**0.54**
SLMAFTAAV	0	1	1	1			856	900		988	899			
LLFNILGGWV	1	3	5	5			870	945		1089	945			
ILDSFDPLV	2	2	3	1			943	921		997	1056			
SLLAPGAKQNV	0	5	7	5			799	824		889	987			
FLU	3	1	34	32	31		878	865	2.4	1890	1937	41.2	38.8	
						R10	744	824		768	891			
						Triton	3461	3604		3489	3407	3554	3437	

Patient A3

	Elispot assay						CTL assay 20:1							
Peptide	neg	neg	pos	pos	pos-neg	ratio to flu	neg	neg	% lysis	pos	pos	% lysis	Sp lysis	Ratio :flu
ADLMGYIPLV	3	0	16	12	12.5	0.68	1146	1078	0.9	2073	2089	15.9	15	0.65
YLLPRRGPRL	2	1	2	1			1333	1226	3.5	1916	1823	12.6	8.5	
ADLMGYIPLV	5	4	19	15	12.5	0.68	1232	1196	2.5	2121	2292	17.8	15.3	0.67
LLCPAGHAV	3	1	2	0			1166	1259	2.5	1618	1696	9.4	6.9	
KLVALGINAV	2	4	2	7			1193	1312		1265	1133			
SLMAFTAAV	0	4	11	12	9.5	0.51	1095	1194	1.4	2227	2341	19	17.6	0.77
LLFNILGGWV	2	7	19	17	13.5	**0.73**	1063	1134	0.7	2215	2035	16.6	15.9	**0.69**
ILDSFDPLV	0	1	2	5			961	1232		1160	1060			
SLLAPGAKQNV	2	2	4	6			1031	1147		1101	1336			
FLU	6	5	27	21	18.5		1205	1237	2.6	2654	2743	25.5	22.9	
							R10	1063	1075	1070	999			
							Triton	7321	7944.3	7321	7414	7526	7597	

Patient C1

	Elispot:						CTL assay 10:01							
Peptide	neg	neg	pos	pos	pos-neg	ratio to flu	neg	neg	% lysis	pos	pos	% lysis	Sp lysis	Ratio to flu
ADLMGYIPLV	2	0	3	1			690	746		678	712			
YLLPRRGPRL	4	5	2	7			756	812		789	802		1	
ADLMGYIPLV	3	1	4	2			689	756		612	780			
LLCPAGHAV	0	0	14	10	12	0.8	726	686		678	746			
KLVALGINAV	2	4	2	1			690	708		789	692			
SLMAFTAAV	3	3	1	3			587	756		645	891			
LLFNILGGWV	4	1	15	12	11	0.7	692	684	1.8	1557	1654	16.34	14.5	0.4
ILDSFDPLV	0	2	1	0			599	712		719	702			
SLLAPGAKQNV	2	1	4	7			674	712		678	812			
FLU	4	5	21	18	15		577	602	0.2	2764	2654	33.8	33.6	
							R10	645	543	517	590			
							Triton	7450	6987	6450	6665			

Patient C2

	Elispot:						CTL assay ratio 20:1							
Peptide	neg	neg	pos	pos	Pos-neg	Ratio to Flu	neg	neg	% lysis	pos	pos	% lysis	Sp. Lysis	Ratio to Flu
ADLMGYIPLV	2	0	1	6			440	521		569	590			
YLLPRRGPRL	5	4	21	18	15	**0.61**	635	490	1.8	879	887	17.4	15.6	**0.34**
ADLMGYIPLV	3	1	0	0			542	580		534	574			
LLCPAGHAV	0	0	0	0			559	477		581	521			
KLVALGINAV	1	0	2	0			696	559		571	702			
SLMAFTAAV	0	0	0	0			578	597		570	623			
LLFNILGGWV	3	2	1	0			637	681		744	574			
ILDSFDPLV	6	8	22	19	13.5	0.55	553	622	3	884	602	10.6	7.6	
SLLAPGAKQNV	2	1	3	4			567	602		598	555			
FLU	3	5	31	26	24.5		578	627	3.8	1562	1543	50	46.2	
							R10	500	505	578	515			
							Triton	2733	2351	2665	2660	2574	2497	

Patient C3

	Elispot:						CTL assay ratio 20:1							
Peptide	neg	neg	pos	pos	Pos-neg	Ratio to Flu	neg	neg	% lysis	pos	pos	% lysis	Sp. Lysis	Ratio to Flu
ADLMGYIPLV	1	0	0	0			567	645		765	654			
YLLPRRGPRL	2	0	1	0			678	632		645	567			
ADLMGYIPLV	0	0	0	0			654	745		645	665			
LLCPAGHAV	2	1	3	1			589	665		689	598			
KLVALGINAV	4	1	18	17	10	**0.38**	598	758	7.6	678	789	9.1	1.5	**0.05**
SLMAFTAAV	0	3	11	9	8.5		690	589		789	605			
LLFNILGGWV	0	2	2	5			765	690		668	756			
ILDSFDPLV	1	0	3	0			567	556		678	545			
SLLAPGAKQNV	1	1	2	1			399	567		456	568			
FLU	5	3	32	29	26.5		678	544	5.8	1890	1567	36.1	30.3	
							R10	378	385	443	390			
							Triton	4423	3890	3998	4002			

Patient C4

	Elispot:						CTL assay ratio 20:1							
Peptide	neg	neg	pos	pos	Pos-neg	Ratio to Flu	neg	neg	% lysis	pos	pos	% lysis	Sp. Lysis	Ratio to Flu
ADLMGYIPLV	1	2	0	0			2498	2580		2451	2394			
YLLPRRGPRL	4	5	14	19	12	**0.5**	2457	2367	1.7	2556	2507	3.2	1.5	**0.07**
ADLMGYIPLV	2	0	1	0			2330	2486		2198	2306			
LLCPAGHAV	1	1	3	0			2681	2494		2135	1973			
KLVALGINAV	2	0	0	0			2648	2357		2265	2763			
SLMAFTAAV	0	0	0	0			2422	2455		2163	2245			
LLFNILGGWV	2	0	0	1			2233	2661		2499	2437			
ILDSFDPLV	2	1	0	2			2291	2592		2186	2391			
SLLAPGAKQNV	1	1	0	3			2425	2686		2352	2396			
FLU	5	3	29	27	24		2279	2314	0.3	4234	4117	23.4	21.1	
							R10	2298	2280	2251	2257			
							Triton	10883	9146	10830	10835			

Control 1

	Elispot:						CTL assay ratio 20:1							
Peptide	neg	neg	pos	pos	Pos-neg	Ratio to Flu	neg	neg	% lysis	pos	pos	% lysis	Sp. Lysis	Ratio to Flu
ADLMGYIPLV	0	0	0	0			567	655		765	654			
YLLPRRGPRL	2	0	0	0			678	632		645	567			
ADLMGYIPLV	0	0	0	0			754	745		645	665			
LLCPAGHAV	2	1	3	1			589	685		689	598			
KLVALGINAV	4	1	3	0			598	658		678	789			
SLMAFTAAV	0	3	1	4			690	589		789	605			
LLFNILGGWV	0	2	2	5			765	690		668	756			
ILDSFDPLV	1	0	3	0			567	556		678	545			
SLLAPGAKQNV	1	1	2	1			399	567		456	568			
FLU	3	3	36	38	**34**		478	544		1990	1767		**37.1**	
							R10	378	365	443	390			
							Triton	4123	3880	3998	4302			

Control 2

	Elispot:						CTL assay ratio 20:1							
Peptide	neg	neg	pos	pos	Pos-neg	Ratio to Flu	neg	neg	% lysis	pos	pos	% lysis	Sp. Lysis	Ratio to Flu
ADLMGYIPLV	1	2	0	0			2490	2564		2451	2394			
YLLPRRGPRL	3	5	3	1			2457	2467		2556	2507			
ADLMGYIPLV	2	0	1	0			2345	2486		2198	2306			
LLCPAGHAV	1	1	3	0			2761	2496		2135	1973			
KLVALGINAV	2	0	0	0			2648	2380		2265	2783			
SLMAFTAAV	0	0	0	0			2422	2455		2163	2745			
LLFNILGGWV	2	0	0	1			2233	2645		2499	2837			
ILDSFDPLV	2	1	0	2			2291	2567		2186	2361			
SLLAPGAKQNV	1	1	0	3			2450	2690		2352	2380			
FLU	5	3	36	30	29		2279	2314		4234	4117		**23.4**	
							R10	2248	2290	2261	2240			
							Triton	10709	9346	10670	10455			

APPENDIX: 2
RAW SEQUENCE DATA

HCV Core: Quasispecies of patient 1 at time point 1

```
1ATGAGCACGAATCCTAAACCTCAAAAAAAAAACAAACGTAACACCAACCGTCGCCCACAGGACGTCAAGTTCCCGGGTGGCGGTCAGATCGTTGGTGGAGTTTACTTGTTGC
2------------------------G-----C--------------------------------T-------------------------------------------
3--------A---------------G-----C--------------------------------T-------------------------------------------
4------------------------G-----C--------------------------------T-------------------------------------------

1CGCGCAGGGGCCCTAGATTGGGTGTGCGCGCGACGAGAAAGACTTCCGAGCGGTCGCAACCTCGAGGTAGACGTCAGCCTATCCCCAAGGCTCGTCGGCCCGAGGGCAGGAC
2-----------------------------------G-----------------------------------------------------A-------------------
3-----------------------------------G-----------------------------------------------------A-------------------
4-----------------------------------G-----------------------------------------------------A-------------------

1CTGGGCTCAGCCCGGGTACCCTTGGCCCCTCTATGGCAATGAGGGCTGCGGGTGGGCGGGATGGCTCCTGTCTCCCCGTGGCCTCGGCCTAGCTGGGGCCCCACAGACCCCC
2------------------------------------------------------------------------------------------------------------
3------------------------------------------------------------------------------------------------------------
4------------------------------------------------------------------------------------------------------------

1GGCGTAGGTCGCGCAATTTGGGTAAGGTCATCGATACCCTTACGTGCCGGCTTCGCCGACCTCATGGGGTACATACCGCTCGTCGGCGCCCCTC
2-----------------------------------------------------------------------------------------
3-----------------------------------------------------------------------------------------
3-------------------------------------------------------------------C---------------------
```

Italic red font indicates amino acid substitution
1= Choo reference sequence(PNAS 1989), 2= quasispecies 1, 3= quasispecies 2, 4 = quasispecies 3
2 = 16/20 clones, 3 = 3/20 clones, 4 = 1/20 clones

HCV core: Quasispecies of patient 2 at time pt 1

```
1ATGAGCACGAATCCTAAACCTCAAAAAAAAAACAAACGTAACACCAACCGTCGCCCACAGGACGTCAAGTTCCCGGGTGGCGGTCAGATCGTTGGTGGAGTTTACTTGTTGC
2-----------------------G-----C-------------------------------T-------------------------------------------

1CGCGCAGGGGCCCTAGATTGGGTGTGCGCGCGACGAGAAAGACTTCCGAGCGGTCGCAACCTCGAGGTAGACGTCAGCCTATCCCCAAGGCTCGTCGGCCCGAGGGCAGGAC
2-----------------------------------G-----------------------------------------------------A-------------------

1CTGGGCTCAGCCCGGGTACCCTTGGCCCCTCTATGGCAATGAGGGCTGCGGGTGGGCGGGATGGCTCCTGTCTCCCCGTGGCCTCGGCCTAGCTGGGGCCCCACAGACCCCC
2-------------------------------------------------------------------------------------------------------------

1GGCGTAGGTCGCGCAATTTGGGTAAGGTCATCGATACCCTTACGTGCCGGCTTCGCCGACCTCATGGGGTACATACCGCTCGTCGGCGCCCCTC
2-----------------------------------------------------------------------------------------
```

Italic red font indicates amino acid substitution
1=Choo refernce sequence , 2 = patient 2 quasispecies 1,
2 = 20/20 clones,

HCV Core: Quasispecies of patient 3 at time pt 1

```
1ATGAGCACGAATCCTAAACCTCAAAAAAAAAACAAACGTAACACCAACCGTCGCCCACAGGACGTCAAGTTCCCGGGTGGCGGTCAGATCGTTGGTGGAGTTTACTTGTTGC
2------------------------G-----C---------------------------------T-------------------------------------------
3--------A---------------G-----C---------------------------------T-------------------------------------------

1CGCGCAGGGGCCCTAGATTGGGTGTGCGCGCGACGAGAAAGACTTCCGAGCGGTCGCAACCTCGAGGTAGACGTCAGCCTATCCCCAAGGCTCGTCGGCCCGAGGGCAGGAC
2-----------------------------------G-----------------------------------------------------A-------------------
3-----------------------------------G-----------------------------------------------------A-------------------

1CTGGGCTCAGCCCGGGTACCCTTGGCCCCTCTATGGCAATGAGGGCTGCGGGTGGGCGGGATGGCTCCTGTCTCCCCGTGGCCTCGGCCTAGCTGGGGCCCCACAGACCCCC
2------------------------------------------------------------------------------------------------------------
3------------------------------------------------------------------------------------------------------------

1GGCGTAGGTCGCGCAATTTGGGTAAGGTCATCGATACCCTTACGTGCCGGCTTCGCCGACCTCATGGGGTACATACCGCTCGTCGGCGCCCCTC
2-----------------------------------------------------------------------------------------
3-----------------------------------------------------------------------------------------
```

Italic red font indicates amino acid substitution
1=Choo reference sequence , 2 = patient 3 quasispecies 1, 3 = quasispecies 2
2 = 15/20 clones, 3 = 5/20 clones

HCV core: Quasispecies of patient 4 at time point 1.

```
1ATGAGCACGAATCCTAAACCTCAAAAAAAAAACAAACGTAACACCAACCGTCGCCCACAGGACGTCAAGTTCCCGGGTGGCGGTCAGATCGTTGGTGGAGTTTACTTGTTGC
2------------------------G-----C-------------------------------------------------------------------------------
3------------------------G-----C---------------------------------T-------------------------------------------
4------------------------G-----C---------------------------------T-------------------------------------------

1CGCGCAGGGGCCCTAGATTGGGTGTGCGCGCGACGAGAAAGACTTCCGAGCGGTCGCAACCTCGAGGTAGACGTCAGCCTATCCCCAAGGCTCGTCGGCCCGAGGGCAGGAC
2--------------------------------------G-----------------A-------------------------------------------------------
3--------------------------------------G-----------------A-------------------------------------------------------
4--------------------------------------G-------------------------------------------------------------------------

1CTGGGCTCAGCCCGGGTACCCTTGGCCCCTCTATGGCAATGAGGGCTGCGGGTGGGCGGGATGGCTCCTGTCTCCCCGTGGCCTCGGCCTAGCTGGGGCCCCACAGACCCCC
2-------------------------------------------------------------------------------------------------------------
3-------------------------------------------------------------------------------------------------------------
4-------------------------------------------------------------------------------------------------------------

1GGCGTAGGTCGCGCAATTTGGGTAAGGTCATCGATACCCTTACGTGCCGGCTTCGCCGACCTCATGGGGTACATACCGCTCGTCGGCGCCCCTC
2-----------------------------------------------------------------------------------------
3-----------------------------------------------------------------------------------------
4-----------------------------------------------------------------------------------------
```

Italic red font indicates amino acid substitution
1 = Choo sequence, 2 = patient 4 quasispecies 1, 3 = quasispecies 2, 4 = quasispecies 3
2 = 14/20 clones, 3 = 2/ 20 clones, 3 = 2/20 clones

HCV Core: Quasispecies of patient 5 at time point 1.

```
1ATGAGCACGAATCCTAAACCTCAAAAAAAAAACAAACGTAACACCAACCGTCGCCCACAGGACGTCAAGTTCCCGGGTGGCGGTCAGATCGTTGGTGGAGTTTACTTGTTGC
2-----------------------G-----C-----------------------------------------------------------C----------------------------
3-----------------------G-----C-----------------------------------------------------------C----------------------------
4-----------------------G-----C-----------------------------------------------------------C----------------------------

1CGCGCAGGGGCCCTAGATTGGGTGTGCGCGCGACGAGAAAGACTTCCGAGCGGTCGCAACCTCGAGGTAGACGTCAGCCTATCCCCAAGGCTCGTCGGCCCGAGGGCAGGAC
2-----------------------------------G-----------------------------------------------------A--------------------
3-----------------------------------G----------------A------------------------------------A--------------------
4-----------------------------------G-------------------------------------------------------------------------------

1CTGGGCTCAGCCCGGGTACCCTTGGCCCCTCTATGGCAATGAGGGCTGCGGGTGGGCGGGATGGCTCCTGTCTCCCCGTGGCCTCGGCCTAGCTGGGGCCCCACAGACCCCC
2-------------------------------------------------------------------------------------------------------------------
3-------------------------------------------------------------------------------------------------------------------
4-----------------------------------------------------------------C-------------------------------------

1GGCGTAGGTCGCGCAATTTGGGTAAGGTCATCGATACCCTTACGTGCCGGCTTCGCCGACCTCATGGGGTACATACCGCTCGTCGGCGCCCCTC
2-----------------------------------------------------------------------------------------
3-----------------------------------------------------------------------------------------
4-----------------------------------------------------------------------------------------
```

Italic red font indicates amino acid substitution
1 = Choo sequence, 2 = patient 5 quasispecies 1, 3 = quasispecies 2, 4 = quasispecies 3
2 = 11/20 clones, 3 = 5/ 20 clones, 3 = 4/20 clones

HCV core: Quasispecies of patient 6 at time point 1.

```
1ATGAGCACGAATCCTAAACCTCAAAAAAAAAACAAACGTAACACCAACCGTCGCCCACAGGACGTCAAGTTCCCGGGTGGCGGTCAGATCGTTGGTGGAGTTTACTTGTTGC
2-------------------------G-----C-----------------------------------------------------------------------------
3--------A---------------G-----C-----------------------------------------------------------------------------

1CGCGCAGGGGCCCTAGATTGGGTGTGCGCGCGACGAGAAAGACTTCCGAGCGGTCGCAACCTCGAGGTAGACGTCAGCCTATCCCCAAGGCTCGTCGGCCCGAGGGCAGGAC
2-----------------------------------G-----------------------------------------------------------------------
3-----------------------------------G-----------------------------------------------------------------------

1CTGGGCTCAGCCCGGGTACCCTTGGCCCCTCTATGGCAATGAGGGCTGCGGGTGGGCGGGATGGCTCCTGTCTCCCCGTGGCCTCGGCCTAGCTGGGGCCCCACAGACCCCC
2-----------------------------------------------------------------------------------------------------------
3-----------------------------------------------------------------------------------------------------------

1GGCGTAGGTCGCGCAATTTGGGTAAGGTCATCGATACCCTTACGTGCCGGCTTCGCCGACCTCATGGGGTACATACCGCTCGTCGGCGCCCCTC
2-------------------------------------------------------------------------------------------
3-------------------------------------------------------------------------------------------
```

Italic red font indicates amino acid substitution
1 = Choo reference sequence, 2 = patient 6 quasispecies 1, 3 = quasispecies 2,
2 = 17/20 clones, 3 = 3/20 clones

HCV Core sequence. Time point 2
Comparison of Choo sequence, gammaglobulin sequence and quasi species of pt 1 at time point 2.

```
1ATGAGCACGAATCCTAAACCTCAAAAAAAAAACAAACGTAACACCAACCGTCGCCCACAGGACGTCAAGTTCCCGGGTGGCGGTCAGATCGTTGGTGGAGTTTACTTGTTGC
2------------------------G-----C-----------------------------------T-----------------------------------------------
3--------A---------------G-----C-----------------------------------C-----------------------------------------------
4--------A---------------G-----C-----------------------------------C-----------------------------------------------
5------------------------G-----C-----------------------------------C-----------------------------------------------
6------------------------G-----C-----------------------------------T-----------------------------------------------

1CGCGCAGGGGCCCTAGATTGGGTGTGCGCGCGACGAGAAAGACTTCCGAGCGGTCGCAACCTCGAGGTAGACGTCAGCCTATCCCCAAGGCTCGTCGGCCCGAGGGCAGGAC
2-----------------------------------G-----------------------------------------------------------A-------------------
3-----------------------------------G-------------------------T-------------------------A-------------------
4--------------------------------C----G-------------------------T-------------------------T-------------------
5--------------------------------C----G-------------------------T-------------------------T-------------------
6-----------------------------------G-----------------------------------------------------------T-------------------

1CTGGGCTCAGCCCGGGTACCCTTGGCCCCTCTATGGCAATGAGGGCTGCGGGTGGGCGGGATGGCTCCTGTCTCCCCGTGGCCTCGGCCTAGCTGGGGCCCCACAGACCCCC
2------------------------------------------------------------------------------------------------------------------
3------------------T-----------------------------------------------------------------------------------------------
4------------------T-----------------------------------------------------------------------------------------------
5------------------------------------------------------------------------------------------------------------------
6------------------------------------------------------------------------------------------------------------------

1GGCGTAGGTCGCGCAATTTGGGTAAGGTCATCGATACCCTTACGTGCCGGCTTCGCCGACCTCATGGGGTACATACCGCTCGTCGGCGCCCCTC
2-----------------------------------------------------------------------------------------------
3-----------------------------------------------------------------------------------------------
4-----------------------------------------------------------------------------------------------
5-----------------------------------------------------------------------------------------------
6-----------------------------------------------------------------------------------------------
```

Italic red font indicates amino acid substitution
1=Choo, 2= GG, 3 =pt1 quasispecies 1, 4 = pt1 quasispecies 2, 5 = pt1 quasispecies 3, 6 = pt1 quasipecies 4
3 = 13/20 clones, 4 = 3/20 clones, 5 = 2/20 clones, 1 = 1/20 clones

HCV core, Time point 2:
Comparison of Choo sequence, gammaglobulin sequence and quasi species of patient 2 at time point 2.

```
1ATGAGCACGAATCCTAAACCTCAAAAAAAAAAACAAACGTAACACCAACCGTCGCCCACAGGACGTCAAGTTCCCGGGTGGCGGTCAGATCGTTGGTGGAGTTTACTTGTTGC
2------------------------G-----C----------------------------------T----------------------------------------------
3--------A---------------G-----C----------------------------------C----------------------------------------------
4------------------------G-----C----------------------------------C----------------------------------------------
5------------------------G-----C----------------------------------T----------------------------------------------

1CGCGCAGGGGCCCTAGATTGGGTGTGCGCGCGACGAGAAAGACTTCCGAGCGGTCGCAACCTCGAGGTAGACGTCAGCCTATCCCCAAGGCTCGTCGGCCCGAGGGCAGGAC
2----------------------------------G-------------------------------------------------------A--------------------
3--------------------------------C-G-------------------------------------------------------T--------------------
4----------------------------------G-------------------------T-------------------------T--------------------
5----------------------------------G-------------------------------------------------------T--------------------

1CTGGGCTCAGCCCGGGTACCCTTGGCCCCTCTATGGCAATGAGGGCTGCGGGTGGGCGGGATGGCTCCTGTCTCCCCGTGGCCTCGGCCTAGCTGGGGCCCCACAGACCCCC
2------------------------------------------------------------------------------------------------------------------
3------------------------------------------------------------------------------------------------------------------
4------------------------------------------------------------------------------------------------------------------
5------------------------------------------------------------------------------------------------------------------

1GGCGTAGGTCGCGCAATTTGGGTAAGGTCATCGATACCCTTACGTGCCGGCTTCGCCGACCTCATGGGGTACATACCGCTCGTCGGCGCCCCTC
2----------------------------------------------------------------------------------------------
3-----------------------------------C----------------------------------------------------------
4----------------------------------------------------------------------------------------------
5----------------------------------------------------------------------------------------------
```

Italic red font indicates amino acid substitution
1= Choo refernce sequence, 2 = GG, 3 = pt2 quasispecies 1, 4 = pt2 quasispecies 2, 5 = pt2 quasispecies 3
3 = 15/20 clones, 4 = 3/20 clones, 5 = 2/20 clones

HCV Core, Time point 2:
Comparison of Choo sequence, gammaglobulin sequence and quasi species of patient 3 at time point 2.

```
1ATGAGCACGAATCCTAAACCTCAAAAAAAAAACAAACGTAACACCAACCGTCGCCCACAGGACGTCAAGTTCCCGGGTGGCGGTCAGATCGTTGGTGGAGTTTACTTGTTGC
2------------------------G-----C-------------------------------T-----------------------------------------------
3--------A---------------G-----C-------------------------------C-----------------------------------------------
4--------A---------------G-----C-------------------------------T-----------------------------------------------
5------------------------G-----C-------------------------------C-----------------------------------------------
6------------------------G-----C-------------------------------T-----------------------------------------------

1CGCGCAGGGGCCCTAGATTGGGTGTGCGCGCGACGAGAAAGACTTCCGAGCGGTCGCAACCTCGAGGTAGACGTCAGCCTATCCCCAAGGCTCGTCGGCCCGAGGGCAGGAC
2-----------------------------------G-----------------------------------------------------------A-----------------
3-------------------------------C---G-------------------------T-------------------------T-----------------
4-------------------------------C---G-------------------------T-------------------------T-----------------
5-----------------------------------G-------------------------T-------------------------T-----------------
6-----------------------------------G-----------------------------------------------------T-----------------

1CTGGGCTCAGCCCGGGTACCCTTGGCCCCTCTATGGCAATGAGGGCTGCGGGTGGGCGGGATGGCTCCTGTCTCCCCGTGGCCTCGGCCTAGCTGGGGCCCCACAGACCCCC
2------------------------------------------------------------------------------------------------------------
3----------------T-------------------------------------------------------------------------------------------
4----------------T-------------------------------------------------------------------------------------------
5------------------------------------------------------------------------------------------------------------
6------------------------------------------------------------------------------------------------------------

1GGCGTAGGTCGCGCAATTTGGGTAAGGTCATCGATACCCTTACGTGCCGGCTTCGCCGACCTCATGGGGTACATACCGCTCGTCGGCGCCCCTC
2-----------------------------------------------------------------------------------------
3-----------------------------------------------------------------------------------------
4-----------------------------------------------------------------------------------------
5-----------------------------------------------------------------------------------------
6-----------------------------------------------------------------------------------------
```

Italic red font indicates amino acid substitution
1=Choo reference sequence, 2= gammaglobulin, 3= quasispecies 1, 4 = quasispecies 2, 5 = pt3 quasispecies 3, 6 = pt3 quasipecies 4
3 = 12/20 clones, 4 = 2 clones, 5 = 2 clones, 6 = 2 clones

HCV Core, Time point 2:
Comparison of Choo sequence and consensus sequence of patient 4 at time point 1 with patient 4 quasispecies at time pt 2

```
1ATGAGCACGAATCCTAAACCTCAAAAAAAAAACAAACGTAACACCAACCGTCGCCCACAGGACGTCAAGTTCCCGGGTGGCGGTCAGATCGTTGGTGGAGTTTACTTGTTGC
2------------------------G-----C-----------------------------------------------------------------------------------
3------------------------G-----C----------------------------------T------------------------------------------------
4--------A---------------G-----C----------------------------------T------------------------------------------------
5------------------------G-----C-----------------------------------------------------------------------------------
6------------------------G-----C-----------------------------------------------------------------------------------

1CGCGCAGGGGCCCTAGATTGGGTGTGCGCGCGACGAGAAAGACTTCCGAGCGGTCGCAACCTCGAGGTAGACGTCAGCCTATCCCCAAGGCTCGTCGGCCCGAGGGCAGGAC
2-----------------------------------G----------------A-----------------------------------------------------------
3-----------------------------------G-----------------------------------------------------------------------------
4-----------------------------------G-----------------------------------------------------------------------------
5-----------------------------------G----------------A-----------------------------------------------------------
6-----------------------------------G-----------------------------------------------------------------------------

1CTGGGCTCAGCCCGGGTACCCTTGGCCCCTCTATGGCAATGAGGGCTGCGGGTGGGCGGGATGGCTCCTGTCTCCCCGTGGCCTCGGCCTAGCTGGGGCCCCACAGACCCCC
2-----------------------------------------------------------------------------------------------------------------
3-------------------------------------------------A---------------------------------------------------------------
4-------------------------------------------------A---------------------------------------------------------------
5-----------------------------------------------------------------------------------------------------------------
6-----------------------------------------------------------------------------------------------------------------

1GGCGTAGGTCGCGCAATTTGGGTAAGGTCATCGATACCCTTACGTGCCGGCTTCGCCGACCTCATGGGGTACATACCGCTCGTCGGCGCCCCTC
2-------------------------------------------------------------------------------------------
3-------------------------------------------------------------------------------------------
4-------------------------------------------------------------------------------------------
5-------------------------------------------------------------------------------------------
6-------------------------------------------------------------------------------------------
```

Italic red font indicates amino acid substitution
1 = Choo sequence, 2 = consensus sequence of patient 4 at time point 1, 3 = quasispecies 1, 4 = quasispecies 2, 5 = quasispecies 3, 6 = quasispecies 4 at time pt 2, 3 = 7/20 clones, 4 = 6/20 clones, 5 = 4/20 clones, 6 = 3/20 clones

HCV Core, time point 2:
Comparison of Choo sequence and sequence of patient 5 at time point 1 with quasispecies of patient 5 at time pt 2

```
1ATGAGCACGAATCCTAAACCTCAAAAAAAAAACAAACGTAACACCAACCGTCGCCCACAGGACGTCAAGTTCCCGGGTGGCGGTCAGATCGTTGGTGGAGTTTACTTGTTGC
2------------------------G-----C-----------------------------------------------------C--------------------------
3--------A---------------G-----C-----------------------------------------------------C--------------------------
4------------------------G-----C-----------------------------------------------------C--------------------------
5------------------------G-----C-----------------------------------------------------C--------------------------

1CGCGCAGGGGCCCTAGATTGGGTGTGCGCGCGACGAGAAAGACTTCCGAGCGGTCGCAACCTCGAGGTAGACGTCAGCCTATCCCCAAGGCTCGTCGGCCCGAGGGCAGGAC
2-----------------------------------G-----------------------------------------------------A--------------------
3-----------------------------------G-----------------------------------------------------A--------------------G
4-----------------------------------G-----------------------------------------------------A--------------------
5-----------------------------------G-----------------------------------------------------A--------------------

1CTGGGCTCAGCCCGGGTACCCTTGGCCCCTCTATGGCAATGAGGGCTGCGGGTGGGCGGGATGGCTCCTGTCTCCCCGTGGCCTCGGCCTAGCTGGGGCCCCACAGACCCCC
2------------------------------------------------------------------------------------------------------------------
3-----------------T------------------------------------------------------------------------------------------------
4-----------------T------------------------------------------------------------------------------------------------
5------------------------------------------------------------------------------------------------------------------

1GGCGTAGGTCGCGCAATTTGGGTAAGGTCATCGATACCCTTACGTGCCGGCTTCGCCGACCTCATGGGGTACATACCGCTCGTCGGCGCCCCTC
2-------------------------------------------------------------------------------------------
3-----------------------------------------------------------------C--------------------
4-----------------------------------------------------------------C--------------------
5-------------------------------------------------------------------------------------------
```

Italic red font indicates amino acid substitution
1 = Choo reference sequence, 2 = sequence of patient 5 at time point 1, 3 = quasispecies 1, 4 = quasispecies 2, 5 = quasispecies 3
3 = 11/20 clones, 4 = 5/20 clones, 5 = 4/ 20 clones

HCV core, Time point 2: Comparison of Choo and consensus sequence of patient 6 at time point 1 with quasispecies at time pt 2

```
1ATGAGCACGAATCCTAAACCTCAAAAAAAAAACAAACGTAACACCAACCGTCGCCCACAGGACGTCAAGTTCCCGGGTGGCGGTCAGATCGTTGGTGGAGTTTACTTGTTGC
2-------------------------G-----C-----------------------------------------------------------------------------------
3--------A----------------G-----C---------------------------------A-----------------------------------------------
4--------A----------------G-----C-----------------------------------------------------------------------------------
5-------------------------G-----C-----------------------------------------------------------------------------------
6-------------------------G-----C-----------------------------------------------------------------------------------
7-------------------------G-----C---------------------------------A-----------------------------------------------

1CGCGCAGGGGCCCTAGATTGGGTGTGCGCGCGACGAGAAAGACTTCCGAGCGGTCGCAACCTCGAGGTAGACGTCAGCCTATCCCCAAGGCTCGTCGGCCCGAGGGCAGGAC
2-----------------------------------G-----------------------------------------------------------------------------
3-----------------------------------A--G--------------------------------------------------------------------------
4-----------------------------------G-----------------------------------------------------------------------------
5-----------------------------------G-----------------------------------------------------------------------------
6-----------------------------------G-----------------------------------------------------------------------------
7-----------------------------------A--G--------------------------------------------------------------------------

1CTGGGCTCAGCCCGGGTACCCTTGGCCCCTCTATGGCAATGAGGGCTGCGGGTGGGCGGGATGGCTCCTGTCTCCCCGTGGCCTCGGCCTAGCTGGGGCCCCACAGACCCCC
2-----------------------------------------------------------------------------------------------------------------
3-------------------------------------------------C---------------------------------------------------------------
4-------------------------------------------------C---------------------------------------------------------------
5-----------------------------------------------------------------------------------------------------------------
6-----------------------------------------------------------------------------------------------------------------
7-----------------------------------------------------------------------------------------------------------------

1GGCGTAGGTCGCGCAATTTGGGTAAGGTCATCGATACCCTTACGTGCCGGCTTCGCCGACCTCATGGGGTACATACCGCTCGTCGGCGCCCCTC
2-------------------------------------------------------------------------------------------
3-------------------------------------------------------------------------------------------
4-------------------------------------------------------------------------------------------
5-------------------------------------------------------------------------------------------
6-------------------------------------------------------------------------------------------
7-------------------------------------------------------------------------------------------
```

1 = Choo sequence, 2 = consensus sequence of patient 6 at time point 1, 3 = quasispecies 1, 4 = quasispecies 2, 5 = quasispecies 3, 6 = quasispecies 4, 7 = quasispecies 5 at time pt 2. 3 = 8/20 clones, 4 = 6/20 clones, 5 = 3/20 clones, 6 = 2/20 clones, 7 = 1/20 clone

Envelope Region 1100 - 1298 nt
Quasispecies found in gammaglobulin batch

```
1.AACTGGGCGAAGGTCCTGGTAGTGCTGCTGCTATTTGCCGGCGTCGACGCGGAAACCCACGTCACCGGGGGAAGTGCCGGCCACACTGTGTCTGGATTTGTTAGCCTCCTCG
2.--------------T--------A---------------------------------G-------------GT--------G-GT-AC---C---C---C-G-----T-T-
3.--------------T--------------------C---------------------G-------------GT--------G-GT-AC---C---C---C-G-----T-T-


1.CACCAGGCGCCAAGCAGAACGTCCAGCTGATCAACACCAACGGCAGTTGGCACCTCAATAGCACGGCCCTGAACTGCAATGATAGC
2.--------------------A-------------------------------A---------------C---------------
3.--------------------A-------------------------A-T----A---------------C---------------
```

1 = Choo consensus sequence and 2 = gammaglobulin batch quasispecies 1, 3 = quasispecies 2
Normal case = synonomous, Blue Italic = non-synonomous
2 = 16/20 clones, 2 = 4/20 clones

Envelope 1100 - 1298 nt
Quasispecies of patient at time point 1

```
1.AACTGGGCGAAGGTCCTGGTAGTGCTGCTGCTATTTGCCGGCGTCGACGCGGAAACCCACGTCACCGGGGGAAGTGCCGGCCACACTGTGTCTGGATTTGTTAGCCTCCTCG
2.--------------T--------A-----------------------------------G-------------GT--------G-GT-AC---C---C---C-G-----T-T-
3.--------------T--------A-----------------------------------G-------------GT--------G-GT-AC---C---C---C-G-----T-T-
4.--------------T--------A-----------------------------------G-------------GT----------GT-AC---C---C---C-G-----T-T-
5.--------------T-------------------------------------------G-------------GT--------G-GT-AC---C---C---C-G-----T-T-

1.CACCAGGCGCCAAGCAGAACGTCCAGCTGATCAACACCAACGGCAGTTGGCACCTCAATAGCACGGCCCTGAACTGCAATGATAGC
2.--------------------A-----------------------------------A----------------C---------------
3.--------------------A-----------------------------------A----------------C---------------
4.--------------------A-----------------------------------A----------------C---------------
5.--------------------A-----------------------------------A----------------C---------------
```

1 = Choo consensus sequence and 2 = Gammaglobulin batch consensus, 3 = patient 1 quasispecies 1, 4 = patient 1 quasispecies 2, 5 = patient 1 quasispecies 3
Normal case = synonymous, Blue Italic = non-synonymous
3 = 18/20 clones, 4 = 1/20 clones, 5 = 1 clone

Envelope 1100 - 1298 nt
Patient 2 quasispecies at time point 1

```
1.AACTGGGCGAAGGTCCTGGTAGTGCTGCTGCTATTTGCCGGCGTCGACGCGGAAACCCACGTCACCGGGGGAAGTGCCGGCCACACTGTGTCTGGATTTGTTAGCCTCCTCG
2.--------------T--------A----------------------------------G-------------GT--------G-GT-AC---C---C---C-G-----T-T-
3.--------------T--------A----------------------------------G-------------GT--------G-GT-AC---C---C---C-G-----T-T-
4.--------------T--------A-----------C----------------------G-------------GT--------G-GT-AC---C---C---C-G-----T-T-

1.CACCAGGCGCCAAGCAGAACGTCCAGCTGATCAACACCAACGGCAGTTGGCACCTCAATAGCACGGCCCTGAACTGCAATGATAGC
2.--------------------A----------------------------------A----------------C---------------
3.--------------------A----------------------------------A----------------C---------------
3.--------------------A----------------------------------A----------------C---------------
```

1 = Choo consensus sequence and 2 = gammaglobulin batch consensus, 3 = patient 2 quasispecies 1, 4 = patient 2 quasispecies 2
Normal case = synonymous, Blue Italic = non-synonymous
3 = 19/20 clones, 4 = 1/20 clones,

Envelope 1100 - 1298 nt
Quasispecies of patient 3 at time point 1

```
1.AACTGGGCGAAGGTCCTGGTAGTGCTGCTGCTATTTGCCGGCGTCGACGCGGAAACCCACGTCACCGGGGGAAGTGCCGGCCACACTGTGTCTGGATTTGTTAGCCTCCTCG
2.--------------T--------A-----------------------------------G-------------GT--------G-GT-AC---C---C---C-G-----T-T-
3.--------------T--------A-----------------------------------G-------------GT--------G-GT-AC---C---C---C-G-----T-T-
4.--------------T--------------------C----------------------G-------------GT--------G-GT-AC---C---C---C-G-----T-T-

1.CACCAGGCGCCAAGCAGAACGTCCAGCTGATCAACACCAACGGCAGTTGGCACCTCAATAGCACGGCCCTGAACTGCAATGATAGC
2.--------------------A-------------------------------A----------------C---------------
3.--------------------A-------------------------------A----------------C---------------
4.--------------------A-----------------------A-T----A----------------C---------------
```

1 = Choo consensus sequence and 2 = gammaglobulin batch consensus sequence, 3 = patient 3 quasispecies 1, 4 = patient 3 quasispecies 2, Normal case = synonomous, Blue Italic = non-synonomous
3 = 18/20 clones, 4 = 2/20 clones

Envelope region 1100 –1298 nt
Quasispecies of patient 4 at time point 1

```
1.AACTGGGCGAAGGTCCTGGTAGTGCTGCTGCTATTTGCCGGCGTCGACGCGGAAACCCACGTCACCGGGGGAAGTGCCGGCCACACTGTGTCTGGATTTGTTAGCCTCCTCG
2.--------------------G----------T---------------------GAA-C-CG-CA-A--------------AG--A-CATCG----AC-TG-C----T--AC
3.--------------------G----------T---------------------GAA---CG-CA-A--------------AG--A-CATCG----AC-TG-C----T--AC
4.--------------------G----------T---------------------GAA-C-CG-CA-A--------------AG--A-CATCG----AC-TG-C----T--AC


1.CACCAGGCGCCAAGCAGAACGTCCAGCTGATCAACACCAACGGCAGTTGGCACCTCAATAGCACGGCCCTGAACTGCAATGATAGC
2.T-A-----C--CC-------A-------------------------------------C-------------------------
3.T-A-----C--CC-------A-------------------------------------C-------------------------
4.T-A--------CC-------A-------------------------------------C-------------T------------
```

1 = Choo consensus sequence , 2 = patient 4 quasispecies 1, 2 = patient 4 quasispecies 2, 3 = paaient 4 quasispecies 3
2 = 15/19 clones, 3 = 2/19 clones, 4 = 2/19 clones
Normal case = synonomous, Blue Italic = non-synonomous

Envelope region 1100-1298nt
Patient 5 quasispecies at time 1

```
1.AACTGGGCGAAGGTCCTGGTAGTGCTGCTGCTATTTGCCGGCGTCGACGCGGAAACCCACGTCACCGGGGGAAGTGCCGGCCACACTGTGTCTGGATTTGTTAGCCTCCTCG
2.---------------A-------------C--G-----------------AC-----------------------------------------A--------------A
3.---------------A-------------C--G-----------------AC---------------------------------C---------A--AA----------A
4.---------------A-------------C--------------------AC-----------------------------------------A--------T-----A
```

```
1.CACCAGGCGCCAAGCAGAACGTCCAGCTGATCAACACCAACGGCAGTTGGCACCTCAATAGCACGGCCCTGAACTGCAATGATAGC
2.--------------------A----------------------------------A-----------------------------
3.--------------------A----------------------------------A-----------------------------
4.--------------------A----------------------------------A-----------------------------
```

1=Choo, 2 = patient 5 quasi species 1, 2= patient 5 quasispecies 2, 3 = patient 5 quasispecies 4
2 = 17/20 clones, 3 = 2/20 clones, 4 = 1 clone
Normal case = synonomous, Blue Italic = non-synonomous

Envelope region 1100-1298 nt
Quasispecies of patient 6 at time point 1

```
1.AACTGGGCGAAGGTCCTGGTAGTGCTGCTGCTATTTGCCGGCGTCGACGCGGAAACCCACGTCACCGGGGGAAGTGCCGGCCACACTGTGTCTGGATTTGTTAGCCTCCTCG
2.----------------------------C--G----------------AC----------------------------------------------------G-----T---
3.----------------------------C--------------------AC----------------------------------C---------------G-----T---
4.--------------A-------------C--------------------AC----------------------------------------------------G-----T---


1.CACCAGGCGCCAAGCAGAACGTCCAGCTGATCAACACCAACGGCAGTTGGCACCTCAATAGCACGGCCCTGAACTGCAATGATAGC
2.--------------------A-------------------------------------------------------------------------------
3.--------------------A-------------------------------------------------------------------------------
4.--------------------A-------------------------------------------------------------------------------
```

1=Choo consensus sequence, 2 = patient 6 quasi species 1, 3= patient 6 quasispecies 2, 4 = patient 6 quasispecies 4
2 = 12/17 clones, 3 = 2/17 clones, 4 = 3/17 clone
Normal case = synonomous, Blue Italic = non-synonomous

Envelope region

Comparison of amino acid sequence of gammagard batch with quasispecies of patient 1 at 2 years

```
1 . NWAKVLVVLLLFAGVDAETHVTGGSAGHTVSGFVSLLAPGAKQNVQLINTNGSWHLNSTALNCNDS

2 . NWAKVLVVLLLFAGVDAETRVTGGVAGRVTSGLAGLFTPGAKQNIQLINTNGSWHINSTALNCNDS
3 . -----M----------H----------------------------S----------------
4 . ----------------R----------------------------T----------------
5 . -----L----------H----------------------------T----------------
```

1. Choo consesus sequence 1a
2. Gammgard batch
3. Patient 1 Quasipecies 1 13/18 Clones
4. Patient 1 Quasipecies 2 3/18 Clones
5. Patient 1 Quasipecies 3 2/18 Clones

Red case = CTL epitope

Envelope region

Comparison of gammagard batch with patient 2 quasispecies at time 2

```
1.NWAKVLVVLLLFAGVDAETHVTGGSAGHTVSGFVSLLAPGAKQNVQLINTNGSWHLNSTALNCNDS

2.NWAKVLVVLLLFAGVDAETRVTGGVAGRVTSGLAGLFTPGAKQNIQLINTNGSWHINSTALNCNDS
3.------------------G-H----A---A---F-----R--Q----------RL-----------
4.------------------G-H--------A---F-----R--Q----------RL-----------
5.--------------------H--------A---F-----R--Q----------RL-----------
6.----------------------------------------------------------------
```

1. Choo consesus sequence 1a
2. Gammgard batch
3. Patient 2 Quasipecies 1 12/19 clones
4. Patient 2 Quasipecies 2 4/19 clones
5. Patient 2 Quasipecies 3 2/19 clones
6. Patient 2 Quasipecies 4 1/19 clones

Red case = CTL epitope

Envelope region

Comparison of amino acid sequence of gammagard batch with quasispecies of patient 3 at time point 2

```
1.NWAKVLVVLLLFAGVDAETHVTGGSAGHTVSGFVSLLAPGAKQNVQLINTNGSWHLNSTALNCNDS

2.NWAKVLVVLLLFAGVDAETRVTGGVAGRVTSGLAGLFTPGAKQNIQLINTNGSWHINSTALNCNDS
3.-----M----------N---------AS--A--------------T----V----RL-----------
4.-----M----------N---------A---A--------------T----V----RL-----------
5.------------------------------------------------------RL-----------
6.-----M----------N-------------A--------------T----V----RL-----------
```

1. Choo consesus sequence 1a
2. Gammgard batch
3. Patient 3 Quasispecies 1 14/20 clones
4. Patient 3 Quasispecies 2 2/20 clones
5. Patient 3 Quasispecies 3 2/20 clones
6. Patient 3 Quasispecies 4 2/20 clones

Red case = CTL epitope

Comparison of amino acid sequence of envelope region of Choo sequence with patient 4 quasispecies at Time 2

```
                          *---------HVR--.----------*
1.NWAKVLVVLLLFAGVDAETHVTGGSAGHTVSGFVSLLAPGAKQNVQLINTNGSWHLNSTALNCNDS
2.--------------------DPRHR----SNHR-TCR--LT-PP--I-----------R--------
3.----------------G-DPRH----VSNHR-TC---P---P-----------I-R--------
4.--------------------DPRH----VSNHR-TC-------P-----------I-R--------
5.--------------------DPRHR----SNHR-TCR--LT-PP--I-----------R--------
6.--------------------D-RHR----SNHR-TCR--LT-PP--I-----------R--------
```

1 = Choo consensus sequence
2 = patient 4 consensus sequence time 1,
3 = patient 4 time 2 quasispecies 1, 14/20 clones
4 = patient 4 time 2 quasispecies 2, 3/20 clones
5 = patient 4 time 2 quasispecies 3, 2/20 clones
6 = patient 4 time 2 quasispecies 4, 1/20 clones
Red case = CTL epitope

Comparison of amino acid sequence of envelope region of Choo sequence with patient 4 quasispecies at Time 2

```
                          *----------HVR------------*
1.NWAKVLVVLLLFAGVDAETHVTGGSAGHTVSGFVSLLAPGAKQNVQLINTNGSWHLNSTALNCNDS
2.-----M----------T---------------L----T------I--------------------
3.------------------T-Y------A-A---L---FT--PR--V----S-----I----------
4.------------------T--------A-----L---FT--PR--V----S-----I----------
5.-----M----------T---------------L---FT------I--------------------
```

1 = Choo consensus sequence
2 = patient 5 consensus sequence time 1,
3 = patient 5 time 2 quasispecies 1, 12/19 clones
4 = patient 5 time 2 quasispecies 2, 4/19 clones
5 = patient 5 time 2 quasispecies 3, 3/19 clones
Red case = CTL epitope

Comparison of amino acid sequence of envelope region of Choo sequence with patient 6 quasispecies at Time 2

```
                          *---------HVR-----------*
1.NWAKVLVVLLLFAGVDAETHVTGGSAGHTVSGFVSLLAPGAKQNVQLINTNGSWHLNSTALNCNDS
2.------------------T----------------G-F-------I--------------------
3.----------------N-T------G-A---F---G-FPL-----I-------------------A-
4.----------------N-T------G---------G-FPL-----I-------------------A-
5.------------------T----------------G-F-------I--------------------
6.-----M----------T----------------G-F-------I--------------------
```

1 = Choo consensus sequence
2 = patient 6 consensus sequence time 1,
3 = patient 6 time 2 quasispecies 1, 12/20 clones
4 = patient 6 time 2 quasispecies 2, 3/20 clones
5 = patient 6 time 2 quasispecies 3, 3/20 clones
6 = patient 6 time 2 quasispecies 4, 2/20 clones
Red case = CTL epitope

CHAPTER 9:
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