THE PATHOLOGY OF HEPATITIS C VIRUS INFECTION

by

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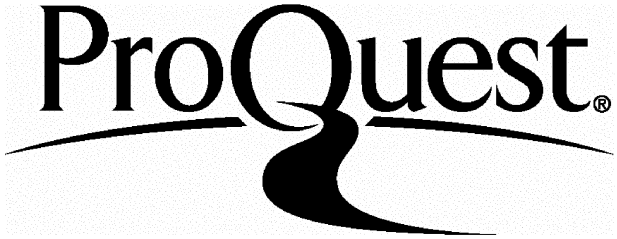

ABSTRACT

Hepatitis C virus is a recently discovered RNA virus which is an important cause of chronic liver disease around the world. This thesis explores several aspects of HCV-induced liver disease using the novel technique of RT-PCR on formalin-fixed, paraffin embedded histological material, and immunocytochemistry for detection of HCV antigen.

The relationship of inflammation within the liver of HCV infected patients to HCV antigen expressing liver cells was investigated using these two techniques. This revealed no direct topographical relationship between inflammation and infected cells, although amount of inflammation within a sample was directly related to amount of HCV immunostaining. Numbers of cytotoxic T lymphocytes appeared inversely linked to the intensity of staining with anti-HCV antibody, implying a role for the immune system in the control of HCV infected cells. This role was supported by observations on HCV patients after liver transplantation. These showed that whilst HCV infection recurred after transplantation, the outcome varied between patients, possibly dependent on the immune status of individuals. HCV antigen expression can be greatly increased in these patients.

The relationship between HCV infection and autoimmune hepatitis (AIH) was explored. It was found that most patients with type I AIH do not have HCV, but a small number of patients with significant levels of autoantibodies but a poor response to steroid treatment do have HCV infection.

Finally, possible inapparent means of HCV transmission were explored by testing for HCV-RNA in extrahepatic sites post mortem. This study revealed that HCV-RNA can be detected in the salivary gland and seminal vesicle of HCV

infected patients, as well as in the thyroid and pancreas. HCV antigen was detected in the salivary gland and thyroid in addition. These observations could help to explain possible non-parenteral or sexual transmission of HCV.

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LIST OF ABBREVIATIONS

A	deoxy-adenosine-triphosphate
aa	amino acids
AIH	autoimmune hepatitis
ALT	alanine amino transferase
ALP	alkaline phosphatase
ANA	anti-nuclear antibodies
APES	aminopropyl triethoxysilane
AST	aspartate amino transferase
bp	base pair
C	deoxy-cytidine-triphosphate
CAH	chronic active hepatitis
CMV	cytomegalovirus
DAB	3,3 diaminobenzidine tetrachloride
DEPC	diethyl pyrocarbonate
cDNA	complementary DNA
CTL	cytotoxic T lymphocytes
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotides
DEPC	diethyl pyrocarbonate
E	envelope
EBV	Epstein Barr virus
ELISA	enzyme linked immmunosorbent assay
FFPE	formalin fixed paraffin embedded

FITC	fluorescein isothiocyanate
G	deoxy-guanidine-triphosphate
gp	glycoprotein
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HIV	human immunodefficiency virus
HLA	human leukocyte antigen
HVR	hypervariable region
ICC	immunocytochemistry
IgG	immunoglobulin G
IOD	integrated optical density
IRES	internal ribosomal entry site
IVDU	intravenous drug users
LFT's	liver function tests
LKM	anti-liver-kidney microsomal antibodies
MHC	major histocompatability complex
MLP	mouse liver powder
M-MLV	mouse maloney leukemia virus
mRNA	messenger RNA
NANB(H)	non-A, non-B (hepatitis)
NS	non-structural
nt	nucleotide
OLT	orthotopic liver transplant

ORF	open reading frame
PAP	peroxidase anti-peroxidase
PBC	primary biliary cirrhosis
PBMC's	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PLL	poly-L-lysine
PT	post transplant
PTH	post transplant hepatitis
RFH	Royal Free Hospital
RFLP	restriction fragment length polymorphism
RIA	radioimmunoassay
RIBA	recombinant immunoblot assay
RNA	ribonucleic acid
RT-PCR	reverse transcription - polymerase chain reaction
SMA	anti-smooth muscle antibodies
SOD	superoxide dismutase
STD	sexually transmitted disease
T	deoxy-thymidine-triphosphate
TBE	tris buffer with EDTA
TBS	tris buffered saline
U	uracil-triphosphate
UV	ultraviolet
UTR	untranslated region

CHAPTER 1

INTRODUCTION

Introduction

Hepatitis C virus (HCV) is a recently discovered virus shown to be the major cause of parenterally transmitted and sporadic or community acquired non-A non-B hepatitis (NANBH). It was identified in 1989 as the result of intensive research by scientists at the Chiron Corporation in the USA (Choo *et al.*, 1989; Kuo *et al.*, 1989). The clinical importance of hepatitis C is that infection can cause chronic hepatitis, defined as inflammation of the liver lasting for six months or longer. Indeed, it is estimated that up to 70% of patients infected develop chronic hepatitis, with at least 20% of these going on to develop cirrhosis, and 10% of these developing hepatocellular carcinoma (Tremolada *et al.*, 1992). The high incidence of HCV worldwide means the virus is a significant cause of morbidity and mortality.

Hepatitis is the general term used to describe inflammation of the liver, and has many aetiologies. It can be caused by toxins, such as alcohol; by idiosyncratic reaction to drugs; by aberrant immune reaction; or by infective agents, the most significant of which are the hepatitis viruses.

To date, six hepatitis viruses have been identified, including HCV, although there are thought to be more. The most common of these are hepatitis A, or infectious hepatitis, and hepatitis B or serum hepatitis. Hepatitis A is an enterically transmitted infection caused by an RNA virus and usually causes an acute, self limiting hepatitis which in rare cases can be fatal; the virus is directly cytopathic, and does not chronically infect the liver (Underwood, 1990; Dienstag, 1980). Hepatitis B is primarily transmitted via the parenteral route, although it can be transmitted by sexual contact, and also vertically. It is associated with a wide

spectrum of liver disease. Infection can be manifest as an acute hepatitis, which can be self limited or lead to fulminant hepatic failure; or it can be subclinical and anicteric. Patients may clear the virus totally or can develop chronic infection. Again, this chronic infection may (rarely) be spontaneously cleared, or can go on to cause cirrhosis and hepatocellular carcinoma. Hepatitis B is not directly cytopathic; liver damage is thought to be due to a HLA class I restricted cytotoxic response to viral antigens presented on the hepatocyte surface in the context of HLA class I molecules (Naoumov and Eddleston, 1994).

Hepatitis D or delta agent is a defective RNA virus that requires the presence of hepatitis B for infection. It can occur either as a coinfection with HBV or as a superinfection, and contributes to chronic liver disease (Lau and Wright, 1993). Hepatitis E, which is epidemiologically similar to hepatitis A, has been identified as the major cause of enterically transmitted NANB, an important disease in the developing world (Zuckerman, 1990). Fulminant hepatitis can be caused by hepatitis A, B and possibly C, but there is much evidence incriminating an as yet unidentified transmissible agent (candidate hepatitis F) as the major cause of this type of hepatitis (Fagan, 1994). The most recent addition to the viral alphabet is hepatitis G, an RNA virus which is virologically similar to hepatitis C virus (Linnen *et al*, 1996).

The pathogenesis of hepatitis C virus-induced liver disease is largely unknown and is the principal concern of this thesis.

Historical perspective: non-A, non-B hepatitis

Hepatitis has long been recognised as a complication of blood transfusion (Beeson, 1943). The principal cause of this hepatitis was thought to be hepatitis B;

hence its original name of serum hepatitis. The discovery of antigenic markers of hepatitis B by Blumberg *et al* (1965, 1968) soon led to the introduction of serological tests for hepatitis B infection. It became apparent that hepatitis B was not the cause of post transfusion hepatitis in as many as 75% of cases (Gocke, 1972); development of other serological tests soon revealed that these cases were not caused by hepatitis A virus (Feinstone *et al.*, 1973), or other known hepatotropic agents such as cytomegalovirus (CMV) or Epstein Barr virus (EBV) (Alter *et al.*, 1975; Feinstone *et al.*, 1975). The term 'non-A non-B hepatitis' (NANBH) was introduced to describe this group of diseases with clinical and epidemiological features suggestive of a hepatotropic virus, but without serological markers for any of the known pathogens - ie diagnosis by exclusion.

The evidence for existence of a different virus or viruses causing hepatitis had been available before serological testing showed their existence, although the significance of this evidence had not been generally appreciated (Dienstag, 1983). Multiple episodes of acute hepatitis in drug addicts (Havens, 1956; Iwarson *et al.*, 1973) and haemophiliacs (Craske *et al.*, 1975) had been described; and it was observed in the 1960's that some episodes of post transfusion hepatitis had an incubation period of approximately 7 weeks, in between those of hepatitis A (3-4 weeks) and hepatitis B (12-14 weeks)(Mosley, 1975).

Recognition of the existence of NANB hepatitis led to intensive research in this area. It was soon realised that infection with the putative NANB agent frequently caused chronic liver disease; that infection was often anicteric and asymptomatic after transfusion; and that 'sporadic' or 'community acquired' hepatitis was also frequently not due to hepatitis A or B (Fagan and Williams,

1984). However, after ten years, there still was no test for the agent, even though more than 40 reports of specific NANB agent assays had been published; none of these assays had proved specific or reproducible for the detection of infectious sera (Dienstag and Alter, 1986). This failure was thought to be due to several elements:

1) The amount of NANBH antigen was low and unmeasurable by existing tests.

2) Most NANBH sera had low infectivity titres of $< 10^3$ chimp infectious doses (CID) per ml, therefore the number of antigen particles was probably $< 10^6$/ml, and not detectable by the techniques used to detect other viral antigens, eg HBsAg.

3) It was therefore probable that antibody to this agent was either not present in serum or present in insufficient titre to serve as a reliable agent to probe for a marker of infection. This inference was based on the high frequency of chronicity after acute NANBH (Dienstag and Alter, 1986).

The most important evidence that NANBH was due to an infectious agent was provided by the work done on experimental transmission of infection to human volunteers and chimpanzees. Indeed, the only way of propagating the unknown virus and proving that NANBH was caused by a transmissible hepatotropic agent was through the use of chimpanzees, the only animal which could be successfully infected and reproduce the symptoms of parenterally transmitted non-A non-B hepatitis.

Re-evaluation of serological data on volunteer studies carried out during the 1950's on post transfusion hepatitis provided the first evidence of experimental transmission of NANBH (Hoofnagle *et al.*, 1977). This work also proved that the

agent caused chronic infection, as serum used for inoculation of volunteers was collected from the donor implicated in transmitting hepatitis 6 months to 1 year after the original donation. Retrospective analysis of a second 'volunteer' study done in 1969 demonstrated serial transmission of NANBH during experiments on transmission of malaria (Dienstag *et al.*, 1981). Only a small amount of inoculum (1-5ml) was used in these experiments, showing that a small volume could still transmit infection.

Two simultaneous reports of successful transmission of NANBH to chimpanzees followed (Tabor *et al.*, 1978b; Alter *et al.*, 1978), which lead to a multitude of studies on NANBH in chimpanzees. These studies implied that there was more than one agent involved in post transfusion hepatitis (Bradley *et al.*, 1983), but established that one of these was a small, enveloped RNA virus (Bradley *et al.*, 1983), which could be inactivated by formalin (Tabor and Gerety, 1980), chloroform (Feinstone *et al.*, 1983), and by a combination of beta-propiolactone and UV irradiation (Prince *et al.*, 1980). It was also demonstrated that there was no heterologous immunity between NANB hepatitis and hepatitis A or B (Tabor *et al.*, 1978a; Trepo *et al.*, 1983).

Study of the histology of NANBH was made difficult by the lack of specific markers. However, several groups described histological features associated with both acute and chronic NANBH. All noted the difficulty in distinguishing acute from chronic NANBH because of the lobular inflammation seen at all stages of the disease (Dienes *et al.*, 1982; Bianchi *et al.*, 1987; Fagan and Williams, 1984). This lobular inflammation varied from case to case, but was sometimes striking; other lobular changes included acidophil body formation, which was notable for the

absence of surrounding inflammatory cells (Bianchi *et al.*, 1987; Dienes *et al.*, 1982); this feature led Dienes *et al* (1982) to conclude that the NANB agent might have a direct cytopathic effect, rather than damage being mediated via the immune system as in hepatitis B infection. Other features considered characteristic of NANBH included lymphocytic infiltration of the portal tracts, with lymphoid aggregates or follicles often seen; steatosis, sinusoidal cell activation, and bile duct damage were noted to differing degrees (Bamber *et al.*, 1981; Schmid *et al.*, 1982; Dienes *et al.*, 1982; Bianchi *et al.*, 1987); piecemeal necrosis and cirrhosis were reported in chronic cases.

Discovery of the hepatitis C virus

Choo *et al* (1989) considered that the failure to identify specific viral antibodies and antigens in NANBH was due to insufficient concentration of antigen rather than lack of antibody. To increase antigen concentrations, they made a cDNA library of infectious material in bacteriophage lamda gt11. The library was screened using serum from a chronic NANBH patient as a presumed source of viral antibodies.

The cDNA library was derived from chimp plasma containing a high infectious titre of virus. The plasma was subjected to extensive ultracentrifugation to ensure pelleting of a small virus, and total nucleic acid was recovered from the pellet. This nucleic acid was denatured, then cDNA synthesised from RNA and DNA using random primers and reverse transcriptase. Total cDNA was expressed using bacteriophage.

Screening of approximately 10^6 plaques of the recombinant phage led to identification of a positive clone, 5-1-1. This clone was then used to isolate a larger

overlapping clone, named 81, from the library. By further experiments, it was revealed that the clones were derived from a single, positive stranded exogenous RNA molecule, of approximately 10,000 nucleotides, which did not replicate via DNA intermediates, and was associated with NANB infection, not with normal chimp or human nucleic acid.

The 5-1-1 clone coded for part of a single open reading frame (ORF) spanning the entire genome. An open reading frame is a continuous DNA coding sequence, defined by the presence of translational start (ATG) and stop (TAA or TAG) codons, and codes for polypeptide (Carman and Thomas, 1990). This clone was expressed in bacteria as a fusion peptide with human superoxide dismutase (SOD), then immunoblots were carried out on total bacterial lysates. The chronic NANBH serum used originally to detect 5-1-1 reacted specifically with this SOD/5-1-1 peptide, but not with SOD alone. Similar results were obtained with serum from seven other NANB patients, but not from 10 normal donors. Four chimps with NANBH converted to antibody to the peptide after acute infection - 7 infected with A or B did not seroconvert. This data showed that 5-1-1 and the larger clone, 81, come from the genome of the blood borne NANBH virus, which was named hepatitis C virus.

The 5-1-1 clone was used to extract a still larger clone from the cDNA library, which expressed a polypeptide (called c100-3) of 363 amino acids. This peptide was expressed as a SOD fusion protein in yeast, and used as the basis for the first screening test for hepatitis C virus (Kuo *et al.*, 1989).

Since the discovery of hepatitis C, and the availability of serological tests for the virus, it has become apparent that at least 80% of cases of parenterally

transmitted NANBH are caused by HCV (Hopf *et al.*, 1990; Di Bisceglie *et al.*, 1991). In addition, HCV has been identified as the major cause of NANBH not associated with any known parenteral risk factor (so-called community acquired or sporadic NANBH) (Hopf *et al.*, 1990; Alter *et al.*, 1992).

Virology of HCV

Research interest soon became focused on the virology of HCV. Choo *et al* (1991) reported the nucleotide sequence of the virus, revealing that the genome of approximately 9.4kb consists of a single large open reading frame, encoding a polyprotein of about 3000 amino acids. The ORF is flanked at the 5' and 3' ends of the genome by short untranslated regions (Feinstone, 1991b; see figure 1.1). Comparison of the nucleotide and amino acid sequences with other viruses revealed similarities with the animal pestiviruses, plant potyviruses and human flaviviruses, which are also single stranded, positive sense RNA viruses (Choo *et al.*, 1991).

The hydrophobicity profiles of the polyproteins from the pesti- and flavi-viruses are also remarkably similar to that of HCV (Choo *et al.*, 1991; Takamizawa *et al.*, 1991); these similarities gave the first clues to the presumed function of the different domains of the HCV polyprotein (Feinstone, 1991a; Bradley, 1992). It was postulated that the structural proteins, which form the viral particle, are found at the amino-terminal part of the polyprotein (coded for by the 5' end of the virus). The non-structural proteins, involved in replication of the virus, are found at the carboxy-terminal (coded for by the 3' end of the genome). The polyprotein encoded by the ORF is subject to post translational processing. It is cleaved into mature viral structural and non-structural proteins by both viral coded protease and host signalase (Feinstone, 1991b). Considerable progress has now been made in characterising the

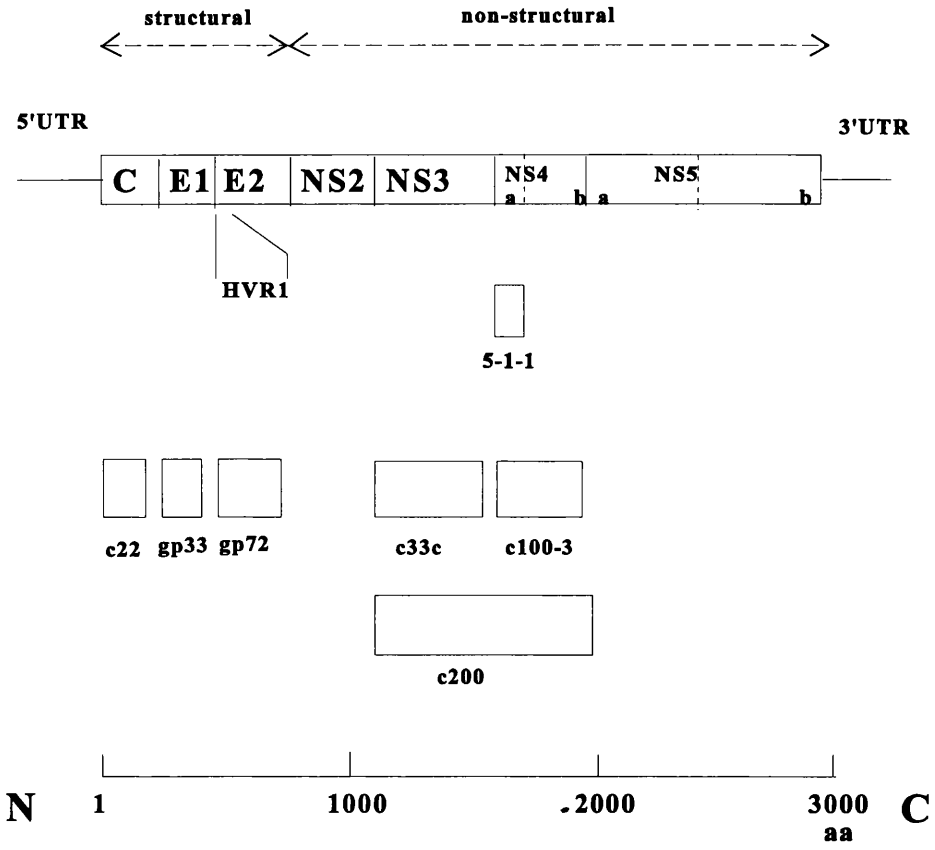


Figure 1.1 Representation of the HCV RNA genome, including the location of several of the cloned antigens used for anti-HCV detection. HVR1 indicates the position of the hypervariable region in E2/NS1 (van Doorn, 1994)
N = N terminal of the polyprotein C = C terminal of the polyprotein aa = amino acid

Table 1.1. HCV encoded proteins (van Doorn, 1994).

Gene	Function	a.a. position	Name/size (kDa)
C	Capsid	1 - 191	p22
E1	Envelope	192 - 383	gp33
E2/NS1	Envelope	384 - 809	gp72
NS2	Metalloproteinase	810 - 1009	p23
NS3	Serine-protease and helicase	1010 - 1619	p72
NS4 (a and b)	?	1620 - 2016	p10 p27
NS5 (a and b)	Replicase/polymerase	2017 - 3033	p58 p70

virus and confirming these assumptions (van Doorn, 1994) (see figure 1.1, table 1.1). Because of the similarities between these viruses, HCV is currently classified within the flaviviridae as a separate genus (Heinz, 1992).

Sequencing of several isolates of HCV from different parts of the world showed that there was significant diversity between these isolates (Choo *et al.*, 1991; Kato *et al.*, 1990; Takamizawa *et al.*, 1991). HCV is an RNA virus replicating via an RNA intermediate, so has a high spontaneous mutation rate due to an error-prone RNA polymerase, without the ability to correct non-lethal mutations (Bukh *et al.*, 1995). The amount of genetic diversity between isolates varies for each gene region (Bukh *et al.*, 1995); some areas of the genome show hypervariability (see figure 1.1), whereas other areas are highly conserved (Han *et al.*, 1991). This variation in diversity of the different domains relates to function.

At the 5' end of the genome, there is an untranslated region (UTR) of 324-341 nucleotides (Kato *et al.*, 1990; see figure 1.1). This region is highly conserved between isolates (Feinstone, 1991a), although some nucleotide differences have been found. Studies by Brown *et al.* (1992b) and Tsukiyama-Kohara *et al.* (1992) have revealed a large conserved stem loop structure within the 5' UTR (see figure 1.2). This folding region is thought to act as an internal ribosomal entry site (IRES) or ribosomal landing pad (Wang *et al.*, 1993; Le *et al.*, 1995); HCV polyprotein translation is thought to initiate at this IRES, proximal to the ATG codon of the ORF (van Doorn, 1994). This is similar to the translation mechanism for picornaviruses (Jang *et al.*, 1989).

The core protein (p22) is probably processed after translation to produce the mature core which is incorporated into the virion (Hijikata *et al.*, 1991b). P22 has

an RNA binding capacity, which allows it to bind genomic HCV-RNA and form nucleocapsid particles (Takahashi *et al.*, 1992). P22 contains several highly conserved immunoreactive epitopes - these have been used for detection of HCV positive sera (Katayama *et al.*, 1992; Hosein *et al.*, 1991) (see figure 1.1).

E1 and E2/NS1 code putative viral envelope proteins, gp33 and gp72 (Spaete *et al.*, 1992; Houghton *et al.*, 1991). gp33 contains an N-terminal stretch of 20 hydrophobic amino acids, which is thought to facilitate transmembrane transport, and could also function as a recognition sequence for host signalase to cleave the core protein p22 from the precursor polyprotein. Another stretch of hydrophobic amino acids (350 - 390) could act as a membrane anchor (Heinz, 1992); E1 contains a moderately variable domain of about 40 amino acids (Weiner *et al.*, 1991).

gp72 has a complex secondary and tertiary structure, which is highly conserved. This protein is membrane associated - the C terminus is membrane anchored, but the N-terminus is located outside, becoming a target for surveillance by the immune system (Spaete *et al.*, 1992). The part of E2/NS1 encoding this N-terminal region is hypervariable (HVR1)(Weiner *et al.*, 1991; Hijikata *et al.*, 1991a); specific antibody reactions against epitopes in HVR1 have been detected. These epitopes have been mapped to the N-terminal region of HVR1, and may be subjected to immune selection (Lesniewski *et al.*, 1993); this immune pressure probably results in the observed variability (Kato *et al.*, 1993). The variability observed in these two structural proteins has significant implications for development of a protective immune response and any vaccine (Weiner *et al.*, 1991; Bukh *et al.*, 1995).

NS2 codes for p23, a putative metalloproteinase thought to cleave NS2 from

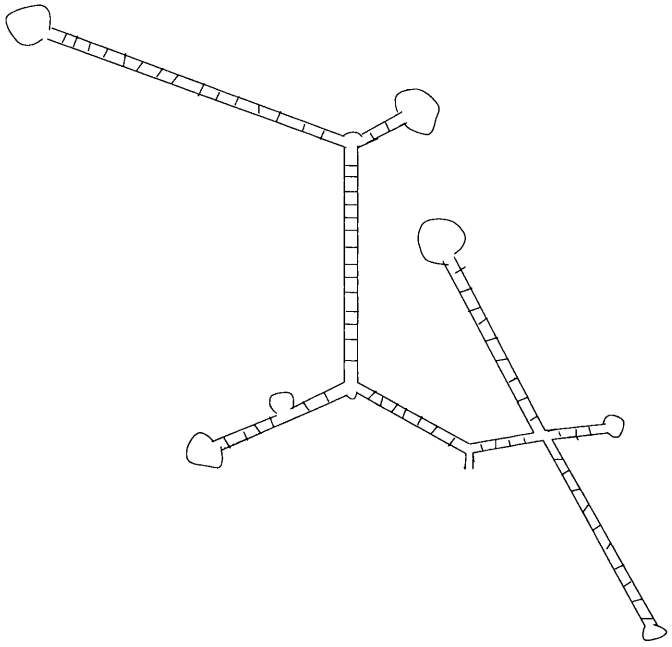


Figure 1.2 Predicted folding pattern of the 5' untranslated region of hepatitis C virus. (Feinstone, 1991a).

NS3. NS3 encodes p72, which resembles a trypsin-like serine protease, similar to that found in flavi- and pesti-viruses. This protein is thought to perform the four cleavages which occur in the NS3, NS4 and NS5 domains. These cleavage sites share several common features, which probably determine substrate specificity of NS3 serine protease (Grakoui *et al.*, 1993). NS3 is also thought to have helicase activity, important in viral replication (Houghton *et al.*, 1991).

The function of the NS4 protein, which is processed into NS4a and NS4b, is unknown, but it contains highly immunogenic epitopes and several predicted transmembrane regions (van Doorn, 1994). The c100-3 antigen, used in most antibody detection assays, is an unnatural antigen, spanning the C terminus of NS3, the whole of NS4a, and the N-terminal region of NS4b.

NS5 is processed into NS5a and NS5b. NS5b shows significant amino acid sequence homology with the putative RNA dependent RNA replicase from carnation mottle virus (Miller and Purcell, 1990), and contains a sequence which is characteristic of RNA-dependent RNA polymerase in positive stranded RNA viruses (Koonin, 1991). The viral replicase activity of this protein has been demonstrated in vitro (Chung and Kaplan, 1992). The function of NS5a is unknown, but the protein contains important B-cell epitopes.

Okamoto *et al* (1992a) have suggested that in some isolates a secondary structure of the genomic RNA exists at the C terminal of NS4 that could form an IRES. This IRES could allow an increased production of NS5-encoded replicase,which might accelerate the replication of the genomic RNA. However, this has yet to be shown experimentally.

Another untranslated region is found at the 3' end of the virus. The 3' UTR

sequences reported to date show significant variation both in length and sequence; the most important difference is the presence of a poly A tail in some isolates (Choo *et al.*, 1991; Han *et al.*, 1991), whereas in others a poly U tail is found (Kato *et al.*, 1990; Takamizawa *et al.*, 1991).

Genetic variation

After the sequencing of the first isolate of HCV, it became clear that isolates of the virus from other parts of the world, in particular Japan, did not have exactly the same sequence as the original isolate, known as HCV-H or HCV-1 (Choo *et al.*, 1991; Takamizawa *et al.*, 1991; Kato *et al.*, 1990). Comparison of the full length sequence of HCV-1 with that of two independent isolates from Japan (Takamizawa *et al.*, 1991; Kato *et al.*, 1990) revealed that whilst the two Japanese isolates were very similar to each other in terms of nucleotide and peptide homology (>90%), they showed much less similarity to HCV-1 (approx. 78%), confirming early indications of distinct HCV types (Kato *et al.*, 1989). The extent of these differences varied in different areas of the genome, with degree of homology for NS5 protein between HCV-1 and the Japanese isolates being only 84-88%, whereas the nucleocapsid protein was highly conserved, with 97-98% homology. The term genotypes was introduced to describe the observed heterogeneity between isolates.

As more isolates from different parts of the world have been sequenced, to date, nine different genotypes of HCV have been identified, which can be divided further into 30 subtypes (Bukh *et al.*, 1995). Several different nomenclatures have been used to describe these genotypes, but the Simmonds classification (Chan *et al.*, 1992) is now generally accepted. This system designates HCV types by arabic numerals in the order of discovery (eg types 1,2,3 etc), and subtypes are designated

by these numerals followed by a letter, also in the order of discovery (eg 1a, 1b, 2a, 2b etc).

Geographic distribution of the different genotypes varies (Bukh *et al.*, 1995). Genotype 1 is widely distributed throughout the world; types 1a and 1b are predominant in the Americas and Europe, whereas 1b is predominant in Asia. Type 1c constitutes more than 20% of infections in Indonesia, but has not been found elsewhere. Type 2 is also widely distributed, but is particularly common in parts of Europe and the Pacific Rim (Bukh *et al.*, 1995). Similarly, type 3 is widely distributed, but certain subtypes have a very specific location; subtype 3b has been found only in Japan, Nepal, Thailand and Indonesia; subtypes 3c - 3f have been found only in Nepal. Distribution in Africa is different, with types 1,2 and 3 rare. Type 4 is predominant in north and central Africa, with type 5 the most common in southern Africa. Type 6 is found mainly in Hong Kong and Vietnam; whilst types 7, 8 and 9 have only been found in Vietnam. It is interesting to note that genotypes differ according to the group of individuals studied. Genotype distribution is different in blood donors than in patients with chronic liver disease (Okamoto *et al.*, 1992b); type 3 is more common in intravenous drug users than in other patients (Pawlotsky *et al.*, 1995); and genotype distribution in haemophiliacs reflects the prevalence of genotypes in the United States because of the use of commercial blood products manufactured there (Okamoto *et al.*, 1992b).

These different genotypes have important diagnostic and clinical implications. Any screening test must use a variety of proteins for detection of antibodies, and preferably ones which are conserved between genotypes (eg core protein). Inclusion of the recombinant core protein c22-3 greatly improved the

sensitivity of second and third generation ELISA's; antibodies to c22-3 are more frequently detected than antibodies to any other protein in patients infected with HCV (Alter and Seeff, 1993). Likewise, primers for PCR must be chosen from a conserved region of the genome to be universally applicable. The 5' UTR is the most highly conserved region of the genome, but even this consists of variable domains interspersed with highly conserved domains (Bukh *et al.*, 1993), so primers and probes must be carefully chosen. Finally, a recent study has indicated that the sensitivity of the quantitative bDNA amplification assay (Chiron) may differ with the genotype analyzed (Kobayashi *et al.*, 1994).

Clinically, the significance of different genotype is more difficult to determine. Workers in Italy, France and Japan have found that genotype 1b is associated with more severe liver disease than infection with other genotypes, and that liver cirrhosis is more common in patients infected with 1b than with 1a, 2a or 2b (Pozzato *et al.*, 1994; Qu *et al.*, 1995). However, the mean age of patients infected with 1b was significantly higher than that of patients infected with other genotypes; this difference may therefore simply reflect longer duration of infection. There is a more convincing link between genotype and response to interferon treatment. It has been shown that patients infected with 1b respond to interferon significantly less well than those infected with other genotypes regardless of age, duration of disease, and histological diagnosis (Qu *et al.*, 1995).

There are several different methods for determining genotype. The most accurate is sequencing, either of the whole genome, or of a selection of appropriate domains of the genome (Bukh *et al.*, 1995). However, this technique is time consuming, difficult and expensive. Type specific antibodies can be detected for

types 1, 2 and 3; this is relatively cheap and easy, but is not very specific, especially for the more unusual genotypes (Zhang *et al.*, 1995). One of the most commonly used methods is the use of PCR to amplify the 5' non-coding region, followed by RFLP analysis (Simmonds *et al.*, 1993). This technique is able to accurately distinguish genotypes 1-6, but cannot resolve subtypes, and may not distinguish types 7-9 from the others; however, this has proved a useful epidemiological tool.

As well as the heterogeneity observed between different isolates of the virus, there is genetic heterogeneity of the HCV population within an infected individual. This phenomenon is commonly seen in RNA viruses; the infecting virus consists of a population of closely related yet heterogenous sequences known as quasispecies, centred around a dominant sequence (referred to as the master sequence) in a single individual. This heterogeneity was discovered when multiple clones generated from the HCV-RNA of an infected individual were sequenced, and found to harbour significant genetic heterogeneity (Martell *et al.*, 1992; Oshima *et al.*, 1991). The master sequence may only constitute a minority of the sequence population, and can be different from the consensus (majority) sequence (Duarte *et al.*, 1994). This consensus sequence has been monitored over time in chronically infected chimpanzees and humans (Ogata *et al.*, 1991; Okamoto *et al.*, 1992a), and has been found to change at a rate of approximately 1.92×10^{-3} base substitutions per genome site per year (Ogata *et al.*, 1991). Once again, different regions of the genome mutate at different rates, with the E1 and E2 regions showing the highest mutation rate, both at the nucleotide and the amino acid level (Ogata *et al.*, 1991). The highest rate of nucleotide change is seen in the HVR1 region, which is within

E2 (see figure 1.1). As discussed earlier, the part of the protein (gp72) coded by the HVR1 region protrudes outside the viral envelope and is under selective pressure by the immune system. It has been shown that HVR1 codes for epitopes that elicit an isolate specific antibody response. Longtitudinal monitoring of chronically infected patients and chimpanzees revealed sequential changes in the consensus sequence resulting in antigenically distinguishable variants (Weiner *et al.*, 1992; Taniguchi *et al.*, 1993). Taniguchi *et al* (1993) showed that at a given time point, antibodies were present that were specific for peptides deduced from the HVR1 consensus sequence found at a previous time point, but not specific for peptides deduced from the sequence of the actual sample. They also showed that the predicted secondary structure was different for the antigenically distinct HVR1 peptides. No such differences were observed in the remainder of the E2 protein (Taniguchi *et al.*, 1993).

These studies demonstrate that chronically infected individuals mount a humoral immune reponse to epitopes of HVR1 of the HCV genome. This sequence change is faster in acute infection (Yamaguchi *et al.*, 1994), and appears accelerated during interferon therapy (Enomoto *et al.*, 1994). It has also been suggested that quasispecies become more divergent as liver disease advances, and that patients with more complex diversity show less response to interferon (Koizumi *et al.*, 1995). This diversity and rapid evolution might represent a mechanism by which HCV evades immune surveillance and maintains persistent infection (Zeldis and Jensen, 1994).

Both genotyping and surveillance of quasispecies can provide valuable information on epidemiology and transmission of HCV. Many studies on vertical

and sexual transmission of HCV have used quasispecies monitoring to determine source of infection; this is discussed in more detail in chapter 6.

Hepatitis C diagnostic assays

The first commercially available assay for anti-HCV antibodies was an enzyme-linked immuno assay which used the artificial c100-3 antigen from the non-structural NS3 and NS4 regions to capture circulating antibody (Kuo *et al*, 1989). Use of this assay revealed that HCV was the cause of post transfusion NANBH in at least 80% of patients from many different countries around the world (Alter *et al.*, 1989a; Esteban *et al.*, 1989; Choo *et al.*, 1990). HCV was also incriminated as the major cause of NANBH in patients with no known parenteral risk factors (ie sporadic NANB) (Hopf *et al.*, 1990; Alter *et al.*, 1992). However, it became apparent that there were problems associated with the specificity and sensitivity of the first generation assays. Testing of low risk blood donors yielded a high rate of false positive results, with as many as 50% of ELISA positive donors subsequently proving negative for HCV (Wong *et al.*, 1990; Weiner *et al.*, 1990b). False positive results were also seen in patients with rheumatoid factor (Theilmann *et al.*, 1990), in patients with antibodies to superoxide dismutase (Ikeda *et al.*, 1990), and in patients with hypergammaglobulinaemia (McFarlane *et al.*, 1990). In addition, not all patients who had NANBH were anti-HCV positive; some of these anti-HCV negative patients were later shown to have HCV viraemia. It also became clear that there was a long seronegative 'window' between infection with HCV, and first detection of anti-c100-3 antibody.

To improve sensitivity and specificity of ELISA testing, the second generation of ELISA assays included the putative core protein c22-3, and c33c from

the NS3 region, together with c100-3 (see figure 1.1). Inclusion of these other antigens improved both the sensitivity and specificity of the assay, and decreased the seronegative 'window' phase before seroconversion (Bresters *et al.*, 1992b). The latest third generation assays contain an antigen from the NS5 region, and the c22-3 and c100-3 are synthetic rather than cloned peptides (De Medina and Schiff, 1995).

To help differentiate between specific and non-specific binding of antibodies in ELISA assays, a variety of supplemental assays were produced. The most commonly used of these is the recombinant immunosorbent blot assay (RIBA) from Ortho Laboratories. For this assay, patient sera are incubated with a nitrocellulose strip, to which are bound bands of HCV antigens, and a control antigen; any antibody binding is detected via an enzyme conjugated anti-human antibody. Intensity of staining is compared with the control bands and graded 1+ to 4+; reaction with more than one HCV antigen is considered positive; reaction with only one HCV antigen is designated indeterminate. The first generation RIBA used E.coli generated 5-1-1 and yeast generated c100-3; SOD was also bound to the strip to test for crossreactivity in sera. This test was relatively insensitive, although it could help distinguish HCV reactive from non-reactive patients. 2nd generation assays included c22-3 and c33c antigens; the more sensitive 3rd generation test consists of c100-3 and c22-3 synthetic peptides, the c33c recombinant antigen, and NS5 recombinant antigen (De Medina and Schiff, 1995).

It should be stressed that these tests are only supplemental, as the same antigens are used as in the ELISA assay. However, they do allow greater confidence in anti-HCV testing as a predictor of infectivity; studies have shown that 80 - 90% of RIBA positive donors transmit hepatitis C to recipients of their blood

(Esteban *et al.*, 1990). Conversely, 10-20% of RIBA reactive donors do not transmit HCV infection; some individuals have true antibody to HCV but have cleared the virus and are no longer infectious.

These findings serve to highlight the shortcomings of anti-HCV testing. Even with a wider range of antigens, there is still a period of approximately 12 weeks between infection and detection of anti-HCV. Moreover, immunosuppressed patients may have infection in the absence of detectable antibody (Lok *et al.*, 1993). At best, anti-HCV detection only reflects immune response and does not indicate active viraemia. The only way to know if a patient is currently infected with hepatitis C virus is by direct detection of viral antigens or RNA.

Detection of viral RNA

The very low concentration of HCV antigens in serum means that they are at the limit of detectability by existing immunoassay technology (Sherlock and Dusheiko, 1991); likewise, conventional molecular biology techniques, such as dot blotting, are not sensitive enough to detect HCV-RNA (Alberti, 1991). Early after the discovery of HCV, researchers began using reverse transcription - polymerase chain reaction (RT-PCR) as a method of amplifying and detecting HCV-RNA in serum (Weiner *et al.*, 1990a).

PCR is a method of artificially amplifying specific sequences of DNA through the use of a thermostable DNA polymerase and short oligonucleotide sequences known as primers (Saiki *et al.*, 1985; Mullis and Faloona, 1987). The target DNA is incubated in the presence of a pair of primers, complementary to short sequences within the target and spanning a length of the target (see figure 1.3). First, the target DNA is denatured by heating to 90 - 95^{0}C. Then the reaction

is cooled to allow annealing of the primers to their complementary sequence. The heat is then increased again to prevent annealing of the two native strands - the thermostable polymerase binds to the double stranded portion between the primer and target, and synthesises a new, complementary DNA (cDNA) strand through extension of the annealed primer. These three steps can be repeated, usually for 30 - 35 cycles; exponential amplification of the region of target DNA flanked by the two primers is achieved.

As HCV is an RNA virus, viral RNA has to be reverse transcribed into DNA to act as a suitable substrate for PCR. Primers for amplification of HCV need to be carefully selected because of sequence diversity between isolates (Choo *et al.*,1991; Bukh *et al.*, 1995). Weiner *et al* (1990a) used primers from the non-structural region; whilst the sensitivity of primers from the non-structural region could be improved by using nested PCR (in which two sets of primers are used, one of which amplifies a sequence within the sequence amplified by the other pair)(Garson *et al.*, 1990a; Farci *et al.*, 1991), other workers proved that the use of nested primers to the highly conserved 5' non-coding region gave the most sensitive and specific results (Inchauspe *et al.*, 1991; Bukh *et al.*, 1992b) .

The high sensitivity and specificity of PCR for detecting HCV-RNA has meant that this test has become the 'gold standard' for diagnosis of HCV infection. HCV-RNA appears earlier in serum during the course of infection than do anti-HCV antibodies (Hilfenhaus *et al.*, 1992), and it has been shown that HCV-RNA negative, anti-HCV positive blood is non-infectious (van der Poel *et al.*, 1994).

The sensitivity and specificity of PCR is also one of its weaknesses. Unless carefully carried out, with appropriate positive and negative controls, PCR is prone

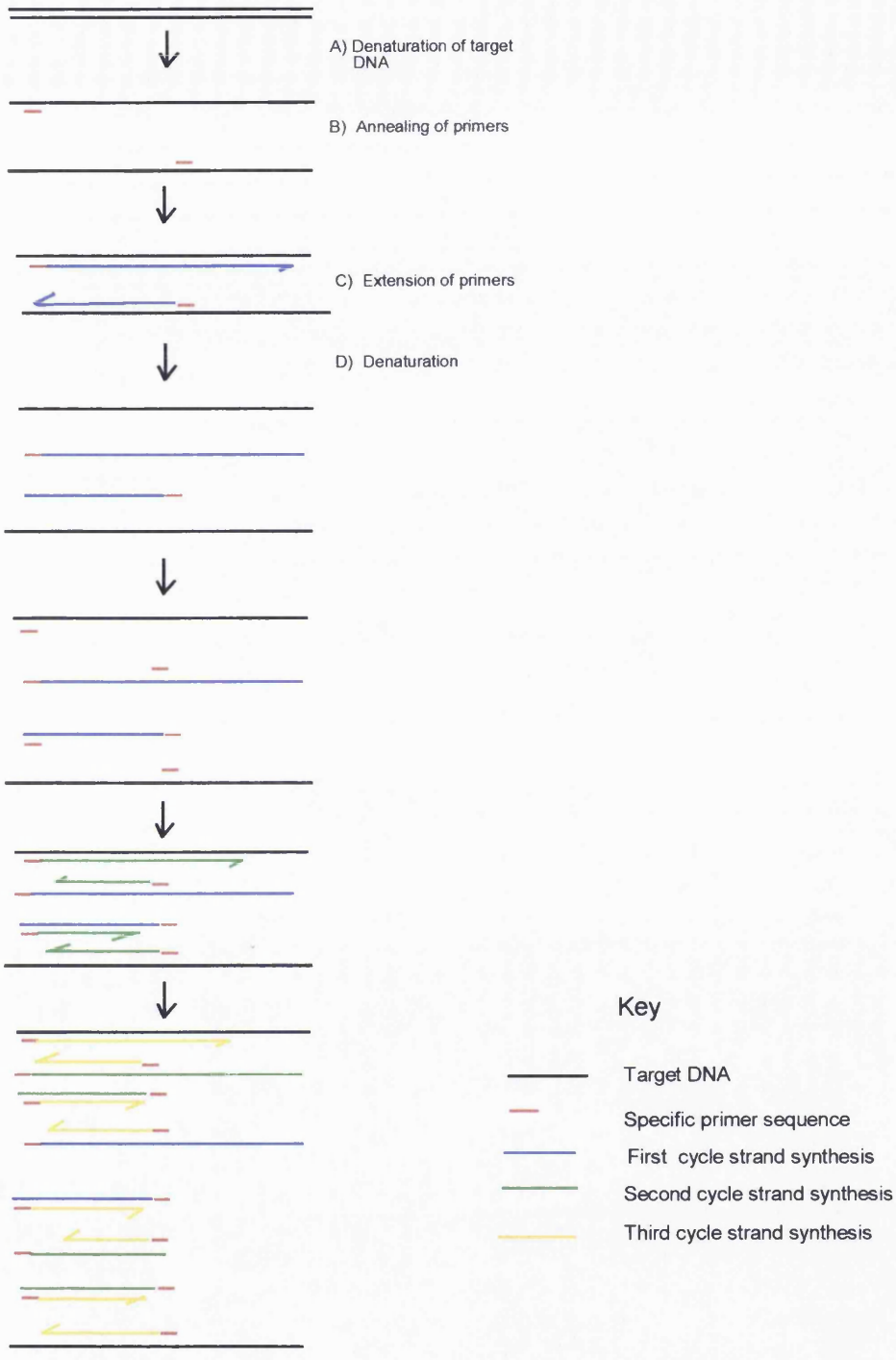


Figure 1.3 Basic principles of PCR. Denaturation of target, annealing and extension of primers are repeated for 30-40 cycles giving exponential amplification of target DNA sequence between the primers. Larger amplimers will only represent a small proportion of total product and will not be detected on the gel.

to contamination and can give false positive results; false negative results can also be obtained without proper technique (Brechot, 1993). A recent study by Zaaijer *et al.*, (1993) revealed that of 31 laboratories regularly performing PCR on serum, only 5 (16%) gave faultless results on a panel of known positive and negative sera. Uses of PCR and methods for control of contamination are discussed in more detail in chapter 2.

Level of viraemia can be quantitated by PCR, but this is a difficult and unreliable technique. An assay by Chiron, called the branched DNA (bDNA) assay has recently been developed. This assay is less technically difficult to perform than PCR and less prone to contamination, and will give a quantitative measure of HCV-RNA within a sample (Suzuki *et al.*, 1995). However, the technique is less sensitive than PCR, and can give inaccurate readings in patients with very low or very high viraemia, for example when immunosuppressed after liver transplantation (Gretch *et al.*, 1995b).

In situ hybridization (ISH) can be used to detect HCV-RNA within liver tissue (Nouri Aria *et al.*, 1993), as can the recently developed technique of *in situ* PCR (Nuovo *et al.*, 1993). These techniques have the advantage of showing the location of HCV-RNA within tissue samples, and so can identify HCV infected cells. In our hands, and in those of others, ISH for HCV was erratic, and it was difficult to prove specific binding (Negro, 1994).

Epidemiology

Epidemiology studies from around the world have demonstrated that HCV is the major cause of both parenterally transmitted and sporadic NANB hepatitis globally (Rassam and Dusheiko, 1991; Alter, 1995; Mansell and Locarnini, 1995),

with an estimated 100-500 million carriers worldwide (Rassam and Dusheiko, 1991). HCV causes both acute and chronic NANBH, and, whilst most studies have focused on transfusion recipients, the majority of HCV infections are acquired outside the transfusion setting (Alter *et al.*, 1992; Alter *et al.*, 1990; Alter, 1995).

Prevalence of anti-HCV ranges from 0.3% in Northern Europe and Canada, to 0.6% in the United States and Central Europe, with southern Europe and Japan showing rates of 1.2% - 1.5% (Alter, 1991). Higher rates of up to 6% have been noted in the Middle East and Africa (Mansell and Locarnini, 1995; Alter, 1995), and a particularly high rate of HCV infection has been found in Egypt, although the reasons for this are not wholly clear (Kamel *et al.*, 1992; Darwish *et al.*, 1993). These studies were performed in the main on blood donors, before the implementation of mass screening, and exclusion of HCV positive donors, altered the prevalences in this group, and so give some indication of prevalence in the general population. However, most studies have used ELISA assays to assess the prevalence of HCV. Even second generation ELISA are known to have a predictive value of only 50% when used to screen low risk populations such as blood donors, so all findings must be interpreted in the light of this information (Mansell and Locarnini, 1995).

It has now been conclusively shown that HCV is the major cause of post transfusion hepatitis (Alter *et al.*, 1989a; van der Poel *et al.*, 1989; Wang *et al.*, 1990). Likewise, prevalence of HCV is very high in patients receiving blood products. Around the world, between 50 and 90% of haemophiliacs are anti-HCV positive (Rollag *et al.*, 1990; Hatzakis *et al.*, 1992); this rate increases to >90% in haemophiliacs who have received large amounts of non-treated blood products

(Alter, 1995). The prevalence of HCV infection is high amongst thalassaemic patients, but varies according to geographic region (Wonke *et al.*, 1990; Lai *et al.*, 1993). Seroconversion has also been reported in patients with hypogammaglobulinaemia treated with intravenous immunoglobulin (Taliani *et al.*, 1995); indeed two patients with common variable immune deficiency have been transplanted for end stage HCV cirrhosis at this centre (Smith *et al.*, 1995).

Also at high risk are intravenous drug users (IVDU). Studies carried out across the world have found anti-HCV antibodies in around 70% of IVDU (van den Hoek *et al.*, 1990; Zeldis *et al.*, 1992; Esteban *et al.*, 1989), rising to >90% in long term (>10 years) abusers (Galeazzi *et al.*, 1995; Alter, 1995).

Haemodialysis patients are at an increased risk for HCV infection. Prevalence of anti-HCV in this patient group averages 20%, but with a wide geographic variation; lowest rates are reported in the UK and Scandinavia (Brind *et al.*, 1990; Nordenfelt *et al.*, 1993); the highest rates (up to 42%) have been reported from Eastern Europe (Mihaljevic *et al.*, 1992; Hruby *et al.*, 1993). This risk is linked to increasing years on dialysis, and is often independent of blood transfusion; this probably represents nosocomial transmission (Almroth *et al.*, 1991).

Health care workers are also a relatively high risk group, with rates of 1% reported among hospital based health care workers (Alter, 1995). Dentists are at a particular risk; in a study on dentists in New York, 2% were found to be anti-HCV positive; those practising oral surgery were at even higher risk (9% positive)(Klein *et al.*, 1991).

Transmission of HCV via blood and blood products is the most efficient

route of infection. Seroconversion occurs in close to 100% of people transfused with a single unit of HCV-PCR positive blood (Mansell and Locarnini, 1995); the high rate of anti-HCV in patients dependent on blood products demonstrates this efficiency. HCV can be transmitted by organ transplantation (Pereira *et al.*, 1991). The natural history of the disease after transplantation is not clear, either in patients who acquire their HCV at time of transplant, or in those transplanted for end stage HCV liver disease. This is discussed in more detail in chapter 5. Other forms of parenteral contact such as intravenous drug use, haemodialysis and occupational needle stick (Serfaty *et al.*, 1995; Sodeyama *et al.*, 1993) are associated with transmission of HCV. Infection has been linked to less obvious forms of parenteral exposure such as tattooing (Kaldor *et al.*, 1992) and acupuncture (Shimoyama *et al.*, 1993). It has also been suggested that certain cultural traditions such as circumcision, scarification and cutting of the umbilical cord by local midwives could play a role in inapparent parenteral transmission in some populations (Rassam and Dusheiko, 1991). In the majority of cases of HCV, however, no obvious parenteral route has been identified (Alter, 1993).

Studies in the USA on acute HCV have revealed that in recent years (1990-1993) transfusion has accounted for only 4% of acute HCV (see figure 1.4). 38% of cases were related to intravenous drug abuse; 2% were associated with health care workers, and 1% were dialysis-associated. 10% of patients had a sexual or household contact with HCV; the remaining 45% of acute hepatitis C occurred in patients with no known risk factor, although acquisition was associated with a low socioeconomic level (Alter, 1995).

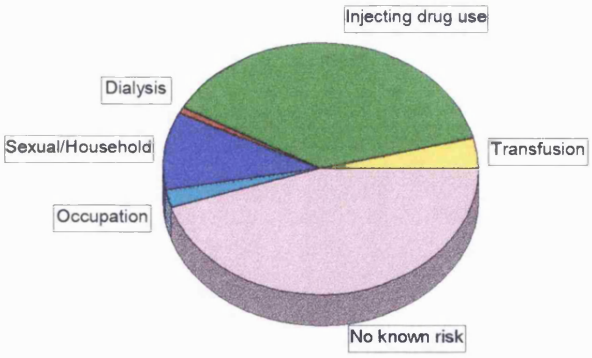


Figure 1.4 Risk factors for acute HCV in the United States (1990-1993). Alter, 1995.

There is some controversy over whether HCV can be sexually transmitted or not. The epidemiological evidence is contradictory. Bresters *et al* (1993) found no evidence of HCV infection in long term partners of 50 HCV-RNA positive haemophiliacs using both ELISA and PCR assays. Other workers have found no increased risk of HCV in prostitutes and STD patients (Hyams *et al.*, 1993), particularly once IVDU's are excluded (Stary *et al.*, 1992). This is in direct contrast with the findings of Garcia-Bengoechea *et al* (1994), who found a small but significant increased incidence of HCV infection in long term partners of HCV index cases; other studies found an increased incidence in both prostitutes and people attending STD clinics, who denied IVDU (Lissen *et al.*, 1993; Nakashima *et al.*, 1992)

Some of strongest epidemiological evidence of sexual transmisson comes from

work done on NANBH. A case controlled study found that patients with no history of parenteral exposures were significantly more likely than control patients without liver disease to have a history of exposure to a sexual or household contact who had had hepatitis (Alter *et al.*, 1989). It also found a significant association between acquiring disease and a history of multiple heterosexual partners (Alter *et al.*, 1989). Likewise, a recent study of HIV infected homosexuals showed a high rate of HCV co-infection; IVDU and blood transfusion was excluded in the majority of these patients, suggesting homosexual transmission of HCV (Wright *et al*, 1994). A concensus is emerging, therefore; that HCV can be transmitted by sexual contact, but at a low frequency. Further evidence to fuel this discussion is presented in chapter 6.

There is a similar discussion over whether HCV can be vertically transmitted, with some studies reporting no transmission of HCV from mothers to babies (Reinus *et al.*, 1992; Roudot-Thoraval *et al.*, 1993), and others reporting rare cases, even among HIV positive women (Manzini *et al,* 1995). Again, it is generally accepted that HCV can be transmitted vertically, although at low frequency. The risk of transmission seems to be related to titre of virus in the mother (Ohto *et al.*, 1994).

Three cases of HCV transmitted by a human bite have been reported, in which it was speculated that the virus was transmitted by saliva (Dusheiko *et al.*, 1990; Hollinger and Lin, 1992; Figueiredo *et al.*, 1994); transmission via inoculation of saliva has been demonstrated in chimpanzees (Abe *et al*, 1987). Reports such as these, and those on supposed sexual and vertical transmission of HCV have led workers to investigate if HCV-RNA can be detected in body fluids

other than blood, such as saliva, semen, urine, breast milk and urine. These studies have also produced contradictory results; this is discussed in more detail in chapter 6.

Altogether, these studies highlight one of the major problems in prevention of HCV infection. Whilst implementation of screening programmes for blood donations has reduced the incidence of post-transfusion hepatitis C (Alter, 1995; Alter, 1994; Gonzalez *et al.*, 1995), the majority of infections are not now acquired via this route. Needle exchange and health education programmes may help reduce spread amongst intravenous drug abusers, but until the non-parenteral routes by which HCV infection is spread become more clear, prevention of that spread will be difficult.

Natural history/clinical course of HCV infection.

The clinical course of HCV has now been well documented, although most is known about post transfusion HCV. Subclinical infection is the rule, with only 10% of patients reporting an acute illness with jaundice (Booth *et al.*, 1995). HCV does not seem to cause fulminant hepatitis in the west, although this has been reported in Japan (Ohnishi *et al.*, 1994). A high proportion of patients go on to develop chronic liver disease, with associated risks of cirrhosis and hepatocellular carcinoma (HCC).

Serum transaminase activities fluctuate during the course of chronic infection (Underwood, 1990), but generally decline slowly from a peak shortly after infection, although they may be transiently normal in as many as 50% of patients (Di Bisceglie *et al.*, 1991; Booth *et al.*, 1995). There is no correlation between ALT and levels of viraemia (Martinot-Peignoux *et al.*, 1994); transaminase levels do not correlate with the development of cirrhosis (Di Bisceglie *et al.*, 1991), or

with histological activity (Patel *et al.*, 1991). The reported incidence of chronic liver disease following HCV infection varies from 40-80% of patients (Bonino *et al.*, 1993; Tremolada *et al.*, 1992). Up to 30% of these can develop cirrhosis (Tremolada *et al.*, 1992), although this proportion may be higher given sufficient follow up. Patients appear to have a long, often symptomless course before presentation, and may present with chronic hepatitis or cirrhosis whilst still relatively symptom free (Takahashi *et al.*, 1993; Patel *et al.*, 1991). However, a proportion of patients do go on to end stage liver disease, and even hepatocellular carcinoma (Di Bisceglie *et al.*, 1991; Gerber, 1993). These patients can only be offered liver transplantation. It is known that HBV can recur post transplant, infecting the graft. HCV in the post transplant situation is discussed in chapter 5.

The relation between HCV and HCC is also complicated. As HCV is an RNA virus without a reverse transcriptase, it is unable to integrate into host DNA. Theoretically, this excludes carcinogenesis by insertion of HCV specific nucleic acid sequences into the genome of hepatocytes (as has been demonstrated with HBV (Lau and Wright, 1993)). Until recently, it was thought that HCV caused hepatocellular carcinoma via chronic necroinflammatory disease with hepatic regeneration and nodule formation; ie development of cirrhosis. However, recent studies by ourselves (El-Refaie *et al.*, 1995) and others (Demitri *et al.*, 1995) have shown that HCC can arise in the non-cirrhotic livers of patients whose only known risk factor is HCV infection, and in whom cryptic HBV infection has been excluded by PCR. These findings suggest that HCV may be oncogenic, and that a more specific mechanism of malignant transformation of hepatocytes is involved than hepatocellular necrosis, regeneration and fibrosis (Gerber, 1993). This view is supported by a recent study

by Ryder *et al* (1995), who investigated the role of HCV in HCC complicating autoimmune hepatitis (AIH). HCC in AIH is generally considered a rare phenomenon, despite the high incidence of cirrhosis in these patients. These investigators found that of eight patients with AIH and HCC, six had HCV-RNA detectable in liver tissue, suggesting a direct oncogenic effect of HCV (Ryder *et al.*, 1995). These findings stress how little is known of the pathogenesis of HCV infection.

Histopathology of HCV

The advent of HCV testing allowed histological study of patients with known hepatitis C infection. These studies in the main supported the earlier observations made on NANBH (see page 23; Goodman and Ishak, 1995); and confirmed that there are characteristic, if not pathognomic, features of HCV infection in the liver. The most notable features of HCV infection appear to be mild portal inflammation, often with lymphoid aggregates in portal tracts; mild interface hepatitis; parenchymal steatosis, bile duct damage, apoptosis and lobular inflammation. Kupffer cell activation, Mallory body-like material and endotheliitis have also been seen, and occasionally, epithelioid granulomas (Dhillon and Dusheiko, 1995; Goodman and Ishak, 1995; Scheuer *et al.*, 1992; Bach *et al.*, 1992; Gerber *et al.*, 1992; Lefkowitch *et al.*, 1993). These features are characteristic of both parenteral and community acquired hepatitis C (Gerber *et al.*, 1992). As noted in earlier studies, it can be difficult to differentiate acute from chronic HCV on histology alone, although chronic disease is more likely if there are portal follicles and interface hepatitis.

Whilst there is some disagreement over the frequency of these different

features in hepatitis C, they do help to differentiate hepatitis C from other causes of chronic hepatitis such as hepatitis B and autoimmune disease. Of particular importance in this respect are lymphoid aggregates, steatosis and bile duct damage (see table 1.2); although, as said before, these features are only characteristic, and not pathognomic, of HCV infection.

The pathogenesis of liver damage in HCV infection is not wholly understood. It is not known if HCV has a direct cytopathic effect, as with hepatitis A, or if liver damage is mediated by via host response to infection, as in HBV infection. There are also some indications that HCV can trigger autoimmunity in patients.

Table 1.2 Comparative histopathology in chronic hepatitis (Goodman and Ishak, 1995)

	Hepatitis C	Hepatitis B	Autoimmune hepatitis
Lymphoid aggregates	45-78%	18-52%	10-42%
Steatosis	31-72%	27-51%	16-19%
Bile duct damage	22-91%	10-55%	10-60%

Some of the early observations on the histology of NANBH suggested that HCV could have a direct cytopathic effect. Workers noted that acidophil bodies were often seen in the liver parenchyma without surrounding inflammatory cells (Bianchi *et al.*, 1987; Dienes *et al.*, 1982; Desmet, 1991); this has also been seen in confirmed HCV infection (Gerber, 1995). HCV is a member of the flaviviridae; other members of this family, in particular yellow fever virus, are known to have a direct cytopathic effect, characterised by hepatocyte apoptosis (Miller and Purcell,

1990; Feinstone, 1991a). However, histologically, HCV infection shares many features with hepatitis B infection, including chronic inflammation and fibrosis of portal tracts, which could suggest a similar type of pathogenesis (Gerber, 1995).

Over half of liver samples with chronic HCV have lymphoid aggregates, often in association with damaged bile ducts. Studies on the nature of these intraportal aggregates/follicles have shown that they consist mainly of T cells, with B cells only seen in follicles with germinal centres (Marrogi *et al.*, 1995); these T cells have a similar distribution of subpopulations (T4:T8 ratio) as is seen in autoimmune hepatitis (Hino *et al.*, 1992; Lee *et al.*, 1994). HCV has also been associated with an autoantibody against a host cellular sequence named GOR. Patients infected with HCV have a high incidence of anti-GOR, (even when negative for anti-HCV), and anti-GOR is not associated with chronic liver disease due to alcohol or hepatitis B infection (Mishiro *et al.*, 1990). Anti-GOR crossreacts with both the host GOR peptide and a viral epitope within the HCV core region. This suggests that anti-GOR is induced by HCV infection (Mishiro *et al.*, 1991). Positivity for anti-GOR does not seem to have prognostic or clinical significance (Lau *et al.*, 1993), but demonstrates the autoimmune-like aspect of HCV pathogenesis (Mishiro *et al.*, 1991). The possible links between HCV infection and autoimmune liver disease are discussed in more detail in chapter 4.

Other evidence in support of the immune hypothesis of liver injury includes the observation that HCV infection is associated with a number of systemic diseases also thought to have an autoimmune diathesis. Not long after the identification of HCV, it was noted that many patients with Sjögrens syndrome, an autoimmune disease affecting the salivary glands, were anti-HCV positive. Whilst there is now

some dispute as to whether patients with primary Sjögrens syndrome have a high incidence of HCV infection (Marrone *et al.*, 1995), prospective studies by Pawlotsky and colleagues have shown that around 50% of patients with chronic HCV have salivary gland lesions resembling those of Sjögrens syndrome (Pawlotsky *et al.*, 1994; Chayama *et al.*, 1994). In a similar way, a link was noted between HCV infection and Hashimoto's thyroiditis (Tran *et al.*, 1992), which lead to the realisation that patients with HCV have a high incidence of anti-thyroid antibodies, some to a pathogenetic degree (Tran *et al.*, 1993), and likewise, that patients with anti-thyroid antibodies have an increased incidence of anti-HCV (Quaranta *et al.*, 1993). HCV infection has also been linked to cryoglobulinaemia (Levey *et al.*, 1994), vasculitis (Shakil and Di Bisceglie, 1994) and glomerulonephritis (Romas *et al.*, 1994), where disease is thought to be mediated via immune complexes (Pawlotsky *et al.*, 1994); to lichen planus and porphyria cutanea tarda, (Pereyo *et al.*, 1995); and to an increased incidence of rheumatoid factor, where a triggering of autoantibody production by the virus could be the route of pathogenesis (Pawlotsky *et al.*, 1994). The possible different modes of pathogenesis of HCV in relationship to these disorders is reflected in the response to interferon therapy; cryoglobulinaemia and glomerulonephritis tend to improve during therapy (Schirren *et al.*, 1995; Johnson *et al.*, 1994), whilst patients may develop anti-thyroid antibodies and thyroid dysfunction during interferon treatment for HCV (Pateron *et al.*, 1992). These aspects of HCV infection are discussed further in chapters 4 and 6.

Conclusions

These considerations raise many questions about how HCV causes liver disease. The aim of this thesis is to explore the pathogenesis of HCV infection; in particular, by looking at the following aspects;

a) The relationship of known histological features of HCV infection to HCV infected cells (see chapter 3).

b) To substantiate any link between autoimmune hepatitis and HCV infection (see chapter 4).

c) To investigate the course of HCV infection after liver transplantation (see chapter 5).

d) To relate extrahepatic sites of HCV infection to possible mechanisms/explore possible inapparent means of viral transmission (chapter 6).

e) To relate the extrahepatic manifestations of HCV disease to presence of the virus (chapter 6).

CHAPTER 2

MATERIALS AND METHODS

Introduction

The principal technique used in this thesis to detect HCV-RNA was reverse transcription polymerase chain reaction (RT-PCR). To detect HCV viraemia, RT-PCR has become the method of choice. However, although detection of HCV-RNA in serum is a useful technique, it does little to further the understanding of the effects of HCV on the liver. It is not yet clear how the presence and level of HCV-RNA in serum relates to its presence in liver, and to any liver damage, although it has been shown that HCV-RNA is present in higher levels in liver tissue than in serum (Yatsuhashi *et al.*, 1995), and that the virus can be detected within the liver when the serum is negative (Lau *et al.*, 1993b). Therefore, direct detection of HCV-RNA within liver tissue is of value. For the purposes of these investigations, RT-PCR was used to detect HCV-RNA in formalin-fixed, paraffin embedded (FFPE) archival material.

The technique used was based on those of Jackson *et al* (1990) and Sallie *et al* (1992). Briefly, total nucleic acid is extracted from tissue blocks, reverse transcribed into cDNA, and amplified using suitable primers. Amplified samples are run on an agarose gel and those containing DNA products of the correct size for the primers used are considered positive. The technique used is described in more detail below.

The primer sequences used in this work were those described by Brown *et al* (1992a) (see table 2.1), and are based on those of Han *et al* (1991). These primers are from the highly conserved 5' untranslated region (UTR) of the virus, which shows little sequence variation between genotypes (Ogata *et al.*, 1991). They have been used routinely at the Royal Free Hospital (RFH) for detection of HCV-RNA in

serum, and have been found to be sensitive and specific (Brown *et al.*, 1992a).

The preliminary experiments described in this chapter established the sensitivity and specificity of these primers and of RT-PCR for detection of HCV-RNA when used on formalin fixed, paraffin embedded material.

Materials

All the paraffin embedded samples used for this work were routine, archival material, obtained for diagnostic purposes. Samples were only used for tissue PCR once all routine requirements had been satisfied. The majority of these samples were FFPE liver biopsies. These are normally fixed for between 2 and 20 hours in 10% neutral buffered formalin (Infrakem Ltd) and processed on the day of receipt to the department. RT-PCR was also performed on post mortem material (see chapter 6).

All samples at RFH are batch processed using a Tissuetek automatic processor (VIP) on a standard overnight programme (see appendix 2.1).

All non-sterile plasticware used for PCR was sterilised before use by baking at 120^0 C for at least 4 hours. Sterile filter tips (Alpha Laboratories) were used for all PCR processes. All chemicals used were of molecular biology/Analar grade.

Methods

Experiment 1

To validate the method, RT-PCR was performed on formalin fixed, paraffin embedded liver tissue from two patients who were positive for HCV-RNA in serum and frozen liver tissue, as established by routine testing by the Department of Medicine. A frozen block from the explant liver of each of these patients was fixed in formalin and processed to paraffin wax. The parallel archival paraffin blocks

from the histology department were tested, to see if HCV-RNA was detectable in routinely processed and stored blocks.

Nucleic acid extraction

A microtome (Bright Ltd) was used to cut sections from each block; a fresh piece of microtome blade (R.A. Lamb) was used for each sample. In parallel, 1 and 10 sections, each 3μm, were cut from each block, and placed into separate, sterile microcentrifuge tubes (Alpha Laboratories). 200μl of digestion buffer was added to each tube, and the contents thoroughly mixed on a whirlimix (Fisons). The digestion buffer (pH 8.4) contains 100mM sodium chloride (NaCl; BDH), 10mM Tris-Cl (BDH), 2.5mM EDTA (Sigma), and 0.5% sodium dodecyl sulphate (SDS; Sigma), to which 0.2mg/ml Proteinase K (Sigma) was added just before use, from a stock solution of 20mg/ml in water (Jackson *et al.*, 1990; Sallie *et al.*, 1992). Samples were incubated for 96 hours at 37^{0}C, then proteins and other contaminating material were removed from the digest by phenol/chloroform extraction. 200μl of phenol/chloroform/isoamyl alcohol (25:24:1; Sigma) was added to each tube and mixed. Tubes were spun for 5 minutes at 12,000g in a microcentrifuge (MSE), then the aqueous supernatant was removed from the phenol layer and put into a fresh, sterile microcentrifuge tube. 200μl of chloroform/isoamyl alcohol (24:1; BDH/Sigma) was added to the supernatant, mixed, and the tubes spun for 5 minutes. A further extraction with chloroform was performed, then 400μl of ice cold ethanol (BDH) added to the supernatant, along with 60μl of 3M sodium acetate (NaAc, pH 5.2, final concentration 0.3M; Sigma). Samples were incubated at -70^{0}C for at least 2h, or overnight, then spun at 12,000g for 30 min. Ethanol was poured off the resulting nucleic acid pellets, which were dried *in vacuuo* for 15 mins then

resuspended overnight, each in 10μl of sterile water containing 25U ribonuclease inhibitor (Amersham International).

Reverse transcription

Extracted RNA from a known HCV-RNA positive serum sample was included for reverse transcription as a positive control; RNA was extracted from 100μl of serum using the method of Garson *et al* (1990a). Water was included as a negative control. Before reverse transcription, samples were boiled for 5 mins to reduce the secondary structure of the 5' region against which the PCR primers are directed (Brown *et al.*, 1992a). cDNA synthesis was performed on resuspended samples in the presence of 0.5mM of each deoxynucleotide (dNTP; Pharmacia), 5μl of reverse transcription buffer (final concentration 50mM Tris-HCl (pH 8.3), 75mM KCl, 3mM $MgCl_2$; Gibco BRL), 10mM dithiothreitol (DTT; Gibco BRL), 25pmol random primers (Pharmacia) and 250U of M-MLV reverse transcriptase (Gibco BRL) in a total volume of 25μl. Samples were incubated at 37^{0}C for 2 hours, then boiled for 5 mins to inactivate reverse transcriptase. Use of random primers means that total RNA within the sample is reverse transcribed into cDNA.

Polymerase chain reaction

Samples were subjected to nested PCR for HCV according to the method described by Brown *et al* (1992a). For first round amplification, 5μl of cDNA from each sample was amplified in the presence of 0.2mM each dNTP, 10μl of 10x PCR buffer (final concentration 20mM Tris-HCl (pH 8.4), 50mM KCl; Gibco BRL), 1.5mM $MgCl_2$ (Gibco BRL) 2.5U of Taq DNA polymerase (Gibco BRL), and 50pmol of each first round primer (see table 2.1), in a total volume of 100μl. The reaction mix is overlaid with sterile mineral oil (Sigma), to prevent evaporation

during PCR. Samples were amplified on a PHC-3 programmable thermal cycler (Techne), according to the programme described by Brown *et al* (1992a) (see fig 2.1). The product produced by first round amplification is 245bp long.

1)	94°C	2 mins	}
2)	50°C	2 mins	} 5 cycles
3)	72°C	3 mins	}
then			
4)	72°C	5 mins	
then			
1)	94°C	1.5 mins	}
2)	60°C	1.5 mins	} 30 cycles
3)	72°C	2 mins	}
finally			
4)	72°C	5 mins	

Figure 2.1 1st round amplification programme. Where 1) = denaturation step, 2) = annealing temp., 3) = primer extension, 4) = final extension

Two microlitres of the first round product were then used in the second round reaction mixture, containing 0.2mM of each dNTP, 5μl of 10x PCR buffer (final concentration 20mM Tris-HCl (pH 8.4), 50mM KCl; Gibco BRL), 1.5mM $MgCl_2$ (Gibco BRL), 2.5U of Taq DNA polymerase (Gibco BRL), and 50 pmole each of the second round primers (see table 2.1), in a total volume of 50μl. Samples were overlaid with sterile mineral oil (Sigma), then amplified using the second round amplification programme (see figure 2.2).

1)	94°C	1 min 20 secs	}
2)	40°C	1 min	} 30 cycles
3)	72°C	1 min	}
then			
4)	72°C	5 mins	

Figure 2.2 2nd round amplification programme. Where 1) = denaturation step, 2) = annealing temp., 3) = primer extension, 4) = final extension

Table 2.1 Sequence of first and second round HCV RT-PCR primers. Nucleotide numbering according to Han *et al*, (1991).

	Primer	Sequences	Position of primers within 5' UTR (nt)	Product size
first round primers	i) 93	sense 5'-TTC GCG GCC GCA CTC CAC CAT GAA TCA CTC CCC-3'	nt 23-45	245bp
	ii) 51	antisense 5'-CCC AAC ACT ACT CGG CTA-3'	nt 251-268	
second round primers	iii) HCVS2	sense 5'-GCG GCC GCA CTG TCT TCA CGC AGA AAG-3'	nt 58-75	200bp
	iv) 52	antisense 5'-AGT CTT GCG GCC GCA GCG CCA AAT C-3'	nt 224-248	

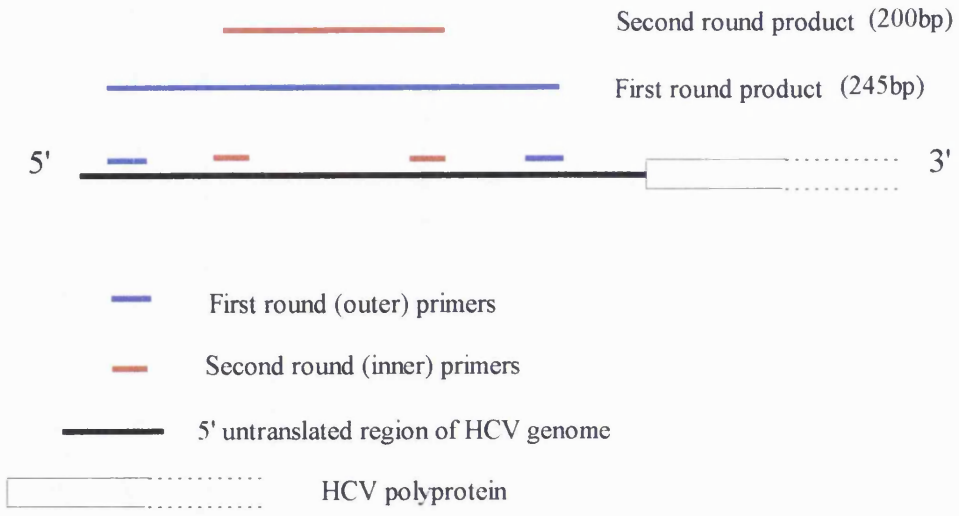


Figure 2.3 Nested PCR for HCV. 2nd round primers amplify a sequence contained within the first round product

Primers amplify a sequence contained within the product of the first round of PCR (see figure 2.3), and produce a 200 base pair (bp) product from HCV-RNA positive samples. Further technical details are given in appendix 2.2. 5μl of second round PCR product were analysed by electrophoresis on a 2% agarose (Sigma) gel, containing 0.2μg/ml ethidium bromide (Sigma), run in TBE buffer (see appendix 2.3). Gels were viewed using a UV transilluminator (UVP), and photographed using a Polaroid land camera with Polaroid film.

Results and conclusions.

All samples showed a band of amplified DNA at 200bp; all negative controls were negative (see figure 2.4). Interestingly, in lane 5, representing tissue extracted from 10 sections from positive case 1, there is only a faint band at 200bp. It has been shown that tissues contain endogenous inhibitors of PCR (An and Fleming, 1991); whilst proteinase K digestion and phenol/chloroform extraction reduce the amount of these inhibitors, this result could demonstrate that with larger amounts of tissue, inhibition of PCR by endogenous substances could be a problem (An and Fleming, 1991).

This experiment demonstrated that HCV-RNA could be successfully extracted and amplified from paraffin embedded liver samples using primers to the 5' end of the virus; that standard processing and storage of archival histopathology blocks does not seem to adversely affect recovery of amplifiable HCV-RNA; and that at least from larger blocks of resected material, one 3μm section contains sufficient material for successful HCV amplification.

Experiments 2 and 3.

To confirm that the amplification was specific for HCV-RNA, two

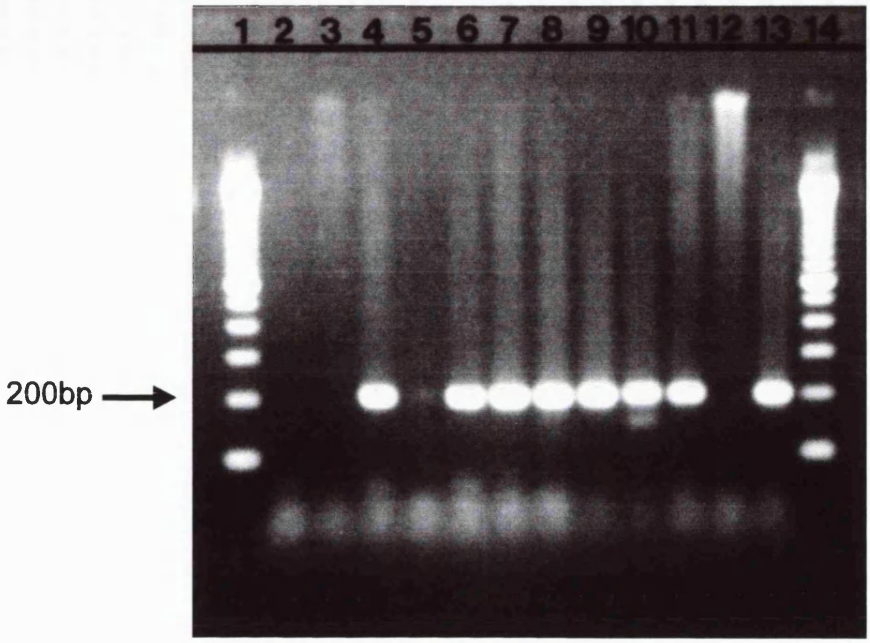


Figure 2.4 Agarose gel showing ethidium bromide labelled products of PCR on formalin fixed liver tissue extracts. HCV products are at the 200bp position.

Lane 1	100 base pair ladder (Gibco/BRL)
Lane 2	Negative control (reverse transcription)
Lane 3	Negative control (1st round PCR)
Lane 4	1 section of case 1a
Lane 5	10 sections of case 1a
Lane 6	1 section of case 2a
Lane 7	10 sections of case 2a
Lane 8	1 section of case 1b
Lane 9	10 sections of case 1b
Lane 10	1 section of case 2b
Lane 11	Positive control (RNA from positive serum sample)
Lane 12	Negative control (2nd round PCR)
Lane 13	10 sections of case 2b
Lane 14	100 base pair ladder

Where 1a =	Routinely stored, FFPE sample from case 1
2a =	Routinely stored, FFPE sample from case 2
1b =	Fresh frozen, FFPE sample from case 1
2b =	Fresh frozen, FFPE sample from case 2

experiments were performed. In the first, total nucleic acid was extracted, reverse transcribed and amplified, using the described method, from the following samples, which should be negative for HCV-RNA:

a) Normal rat liver (x1)

b) Human liver biopsies with 'normal' histology (x2)

c) Human liver biopsies from patients with Gilbert's disease. (x2)

A known positive tissue block was included as positive control.

To confirm that bands seen at 200bp represented amplified HCV-RNA, and not just primers or another component of infected liver, a southern blot and hybridisation was performed on a selection of positive samples. Samples were run on an agarose gel; the gel was blotted, to attach sample DNA to a nitrocellulose filter (Sigma) and the filter hybridised overnight with a ^{32}P-labelled oligonucleotide probe, ALX89 (see appendices 2.4 and 2.5). The probe is complementary to an internal part of the HCV-cDNA sequence amplified by these primers (see figure 2.5), ensuring that the probe will only bind to amplified HCV cDNA sequences, and not simply to the primer sequences.

Results and conclusions.

PCR of negative tissue samples showed no band at 200bp (see figure 2.6). This indicated that the PCR product was not just a normal component of liver.

Autoradiography of the southern blot showed that the probe hybridised to the 200bp product in the positive samples, and not to the negative samples, so confirming that this product is amplified from the hepatitis C virus (see figures 2.7 and 2.8).

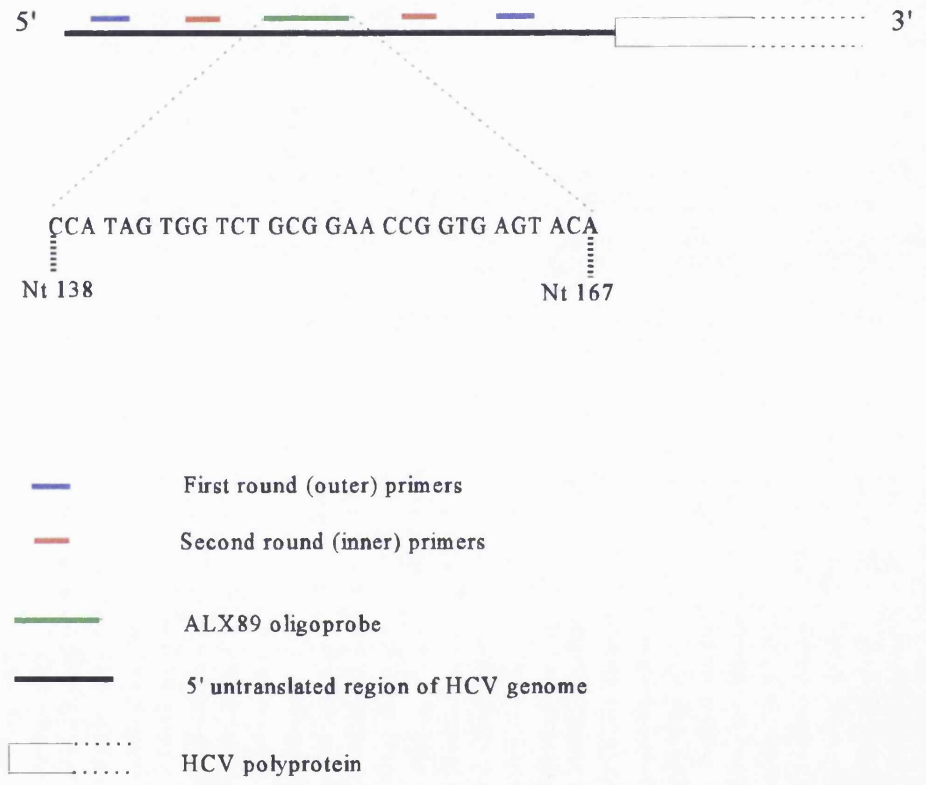


Figure 2.5 Sequence and position of ALX89 oligoprobe and its relation to first and second round HCV primers in the 5' untranslated region of the HCV genome.

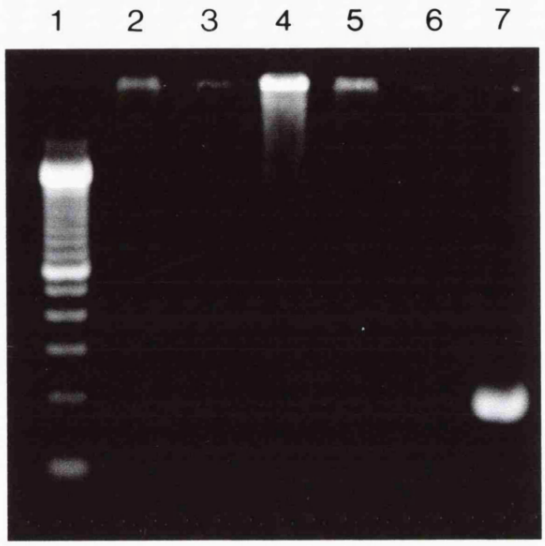


Figure 2.6 Ethidium bromide stained agarose gel of HCV RT-PCR products

Lane 1 100bp ladder
Lane 2 Rat liver (showing no PCR product)
Lane 3 Normal liver biopsy "
Lane 4 Normal liver biopsy "
Lane 5 Biopsy from patient with Gilberts disease "
Lane 6 Biopsy from patient with Gilberts disease "
Lane 7 HCV-RNA positive biopsy (showing PCR product band at 200bp)

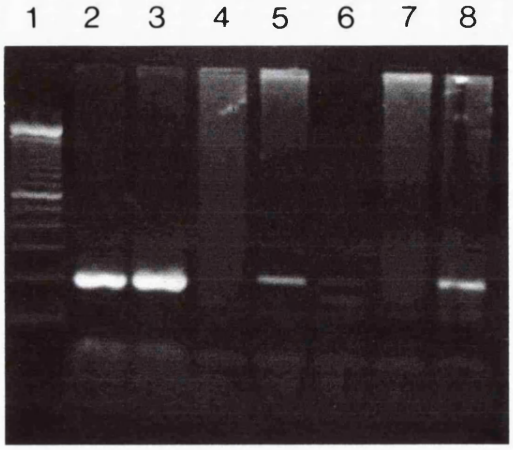


Figure 2.7 Ethidium bromide stained agarose gel of PCR

Lane 1 100bp ladder
Lane 2 HCV-RNA positive tissue, showing band at 200bp
Lane 3 HCV-RNA positive tissue
Lane 4 HCV-RNA negative tissue
Lane 5 HCV-RNA positive tissue
Lane 6 HCV-RNA positive tissue (faint)
Lane 7 Negative control
Lane 8 Positive control (HCV-RNA positive serum sample)

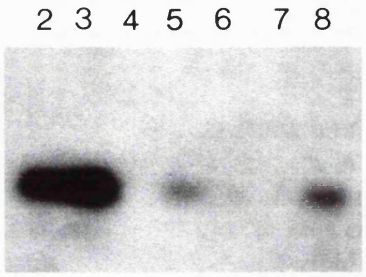


Figure 2.8 Autoradiograph of corresponding Southern blot products hybridised with ALX89

Showing binding of probe to HCV-RNA positive samples in lanes 2, 3, 5, 6 and 8, but no binding to negative tissue sample in lane 4, or to the negative control in lane 7.

Experiment 4

There has been some debate in the literature as to whether deparaffinisation of sections is necessary or not for PCR (Sallie *et al.*, 1992; Jackson *et al.*, 1990). In addition, the length of digestion reported has varied. This experiment was performed to assess the effect of a shorter digestion time and of deparaffinisation of sections on the sensitivity of PCR for HCV. As a positive control for extraction and reverse transcription, PCR for albumin mRNA, a gene constitutively expressed in liver, was performed on all liver samples. The same conditions and amplification programme were used as for first round HCV amplification; sequences of the primers used are shown in table 2.2. These primers span an intron within the albumin gene, allowing differentiation of RNA/DNA amplification (Sallie *et al.*, 1992).

Table 2.2 Sequence of albumin primers. (Sallie *et al.*, 1992)

	Primer sequences	Product size
i) sense	5'-TGA AAT GGC TGA CTG CTG TG-3'	270bp
ii) antisense	5'-GCA GCT TTA TCA GCA GCT TG-3'	

Five 3μm sections from a HCV-RNA positive tissue block were put into each of four sterile eppendorf tubes. The sections in two of the tubes were dewaxed using xylene (Chemix) at 50^{0}C for 10mins. 50μl of DEPC-treated water was added, then samples were centrifuged for 5mins at 12,000g, and the xylene removed. The pellet of cell debris was washed in 70% ethanol (BDH), then resusupended in 200μl of digestion buffer containing proteinase K (0.2mg/ml). The other two tubes, containing non-dewaxed sections, were resuspended in digestion buffer as previously described. One tube of dewaxed sections, along with a non-dewaxed set, was digested for 60 hours. The other tubes of dewaxed/waxed sections were digested for

96 hours, as before. All samples were reverse transcribed together. Each reverse transcription was diluted 1:10 and 1:50 to allow semi-quantitative analysis of the amounts of cDNA in each sample (Simmonds *et al.*, 1990); all dilutions were amplified together. The samples were cut and tested in triplicate on different days: the results are shown in Table 2.3.

Results and conclusions.

Table 2.3 Results of waxed vs dewaxing and digestion time experiments.

		Waxed HCV	Waxed Albumin	Dewaxed HCV	Dewaxed Albumin
60 h digest	neat	+	+	+	+
	1:10	+	+	+	+/-
	1:50	+	+	-	-
96 h digest	neat	+	+/-	-	-
	1:10	+	-	-	-
	1:50	-	-	-	-

As shown by the table, dewaxing actually decreased the amount of HCV-RNA retrieved, and therefore the sensitivity of the technique. Likewise, 96h of digestion resulted in a lower titre of HCV and albumin on PCR than did 60h digestion. This protocol of not dewaxing the sections and digesting for 60h only was therefore adopted for all further experiments. The procedure is fully described in appendix 2.6.

Experiment 5

It has been shown that PCR for HCV on formalin fixed tissue is less sensitive than PCR on frozen material (Sallie *et al.*, 1992). However, the specificity and sensitivity of the technique on routine FFPE material has not been fully

established. In order to do this, RT-PCR was performed on diagnostic liver biopsies from 43 patients which had been routinely fixed, processed and stored. All biopsies were tested for HCV-RNA by tissue RT-PCR using the technique described. Samples were cut and tested on three separate occasions; the recommendations of Kwok and Higuchi (Kwok and Higuchi, 1989) for the control of contamination were strictly adhered to (see below). This work was done in collaboration with Dr Mohamed El Batanony (El-Batanony *et al.*, 1994).

Sera from the 43 patients taken at around the same time as the biopsy were tested for HCV-RNA by RT-PCR using the same primers as used on the tissue. RNA was extracted from sera using the method of Garson *et al* (1990a); all serological testing was performed by the Medical Unit, RFH.

Anti-contamination measures

PCR is a very powerful technique, allowing the detection of very small quantities of DNA, where only parts of the sequence are known. This strength of the technique is, however, also one of it s weaknesses. The sensitivity of the technique means that it is very easy to contaminate samples, reagents and even work areas with the amplified product being detected, so leading to false positive results. To minimise this problem, all experiments described in this thesis were carried out according to the procedures recommended by Kwok and Higuchi (1989) for minimising contamination. These precautions included the use of separate work areas for isolation of RNA, for preparation and storage of reagents, and for handling of PCR products and running of gels. Separate pipettes were used for each stage of the procedure, and filter tips were used to avoid contaminating pipettes with aerosols containing either HCV-RNA or PCR products. In addition, all

mixtures for reverse transcription and PCR are made up in a laminar air flow cabinet, and treated with UV light for 5 mins before addition of primers and enzymes. As already stated, samples were cut using a fresh piece of microtome blade for each sample, and gloves are worn (and changed frequently) to avoid contaminating samples with RNAase or cross-contaminating with HCV-RNA of PCR products.

Data analysis.

The data were analysed statistically using Fisher's exact test, suitable for the small numbers.

To evaluate the validity of tissue PCR, the serum-PCR results were considered as the reference. The test indices were calculated as follows:

$$\text{Sensitivity} = \frac{\text{true positives}}{\text{true positives} + \text{false negatives}} \times 100$$

$$\text{Specificity} = \frac{\text{true negatives}}{\text{true negative} + \text{false positives}} \times 100$$

$$\text{False positive rate} = \frac{\text{false positives}}{\text{false positives} + \text{true positives}} \times 100$$

$$\text{False negative rate} = \frac{\text{false negatives}}{\text{false negatives} + \text{true negatives}} \times 100$$

$$\text{Efficiency} = \frac{\text{true positives} + \text{true negatives}}{\text{true positives} + \text{true negatives} + \text{false positives} + \text{false negatives}} \times 100$$

Results and conclusions

The histological diagnosis of the forty-three cases are correlated with HCV viral markers in serum and liver tissue in table 2.4. Twenty nine cases had HCV-RNA in serum; 17 of these had HCV-RNA detectable in biopsies by tissue-PCR. Fourteen cases were negative in serum for HCV-RNA; none of these was positive by tissue-PCR. All cases were positive for albumin mRNA by PCR.

Table 2.4. Histological diagnosis and hepatitis C viral markers in serum and liver tissue of the studied cases.

Histological diagnosis	Total	HCV-RNA			
		SERUM		LIVER	
		+	-	+	-
Non-hepatitic	6	0	6	0	6
Cellular rejection	4	3	1	3	1
Acute hepatitis	3	1	2	0	3
Chronic hepatitis	18	14	4	7	11
Cirrhosis	12	11	1	7	5
Total	43	29	14	17	26

The effect of storage of liver biopsies on the success of HCV amplification was assessed by looking at the time interval between taking and testing liver biopsies, and its effect on tissue PCR results in 29 serum PCR positive patients (table 2.5). Tissue PCR was performed within 12 months of taking the biopsy in 11 cases; nine of these (81.1%) were tissue PCR positive. In 18 cases, tissue PCR was performed more than 12 months after taking the biopsy; 8 of these 18 cases (44.4%) were tissue PCR positive. This difference between the two groups was significant ($p = 0.046$).

The importance of biopsy length on tissue PCR results was also assessed (see

table 2.6). 11 biopsies from serum PCR positive patients were <0.5 cm long; 6 of these (54.5%) were tissue PCR positive. 18 biopsies were >0.5cm long; 11 of these (61%) were tissue PCR positive. This difference was not statistically significant (p = 0.28).

Table 2.5 Time interval between taking liver biopsy and testing by PCR and its effect on tissue PCR results in serum PCR positive patients.

Time between taking liver biopsy and testing by PCR	Total No (%)	Tissue PCR results		P
		Positive No (%)	Negative No (%)	
<12 months (mean 6.8 months)	11 (100%)	9 (81.8%)	2 (18.2%)	0.046
> 12 months (mean 45 months)	18 (100%)	8 (44.4%)	10 (55.6%)	

Table 2.6 Effect of the length of biopsy on tissue-PCR results in serum-PCR positive patients.

Size (cm)	Total	TISSUE PCR RESULTS		p
		POSITIVE	NEGATIVE	
<0.5	11	6	5	0.28
>0.5	18	11	7	

Table 2.7 Performance of HCV-PCR testing of routinely processed formalin-fixed diagnostic liver biopsies and the effect of biopsy storage.

	Time interval between liver biopsy and tissue-PCR		Total (n=43)
	<12 months (n=22)	>12 months (n=21)	
Sensitivity	81.8%	44.4%	58.6%
Specificity	100%	100%	100%
False positive rate	0%	0%	0%
False negative rate	15.4%	76.9%	46.2%
Efficiency	90.9%	52.4%	72%

Overall, when compared to serum PCR for HCV-RNA, tissue PCR was 58.6% sensitive, 100% specific and 72% efficient. The false positive rate was 0%, and the false negative rate was 46.2%. Tissue PCR sensitivity and efficiency was significantly higher and false negative rate lower in biopsies tested within 12 months of taking (81.8%, 90.0% and 15.4% respectively) than in those tested more than 12 months afterwards (44.4%, 52.4% and 76.9% respectively). See table 2.7.

These results show that HCV-RNA can be successfully detected in routinely processed and stored FFPE material. When carried out under the strict anti-contamination procedures described, the technique is highly specific and sensitive, although the sensitivity is reduced on material stored for longer than 12 months before testing. The size of a biopsy has no significant effect on the PCR result, confirming that PCR can successfully amplify HCV from small amounts of material (Garson *et al*, 1990a).

Conclusions

Overall, these experiments show several important things about PCR on FFPE tissue. They show that it is possible to successfully extract and amplify HCV-RNA from formalin fixed, paraffin embedded liver samples, with a high degree of specificity when stringent anti-contamination procedures are employed. Tissue PCR is very sensitive when applied to liver biopsies taken within 12 months of testing, although it is less sensitive when applied to specimens which have been stored for a long period (El-Batanony *et al*, 1994). Bearing these qualifications in mind, tissue PCR for detection of HCV-RNA provides a valuable adjunct to existing serological assays, indicates the presence of HCV in a given liver sample, and provides a measure against which other techniques, eg immunostaining, can be assessed.

CHAPTER 3

DETECTION OF HEPATITIS C VIRAL ANTIGEN WITHIN LIVER TISSUE

Introduction

Since the discovery of the hepatitis C virus, and the development of reliable screening and diagnostic techniques such as ELISA and RT-PCR, an important goal for many workers has been the development of an antibody that can detect HCV antigen within liver tissue sections. While RT-PCR and ELISA allow for sensitive detection of HCV infected patients, the relationship between antibodies in serum and damage to liver tissue remains uncertain, and detection of HCV-RNA does not provide information on viral expression, or its relation to tissue damage, inflammation and disease pathogenesis (Dhillon and Dusheiko, 1995).

Several histological features have been associated with liver biopsies from HCV-infected patients. These include the presence of lymphoid aggregates or follicles within portal tracts, steatosis, apoptosis and lobular inflammation and mild periportal interface hepatitis (Scheuer *et al.*, 1992; Dhillon and Dusheiko, 1995). Bile duct inflammation and damage have also been reported, but the relative frequency of these and the other features of hepatitis C have been the subject of some controversy (Bach *et al.*, 1992; Czaja and Carpenter, 1993; Goodman and Ishak, 1995). Indeed, all of these features, whilst characteristic of HCV infection, are not pathognomic.

Detection of HCV-antigen within tissue sections would allow investigators to see any correlation between HCV infected cells and these observed histological features. It would also aid assessment of viral load and expression within the liver of patients, and relation of this to disease progression as well as to efficacy of any treatment (Gonzalez-Peralta *et al.*, 1994).

The pathogenesis of HCV-induced liver disease is unclear. The virus persists

in over 60% infected patients (Alter *et al.*, 1992); this persistent infection is linked to chronic hepatitis which progresses to cirrhosis in a large number of these patients. It is not known if the virus is directly cytopathic, or if liver damage is mediated via the immune system (Marrogi *et al.*, 1995). This immune mediated damage could be caused by a direct response to viral infected cells, usually effected by cytotoxic T cells, or it could be that viral infection breaks immune tolerance and leads to an autoimmune reaction. Viral clearance by cytotoxic T cells can be associated with severe pathology; and viral infections have been thought to trigger autoimmunity, possibly through molecular mimicry, although no link has yet been proven in humans (Askonas, 1994). These scenarios are not mutually exclusive, and may differ in individual patients, influenced by factors such as host tissue type, mode of acquisition, viral load, viral genotype and stage of infection.

Immunocytochemistry (ICC) has been defined as 'the identification of a tissue constituent *in situ* by means of a specific antigen-antibody reaction tagged by a visible label' (Van Noorden and Polak, 1983). In practice this tissue constituent could be a tumour marker, a hormone, or, as in this case, a viral protein. The technique allows specific detection and visualisation of such proteins within tissue sections, by exploiting the ability of antibodies to specifically recognise and bind to the epitope against which they are targeted.

The first workers to successfully detect particular proteins within sections used antibodies directly conjugated with fluorescent markers (Coons *et al.*, 1941). As techniques developed and became more sophisticated, indirect methods were used in which a second antibody, raised against the immunoglobulin of the animal donating the first antibody was added - this second layer could be labelled or could

be used to link a third layer, conjugated to the label. This amplification of antibody binding increases the sensitivity of the technique (Sternberger, 1979). A more varied range of labels also became available, chiefly enzymes such as peroxidase and alkaline phosphatase, which could be used to produce a coloured product, visible under ordinary light microscopy (Van Noorden and Polak, 1983). Peroxidase and alkaline phosphatase are still the most commonly used reporter molecules today.

Development of a reliable method for immunocytochemical detection of HCV antigen within routine histological material would be a valuable adjunct to the diagnostic techniques currently available. RT-PCR in particular is a technically difficult and expensive technique, not routinely available outside specialist centres. Conversely, immunocytochemistry is relatively cheap, and can be performed in most hospitals.

One of the earliest reports of immunodetection of hepatitis C virus was that of Infantolino *et al*, (1990) who reported using human and rabbit polyclonal antibodies to detect HCV antigen within formalin fixed paraffin material. More recently, Krawczynski *et al.* (1992a) described a method using a human polyclonal antibody to detect HCV antigen within frozen material. The aim of these experiments was to determine the sensitivity and specificity of a panel of anti-HCV antibodies including these on material obtained at our institution; and to examine the relationship of any positive staining to stage of liver disease, to inflammation within the sample, and to histological features commonly found in chronic hepatitis C infection.

Materials and methods

Rabbit polyclonal and mouse monoclonal antibody testing

Two rabbit polyclonal and 24 mouse monoclonal antibodies were tested. Rabbit antibodies were raised against the synthetic peptide c100-3 of HCV. Three purified mouse monoclonals against c100-3 were also tested; the remaining 21 antibodies were hybridoma supernatants against the synthetic peptides c22-3 or c33c. The rabbit polyclonal antibodies were donated by Infantolino; all monoclonal antibodies were obtained from Ortho Diagnostics Inc.

All liver biopsies used for antibody testing were obtained by percutaneous needle biopsy as part of a routine diagnostic procedure. Anti-HCV status of the patients was determined using ELISA II (Ortho, Raritan, New Jersey) testing of serum samples.

i) Rabbit polyclonal antibodies

The rabbit polyclonal antibodies were tested on 59 formalin fixed, paraffin embedded (FFPE) liver biopsies. Eighteen of these biopsies were from anti-HCV negative patients (3 normal, 4 PBC, 2 drug related hepatitis, 3 Gilberts disease, 2 alpha-1-antitrypsin deficiency, 4 hepatitis B); 22 were from NANB hepatitis cases positive for anti-HCV; 10 were from NANBH cases negative for anti-HCV; 3 from anti-HCV positive alcoholics; and 6 from anti-HCV negative alcoholics.

Sections (3μm) were cut using a microtome (Anglia Scientific) and picked up onto slides coated with 0.1% poly-l-lysine (PLL - Sigma). They were air dried at 40^{0}C overnight, then stored at room temperature until stained. .

All antibodies were tested using an optimised ICC technique based on that of Infantolino *et al.* (1990). This is detailed in appendix 3.1, but briefly, endogenous

peroxidase activity was blocked with 0.5% H_2O_2 (BDH) in methanol (BDH); non-specific binding of antibody was blocked with 50% normal swine serum (Dako). Sections were incubated with optimally diluted primary antibody for 30 minutes, and any antibody binding detected using a double PAP technique, with diaminobenzidine (DAB; Sigma) as chromogen. Pretreatment with trypsin (0.1% in 0.1% $CaCl_2$, pH7.8) at 37^{0}C for varying times was performed to improve sensitivity and specificity. Liver tissue sections from anti-HCV positive patients were included as positive controls; negative controls included sections from liver samples of patients negative for hepatitis C; incubation with preimmune rabbit serum instead of primary antibody; and incubating in tris buffered saline (TBS) instead of primary antibody.

ii) Mouse monoclonal antibodies

Mouse monoclonal antibodies were screened using a composite block of formalin fixed, paraffin embedded biopsies. This block contained two biopsies from anti-HCV positive patients, and three from anti-HCV negative patients (1 PBC, 1 alcoholic and 1 hepatitis B positive biopsy.) Again, all antibodies were tested using an optimised ICC technique (see appendix 3.2). All antibodies were tested at dilutions ranging from neat to 1/50; incubations were carried out for 1 hour at room temperature in the first instance, then at 4^{0}C overnight. Positive and negative controls were as detailed above.

Human polyclonal antibody testing

A patient with a high titre of anti-HCV on ELISA II (Abbott Labs, Nth Chicago, IL), who was also positive on RIBA II (Chiron, Emeryville, Ca.) testing was selected and a blood donation taken, with informed consent. Total IgG was

removed from the serum and labelled with FITC by Dr K. Krawczynski of Centers for Disease Control (CDC), Atlanta, using ammonium sulphate precipitation to extract immunoglobulin, and a dialysis method for labelling the antibody with FITC (Krawczynski *et al.*, 1992a). The labelled antibody, at a protein concentration of 12.9 mg/ml, was diluted 1/10 in normal human serum, absorbed with mouse acetone liver powder (MLP; Sigma) at a concentration of 75mg/ml, and aliquots stored at -70^{0}C until use.

i) Staining of frozen material

The labelled antibody was used to stain 22 frozen liver tissue samples. Ten of these samples were from patients with chronic HCV infection, all of whom were seropositive for anti-HCV using a second generation ELISA (Ortho, Raritan, New Jersey). Nine were also seropositive for HCV-RNA by RT-PCR (Brown *et al.*, 1992a). Two samples were post transplant biopsies from patients transplanted for HCV cirrhosis, and were taken whilst patients were on immunosuppressive therapy. Both of these patients were anti-HCV positive and positive for HCV-RNA in serum. The other ten samples were from anti-HCV negative patients (see table 3.1 for summary of clinical data). Fourteen of the samples were obtained by percutaneous needle biopsy as part of a routine diagnostic procedure. Standard practice (with ethical committee approval) at the RFH is that given a sufficiently large routine diagnostic liver biopsy ($>$1.5cm), part of the biopsy is frozen. The remaining 8 samples were taken from resected liver at transplantation. A small portion of each sample was embedded in OCT (R.A. Lamb), then snap frozen in liquid nitrogen and stored at -70^{0}C; the remainder was fixed in 10% buffered formalin and processed for routine histology.

Table 3.1. Summary of clinical details of patients tested with anti-HCV^{FITC}

Patient	Histology	Clinical	α-HCV	Serum PCR
1	Mild CAH	Chronic HCV	Pos	Pos
2	Mild hepatitis	Chronic HCV	Pos	Pos
3	Cirrhosis	Chronic HCV	Pos	Pos
4	Mild CAH	Chronic HCV	Pos	Neg
5	Mild inflammation	Chronic HCV & B	Pos	Pos
6	Cirrhosis	Chronic HCV	Pos	Pos
7	Cirrhosis	Chronic HCV	Pos	Pos
8	Mod CAH	Chronic HCV	Pos	Pos
9	Mild inflammation	Chronic HCV	Pos	Pos
10	Cirrhosis	Chronic HCV	Pos	Pos
11	Mod CAH, recurrent HCV	Post transplant for HCV cirrhosis	Pos	Pos
12	Mild cellular rejection	Post transplant for HCV cirrhosis	Pos	Pos
13	Minimal inflammation	Hepatitis B positive	Neg	Neg
14	Steatohepatitis	Alcoholic	Neg	Neg
15	Steatohepatitis	Hepatitis B positive	Neg	Neg
16	PT- Mild rejection	Primary biliary cirrhosis	Neg	Neg
17	Cirrhosis	Wilson's disease	Neg	Neg
18	Normal	Unused lobe of donor liver	Neg	Neg
19	Cirrhosis-like	Budd-Chiari syndrome	Neg	Neg
20	Near normal	Unused lobe of donor liver	Neg	Neg
21	Cholestasis and duct proliferation	Primary sclerosing cholangitis	Neg	Neg
22	Normal liver	Unused lobe of donor liver	Neg	Neg

All FFPE biopsies were tested for HCV-RNA using tissue PCR, as described in chapter 2, and appendices 2.2 and 2.7, once all routine diagnostic requirements had been satisfied.

Sections (5μm) were cut from each frozen sample using a cryostat (Bright Ltd) and picked up onto slides coated with PLL. Sections were air dried for 20 minutes, fixed in chloroform for 5 minutes, then incubated with optimally diluted FITC-conjugated human anti-HCV antibody for forty minutes. An aliquot of the antibody was diluted 1/300 in normal human serum, spun at 12000g in a microcentrifuge (MSE) to separate the MLP; the supernatant was used for staining. After incubation, the slides were washed in phosphate buffered saline, pH 7.2 (PBS; Oxoid) for 3 x 7 minutes, and mounted in citifluor (UKC Chem. Lab.; pH 9). All experiments included negative and positive controls; negative controls included a known HCV negative case, and sections incubated in normal human serum instead of antibody; a known HCV-antigen positive case (tested by Dr Krawczynski) was used as a positive control. All sections were viewed in a blind fashion at high power (x63) using a direct view line scanning confocal laser imaging system (DVC 250; Biorad), and a Zeiss standard 14 epifluorescence microscope with an HBO 50W high pressure mercury source, and Zeiss filter set no. 9. Samples in which staining was observed were graded in a semi-quantitative manner for the area and intensity of that staining (see table 3.2)

Table 3.2. Semiquantitative assessment of staining

Area of staining		Intensity of staining	
+	≤ 5% of hepatocytes stained	+	Pale staining
++	5 - 50% of hepatocytes stained	++	Medium staining
+++	≥ 50% of hepatocytes stained	+++	Intense staining

Eleven of the 12 HCV positive cases were stained with TIA-1 antibody (Coulter Clone); there was not sufficient tissue remaining in the 12th biopsy to allow staining. Three HCV negative cases were also stained with the antibody. TIA-1 is a mouse monoclonal antibody against a cytoplasmic granule associated protein, which appears to distinguish cells possessing cytolytic potential (Anderson *et al.*, 1990). These are mainly cytotoxic T lymphocytes (CTL), although the protein is also expressed in natural killer cells. It is not expressed by B cells. Using this antibody, double labelling was performed on sections, to compare localisation of the anti-HCV^{FITC} to the presence of cytotoxic T cells. Anti-HCV staining was performed as previously described, then slides were incubated with TIA-1, and finally with rhodamine-conjugated rabbit anti-mouse antibody (Dako) - see appendix 3.3. Numbers of TIA-1 positive cells in each case were estimated by counting the number of positive cells in ten high power (x63) fields of parenchyma from each sample.

The ten biopsies from patients with chronic hepatitis C (ie not including biopsies from the two post transplant patients) were assigned a histological activity index (HAI) using the method of Knodell *et al.* (1981). The HAI was used to compare inflammation and fibrosis in biopsies (table 3.3) to the amount of HCV staining within the same biopsy.

Sections were cut from the formalin fixed, paraffin embedded portion of each biopsy, and stained with haematoxylin and eosin (Stevens, 1982). HAI scores were assigned by this observer (see appendix 3.4), then slides were reassessed by a pathologist, any differences discussed, and a consensus reached. Haematoxylin and eosin stained sections of the frozen material were also reviewed to ensure that activity was comparable in both portions of the biopsy. In addition, each frozen

Table 3.3 HAI for numerical scoring of liver biopsy specimens (From Knodell *et al.*, 1981)

I.Periportal +/- bridging necrosis	Score	II. Intralobular degeneration and focal necrosis[a]	Score	III. Portal inflammation	Score	IV. Fibrosis	Score
None	0	None	0	No portal inflammation	0	No fibrosis	0
Mild piecemeal necrosis*	1	Mild (acidophilic bodies, ballooning degeneration and/ or scattered foci of hepatocellular necrosis in $<^1/_3$ of lobules or nodules	1	Mild (sprinkling of inflammatory cells in $<^1/_3$ of portal tracts)	1	Fibrous portal expansion	1
Moderate piecemeal necrosis (involves less than 50% of the circumference of most portal tracts	3	Moderate (involvement of $^1/_3$ - $^2/_3$ of lobules or nodules)	3	Moderate (increased inflammatory cell in $^1/_3$ - $^2/_3$ of portal tracts)	3	Bridging fibrosis (portal-portal or portal-central linkage)	3
Marked piecemeal necrosis (involves more than 50% of the circumference of most portal tracts.	4	Marked (involvement of $>^2/_3$ of lobules or nodules)	4	Marked (dense packing of inflammatory cells $>^2/_3$ in of portal tracts)	4	Cirrhosis[b]	4
Moderate piecemeal necrosis plus bridging necrosis[c]	5						
Marked piecemeal necrosis plus bridging necrosis[c]	6						
Multilobular necrosis[d]	10						

a) Degeneration - acidophilic bodies, ballooning; focal necrosis - scattered foci of hepatocellular necrosis.

b) Cirrhosis - loss of normal hepatic lobular architecture with fibrous septae separating and surrounding nodules.

c) Bridging is defined as 2 or more bridges in the liver biopsy specimen; no distinction is made between portal-portal and portal-central linkage.

d) Multilobular necrosis - 2 or more contiguous lobules with panlobular necrosis.

* Many people now use the term "interface hepatitis" instead of "piecemeal necrosis" to describe inflammation of the portal tract which spills over the limiting plate into the parenchyma.

biopsy was assessed for the presence or absence of lymphoid aggregates or follicles.

ii) Staining of routinely fixed and processed material

The anti-HCV^{FITC} was also tried on FFPE material. To increase the chances of success, experiments were performed on a biopsy from a patient transplanted six months previously for end stage HCV positive cirrhosis, with hepatocellular carcinoma. The frozen portion of this biopsy had been stained with anti-HCV^{FITC}, and found to contain particularly high levels of HCV antigen. 3μm sections were cut and picked up on APES (Sigma) coated slides. Sections were dewaxed and in parallel, either post fixed in chloroform or not. Sections were then subjected to 0, 5, 10 or 15 minutes microwave treatment (see table 3.4). Microwave treatment can reveal antigens obscured by fixation, and enhance antibody binding (Shi *et al.*, 1991).

Table 3.4 Treatment of paraffin sections for anti-HCV testing.

	Post fixed in chloroform	No post fixation
No microwave	2 slides	2 slides
5 mins microwave	2	2
10 mins microwave	2	2
15 mins microwave	2	2

Slides were placed in plastic sealable coplin jars (R.A. Lamb) and covered in 0.01M citric acid buffer (pH 6.0). They were microwaved in five minute bursts, till the required time; buffer levels were topped up after each 5 minutes. Slides were heated at full power in a 700W microwave oven (Panasonic). After cooling, sections were rinsed in PBS, then incubated in anti-HCV ^{FITC} diluted 1/300 in normal human serum overnight at 4^{0}C. Slides were rinsed in PBS, then mounted in citifluor and examined using a Zeiss Standard 14 epifluorescence microscope.

The optimal conditions as determined by this experiment were then used on sections from this post transplant biopsy, and from the FFPE part of the sample from patient 10. The method used was based on that of Ballardini *et al.* (1995). Samples were stained as described above, then blocked in 10% normal goat serum (DAKO) for 15 minutes, and incubated in monoclonal anti-FITC antibody (DAKO) diluted 1/50 in TBS for 1 hour at room temperature. Alkaline phosphatase labelled sheep anti-mouse antibody (Sigma), diluted 1/100 in TBS, 1 hour at room temperature, was used to detect any antibody binding. Slides were developed using fast red tablets (Sigma) as chromogen, then counterstained in Carazzi's Haematoxylin (Stevens, 1982), air dried and mounted in UV setting adhesive (Locktite UK Ltd).

Photomicrography

Fluorescence photomicrographs of anti-HCV^{FITC} were taken on the DVC 250 confocal microscope, using a Nikon F-601M camera. Fujichrome 1600 slide film was used, and films were developed by 1 stop push processing. Pictures of double immunostaining were taken on the Zeiss standard 14 microscope with the Zeiss MC63/M35 camera setup, using Fujichrome 1600 slide film. Films were developed using 2 stop push processing.

Transmitted light photomicrographs were taken on Fujichrome Velvia film using a Zeiss 68069 photomicroscope. Films were developed using E6 processing. After developing, all transparencies were transferred onto a photo CD (Kodak). They were then imported onto a Dan multimedia PC and printed via Corel Multimedia onto HQ gloss paper (Epson) using an Epson stylus colour printer.

Statistics

Mean and standard deviations were calculated from TIA cell counts taken over a representative area for each specimen and from area, intensity and total HCV staining.

Results

Rabbit polyclonal and mouse monoclonal antibodies

None of the rabbit polyclonal or mouse monoclonal antibodies gave any specific staining on this material. The rabbit polyclonal antibodies gave some staining which was non-specific and did not relate to whether biopsies were HCV negative or HCV positive (see table 3.5). In most cases, the staining obtained related more to the nature of the biopsy, ie if the patient was cirrhotic (see figures 3.1 and 3.2). All negative controls without primary antibody were negative.

Table 3.5. Results of staining using rabbit polyclonal anti c100-3 antibodies

Disease category	No. of biopsies studied	Results of staining	
		Pos	Neg
Anti-HCV negative controls	14	6	8
NANB, anti-HCV positive	22	10	12
NANB, anti-HCV negative	10	4	6
Primary biliary cirrhosis	4	1	3
Anti-HCV positive alcoholic	3	2	1
Anti-HCV negative alcoholic	6	3	3

None of the mouse monoclonal antibodies gave any specific staining at any concentration. Five of the twenty one antibodies tested gave weak staining of occasional hepatocytes (when used neat with overnight incubation), but staining did not relate to HCV status of patients. Eight of the antibodies gave no liver cell staining at all, but stained the connective tissue of cirrhotic liver. The remaining

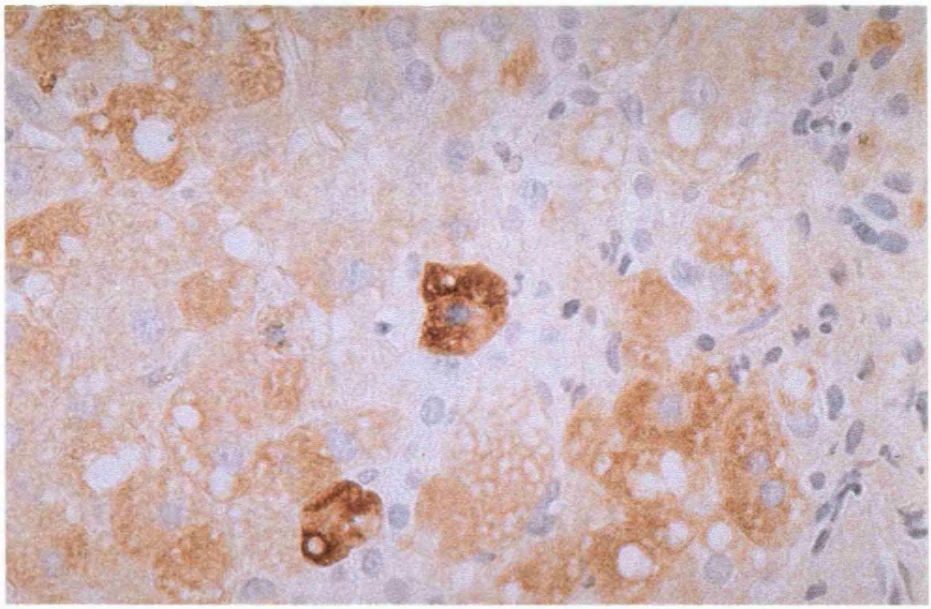


Figure 3.1. Biopsy from patient with chronic HCV infection, stained with anti-c100-3 antibody. Several hepatocytes show staining with the antibody, while other cells, including lymphocytes, are negative. Mag x250

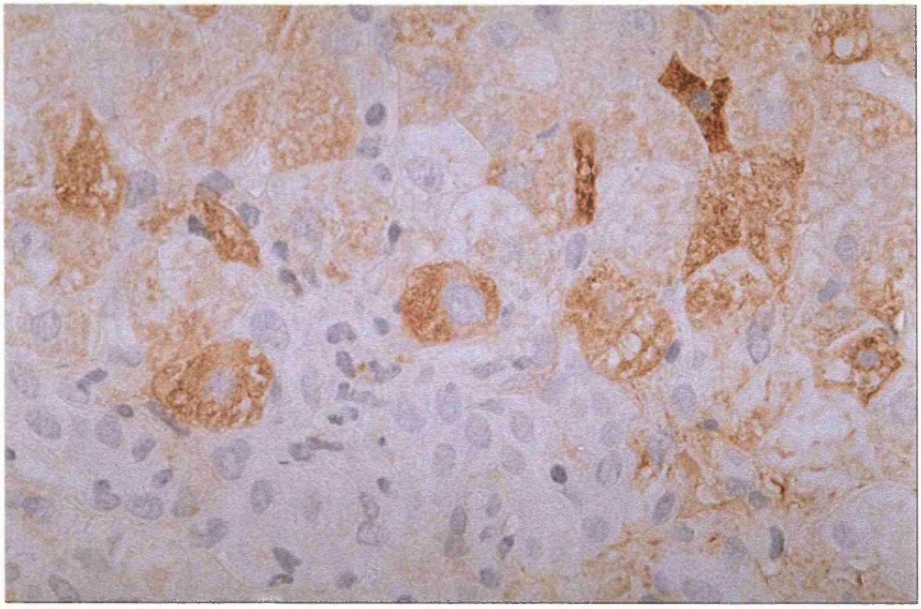


Figure 3.2. Biopsy from HCV negative alcoholic stained with anti-c100-3. Again, hepatocytes are stained with the antibody, and lymphocytes are negative, showing that the antibody is not specifically staining HCV infected cells. Mag x250.

eight antibodies showed staining of virtually all liver cells, again in biopsies from both anti-HCV positive and negative patients. All negative controls without primary antibody were negative.

Human polyclonal antibody testing

i) Testing of FFPE biopsies by tissue PCR

Eleven out of 12 anti-HCV positive patients were positive for HCV-RNA in their liver biopsies by tissue PCR (see table 3.6, figure 3.3). These results showed a 100% correlation with serum HCV PCR results. None of 10 anti-HCV negative patients had HCV-RNA in their liver biopsies detected by tissue PCR.

ii) Staining of frozen material

In the frozen liver biopsies tested with anti-HCV^{FITC}, HCV-antigen was detected in all twelve (100%) of the anti-HCV positive cases. Staining was not seen in any of the anti-HCV negative cases, nor in any sections where the primary antibody was omitted (see table 3.6). Only hepatocytes were stained with the antibody; staining was cytoplasmic, and no nuclear staining was seen. In the majority of cases (10/12), less than 50% of hepatocytes contained HCV antigen, with some cases showing only a few positive cells per biopsy. In all cases, antigen positive cells were scattered singly or in small groups throughout the parenchyma, with great lobule to lobule variation. No relation was seen between antigen positive cells and piecemeal necrosis (interface hepatitis).

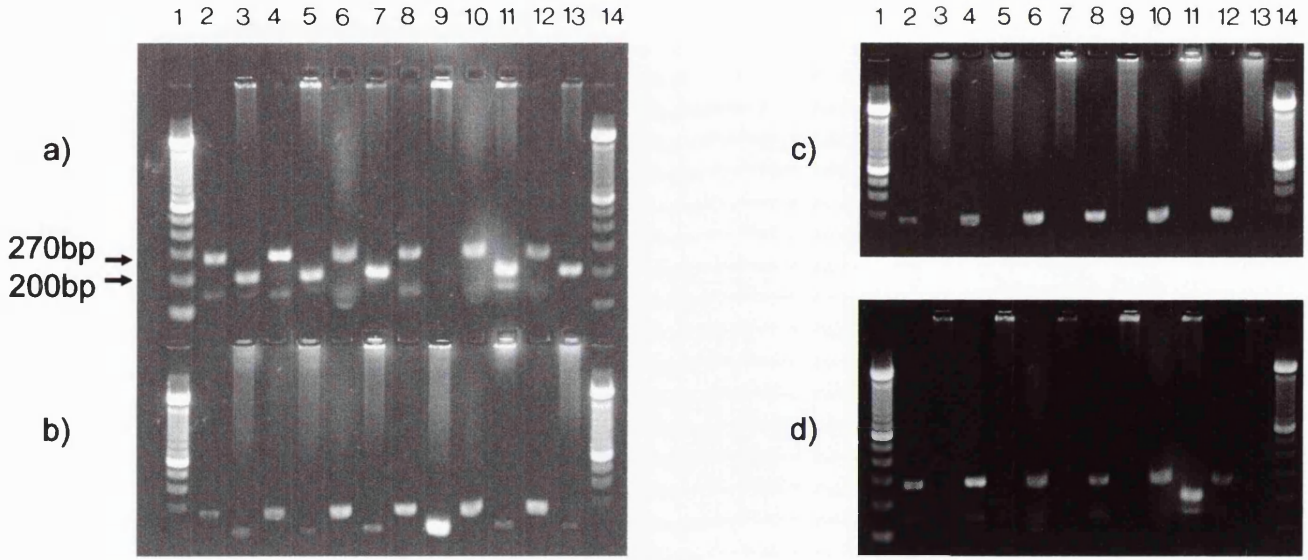
A haematoxylin and eosin section from each frozen biopsy was assessed for the presence or absence of lymphoid aggregates or follicles. Eight of the 12 (67%) HCV positive biopsies had at least one lymphoid aggregate, compared to 2/10 (20%) HCV negative cases. No staining with anti-HCV^{FITC} of lymphocytes or

Table 3.6. Summary of immunostaining results versus serum and tissue HCV PCR results and anti-HCV results on patients tested with anti-HCV ^{FITC}.

Patient	α-HCV	Serum PCR	Tissue PCR	HCV-IF	Area*	Intensity
1	Pos	Pos	Pos	Pos	++	++
2	Pos	Pos	Pos	Pos	+	+
3	Pos	Pos	Pos	Pos	++	+++
4	Pos	Neg	Neg	Pos	+	+
5	Pos	Pos	Pos	Pos	++	+
6	Pos	Pos	Pos	Pos	+	++
7	Pos	Pos	Pos	Pos	+++	++
8	Pos	Pos	Pos	Pos	+	+++
9	Pos	Pos	Pos	Pos	++	+
10	Pos	Pos	Pos	Pos	+++	+++
11	Pos	Pos	Pos	Pos	++	+++
12	Pos	Pos	Pos	Pos	++	+++
13	Neg	Neg	Neg	Neg	--	--
14	Neg	Neg	Neg	Neg	--	--
15	Neg	Neg	Neg	Neg	--	--
16	Neg	Neg	Neg	Neg	--	--
17	Neg	Neg	Neg	Neg	--	--
18	Neg	Neg	Neg	Neg	--	--
19	Neg	Neg	Neg	Neg	--	--
20	Neg	Neg	Neg	Neg	--	--
21	Neg	Neg	Neg	Neg	--	--
22	Neg	Neg	Neg	Neg	--	--

*Area of staining:

+ ≤ 5% of hepatocytes stained
++ 5 - 50% of hepatocytes stained
+++ ≥50% of hepatocytes stained



a) Lane 1 contains the 100bp ladder. Samples in lanes 2 - 13 are PCR products for albumin (270bp) and HCV (200bp) from patients 1-6. Patient 4 (lanes 8 and 9) is positive for albumin but negative for HCV.

b) Samples in lanes 2 - 13 are PCR products from patients 7 - 12. All were positive for albumin and HCV.

c) Samples in lanes 2 - 13 are PCR products for albumin and HCV for patients 13 - 18. All samples were positive for albumin but negative for HCV.

d) Samples in lanes 2 - 9 are from patients 19 - 22. All samples were positive for albumin, but negative for HCV. The sample in lanes 10 and 11 was the HCV positive control tissue sample. The sample in lanes 12 and 13 was the HCV negative control tissue (positve for albumin).

Figure 3.3 Photograph of gel showing PCR products from liver biopsies tested with HCV-FITC

lymphoid follicles was identified; there was no marked relationship between HCV antigen positive cells and lymphoid aggregates. No staining of bile ducts was observed, or of any other cellular elements in the liver.

Two types of staining were seen:

a) A diffuse, cytoplasmic staining of variable intensity. This intensity varied both within and between biopsies (see Table 3.6, figures 3.4 - 3.7).

b) Focal areas of intense staining seen within the cytoplasm (figures 3.8, 3.11). This type of staining was seen only in biopsies with very intensely stained cells.

In general, the area of staining did not correlate well with the stage of liver disease, although the two cases with the greatest number of cells stained (>50%) of hepatocytes were both cirrhotic (figures 3.6, 3.7). Likewise, the intensity of staining was not directly related to the stage of liver disease, although the two post-transplant cases, who were both receiving immunosuppressive treatment at time of biopsy, both showed a high intensity of staining (figures 3.8 and 3.9).

Figures 3.4 to 3.9 are on the following pages.

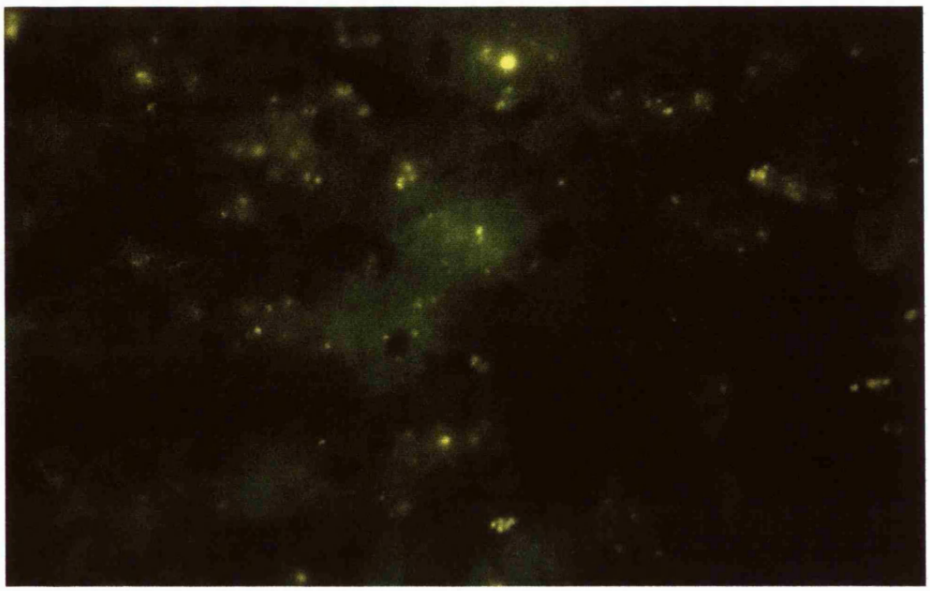


Figure 3.4. Section from Patient 7 showing pale staining cells in the centre and top of the field. This cirrhotic patient had large numbers of pale staining cells, positive for HCV antigen. Mag. X660.

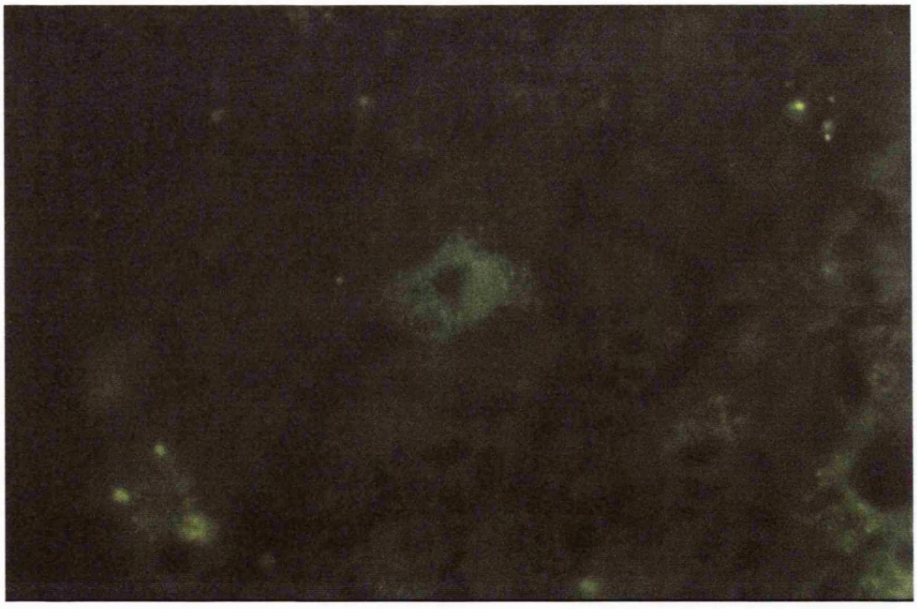


Figure 3.5. Biopsy from patient 1, who had mild hepatitis due to HCV. A single pale staining cell in the centre of the field shows green diffuse cytoplasmic immunofluorescence for HCV. Mag x660.

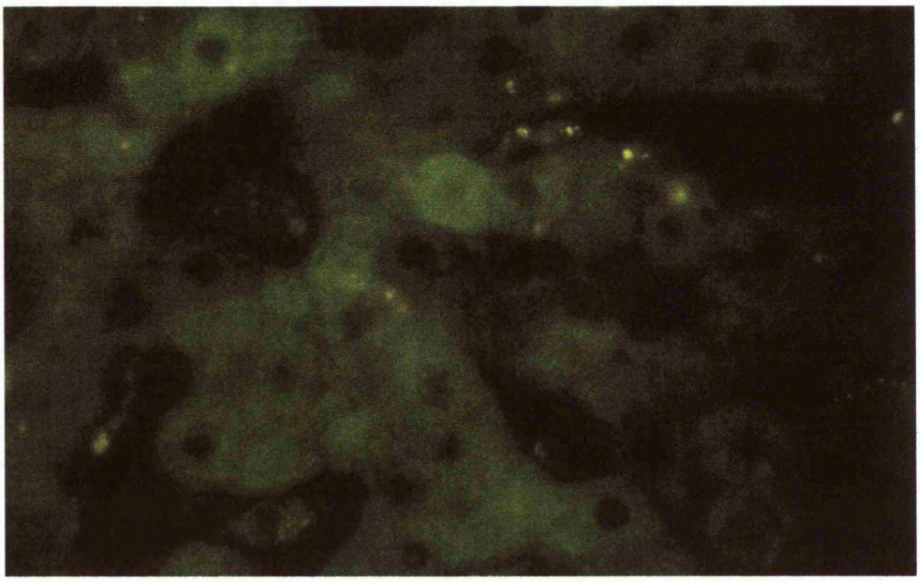


Figure 3.6 Biopsy from cirrhotic patient (patient 10) showing staining of a high percentage of hepatocytes with the anti-HCV antibody. The hepatocytes show the great variation in intensity of staining seen in many cases. Mag x660.

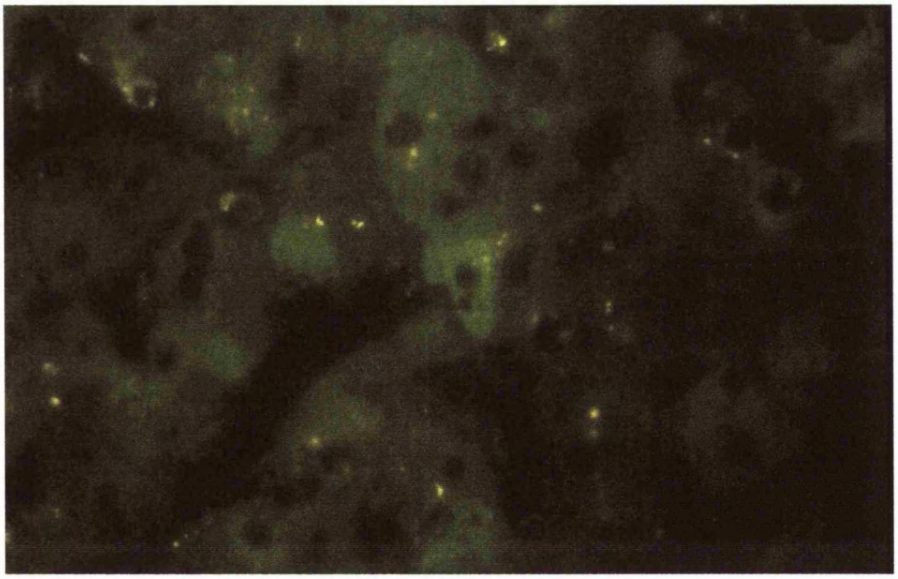


Figure 3.7. Photomicrograph showing different field of biopsy from patient 10,with a brightly stained hepatocyte in the centre of the field. Mag x660.

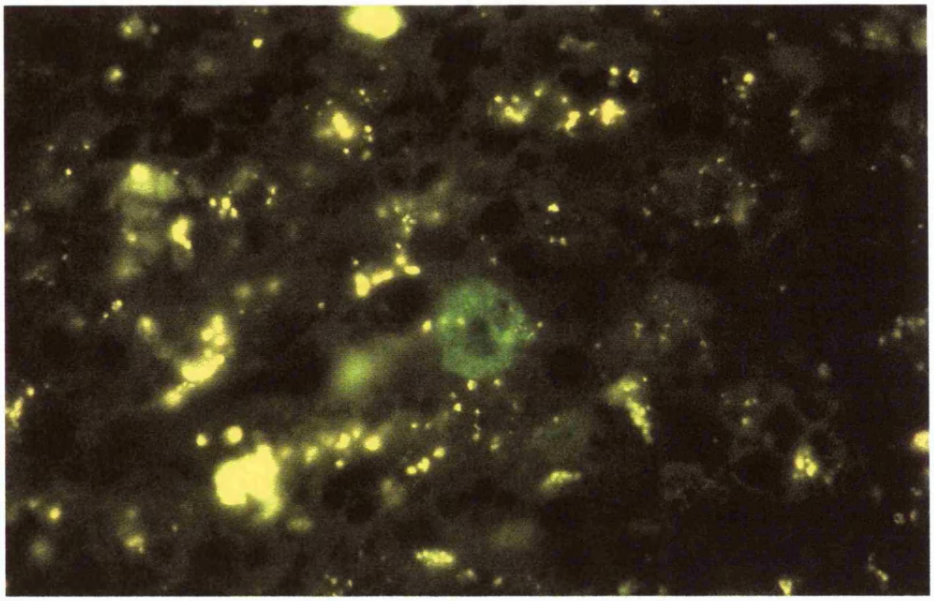


Figure 3.8. Photomicrograph of post transplant biopsy (patient 11) showing very brightly stained hepatocyte in centre of field. Focal areas of punctate staining can be seen within the cytoplasm of this cell. The yellow fluorescence in this biopsy represents autofluorescent lipofuscin. Mag x660.

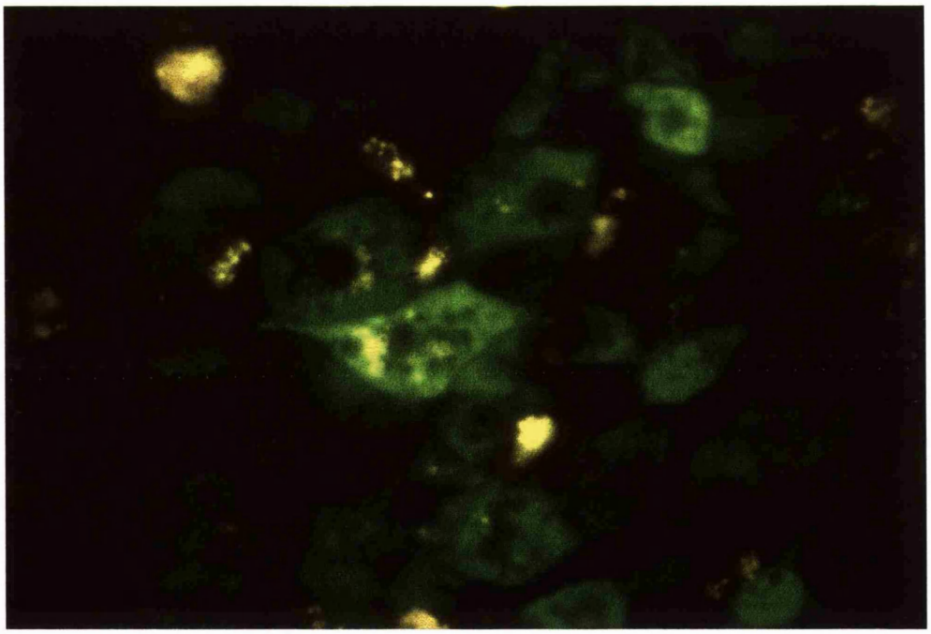


Figure 3.9. Post transplant biopsy in which many hepatocytes are positive with anti-HCV. Very strong staining of some hepatocytes is seen. Mag x660.

Total HCV staining was assessed as percentage of hepatocytes stained times intensity (a crude form of integrated optical density), and compared to the amount and type of inflammation within the biopsy as assessed by Knodell scoring (see tables 3.7 and 3.8; figure 3.10). The biopsies from the two post transplant cases were excluded from this analysis as inflammation in their biopsies would have been affected by any immunosuppressive therapy.

Total HCV staining (IOD) appeared to correlate with total inflammation within the biopsy (see figure 3.10a). There was no obvious correlation between IOD and fibrosis, although the two cases with the most staining were cirrhotic (see figure 3.10b).

Table 3.7. Histological activity indexes of chronic hepatitis C biopsies.

Patient	I Periportal +/- bridging necrosis	II Intralobular degeneration and focal necrosis	III Portal inflammation	IV Fibrosis	Total inflammation (fibrosis)
1	3	3	3	1	9 (1)
2	3	1	3	3	7 (3)
3	3	1	3	4	7 (4)
4	3	1	3	3	7 (3)
5	3	3	3	0	9 (0)
6	3	3	3	4	9 (4)
7	3	3	3	4	9 (4)
8	3	3	3	3	9 (3)
9	1	1	1	1	3 (1)
10	3	4	3	4	10 (4)

Amount and type of lobular inflammation were further characterised.

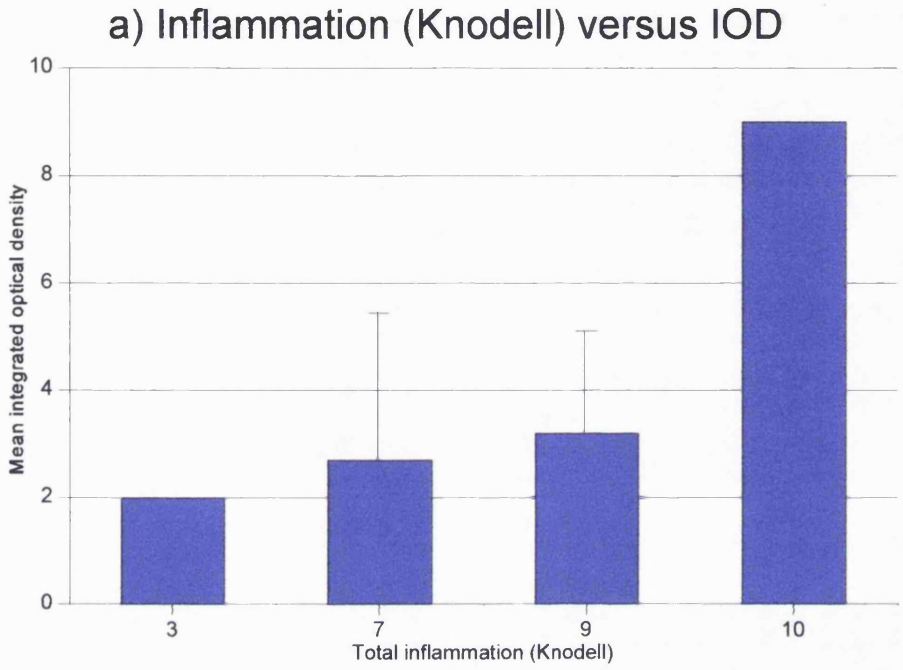
Sections from three HCV and anti-HBV negative cases (patients 14, 20, and 22, table 3.6), along with sections from 11 of the HCV positive biopsies (excepting that from patient 5) were double stained with anti-HCV, and TIA-1 antibody to demonstrate cytotoxic T cells (CTL). This staining revealed much higher numbers of CTL in HCV infected livers than in liver samples from patients negative for HCV and HBV. There was an average of 8.75 TIA positive cells per high power field for HCV positive biopsies, compared to an average of 0.66 positive cells per field for HCV negative biopsies (see table 3.8).

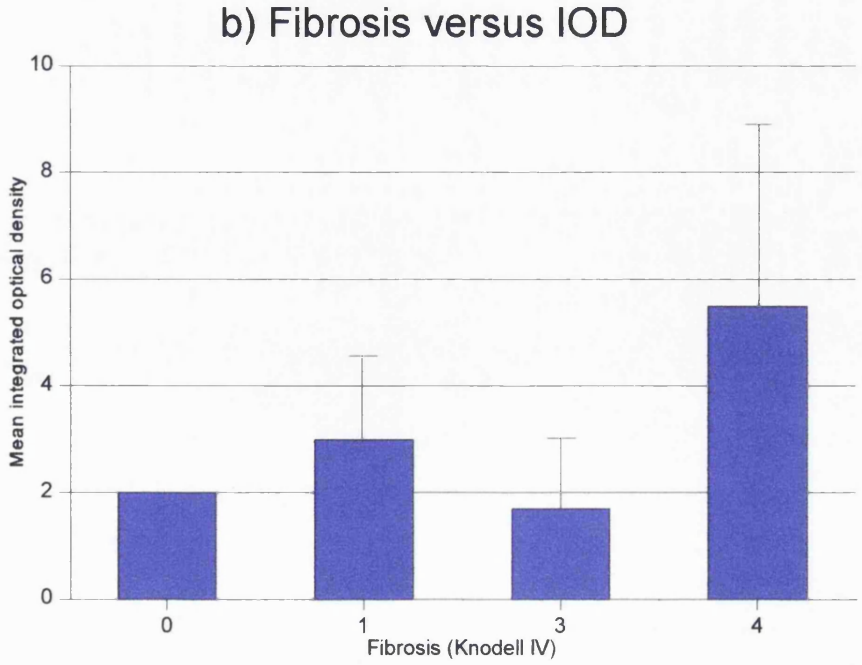
Table 3.8 Correlation of HCV^{FITC} staining with inflammation.

Patient	HCV^{FITC} Area	staining Intensity	Integrated optical density	TIA-1 positive cells *	Total inflammation (fibrosis)
1	++	++	4	2	9 (1)
2	+	+	1	33	7 (3)
3	++	+++	6	3.9	7 (4)
4	+	+	1	10	7 (3)
5	++	+	2	ND	9 (0)
6	+	++	1	8	9 (4)
7	+++	++	6	5.8	9 (4)
8	+	+++	3	5	9 (3)
9	++	+	2	13	3 (1)
10	+++	+++	9	10.5	10 (4)
11	++	+++	6	0	ND
12	++	+++	6	5.1	ND
14	--	--	0	0	ND
20	--	--	0	1	ND
22	--	--	0	<1	ND

* Mean number of TIA-1 positive cells per high power field - ten fields counted per slide. (ND = not done; NS = not scored)

Figure 3.10. Total HCV staining measured by integrated optical density (IOD) versus a) inflammation and b) fibrosis. Results are expressed as mean IOD plus standard deviation.





In general, whilst cytotoxic T lymphocytes were sometimes seen in apposition to HCV-infected cells (see figure 3.11), distribution of CTL did not follow the distribution of HCV antigen staining cells. This was very apparent because of the marked lobule to lobule variation seen in HCV staining. TIA-1 positive cells were seen mainly in the parenchyma distributed more evenly throughout the samples, and did not cluster in areas with high levels of HCV staining. Interestingly, the case with the highest number of cytotoxic T cells had the least amount of HCV staining and the lowest Knodell score. Indeed, numbers of cytotoxic T cells appeared to be inversely related to the intensity of staining (see figure 3.12a) - ie, where high numbers of cytotoxic T cells were present, individual hepatocytes expressed HCV antigen at lower levels, and vice versa, although IOD did not show this same clear relationship (see figure 3.12b).

iii) Staining of routinely fixed and processed material

The anti-HCV^{FITC} antibody was also applied to the paraffin embedded portion of a biopsy containing a large amount of HCV antigen (see figures 3.13, 3.14). Sections from this biopsy were pretreated in a variety of ways to try and reveal HCV antigen (see table 3.4). Without microwave pretreatment, the antibody did not stain any cells within the biopsy, either with or without post fixation in chloroform. With five minutes microwave treatment, some cells at the edge of the biopsy showed staining. This resembled true staining, giving the same pattern and colour of staining as seen in the frozen biopsies; positive cells and negative cells were seen next to each other (figure 3.15). This staining was slightly more intense on the duplicate slide post-fixed in chloroform. After ten minutes microwave treatment, more cells stained with the antibody, mostly in a rim towards the edge of the

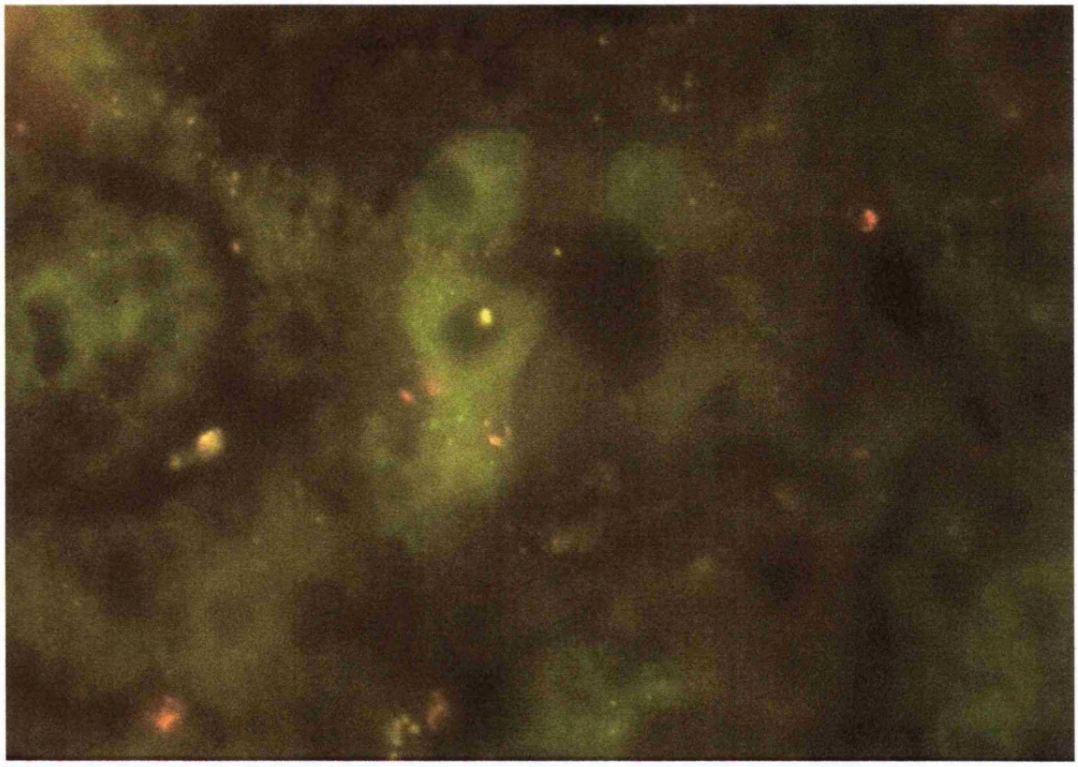
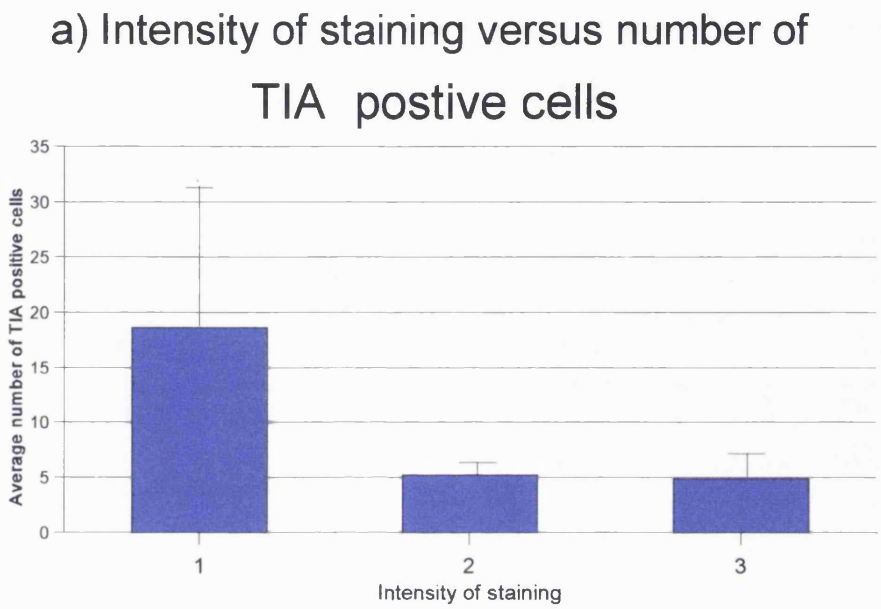
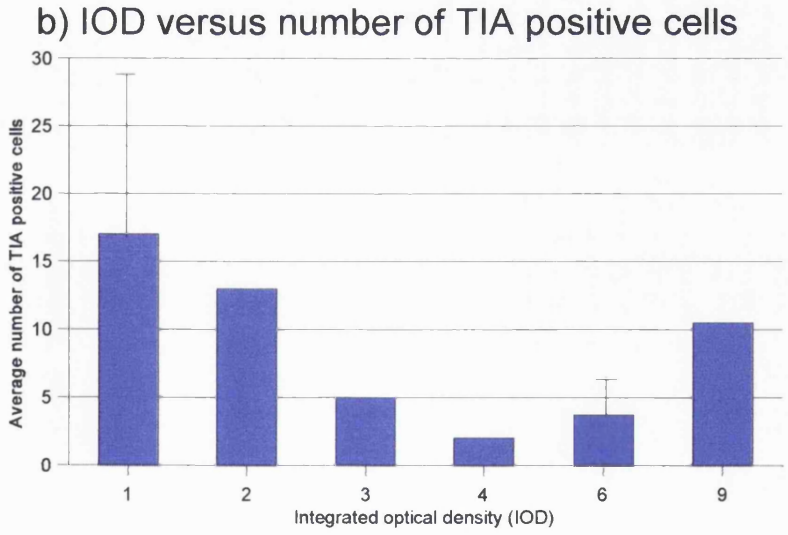


Figure 3.11 Photomicrograph showing the biopsy from a cirrhotic patient transplanted for end stage HCV associated liver disease. This biopsy was double stained for hepatitis C virus (green fluorescence) and for cytotoxic T cells using the TIA antibody (red fluorescence). Two TIA positive cells, showing red granular staining, can be seen in apposition to an intensely stained hepatocyte in the centre of this picture. This hepatocyte also shows focal cytoplasmic staining. Mag x660.

Figure 3.12. Average number of TIA cells versus a) intensity of staining and b) total HCV staining (IOD).





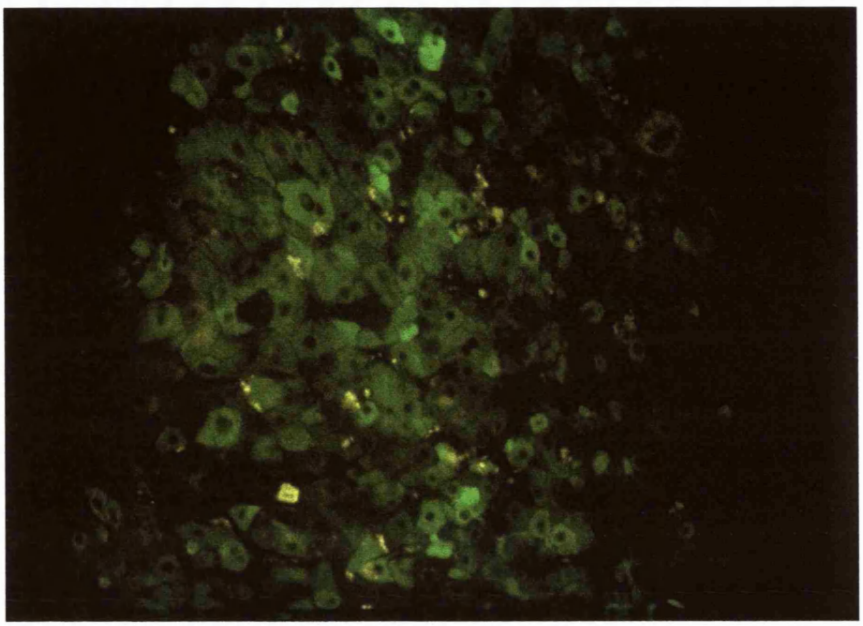


Figure 3.13 Post transplant biopsy showing particularly high levels of antigen expression. The paraffin embedded part of this biopsy was stained with the anti-HCV antibody to see if the antigen was retained and detectable after routine processing (see figures 3.15-3.17). Mag x400

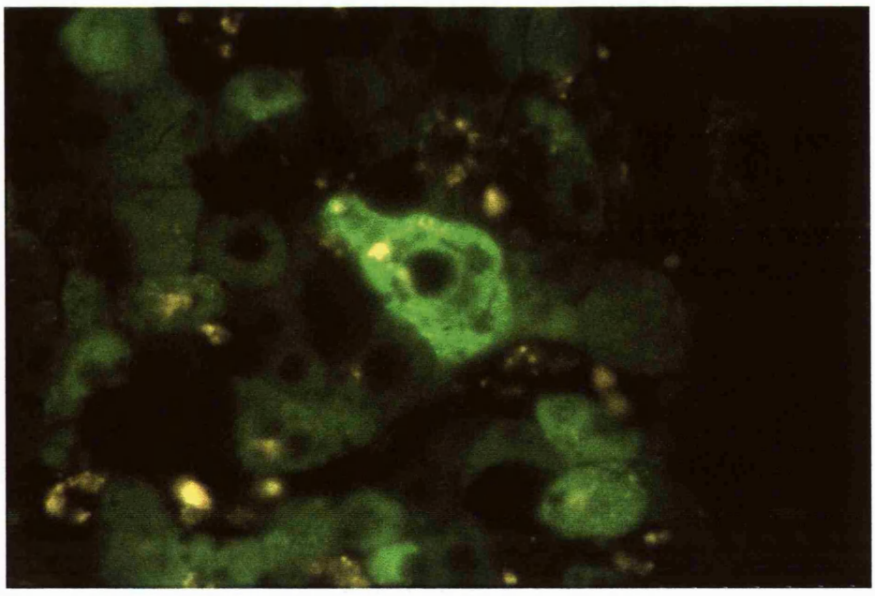


Figure 3.14 Photomicrograph of same specimen showing intensely stained hepatocyte. Many of the parenchymal cells in this biopsy showed high levels of antigen expression. Mag x660

biopsy, but some within the main part of the biopsy (figure 3.15). Background was lower in the chloroform fixed biopsy, and slightly more positive cells were seen. After fifteen minutes of microwave treatment, more cells had stained, but less brightly. Again, the chloroform fixed slide showed better staining.

As with the frozen biopsies, only hepatocyte cytoplasm stained with the antibody. Both the diffuse, cytoplasmic and the bright, punctate staining were seen (see figures 3.15 and 3.16).

However, all sections had a very high level of background fluorescence, making it difficult to distinguish staining. This background increased as length of microwaving increased. This artefact appeared to be caused by formalin fixation/paraffin processing, as it was identical in sections without primary antibody. Whilst it was possible in this case, with very high levels of staining, to assess the affects of the different treatments, in a biopsy with fewer antigen expressing cells (eg patient 4, table 3.6), this background could have obscured any staining, making any assessment impossible.

Sections from the biopsy of this post transplant patient, and from the paraffin block corresponding to the sample from patient 10 were post fixed in chloroform, treated for 15 minutes in the microwave, and processed for light microscopy.

The sample from the post transplant patient showed positive staining using this technique (see figure 3.17). The staining pattern was similar to that seen in the frozen material (figures 3.13 and 3.14), but amount and intensity of staining was reduced. No satisfactory staining was obtained on the FFPE sample from patient 10.

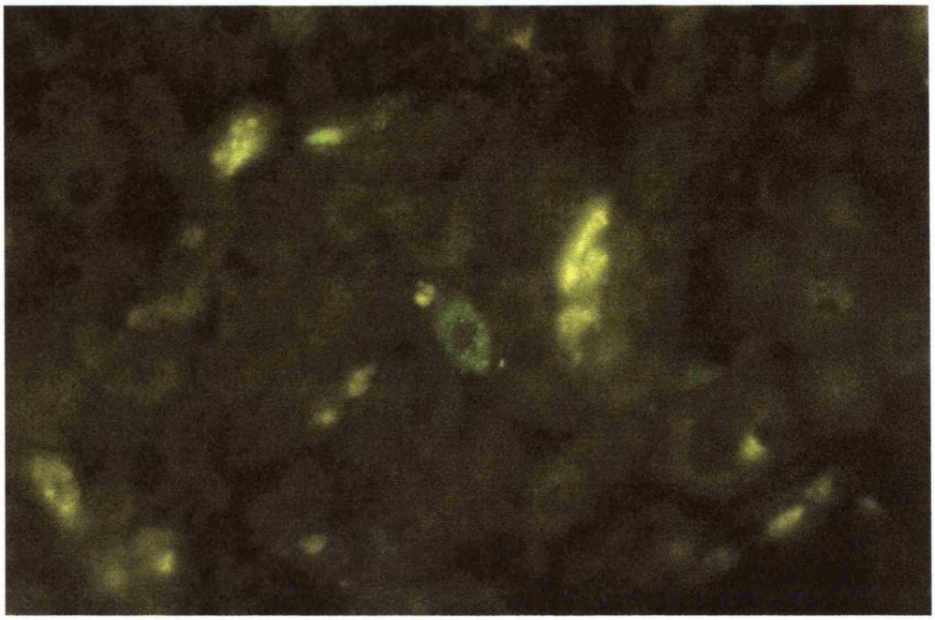


Figure 3.15 Section from FFPE post transplant biopsy stained with anti-HCV, showing a faintly stained hepatocyte in the centre of the field. Both intensity and amount of HCV staining was reduced when compared to the frozen material from the same biopsy stained with anti-HCV (see figures 3.13 and 3.14). Mag x660.

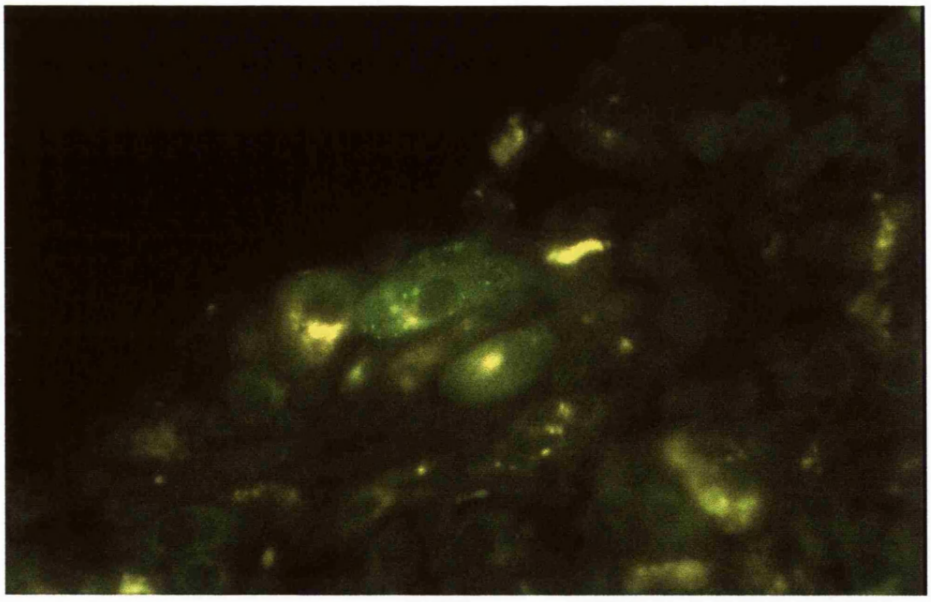


Figure 3.16 FFPE post transplant biopsy showing HCV postive hepatocyte with both diffuse cytoplasmic and granular HCV staining. Mag x660.

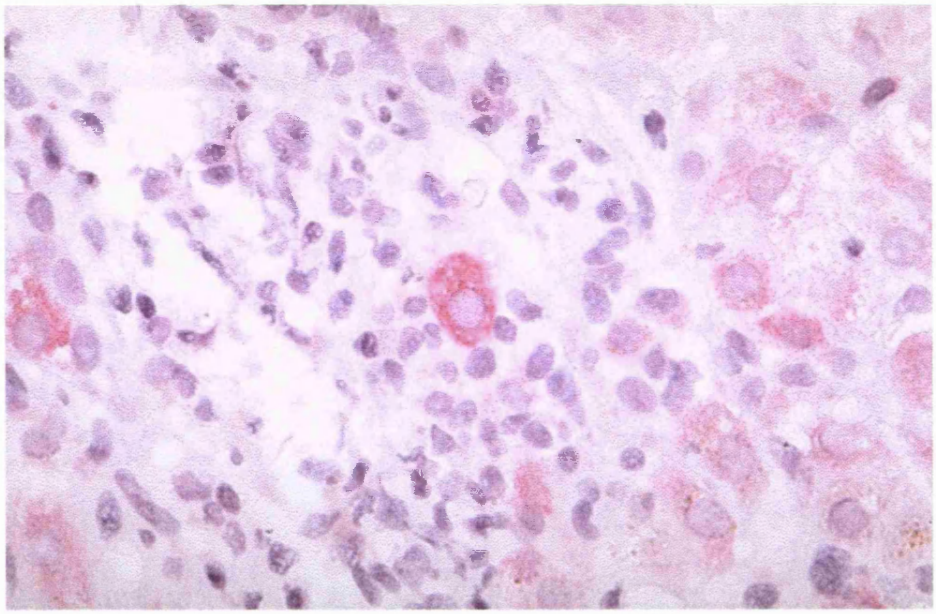


Figure 3.17 FFPE post transplant biopsy stained with anti-HCV then developed for light microscopy, using an alkaline phosphatase system with fast red as chromogen. In the centre of the field is an HCV positive hepatocyte (red) surrounded by inflammatory cells. Mag x250

Discussion

None of the rabbit polyclonal or mouse monoclonal antibodies gave any specific staining. Sixteen of the twenty one monoclonal antibodies, raised against synthetically produced HCV antigens, stained up connective and liver cell elements of the liver non specifically. In theory, monoclonal antibodies are very specific reagents and will only bind to the epitope against which they were raised. There is usually greater specificity of antibodies raised using culture techniques, as these antibodies were, than via mouse ascites. However, it is well known that monoclonal antibodies can also have an intrinsic cross-reactivity (Elias, 1990). Epitopes generally consist of three to eight amino acid sequences, so it is possible that sequences can be shared between molecules. Formalin fixation and paraffin processing are known to alter and unmask proteins within the cell; this may increase the chances of an antigenic determinant being mimicked or duplicated by another (Warnke, 1979).

For this part of the study, patients were assigned to HCV positive or negative groups on the basis of anti-HCV testing. The indiscriminate results obtained both by anti-HCV immunostaining and by *in situ* hybridisation experiments performed at this same time led to a realisation that a more sensitive and specific test was required to definitively identify HCV infected liver biopsies. It had already become clear that not all HCV infected patients were detectable by second generation anti-HCV testing; these tests also had a relatively high false positive rate, making interpretation of these indiscriminate results difficult (Alter *et al*, 1992). In order to reliably identify samples infected by HCV, the tissue PCR for HCV-RNA detection within individual patient biopsies was developed.

Overall, the results of the monoclonal antibody staining suggest that there may be some shared sequences between HCV antigens and endogenous liver antigens. HCV infection has been linked to production of an autoantibody to a host protein called GOR (Mishiro *et al*, 1990). These results led to further investigations of the links between HCV and autoimmune liver disease, which are discussed in more detail in Chapter 4.

Other workers have reported specific detection of HCV antigens using rabbit polyclonal and mouse monoclonal antibodies. Using the antibodies available for this study, no discriminant staining was found. This is not, in theory, surprising, as all the antibodies were raised against synthetic HCV peptides; recognition of antigen by antibody can depend on the 3-D structure of the antigen (Boon and Kok, 1987). There may be unsuspected differences in secondary or tertiary structure between these antigens and the naturally expressed antigens present in infected liver. Whilst patient polyclonal antibodies to these naturally expressed antigens are able to recognise these synthetically produced antigens, it does not necessarily follow that antibodies raised against these synthetic peptides will recognise the natural antigen. As monoclonal antibodies generally recognise such small amino acid sequences (Elias, 1990), different antibodies raised against the same peptide may recognise different epitopes. This, together with the fact that some groups have used recombinantly expressed rather than synthetic peptides (Hiramatsu *et al.*, 1992; Nouri-Aria *et al.*, 1995; Sansonno and Dammacco, 1993), could explain the discrepancy between these results and those of other workers who have obtained discriminant staining with mouse monoclonal and rabbit polyclonal antibodies (Yamada *et al.*, 1993; Tsutsumi *et al.*, 1994; Blight *et al.*, 1993b). Gonzalez-

Peralta *et al.* (1994), who also described using rabbit polyclonal antibodies raised against synthetic peptides of HCV, obtained only non-specific staining with these antibodies. The fact that, at time of writing, there is no commercially available antibody giving sensitive and specific staining in either frozen or paraffin embedded material only emphasises the difficulties in producing such antibodies with these synthetic peptides.

These ideas are supported by the fact that the human polyclonal antibody used in this study, raised against naturally expressed antigen, gave sensitive, specific staining. The results obtained in this part of the study agree with those of Krawczynski *et al.* (1992a), and confirm the specificity and sensitivity of this antibody. The slightly higher sensitivity in our series (100% c.f. 92%) is probably due to the small number of samples tested in this series.

The immunofluorescence observations were greatly facilitated by the direct view confocal microscope because of the greater intensity of the fluorescence and greater resolution of the optical system, compared to conventional fluorescence microscope. The contrast between the specific green immunofluorescence and the yellow-green of endogenous liver lipofuscin was also greater using the confocal microscope. Indeed, the immunostaining required use of the correct filter before accurate distinction of positive staining from background could be obtained on the ordinary fluorescence microscope.

No direct relationship was seen between HCV positive hepatocytes and portal inflammation or interface hepatitis. The presence of lymphoid aggregates (seen in 67% of HCV positive cases) did not appear to relate to the position of HCV infected cells. HCV antigen was only detected in the cytoplasm of

hepatocytes; no other cells within the liver were stained, including bile duct epithelial cells, endothelial cells, macrophages, monocytes and lymphocytes. These results correspond well to those of other workers, who in general have only detected HCV antigen in hepatocyte cytoplasm, although Nouri-Aria *et al.* (1995) also detected antigen within bile duct epithelium and mononuclear cells in a small proportion of patients.

The degree of inflammation as assessed by HAI correlated directly with amount of HCV staining suggesting an immunological response to HCV infected cells. No correlation was found between the degree of staining and stage of disease, although it is interesting that the two cases showing the greatest amount of staining were cirrhotic. The two post-transplant biopsies stained (see table 3.6, patient 11 & 12) showed very intense staining of positive hepatocytes, although the number of positive cells was not as high as in some of the other non-immunosuppressed cases (table 3.6, patients 7 & 10). This provides further evidence that the immune system plays a role in the control of HCV infected hepatocytes.

The relationship of HCV antigen containing cells to lobular inflammation was less clear, partly because of the limitations of fluorescence microscopy, which does not allow visualisation of histological detail. The cases which were counterstained with TIA-1 for cytotoxic T lymphocytes generally showed no co-distribution of CTL and HCV infected cells, as lobules with a high number of HCV antigen positive cells did not have higher numbers of CTL compared with surrounding lobules. However, CTL were seen in apposition to HCV positive cells in some instances (see figure 3.11), and it is interesting that there appears to be an inverse relationship between numbers of TIA positive cells and intensity of HCV

immunostaining (see table 3.8, figure 3.12). As cytotoxic T cells are a "hit and run" type of effector cell, a lack of a notable morphological co-distribution of CTL and HCV infected cells does not preclude this type of response. The immunophenotype of hepatitis C has been the subject of several studies, including two at the Royal Free (Hudson *et al.*, 1993; Khakoo *et al.*, 1995). These have shown that the predominant type of inflammatory cells within portal tracts and hepatic lobules are T cell (Liaw *et al.*, 1995; Hino *et al.*, 1992; Marrogi *et al.*, 1995), which suggests that a cell mediated immune response is important in hepatitis C. However, there is some controversy as to the type of T cells found in the parenchyma; Khakoo *et al.* (1995) found a predominance of CD4+ T cells in severe HCV-related liver disease, and have suggested an autoimmune type of hepatocyte injury, whereas other workers have found CD8+ T cells to predominate in the parenchyma (Liaw *et al.*, 1995), and have successfully extracted HCV-specific CTL from patients with chronic HCV (Koziel *et al.*, 1992) which show an HLA class I restricted response (Koziel *et al.*, 1995). The two types of reaction are not mutually exclusive. It is not possible to draw statistically significant conclusions from the small number of biopsies studied here, but it is tempting to speculate that the higher numbers of CTL observed within biopsies with less intense immunostaining (see table 3.8) are controlling hepatocyte antigen expression, and that this control has somehow broken down in the biopsies with lower CTL numbers and higher antigen expression. This escape from CTL immune surveillance could be precipitated by changes in the quasispecies during the course of infection (Zeldis and Jensen, 1994). The increased inflammation seen in biopsies with more HCV staining could represent a secondary, CD4 mediated response. The absence of degenerative changes in many HCV

infected hepatocytes, and lack of correlation between presence of HCV and liver cell injury supports the hypothesis that hepatocellular necrosis is immune mediated in hepatitis C (Uchida *et al.*, 1994; Krawczynski *et al.*, 1992b). The reason why apoptosis is precipitated in some HCV infected hepatocytes remains mysterious.

The sensitivity of the human polyclonal antibody used in this study was generally higher than that of mouse or rabbit antibodies used elsewhere. This finding is supported by those of other workers who have used polyclonal human antibodies (Krawczynski *et al.*, 1992a; Di Bisceglie *et al.*, 1993; Nouri-Aria *et al.*, 1995; Ballardini *et al.*, 1995). This may be explained by the observation that each HCV infected cell appears to express multiple HCV-antigens (Ballardini *et al.*, 1995; Gonzalez-Peralta *et al.*, 1994; Hiramatsu *et al.*, 1992), each of which can be detected by the human polyclonal antibody, so making staining stronger and more sensitive. The distinctive pattern of HCV-antigen staining seen here has also been described by other workers, including the presence of bright, punctate staining (see figures 3.7, 3.11) in some densely stained cells (Krawczynski *et al.*, 1992a; Sansonno and Dammacco, 1993; Blight *et al.*, 1993a). No precise subcellular localisation of this punctate staining has been described, but it has been suggested that these could be foci of replicative virus (Blight *et al.*, 1993b).

One case (see table 3.6, patient 4) which was negative for HCV-RNA was positive for HCV antigen. This could represent a false positive result with the anti-HCV antibody. However, this patient had chronic liver disease, was negative for all other hepatitis viruses and had histological features of chronic hepatitis C. It is known that HCV viraemia can fluctuate (Alter *et al*, 1992); other groups have found that HCV antigen could be detected in the absence of detectable HCV-RNA in

serum in some patients (Gonzalez-Peralta *et al.*, 1994; Di Bisceglie *et al.*, 1993; Hiramatsu *et al.*, 1992). The patient in this study had only a low level of HCV antigen in the biopsy examined, with only 4 hepatocytes staining with the antibody. It may be that this patient was clearing the virus, as he was asymptomatic at the time of biopsy, or that viral replication had fallen to a level undetectable by PCR. This does show that detection of HCV-Ag can be very sensitive, and is a useful adjunct to other tests, perhaps in particular when judging clearance of HCV after therapy (Di Bisceglie *et al.*, 1993).

The anti-HCV^{FITC} also successfully stained FFPE material, although at a reduced sensitivity compared to the frozen material. Whilst this is obviously a preliminary result, it shows that HCV antigen is both preserved and detectable in FFPE material, something which has been a matter of some debate (Gonzalez-Peralta *et al.*, 1994; Nouri-Aria *et al.*, 1995; Blight *et al.*, 1993b). This result has important implications, as it releases this technique from many of its limitations. However the use of a fluorescent antibody limits the amount of histological detail which can be seen; the optical quality of frozen material is not optimal, and frozen material may not be routinely available. An anti-HCV antibody which could detect HCV antigen within routine material would be transformed from solely a research tool to a reagent which would provide all pathologists with a valuable diagnostic test which could facilitate the study of the natural history and evolution of this disease, as well as success of any treatments, on patients worldwide.

CHAPTER 4

HCV AND AUTOIMMUNE HEPATITIS

Introduction

There has been controversy over the relationship between autoimmune hepatitis (AIH) and the hepatitis C virus (HCV). Autoimmune hepatitis is a disease in which the patient suffers a chronic active hepatitis (CAH) of essentially unknown aetiology, associated with the production of autoantibodies to a variety of self antigens. The principal pathogenetic mechanism of AIH is thought to be the loss of immune tolerance to autologous liver tissue, in which the primary target for tissue destruction is the hepatocyte (Manns and Meyer zum Buschenfelde, 1994). Whilst the definition and diagnosis of autoimmune hepatitis has classically been of CAH in young women with high titres of autoantibodies, hypergammaglobulinaemia, and an absence of other aetiological factors, it has long been suspected that a hepatotropic virus could be responsible for the initial "insult" in AIH, resulting in an inappropriate host response leading to the production of autoantibodies, and to chronic disease (Askonas, 1994; McFarlane, 1991).

AIH can be associated with other autoimmune diseases such as autoimmune thyroiditis, and ulcerative colitis (Sheron and Eddleston, 1991); and the efficacy of immunosuppressive drugs in treatment of these patients has added weight to claims that the condition has an autoimmune basis (Cook *et al.*, 1971). AIH is largely classified into two types, according to which species of autoantibodies are detected. Patients with type I AIH have non-organ specific anti-nuclear (ANA) and/or tissue specific anti-smooth muscle (SMA) antibodies. In type II AIH, patients have antibodies directed against a liver kidney microsomal (LKM1) antigen (Johnson *et al.*, 1991; Homberg *et al.*, 1987). It is now recognised that the disease can occur in men and women, and can present over a wide age range, with many patients not

developing overt disease until the fourth or fifth decade of life (McFarlane, 1991). Environmental or hormonal factors could be involved in triggering the disease in genetically susceptible individuals; viruses have long been a prime suspect for this role.

The discovery of hepatitis C virus led to many of these patients being tested for anti-HCV antibodies. HCV was a likely candidate as an environmental trigger since, as well as being a hepatotropic virus, it has been shown to be closely associated with the production of an autoantibody to a host protein named GOR (Mishiro *et al.*, 1990). Indeed, the observations noted earlier (see chapter 3), of a marked cross reactivity between monoclonal and polyclonal antibodies raised against synthetic HCV antigens and HCV negative liver tissue suggested that these epitopes might share a similar structure with certain host antigens.

In early studies, different groups reported markedly different prevalences of anti-HCV antibodies in patients with autoimmune hepatitis, with rates of between 5 and 78% noted (Esteban *et al.*, 1989; Craxi *et al.*, 1991; McHutchinson *et al.*, 1989; Lenzi *et al.*, 1990). The first studies performed on patients with AIH used first generation radioimmunoassay (RIA) or enzyme linked immunoassay (ELISA) tests to detect anti-c100-3 antibodies. These tests were prone to false positive results in patients with AIH, owing to cross-reactions with a suspected serum factor against yeast proteins, and to the hypergammaglobulinaemia prevalent in these patients (Nishiguchi *et al.*, 1992; McFarlane *et al.*, 1990). Geographic differences have been noted, with patients from high risk areas for HCV infection (eg Italy) having a higher co-incidence of HCV markers and type I or II autoimmune hepatitis than AIH patients from the UK (Lenzi *et al.*, 1991). HCV-RNA has been detected in

sera of autoantibody positive patients using the polymerase chain reaction (PCR) technique (Magrin *et al.*, 1991a).

Histologically, AIH has the characteristic features of a chronic active hepatitis, ie. periportal piecemeal necrosis, with a predominantly lympho-plasmacytic infiltrate. These features are not diagnostic of AIH, and are seen in CAH with different aetiologies. The additional association with lobular hepatitis of intense activity, numerous plasma cells and the formation of liver cell rosettes is more suggestive of AIH (Johnson *et al.*, 1991; Johnson and McFarlane, 1993). Whilst AIH and HCV can be distinguished histologically, they have certain features in common; in particular, the presence of lymphoid aggregates. It has been shown that these aggregates have a similar immunophenotype in AIH and HCV, which has lead to speculation that this could reflect a similar disease pathogenesis (Mosnier *et al.*, 1993). Clarification of the links between autoimmune hepatitis and hepatitis C could therefore throw light on the pathogenesis of both diseases. To examine this relationship between AIH and HCV in patients treated at the Royal Free Hospital, diagnostic liver biopsies from 19 patients originally diagnosed with autoimmune chronic hepatitis were analysed.

Materials and methods.

All patients coded in our files as having autoimmune hepatitis in whom there was archival biopsy material available were reviewed. These patients had been labelled as having autoimmune chronic active hepatitis based on clinical presentation, circulating autoantibodies, compatible histology, IgG levels, and absence of hepatitis B serology markers. Every specimen received in the histopathology department is examined by a pathologist and allocated a code by the pathologist according to the SNOMED system (Systemised nomenclature of medicine - College of American Pathologists, 1980). Each specimen is given a code which takes into account topography and morphology of the specimen; aetiology (if known); functional abnormalities and symptoms; disease; diagnostic and therapeutic procedures; and occupation of the patient. A database of these codes for every specimen is maintained in the department, allowing samples from patients with a particular disease to be retrieved. Using this database, a total of 36 biopsies from 23 patients with AIH were retrieved. However, on review of patient notes and results, not all of these patients had detectable ANA, SMA or LKM1 antibodies, and on one patient only post mortem material was available for testing. These patients were not included in the reported data. A total of 19 patients (27 biopsies) were fully investigated.

HCV testing of liver tissue and serum.

All human liver biopsies analysed in this study were taken for routine histopathological examination as clinically indicated. All biopsies were tested for HCV-RNA and albumin mRNA using RT-PCR as previously described (see chapter 2; appendices 2.2, 2.3 & 2.7). Testing was performed on at least three separate

occasions to ensure reliable results; only results in which the samples were positive for albumin were interpreted. Known positive and negative control material was included with each PCR batch (Kwok and Higuchi, 1989; Sallie *et al.*, 1992); experiments were only interpreted if all controls gave the correct result.

Where available, serum samples taken at the time of each biopsy were tested by the department of Medicine for anti-HCV antibodies and HCV-RNA. Measurement of anti-HCV antibodies in serum was performed using ELISA II (Ortho, Raritan, New Jersey) and by RIBA II (Chiron, Emeryville Ca. USA) assays. RNA was extracted from 100μl of serum using the method of Garson et al (1990), and tested for HCV-RNA using the same primers and methodology as were used for the tissue PCR analysis (Brown *et al.*, 1992a).

Clinical details of patients

Autoantibody results for each patient were reviewed. Testing was performed on patient serum by the Immunology department (RFH), using an indirect immunofluorescence technique with frozen rat liver and kidney as substrate (Thompson, 1991). Fourteen of the patients were seropositive for anti-nuclear (ANA) and anti-smooth muscle antibodies (SMA); 2 were positive for ANA only; 2 were positive for SMA only; and one was positive for anti-liver-kidney-microsomal (LKM1) antibody only (see Table 4.1). All patients were negative for other autoantibodies (eg anti-mitochondrial antibodies). Age at onset of disease, sex, levels of circulating immunoglobulin and histology of presenting biopsy are also detailed in Table 4.1. Each patient history was also checked for evidence of other autoimmune diseases, ethnic origin of patient and for any history of blood transfusion.

Table 4.1. Clinical and histological details of patients whose biopsies were tested for HCV-RNA by tissue PCR.

patient	sex	age at onset	Autoantibody profile	IgG (g/dm^3)	Histology
1	M	18	ANA 1:320 SMA >1:90	16.3 (N)	Cirrhosis
2	F	57	ANA 1:160	22.5 (↑)	Cirrhosis
3	F	22	ANA 1:40	6.2 (N)	CAH
4	F	35	ANA >1:40 SMA 1:40	23 (↑)	CAH
5	F	35	ANA 1:1280 SMA >1:90	29.9 (↑)	CAH
6	F	17	ANA 1:10 SMA >1:40	18.8 (↑)	Cirrhosis
7	F	44	ANA 1:40 SMA 1:40	33.6 (↑)	Acute hepatitis
8	F	29	ANA 1:40 SMA 1:40	11.2 (N)	Cirrhosis
9	F	30	SMA pos	14.6 (N)	Acute hepatitis
10	F	62	ANA 1:2560 SMA 1:640	10.6(N)	CAH
11	M	34	ANA >1:2560 SMA >1:90	32.7 (↑)	Cirrhosis
12	M	17	ANA 1:40 SMA >1:40	20.5 (↑)	Cirrhosis
13	M	31	LKM1 pos	15.9 (N)	Cirrhosis
14	F	11	ANA 1:2560 SMA 1:40	26.9 (↑)	Cirrhosis
15	F	22	ANA 1:640 SMA 1:320	20.9 (↑)	CAH
16	F	36	ANA 1:640 SMA 1:160	24.6 (↑)	CAH
17	M	39	ANA 1:160 SMA 1:90	20.8 (↑)	CAH
18	F	22	ANA 1:40 SMA 1:10	25 (↑)	CAH
19	F	25	SMA 1:160	11.3 (N)	Acute hepatitis

KEY: N = normal ↑ = raised CAH = chronic active hepatitis

Patient response to corticosteroid therapy was evaluated retrospectively by a consultant hepatologist (Dr G. Dusheiko) who was blinded to the HCV results. Each patient was allocated to responder or partial responder categories according to biochemical and symptomatic response to treatment. Responders were those patients with rapid normalisation of liver function tests (LFT's), together with a marked improvement in symptoms; partial responders were those with some decline in LFT's, but not a sustained normalisation.

Histopathology

Routine histological sections from the presenting biopsies of each patient were examined by two pathologists without knowing the results of any HCV tests. Features characteristic of HCV infection or AIH were looked for in each of the biopsies. In particular, inflammation, steatosis, lymphocytic infiltration and cholestasis were assessed and graded; as was presence or absence of hepatic rosettes. Presence of any lymphoid collections was described, along with presence and degree of bile duct damage, necroinflammatory parenchymal changes, and type of portal inflammatory infiltrate. The presence or absence of multinucleated hepatocytes was also noted. These observations were then correlated with HCV results and with patient response to steroids, to see if particular features could differentiate HCV infected biopsies from those with 'pure' AIH. The findings were tabulated and analyzed statistically using Fisher's exact test (suitable for the small number of cases).

Results

Five patients (26%) were positive in liver tissue for HCV-RNA by PCR (see table 4.2). Of these five, two patients were serologically positive for anti-HCV by second generation ELISA and RIBA assays, had HCV-RNA in serum, and were not treated with steroids. Both of these patients came from a 'high risk' area for HCV infection; neither had a history of blood transfusion or high risk behaviour; neither showed extrahepatic manifestations of autoimmune disease (see table 4.3).

One patient was positive for HCV-RNA in tissue only, and had no detectable antibodies or HCV-RNA in serum. This patient showed a partial response to corticosteroid treatment. She did not come from an endemic area for HCV; had no history of transfusion or high risk behaviour, but was an insulin dependent diabetic (a possible autoimmune disease).

For the remaining two tissue PCR positive patients, no serum was available for testing. Both of these patients showed a partial response to corticosteroid treatment. Neither came from a high risk area, but one had received a blood transfusion. This patient had no systemic autoimmune manifestations; the other patient had psoriasis and hypothyroidism (see table 4.3).

The remaining fourteen patients (73%), including the only patient with anti-LKM1 antibodies, were negative for HCV-RNA by tissue PCR in all of their biopsies tested. Eleven of these patients were also negative by serum PCR and RIBA II, although one gave a false positive result by ELISA II assay. Serum was unavailable for three of these patients. Twelve of the patients responded to treatment with corticosteroids; two showed a partial response. Only one of these patients had a possible history of blood transfusion; five (26%) had systemic manifestations of

autoimmune disease. Seven (50%) of these HCV negative patients came from areas with a high rate of HCV infection.

Table 4.2. Comparison of tissue PCR result to serum HCV test results and steroid responsiveness of the patient.

Patient	ELISA	RIBA	Serum PCR	Tissue PCR	steroid response
1	pos	pos	pos	pos	not treated
2	pos	pos	pos	pos	not treated
3	n.t.	n.t.	n.t.	pos	partial resp.
4	n.t.	n.t	n.t.	pos	partial resp.
5	neg	neg	neg	pos	partial resp.
6	neg	neg	neg	neg	resp.
7	n.t.	n.t.	n.t.	neg	resp.
8	n.t.	n.t.	n.t.	neg	resp.
9	neg	neg	neg	neg	resp.
10	neg	neg	neg	neg	resp.
11	pos	neg	neg	neg	resp.
12	neg	neg	neg	neg	partial resp.
13	neg	neg	neg	neg	resp.
14	n.t.	n.t.	n.t.	neg	resp.
15	neg	neg	neg	neg	resp.
16	neg	neg	neg	neg	resp.
17	neg	neg	neg	neg	partial resp.
18	neg	neg	neg	neg	resp.
19	neg	neg	neg	neg	resp.

KEY: n.t. = not tested

resp = response

partial response = decline in serum ALT in treatment

response = normalisation or near normalisation in serum ALT on treatment.

Table 4.3. Clinical history of each patient, and number of biopsies tested from each.

Patient	sex	age at onset	Other autoimmune disease	History of transfusion	Ethnic Origin	No of biopsies tested
1	M	18	None	None	ME	1
2	F	57	None	None	ME	1
3	F	22	None	yes	NE	1
4	F	35	Psoriasis, hypothyroid	None	NE	1
5	F	35	IDDM	None	NE	1
6	F	17	None	None	SA	1
7	F	44	IDDM, hypothyroid	None	NE	3
8	F	29	Arthralgia	None	SE	1
9	F	30	None	None	NE	1
10	F	62	None	None	SE	2
11	M	34	Ulcerative colitis	Possible	SE	1
12	M	17	None	None	NE	3
13	M	31	None	None	AC	1
14	F	11	None	None	AC	1
15	F	22	Pruritus	None	A	1
16	F	36	Excema, family history	None	NE	1
17	M	39	None	None	NE	2
18	F	22	None	None	NE	3
19	F	25	None	None	NE	1

KEY: IDDM = Insulin dependent diabetes mellitus
NE = Northern European SE = Southern European SA = South American AC = Afrocaribean A = Asian ME = Middle Eastern

Histopathology

HCV testing of liver biopsies allowed the differences between HCV positive and HCV negative biopsies to be studied in detail, although histological examination of the biopsy was done in a blind fashion.

At the time of biopsy, the average age was 48 years for patients with HCV positive biopsies and 33 years for patients with HCV negative biopsies. Among cirrhotic patients, the average age was 52 years in HCV positive and 26 years in HCV negative cases. One of the HCV negative cirrhotics was a 12 year old child. The histological changes in tissue HCV positive and HCV negative biopsies are summarised in Table 4.4. These results are shown in more detail in the publication which arose from this work (Savage *et al*, 1995).

Using Fisher's exact test, no significant differences were shown between HCV negative and HCV positive biopsies. However, some interesting linkages, though not statistically significant, were apparent. Lymphoid aggregates or follicles were seen in 80% of HCV positive biopsies, but in only 43% of HCV negative ones. Spotty necrosis was seen more in HCV positive cases (80%; compared to 36% in HCV negative); as was steatosis (60% vs 21%) and chronic hepatitis (60% vs 29%).

Bridging necrosis was more common in HCV negative biopsies (57% vs 20%), and whilst bile duct damage and periportal inflammation was seen in HCV positive biopsies, severe degrees of damage were only seen in the HCV negative cases. In addition, acute hepatitis, and presence of multinucleated giant hepatocytes was only found in HCV negative biopsies.

Cholestasis was seen more often in HCV negative biopsies; apoptosis,

Table 4.4. Histologic changes in tissue-HCV-positive and HCV-negative cases.

HISTOLOGIC CHANGES		HCV-positive n=5 (%)	HCV-negative n=14 (%)
Portal inflammation:			
Degree:	None	0 (0)	0 (0)
	Mild	2 (40)	3 (21)
	Moderate	3 (60)	9 (64)
	Severe	0 (0)	2 (14)
Type:	Plasmalymphocytic	2 (40)	7 (50)
	Lymphohistiocytic	2 (40)	5 (36)
	Mixed	1 (20)	2 (14)
Portal lymphoid aggregates:		4 (80)	6 (43)
Small bile duct damage:		3 (40)	16 (71)
Periportal inflammation:		4 (80)	13 (93)
Parenchymal Inflammation:			
Degree:	None	0 (0)	2 (14)
	Mild	2 (40)	3 (21)
	Moderate	2 (40)	6 (43)
	Severe	1 (20)	3 (21)
Type:*	Apoptosis	4 (80)	7 (50)
	Spotty necrosis	4 (80)	5 (36)
	Zone 3 necrosis	3 (60)	5 (36)
	Bridging necrosis	1 (20)	8 (57)
Steatosis:		3 (60)	3 (21)
Sinusoidal lymphocytic infiltration:		3 (60)	8 (57)
Cholestasis:		1 (20)	5 (36)
Giant hepatocytes		0 (0)	4 (29)
Hepatitic rosettes:		3 (60)	8 (57)
Overall diagnosis:			
	Acute Hepatitis	0 (0)	4 (29)
	Chronic Hepatitis	3 (60)	4 (29)
	Developing Cirrhosis	1 (20)	2 (14)
	Established Cirrhosis	1 (20)	4 (29)

*Each biopsy may show more than one of these features.

parenchymal inflammation and zone 3 necrosis were seen more in HCV positive samples; but these differences were not statistically significant.

Patients were also compared on the basis of their response to steroid treatment.

At time of biopsy, patients who had a good biochemical response to steroid treatment had an average age of 32 years while those who partially responded had an average age of 42 years.

The different histologic changes, as well as HCV positivity, in biopsies from patients who responded or partially responded to steroid treatment are shown in Table 4.5.

Two significant differences were found between those patients who responded to treatment with steroids and those who did not. Partial responders were more often HCV positive than responders (60% vs 0%, p= 0.01); and more of the partial responders had chronic active hepatitis than did the responders (80% vs 25%, p=0.05). Conversely, cirrhosis and acute hepatitis were more frequent in the responders (see table 4.5), although these differences were not statistically significant. Additionally, spotty necrosis was seen more often in partial responders, whereas cholestasis, giant hepatocytes and rosette formation were noted more often in responders (see table 4.5).

Table 4.5. Tissue PCR positivity and histologic changes in biopsies from responders and partial responders.

PCR POSITIVITY AND HISTOLOGIC CHANGES		Partial responders n=5 (%)	Responders n=12 (%)	p
PCR positivity		3 (60)	0 (0)	0.01
Portal inflammation:				
Degree:	None	0 (0)	0 (0)	
	Mild	2 (40)	2 (17)	
	Moderate	3 (60)	8 (67)	
	Severe	0 (0)	2 (17)	
Type:	Plasmalymphocytic	2 (40)	7 (58)	
	Lymphohistiocytic	1 (20)	4 (33)	
	Mixed	2 (40)	1 (8)	
Portal lymphoid aggregates:		4 (80)	9 (75)	
Small bile duct damage:		4 (80)	8 (67)	
Periportal inflammation:		4 (80)	11 (92)	
Parenchymal Inflammation:				
Degree:	None	0 (0)	2 (17)	
	Mild	2 (40)	2 (27)	
	Moderate	2 (40)	5 (42)	
	Severe	1 (20)	3 (25)	
Type:*	Apoptosis	3 (60)	7 (58)	
	Spotty necrosis	4 (80)	4 (33)	
	Zone 3 necrosis	3 (60)	4 (33)	
	Bridging necrosis	2 (40)	7 (58)	
Steatosis:		2 (40)	3 (25)	
Sinusoidal lymphocytic infiltration:		3 (60)	7 (58)	
Cholestasis:		0 (0)	5 (42)	
Giant hepatocytes		0 (0)	4 (33)	
Hepatitic rosettes:		1 (20)	8 (67)	
Overall diagnosis:				
	Acute Hepatitis	0 (0)	4 (33)	
	Chronic Hepatitis	4 (80)	3 (25)	0.05
	Developing Cirrhosis	1 (20)	1 (8)	
	Established Cirrhosis	0 (0)	4 (33)	

*Each biopsy may show more than one of these features.

Discussion

A cohort of 19 patients with autoantibody positive chronic hepatitis was examined. These patients had previously been labelled as having autoimmune chronic active hepatitis based on clinical presentation, circulating autoantibodies, compatible histology, IgG levels, and absence of Hepatitis B serology markers. The advent of HCV testing has allowed reappraisal of such patients, in whom the diagnosis of autoimmune hepatitis was sometimes made on the basis of a clinical "best fit" in the absence of a definitive marker.

Five of these 19 patients were positive for HCV RNA in their liver biopsies. Three of these five patients were treated with steroids, and showed a partial response to therapy. These results agree with Silva *et al* (1993) and Magrin *et al* (1991b), in that patients with "classical" type I autoimmune hepatitis, ie young females with severe disease responsive to steroid treatment, are not usually HCV-RNA positive. However these five HCV RNA positive patients all had significant levels of autoantibodies ($\geq$ 1:40); three patients had both ANA and SMA antibodies, considered a good indication of true autoimmune hepatitis (Cassani *et al.*, 1992), and two had extrahepatic manifestations of autoimmune disease. These findings emphasize the difficulty of differentiating autoimmune hepatitis with viral markers from chronic viral hepatitis with autoimmune features (Czaja, 1991). Similar results have been reported by Pawlotsky *et al* (1993), who described five patients with presenting features of AIH, including extrahepatic autoimmune disease, in whom steroid therapy was ineffective, and by Czaja and co-workers, who found in a retrospective study that patients with AIH and markers of HCV infection could respond to steroid treatment, but would relapse when treatment was

withdrawn (Czaja *et al.*, 1995).

The temporal relationship of HCV infection to autoantibody production is complex, and will require further prospective study. It was not possible in this study to ascertain whether autoantibodies were triggered by HCV infection in these patients, or if HCV was superimposed on an underlying autoimmune disease. It was also not clear what influence each factor had on the pathogenesis of their liver disease. Two of the patients described here had anti-HCV antibodies and were serum HCV-RNA positive; both of these patients came from areas with high rates of HCV infection and probably had HCV-induced liver disease with associated autoantibodies. Chronic HCV-hepatitis is usually characterised by a lack of ANA and SMA (Manns, 1989) although presence of these antibodies is occasionally associated with infection (Abuaf *et al.*, 1993).

Hepatitis C virus can induce an autoantibody response to a host epitope named GOR (Mishiro *et al.*, 1990; Lau *et al.*, 1993a). This anti-GOR antibody has been particularly linked to type II, anti-LKM1 positive hepatitis (Lunel *et al.*, 1992; Michel *et al.*, 1992; Todros *et al.*, 1991; Strassburg and Manns, 1995); it has been suggested that HCV infection can trigger anti-LKM1 antibodies because of a cross-recognition between cytochrome P-450 IID6 (the antigen for anti-LKM1) and the HCV polyprotein (Lunel *et al.*, 1992). Whilst the only anti-LKM1 positive patient in this group was HCV-RNA negative, it is significant that Lunel *et al* (1992) have proposed that patients with anti-LKM1 antibody positive chronic hepatitis be divided into true autoimmune hepatitis type 2 and anti-LKM1 positive HCV-virus associated hepatitis. Indeed, Magrin *et al.* (1991a) proposed that a subgroup of type I autoantibody positive patients who do not respond to corticosteroids and who are

HCV positive must be distinguished from AIH and may need to be considered for other treatment such as interferon. However, therapeutic response to corticosteroid does not necessarily separate AIH from HCV hepatitis. Steroids may suppress ALT's in HCV disease, and interferon may aggravate autoimmune disease (Wright and Millward-Sadler, 1985; Vento *et al.*, 1989; Shindo *et al.*, 1992). Two of the HCV negative patients in this group showed only partial response to corticosteroids; all of the HCV positive patients treated with corticosteroids showed a partial response to treatment.

All these findings show that patients with type I AIH who do not respond well to treatment with corticosteroids must be thoroughly investigated for HCV infection; these tests should include tissue PCR analysis since, as demonstrated by these results, the liver can be HCV positive without detection of HCV markers in serum (Brechot, 1993).

In general, the tissue PCR results agreed with serum antibody and PCR results; all tissue PCR negative patients tested were negative by RIBA II and serum PCR. Two of the patients found to be tissue PCR positive were also positive by serum tests; however, one patient was positive only by tissue PCR, and was negative for structural and non-structural anti-HCV antibodies, and for HCV-RNA in serum. Circulating HCV-RNA has been detected in patients sero-positive for anti-GOR but negative for anti-HCV (Mishiro *et al.*, 1990); viraemia has also been noted to be intermittent in patients with chronic hepatitis C (Garson *et al.*, 1990b; Abe *et al.*, 1992). In addition, Brechot (1993) has reported that HCV-RNA can be detected in some liver biopsies even when serum tests are negative; recent quantitative testing has shown that HCV-RNA is present at a higher concentration in

the liver than in the serum of an infected individual (Yatsuhashi *et al.*, 1995). A contributing factor in this result may be that as this was a retrospective study, much of the serum antibody and RNA testing was carried out on stored sera; it is possible that the serum from this patient was not stored correctly leading to the discrepant result. Bresters *et al.* (1992a) have used a similar method to the one described here for detection of HCV-RNA in paraffin embedded material and found that the sensitivity of detection compares well with that of frozen liver material and serum RNA detection.

Importantly, this study of tissue HCV-RNA also allowed the direct investigation of HCV infection in five patients from whom serum taken at the time of biopsy was not available. Three of these patients were negative for HCV-RNA in tissue; two were positive. Testing for HCV-RNA by tissue PCR on formalin fixed, paraffin embedded material provides a further predictable means of establishing the presence of HCV infection in archival material; a valuable additional test which may be more sensitive or appropriate in some circumstances.

Testing of the biopsies by tissue PCR allowed the assessment of any differences between HCV positive and HCV negative cases, and indicated that these biopsies could be characterised by two separate sets of histological features. The HCV negative group showed an overall histological picture characterized by more severe portal, periportal and parenchymal inflammation. Plasma cells predominated in the inflammatory infiltrate, and bridging necrosis rather than spotty necrosis or apoptosis was the predominant type of parenchymal necrosis. Multinucleated giant hepatocytes, severe bile duct damage and cholestasis were more commonly seen in this group than in the HCV positive group. Conversely, the HCV positive group

showed a histological picture characterized by less severe portal, periportal and parenchymal inflammation, by lymphoid aggregates in portal tracts and by mild macrovesicular steatosis. Apoptosis, spotty necrosis and zone 3 necrosis were the predominant types of parenchymal necrosis.

In agreement with these findings, Bach *et al.* (1992) described a severe degree of piecemeal necrosis in 81% of biopsy specimens from patients with autoimmune hepatitis (AIH) but in only 10% of biopsies from patients with chronic hepatitis C (CH-C). They also reported that more severe lobular inflammation was seen in AIH (70%) compared to CH-C (38%), and that bridging hepatic necrosis (BHN) was the predominant form seen in AIH cases. Lymphoid aggregates/follicles in portal tracts were present in 49% of patients with CH-C compared with 10% of patients with AIH (Bach *et al.*, 1992). Scheuer *et al.* (1992) found easily recognisable lymphoid aggregates or follicles with germinal centres in 78% of hepatitis C patients as compared to 52% of hepatitis B cases. They detected follicles with germinal centres in 11% of patients with hepatitis C but in none of hepatitis B patients. In agreement with these findings, the present study showed definite lymphoid aggregates in 80% of tissue HCV-positive cases, but in only 43% of HCV-negative ones.

In conclusion, histologic features, suggestive but not conclusive of, HCV infection, as well as of poor response to steroids include: mild portal and periportal inflammation with few, if any, plasma cells; portal lymphoid aggregates or follicles; parenchymal necrosis in the form of apoptosis, spotty and zone 3 necrosis, and mild macrovesicular steatosis. Steroid responsive autoimmune disease is indicated by severe and confluent necrosis, bridging necrosis, plasma cells, hepatic rosettes,

giant cell transformation of liver cells and histological cholestasis.

Conclusions

These results support the general view on HCV and autoimmune hepatitis in this country: that in the majority of patients with classical AIH (young females, responsive to corticosteroid treatment) hepatitis C virus is not a common factor. However, HCV can be present in some patients with significant levels of autoantibodies. If the response to steroids is poor, it is important to establish that HCV is not a contributing factor to liver disease. A subgroup of patients testing positive for HCV-RNA do have antibodies to nuclei, liver kidney microsomes and smooth muscles. This subgroup respond poorly to prednisolone but may benefit from interferon (Sherlock, 1993).

CHAPTER 5

RECURRENCE OF HCV INFECTION AFTER ORTHOTOPIC LIVER TRANSPLANT

Introduction

HCV-associated end stage liver disease has now become an important indication for OLT (Smedile *et al.*, 1991). From the earliest studies of HCV in the transplant situation, it became clear that HCV infection recurred post transplant. However, there was some controversy about how often HCV recurred, and how significant recurrence was, particularly in studies using only ELISA testing post transplant. Martin *et al* (1991) studied 6 patients who were anti-HCV positive before transplant. One year after transplant, 5 remained positive for anti-HCV, but only 2 of these had clinical and histological evidence of hepatitis. Read *et al* (1991) studied the incidence of anti-HCV post transplant and its relation to hepatitis. They reported that loss of anti-HCV was frequent, and in their series only 4/13 patients (31%) developed post transplant hepatitis. With the development of PCR for detection of HCV-RNA, Porterucha *et al* (1992) looked at the relevance of HCV in post transplant hepatitis of uncertain origin. They found that 60% of patients with chronic hepatitis of unknown origin post transplant were HCV-RNA positive. However, only 47% of these had detectable anti-HCV. This data showed the unreliability of anti-HCV testing in these immunosuppressed patients. Indeed, it has recently been shown that anti-HCV production is suppressed more than other anti-viral antibodies in immunosuppressed patients (Kudo *et al.*, 1995). Because of this, polymerase chain reaction detection of HCV-RNA has become the method of choice for studying this group of patients. Most often this is assessed in the serum, and direct evaluation of liver tissue post OLT has been infrequent.

In this study, the recurrence of HCV post transplant was investigated by testing sequential post transplant biopsies for HCV-RNA by tissue PCR. Although

much work has been done on HCV associated hepatitis post transplant, the timing of reinfection of the liver has rarely been investigated, and histology at time of detection of HCV-RNA has not been looked at. The characteristic features of chronic HCV infection, including mononuclear portal inflammation and portal lymphoid aggregates or follicles, have been reported in the post transplant situation (Ferrell *et al.*, 1992; Thung *et al.*, 1993). Atypical features, such as extensive hepatocyte swelling (Ferrell *et al.*, 1992) have also been noted. It is thus difficult to distinguish recurrent hepatitis C from other common features of post transplant liver biopsies, such as rejection, drug related injury and sepsis (Hertzler and Millikan, 1991). The evolution of the histological changes caused by HCV reinfection post transplant remains to be clarified.

The post transplant situation provides an opportunity to look at *de novo* infection of a liver and potentially to characterise acute infection with HCV. Hence, useful data on the natural history and histology of HCV could be obtained, even though these patients are immunosuppressed and may show a different evolution of disease. In addition, the course of HCV infection in these immunosuppressed patients could throw light on the pathogenesis of HCV-induced liver damage and could clarify whether HCV is directly cytopathic or if all damage is immune mediated.

The objectives of this study were to determine at what point HCV-RNA and HCV antigen could be detected in liver biopsies post transplant; to assess any specific histological features of graft reinfection, and to study the evolution of these features following recurrence of HCV infection.

Materials and Methods

The total number of liver transplants carried out between November 1990 and October 1992 at RFH was 79. Thirteen of these patients had end-stage HCV cirrhosis. Four patients were excluded from the study due to insufficient follow-up; nine patients (1 - 9) were considered further. One additional patient (patient 10), transplanted in 1994, has also been included, as frozen material was available on this patient's post transplant biopsies. All patients were serum PCR positive for HCV-RNA prior to transplantation. Nine were anti-HCV positive by second generation ELISA (Abbott); the remaining patient had common variable immunodeficiency, and was anti-HCV negative. HBV infection was excluded by serological testing in all patients. One patient underwent a second transplant 5 days post operatively for hepatic arterial thrombosis; only data on the second transplant is reported here. All patients received a standard immunosuppressive regime post transplant, including methylprednisolone, azathioprine and cyclosporine, with additional immunosuppressive therapy as necessary. Mild rejection was not routinely treated.

Liver biopsies were taken post transplant according to protocol, or as clinically indicated. For patients 1 - 9, all biopsies were formalin fixed and routinely processed to paraffin wax. In patient 10, where the biopsy was big enough, the biopsy was split, and the first 2cm put into formalin, and processed routinely. The remainder was put onto saline soaked gauze, then transported immediately to the laboratory, mounted on cork with OCT (RA Lamb), and frozen via cooled isopentane (BDH) in liquid nitrogen (BOC). Frozen biopsies were stored at -70°C until required.

A total of 81 samples taken from these patients were tested for HCV-RNA, including 70 post transplant biopsies. The number of biopsies tested from each patient ranged from 3 - 12. All samples were cut and tested in duplicate, on two separate occasions. HCV-RNA positive and negative tissue controls were included in each experiment; only experiments in which the positive control was positive and all negative controls negative were assessed. If a discordant result was obtained for the duplicate tests, the sample was tested for a third time. RT-PCR for albumin mRNA was performed on all samples; only samples which were positive for albumin were assessed. The standard tissue RT-PCR technique was used to detect the virus, as described in chapter 2. Tissue PCR was only carried out once all diagnostic requirements for the biopsy had been satisfied.

Testing for HCV-RNA in serum was performed by the department of Medicine, RFH, on samples collected both before and after transplant. Serum samples were stored at -70^{0}C until testing; RNA was extracted using the method of Garson *et al* (1990a), and reverse transcription and amplification performed using the same primers as used for the tissue PCR (see chapter 2).

Six frozen tissue samples from patient 10 were tested for the presence of HCV antigen using human anti-HCV^{FITC}. Cryostat sections (5μm) were post fixed in chloroform (BDH) for 5 minutes, then incubated for 40 minutes with FITC conjugated human anti-HCV antibody (see chapter 3). Slides were reviewed on a Zeiss standard 14 fluorescence microscope. Positive slides were scored as described in chapter 3, according to area and intensity of staining. Photomicrographs were taken using a Zeiss MC63/35 camera on Fujichrome 1600 slide film, and developed by 2 stop push processing. After developing, all transparencies were transferred

onto a photo CD (Kodak). They were then imported onto a Dan multimedia PC and printed via Corel Multimedia onto HQ gloss paper (Epson) using an Epson stylus colour printer.

Routine histological reports on the first biopsies for each patient in which HCV-RNA was detected were reviewed to see what the original diagnosis had been. In addition, all reports on post transplant biopsies were reviewed to see how many were diagnosed as showing post transplant hepatitis; how many showed features typical of HCV; and at what point recurrence of HCV was suspected on histological grounds.

Routine liver function tests (alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP)) were reviewed for each patient to see if changes occurred at the time of first detection of HCV-RNA in a liver biopsy, and to see if these changes were typical of hepatitis outside the transplant situation - ie transaminases raised with ALT > AST (McIntyre and Rosalki, 1991).

A total of 107 liver samples (including 96 post transplant biopsies) from the ten patients were reviewed histologically by this observer and a pathologist in a blind fashion. Biopsies were scored for the presence or absence of a number of features important in HCV infection or rejection. These features were; the presence and type of portal inflammation; eosinophils; interface hepatitis; lobular inflammation; acidophil bodies; vacuolation of hepatocytes; and endotheliitis. Zone 3 necrosis, ballooning and fibrosis were noted, as was bile duct damage, defined by the presence of either inflammatory infiltration of epithelial cells, stratification, vacuolation or degeneration of epithelial cells, or mitotic activity (Portmann and

MacSween, 1994). The biopsies in which HCV was first detected were reviewed a second time in the light of the PCR results to see if any features were visible to help distinguish HCV recurrence from other conditions.

RESULTS

Detection of HCV-RNA by PCR

Recurrence of HCV infection was detected in the liver tissue of all ten patients (see figure 5.1). One of these patients did not have recurrence of HCV-RNA detected in serum during the follow up period (see table 5.1). Patients who were anti-HCV positive before transplant (9/10) remained anti-HCV positive after OLT; the one patient with CVID who was anti-HCV negative before OLT remained anti-HCV negative throughout. Patients were followed up for between 43 and 1185 days, with a mean of 656 days. The interval between transplant and biopsy detected HCV-RNA recurrence ranged from 0 to 571 days with a mean of 84 days.

Table 5.1 HCV recurrence in liver and serum

Patient	Follow up (days) post transplant	Liver HCV-RNA recurrence (days)	Serum HCV-RNA recurrence
1	212	10	+
2	1195	80	+
3	306	117	-
4	701	5	+
5	1088	12	+
6	43	15	+
7	926	22	+
8	1109	571	+
9	748	0	+
10	243	8	+
	Mean 656	Mean 84	

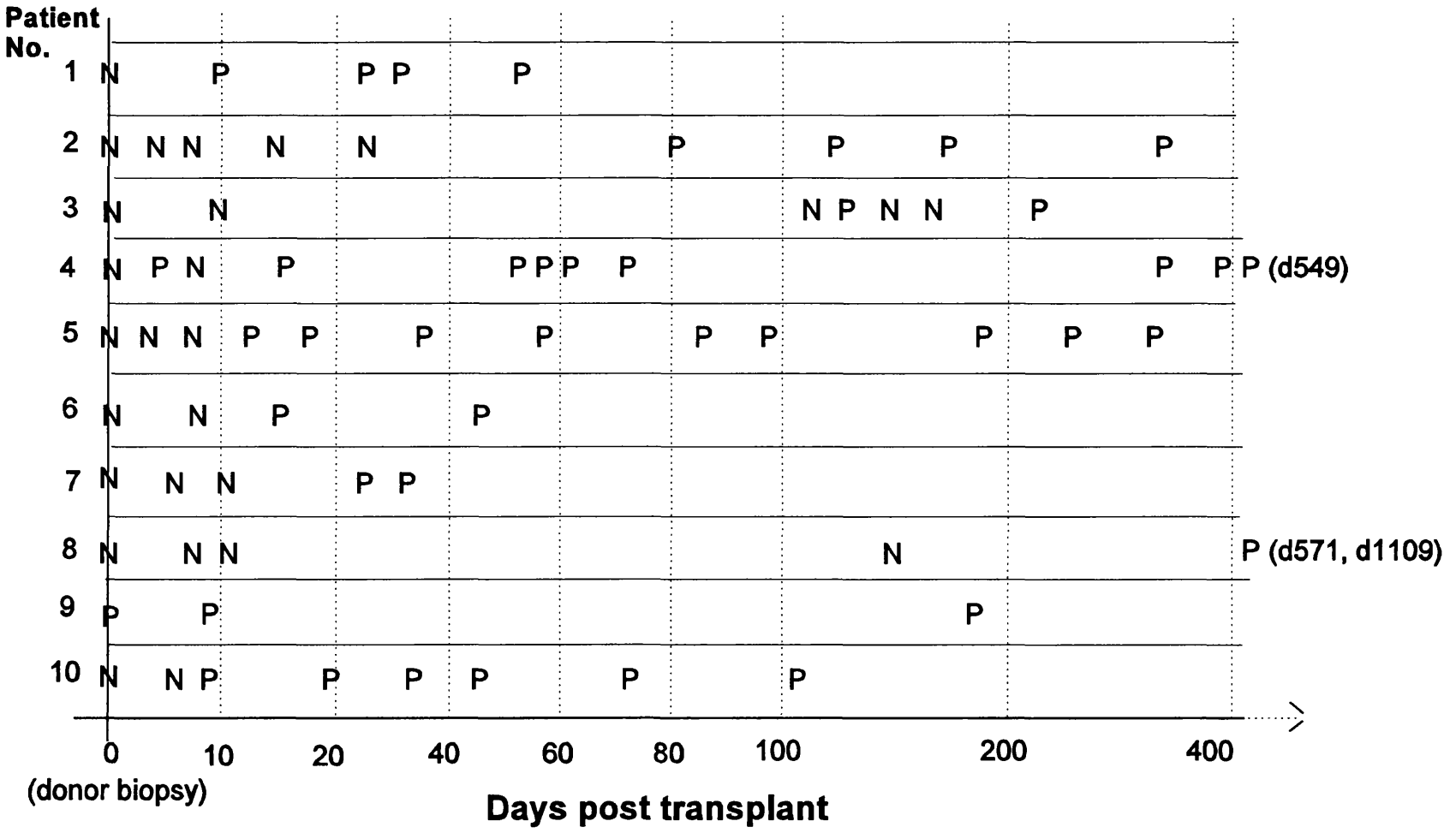


Figure 5.1 HCV RT-PCR results for post transplant liver biopsies against time. Key: P = positive for HCV-RNA N = negative for HCV-RNA

The number of post transplant biopsies tested per patient ranged between 3 and 12 (mean of seven per patient). In general, once HCV was detected in the transplant liver, subsequent biopsies were also HCV-RNA positive, although two patients (patients 3 and 4) were intermittently positive (see figure 5.1). Seven out of 10 patients had HCV-RNA detectable in their own liver; PCR failed on 3/10 explants, which were negative for both HCV-RNA and albumin mRNA. All ten organ donors were anti-HCV negative; 9/10 donor biopsies were HCV-RNA negative by tissue PCR. Nine out of ten patients had HCV recurrence detected in serum (see table 5.1); in one of these patients, from whom sequential serum samples were available, HCV-RNA was detected in the liver before it was detectable in the serum (see figure 5.2)

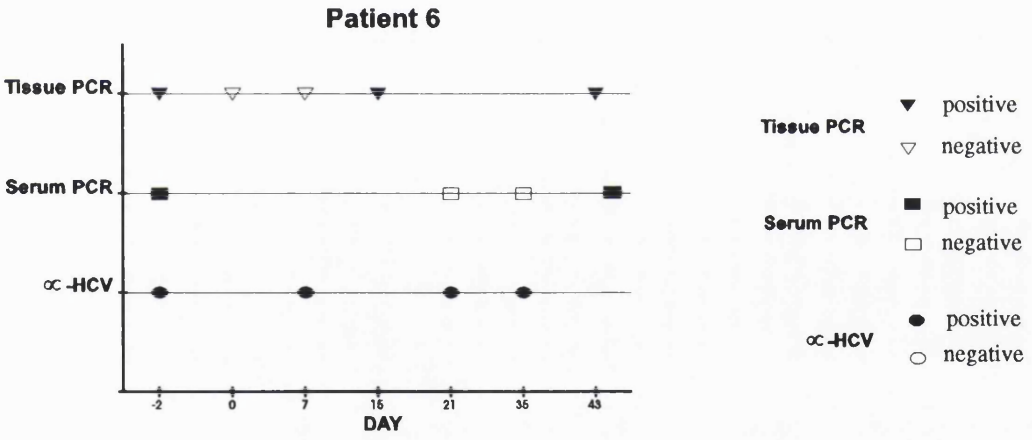


Figure 5.2 Comparison of HCV testing in tissue and serum from patient 6.

HCV antigen detection

Six samples from patient 10 were tested for the presence of HCV antigen using a human anti-HCV^{FITC} antibody. These samples included tissue from the recipient liver, and five post transplant biopsies. The recipient liver, and 4/5 post transplant biopsies, showed expression of HCV antigen (see table 5.2).

Table 5.2 Summary of HCV-RNA and immunostaining results on samples tested with anti-HCV^{FITC}.

	HCV-RNA by tissue PCR	Results with anti-HCV^{FITC}	
		Area	Intensity
Explant liver	+	++	+++
day 8 biopsy	+	--	--
day 20 biopsy	+	+	+++
day 33 biopsy	+	+++	+++
day 42 biopsy	+	+++	+++
day 69 biopsy	+	++++	++++

Key:

Area of staining		Intensity of staining	
+	≤ 5% of hepatocytes stained	+	Pale staining
++	5 - 50% of hepatocytes stained	++	Medium staining
+++	≥ 50% of hepatocytes stained	+++	Intense staining
++++	~100% of hepatocytes stained	++++	Very intense staining

As shown in table 5.2, HCV antigen was detectable by day 20 post transplant, and by day 33 had exceeded the total amount of staining seen pre-transplant. By day 69, virtually all hepatocytes had detectable HCV antigen, and most were very intensely stained by the anti-HCV^{FITC} (see figures 5.3 - 5.8)

Histopathology of post transplant biopsies

The routine histopathology reports (assessed previously in ignorance of these HCV tests) were reviewed for the diagnosis in the first biopsy in which HCV-RNA was detected, the time of first suspected recurrence of HCV, and the number of post transplant biopsies showing features of hepatitis C. The results are summarised in table 5.3. In the biopsy in which HCV-RNA was first detected, 7/10 biopsies showed rejection of some degree, one biopsy showed possible biliary obstruction, one biopsy was near normal liver, and only one biopsy showed acute hepatitis. Post transplant hepatitis was seen in 7/10 patients. In 6/10 patients this hepatitis was

thought to be recurrent HCV on morphological grounds. This histological recurrence of HCV was reported at a mean time of 134 days post transplant (range 72 - 286 days).

The diagnosis on the most recent biopsy available was also reviewed to assess the outcome for each patient. The results are summarised in table 5.4. Six out of ten patients had histology suggestive of or consistent with recurrent HCV in their most recent biopsy. These biopsies were taken at a range of 43 - 1195 days post transplant. Two of the ten patients (20%) died post transplant - both had features suggesting severe recurrent hepatitis C (patients 4 and 5). One patient (4) had severe fibrosis but without cirrhosis; the other (5) had established cirrhosis. Patient 4 died of severe respiratory insufficiency at day 701 post transplant; patient 5 died of pneumonia at day 1088 post transplant.

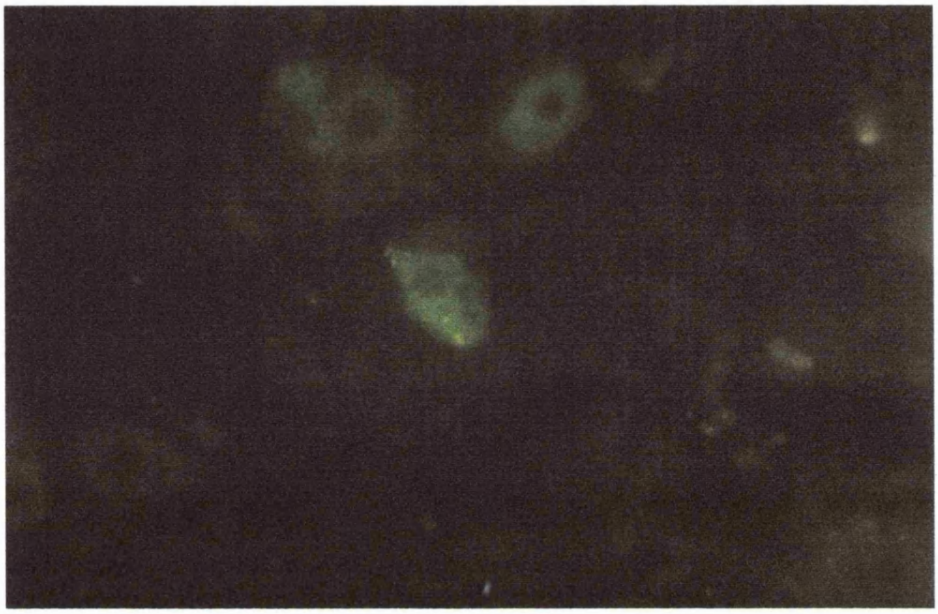


Figure 5.3 Photomicrograph of frozen section from the cirrhotic explant liver of patient 10, stained with anti-HCV. Three positive hepatocytes can be seen. Mag x660.

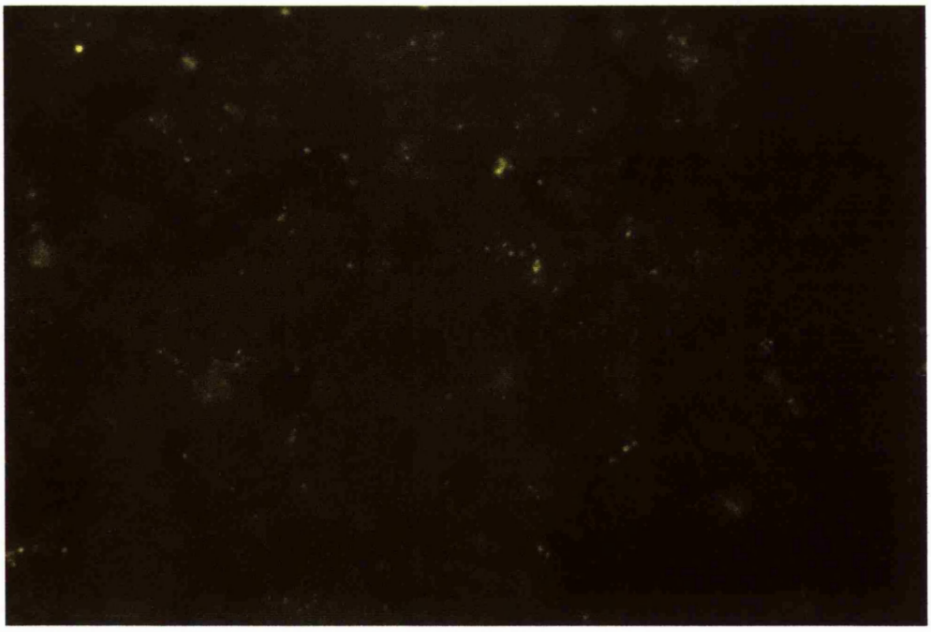


Figure 5.4 Section from same specimen without antibody. Some spots endogenous fluorescence can be seen, but no fluorescent staining of hepatocytes is observed. Mag x660.

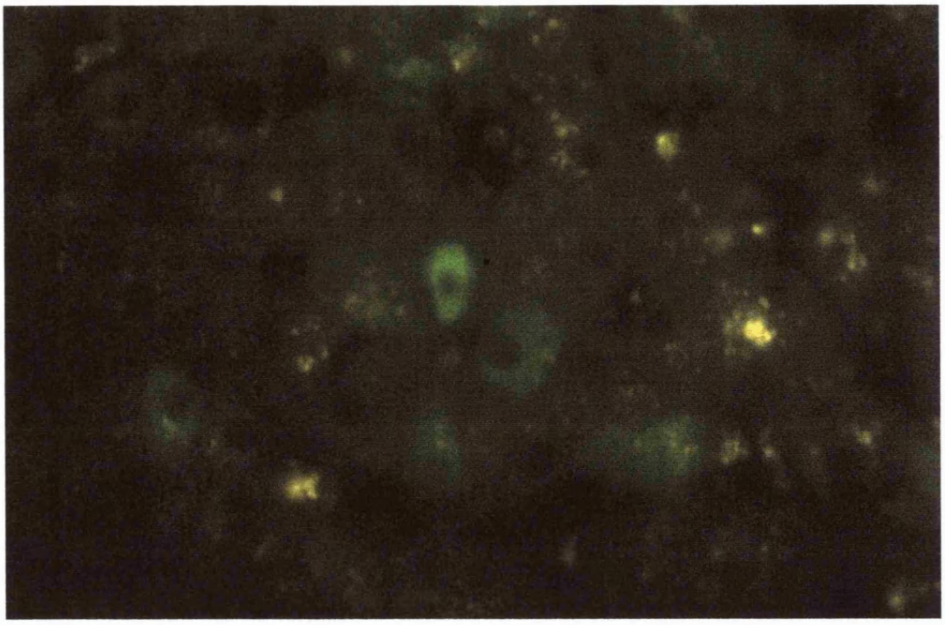


Figure 5.5 Biopsy taken 20 days after patient 10 had a liver transplant for end stage HCV cirrhosis. The biopsy is stained with anti-HCV. One brightly stained hepatocyte is seen in the centre of the picture. Several other palely stained hepatocytes can also be seen. Mag x660.

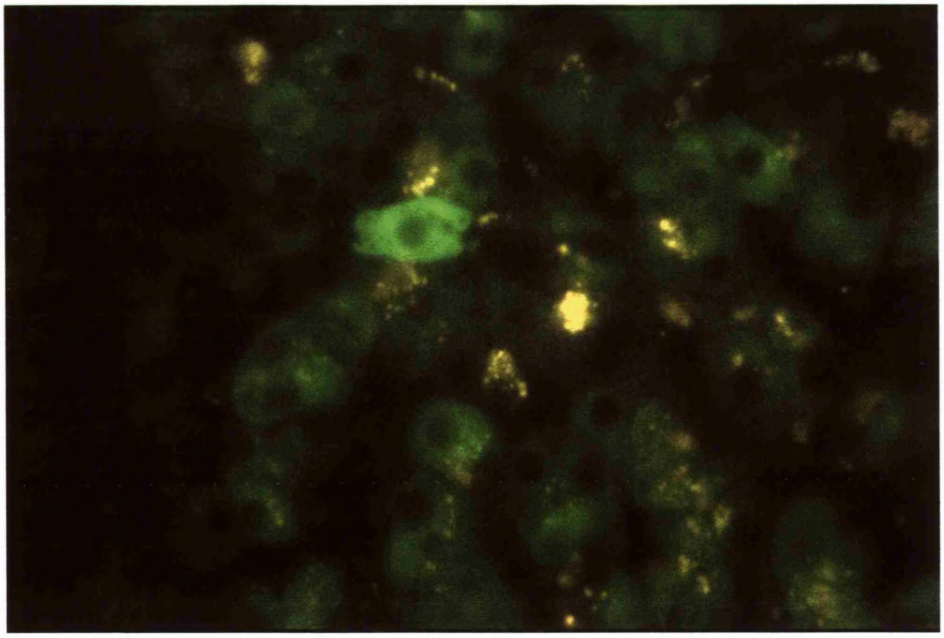


Figure 5.6 Biopsy taken at day 33 post transplant. One very brightly stained hepatocyte can be seen. Most of the hepatocytes in this field are positive for HCV antigen. Both diffuse and spotty cytoplasmic staining are seen. Mag x660.

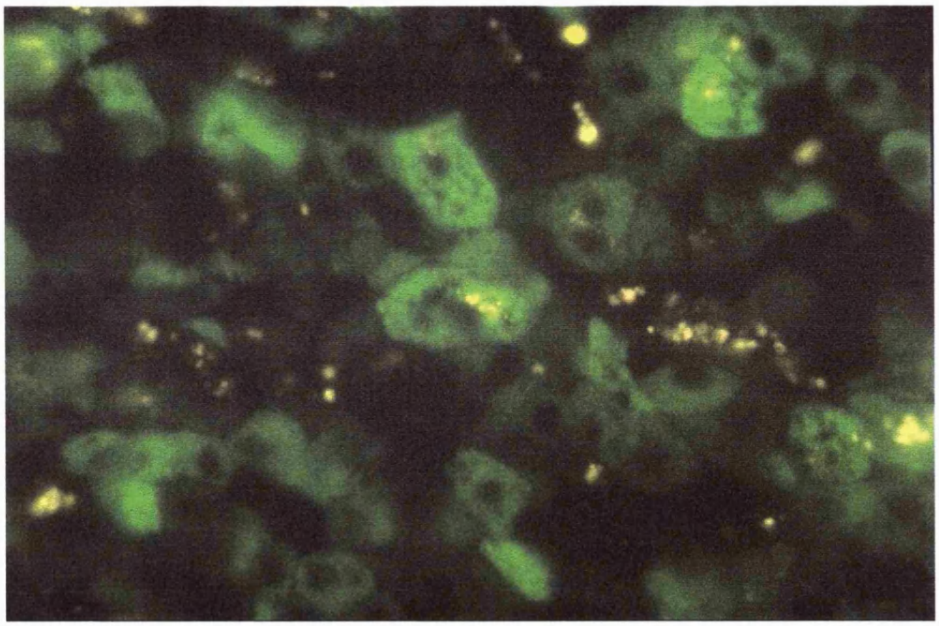


Figure 5.7 Day 42 post transplant biopsy stained with anti-HCV. More of the hepatocytes in this biopsy showed strong staining than in that taken at day 33 PT. Mag x660.

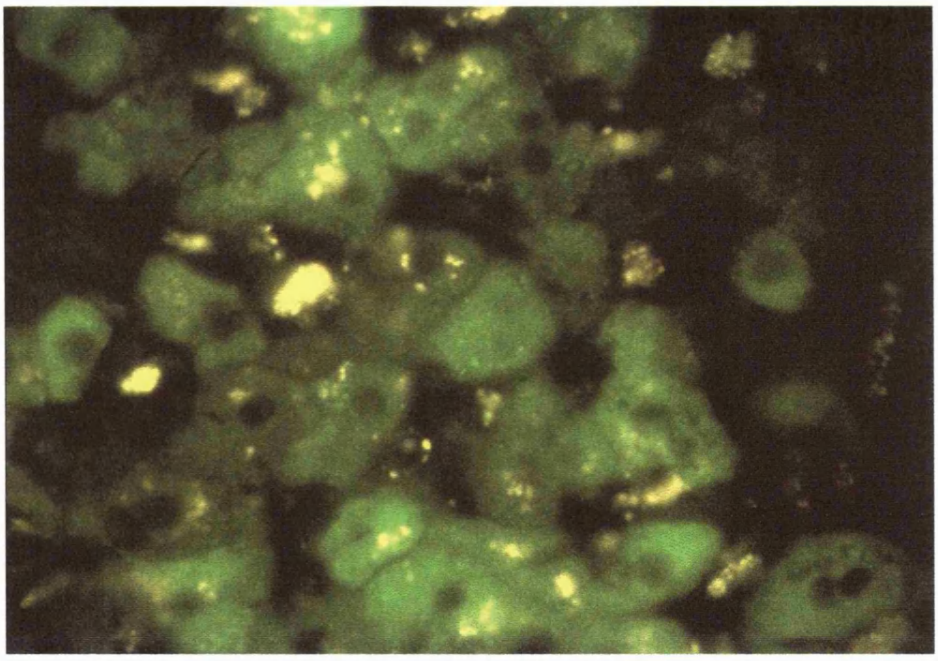


Figure 5.8 By day 69 post transplant, the majority of the hepatocytes were intensely stained with the anti-HCV antibody. Mag x660.

Table 5.3 Summary of PCR and histology results for each patient.

Patient	Day of first detected HCV-RNA recurrence in liver	Routine diagnosis at time of HCV-RNA recurrence	Day of reported histological recurrence of hepatitis C	Total number of post transplant biopsies	Number of biopsies showing features of hepatitis C
1	10	Moderate cellular rejection	72	7	2
2	80	Mild cellular rejection	N/R	12	N/R
3	117	Acute viral hepatitis	117	8	1
4	5	Moderate cellular rejection	77	15	3
5	12	?Biliary obstruction	93	17	5
6	15	Mild cellular rejection	N/R	4	N/R
7	22	Mild cellular rejection	286	14	4
8	571	Moderate cellular rejection	N/R	6	N/R
9	0	Near normal liver	196	5	2
10	8	Mild cellular rejection	79	16	4

Table 5.4 Histology of most recent biopsy from patients

Patient	Histology of most recent biopsy (days post transplant)
1	Expansion of portal tracts with a moderate portal inflammation and occasional lymphoid aggregates. (day 212) **Suggestive of recurrent HCV**
2	Mild lymphocytic infiltration in some portal tracts. Nuclear vacuolation and mild steatosis - ?diabetes (day 1195)
3	Canalicular cholestasis, foci of parenchymal necrosis. Aggregates of foamy cells; bile duct loss. (day 306) **Suggestive of CMV**
4	Severe hepatitis with marked lobular activity. Fibrosis, portal-portal linking, and portal-central bridging seen, with portal lymphoid follicles. (day 641) **Consistent with recurrent HCV.**
5	Established cirrhosis with moderate inflammation and occasional lymphoid aggregates. (day 1058) **Consistent with recurrent HCV**
6	Mild cellular rejection. (day 43)
7	Dense mononuclear portal infiltrate. Mild fatty parenchymal change, with occasional apoptotic bodies. (day 926) **Suggestive of recurrent HCV**
8	Mild non-specific hepatitis with minimal rejection. (day 1109)
9	Portal tracts with lymphocytic infiltration. Mild parenchymal infiltrates seen. (day 748) **Consistent with recurrent HCV**
10	Portal expansion with chronic inflammation and lymphoid aggregates. Mild interface hepatitis and moderate parenchymal inflammation. (day 243) **Suggestive of recurrent HCV**

Review of all liver biopsies from the patients was carried out in a blind fashion by two observers (this observer and a histopathologist). This revealed the detailed chronology of changes in biopsies post transplant. Features typical of chronic hepatitis C developed in most cases (Table 5.5). A lymphoid portal infiltrate was seen in 21/30 (70%) of biopsies > 6 months. Lymphoid aggregates were seen in 16/30 (53%) of biopsies > 6 months compared with 2/66 (3%) < 6 months. Interface hepatitis was seen in 9/66 (14%) biopsies taken within 6 months

Table 5.5. Temporal sequence of histopathological changes

Histological Features (n =number of biopsies)	**Pre-transplant (%)** (11)	**0-1 mth (%)** (36)	**1-2 mth (%)** (7)	**2-4 mth (%)** (15)	**4-6 mth (%)** (8)	**>6mth (%)** (30)
Lymphoid portal inflammation	11 (100)	0 (0)	2 (29)	10 (66)	3 (37)	21 (70)
Lymphoid aggregates	8 (73)	0 (0)	1 (14)	1 (7)	0 (0)	16 (53)
Bile duct damage	0 (0)	11 (31)	0 (0)	4 (27)	4 (50)	4 (13)
Interface hepatitis	11 (100)	0 (0)	0 (0)	5 (33)	4 (50)	18 (60)
Lobular inflammation	7 (64)	20 (56)	6 (86)	15 (100)	8 (100)	28 (93)
Liver cell vacuolation	5 (45)	17 (47)	1 (14)	9 (60)	5 (63)	22 (73)
Acidophil bodies	9 (82)	22 (61)	5 (71)	15 (100)	8 (100)	21 (70)
Endotheliitis	0 (0)	13 (39)	3 (43)	8 (53)	3 (38)	8 (27)
Cholestasis	4 (36)	13 (36)	2 (29)	10 (66)	7 (88)	4 (13)
Zone 3 damage	1 (9)	17 (47)	4 (57)	12 (80)	6 (75)	13 (43)
Eosinophils	3 (27)	24 (66)	3 (43)	5 (33)	5 (63)	13 (43)

of transplantation compared with 18/30 (58%) biopsies > 6 months. Eosinophils were identified within portal tracts at all stages after transplantation, but most commonly between 0 and 1 month (66%). In 3/11 (27%) biopsies taken before transplant (including the explanted liver), eosinophils were identified. On an individual biopsy basis, eosinophils did not discriminate between cellular rejection and HCV recurrence.

None of the cases showed chronic ductopenic rejection. Hepatocyte vacuolation, lobular inflammation and acidophil bodies were seen at all stages post-transplantation, as was zone 3 damage (hepatocellular ballooning, necrosis and zone 3 haemorrhage). 2/10 patients showed moderate or severe zone 3 fibrosis by the end of the follow up period (641 days and 395 days respectively). Cholestasis was also seen at all stages post-transplantation and was present in up to 66% of biopsies taken at 2-4 months and 88% between 4 and 6 months. Bile duct damage was seen most commonly between 4 and 6 months (50%), and declined in biopsies taken > 6 months (13%). Endotheliitis was seen most commonly in biopsies taken within 6 months and declined after 6 months.

The first post transplant liver biopsy in which HCV-RNA was detected was reviewed for each patient in the light of the HCV-RNA results (see table 5.6). A mononuclear portal infiltrate was only seen in 3/10 patients (30%), whereas a mixed infiltrate containing eosinophils was seen in 7/10 biopsies (70%). Lymphoid aggregates and interface hepatitis were absent. Seven out of ten biopsies (70%) showed mild/moderate or severe lobular inflammation and 6/10 (60%) showed acidophil bodies. Zone 3 damage was also common, and present in 6/10 biopsies (60%). Bile duct damage was seen in 2/10 cases (20%); enotheliitis in 3/10 (30%) and cholestasis in 5/10 biopsies (50%).

Table 5.6. Assessment of histological features in first biopsy shown subsequently to contain HCV-RNA by tissue PCR.

Histological features	Biopsy at time of HCV recurrence
Mononuclear portal infiltrate	3/10 (30%)
Lymphoid aggregate	0/10 (0%)
Bile duct damage	2/10 (20%)
Cholestasis	5/10 (50%)
Interface hepatitis	0/10 (0%)
Lobular inflammation	7/10 (70%)
Liver cell vacuolation	5/10 (50%)
Acidophil bodies	6/10 (60%)
Endotheliitis	3/10 (30%)
Zone 3 damage	6/10 (60%)
Eosinophils	7/10 (70%)

Biochemical liver function tests

The results of the routine liver function tests against time were correlated with when HCV-RNA was detected in liver biopsies (see figures 5.9 - 5.19). These figures show that 5/10 patients (50%) showed a rise in transaminases around the time of reinfection; however, all of these patients showed a concomitant rise in alkaline phosphatase levels. Indeed, 8/10 patients (80%) showed chronic, fluctuating elevation of transaminases (see figures 5.9 - 5.14, 5.17-5.18), with only 2/10 patients (20%) showing raised enzymes which intermittently became normal (5.15 and 5.16). 6/10 patients (60%) showed chronically raised alkaline phosphatase levels, with 3/10 patients (30%) showing fluctuating ALP levels, and only one patient (9; see figure 5.17) showing normal levels of ALP, although only a very short amount of follow up data was available for this patient.

Figure 5.9. LFT s against time post transplant in relation to HCV-RNA results on liver biopsies for patient 1

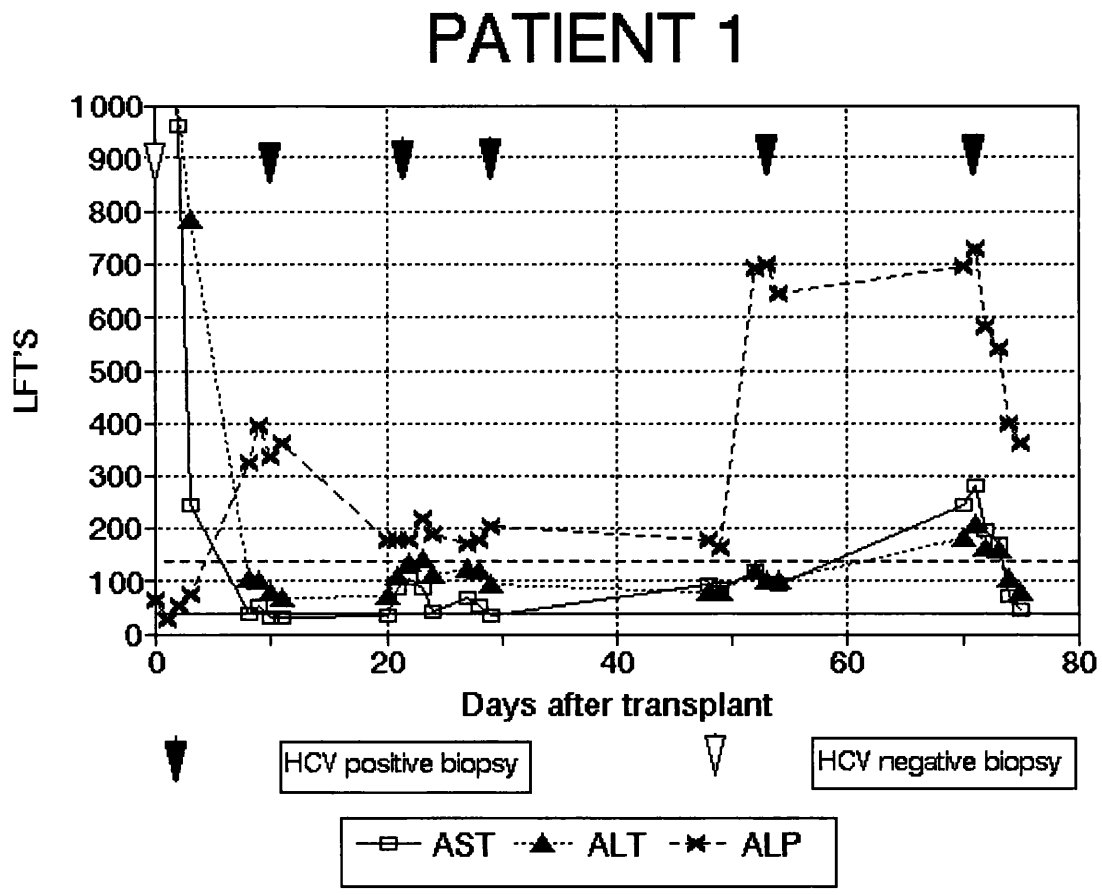


Figure 5.10. LFT s against time post transplant in relation to HCV-RNA results on liver biopsies for patient 2

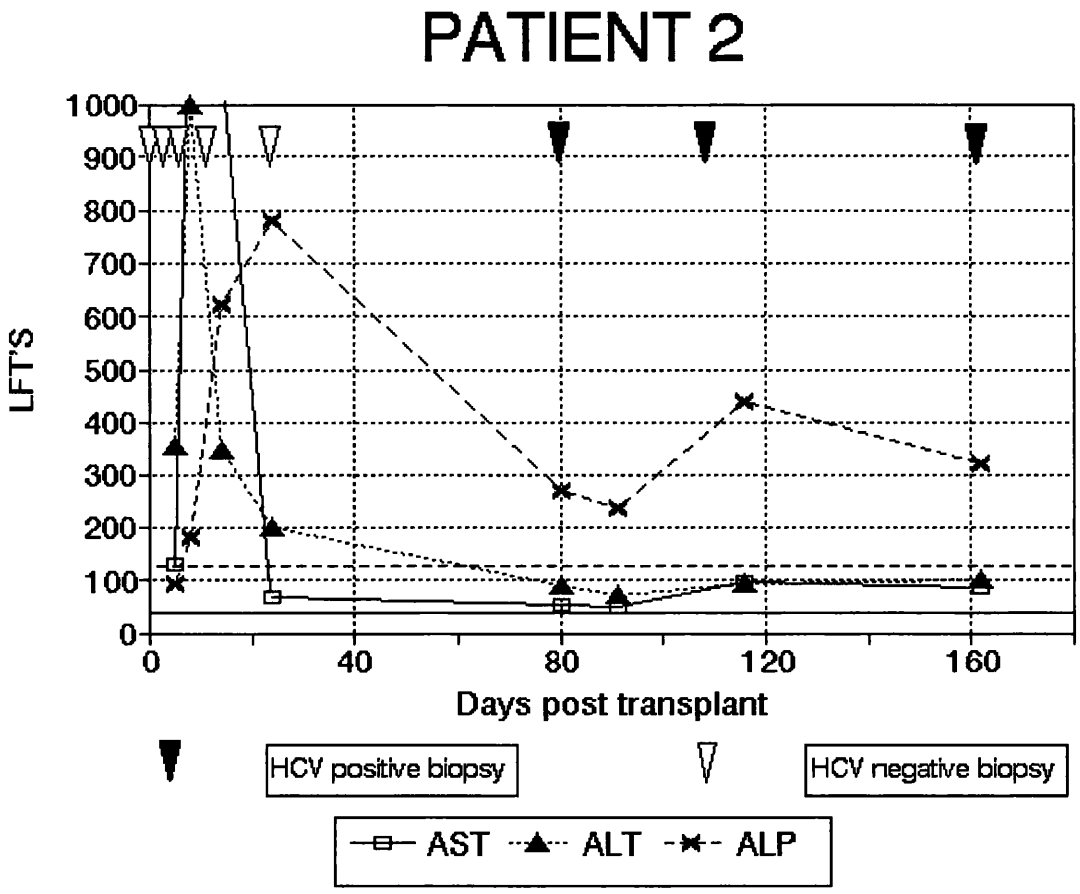


Figure 5.11. LFT s against time post transplant in relation to HCV-RNA results on liver biopsies for patient 3

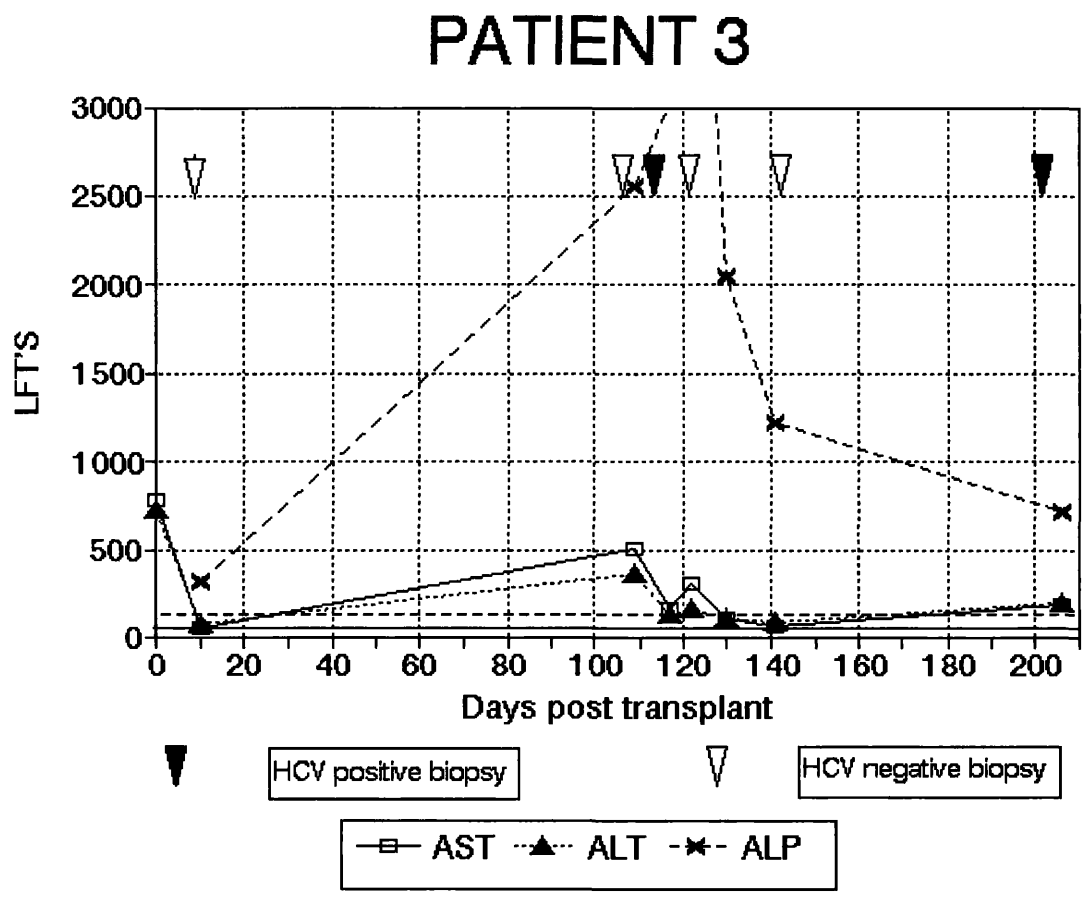


Figure 5.12. LFT s against time post transplant in relation to HCV-RNA results on liver biopsies for patient 4

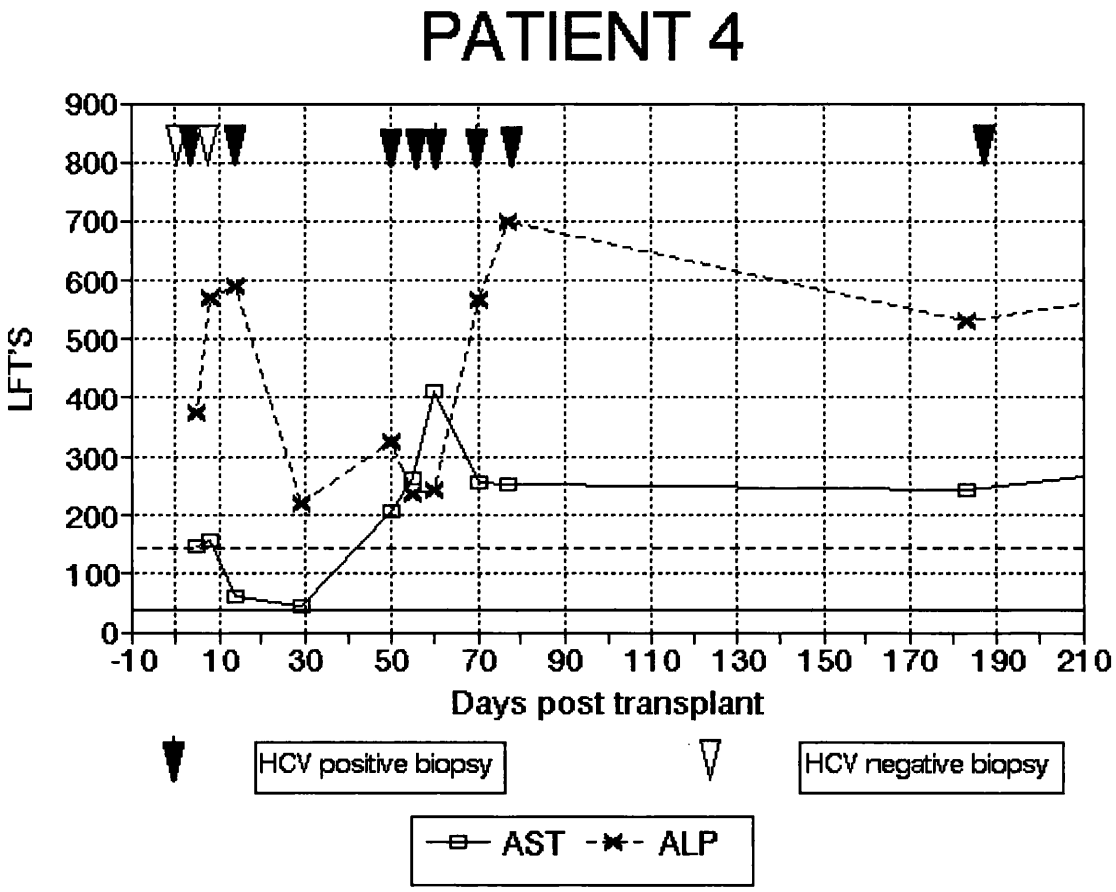


Figure 5.13. LFT s against time post transplant in relation to HCV-RNA results on liver biopsies for patient 5

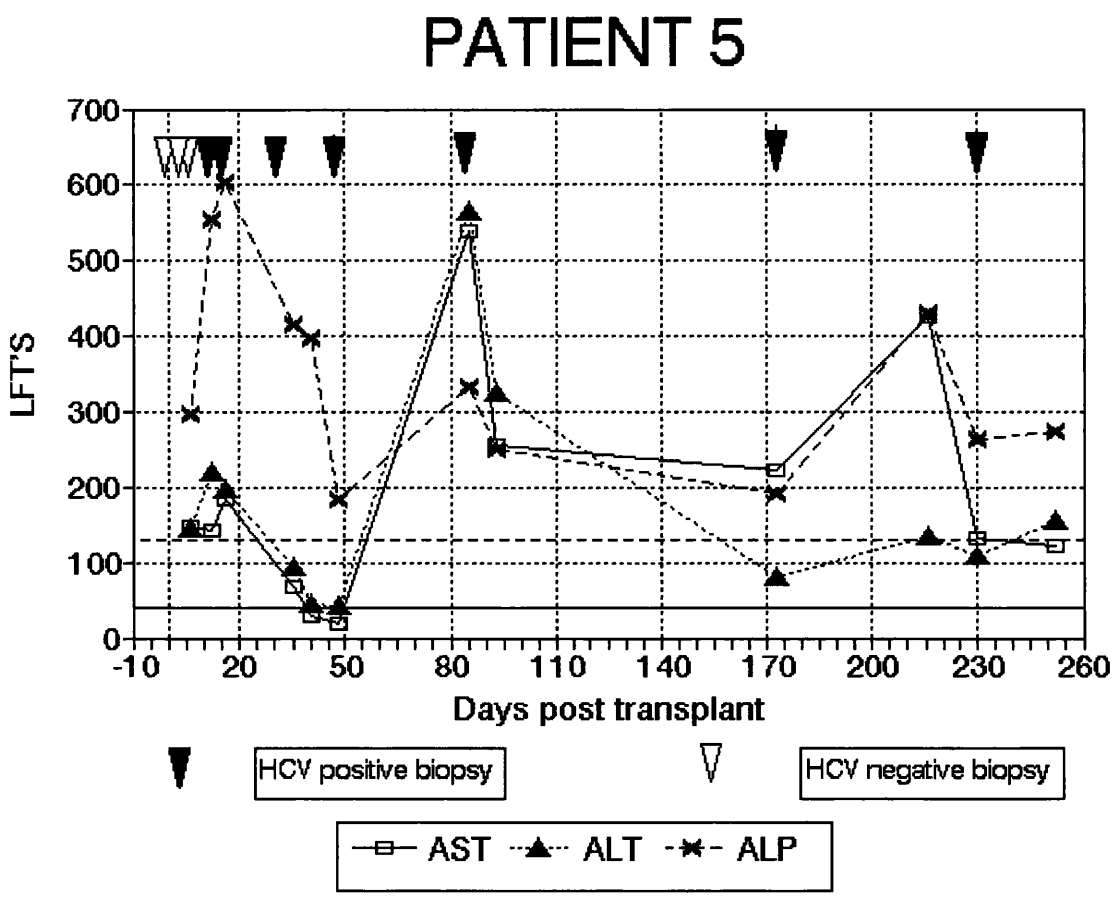


Figure 5.14. LFT s against time post transplant in relation to HCV-RNA results on liver biopsies for patient 6

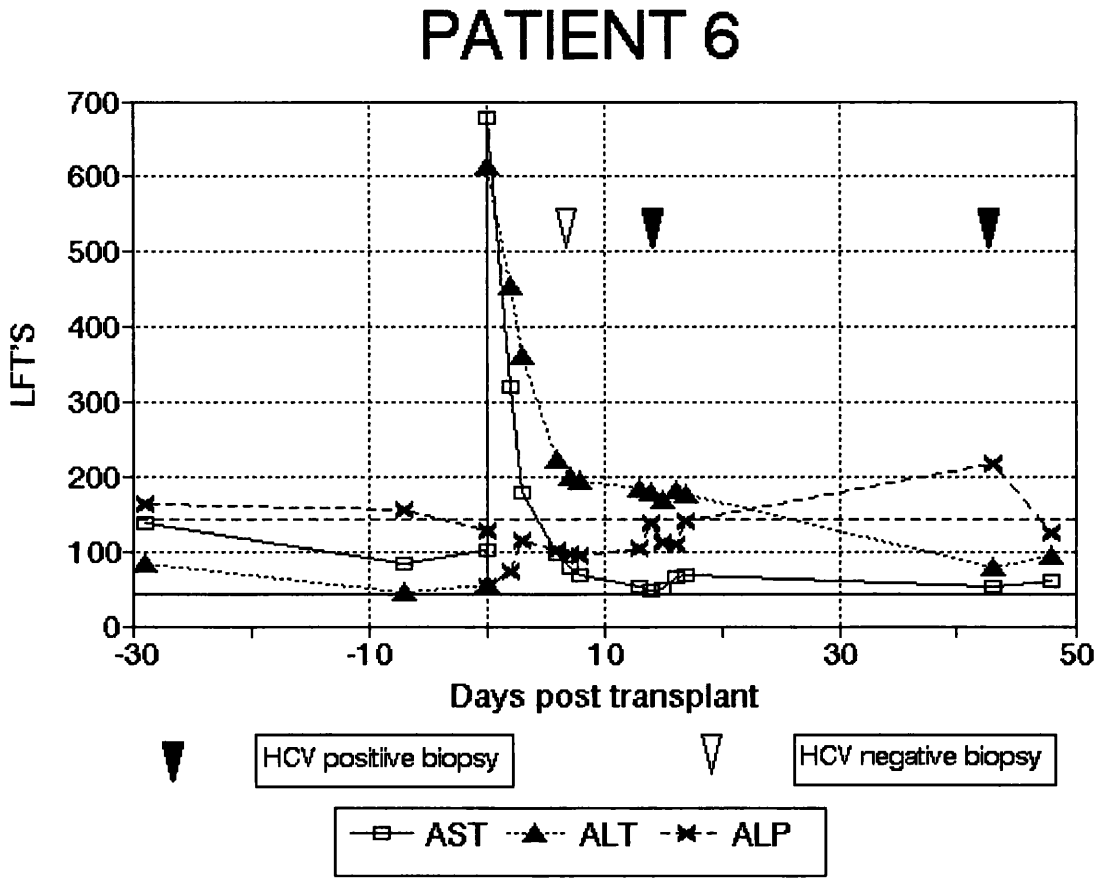


Figure 5.15. LFT s against time post transplant in relation to HCV-RNA results on liver biopsies for patient 7

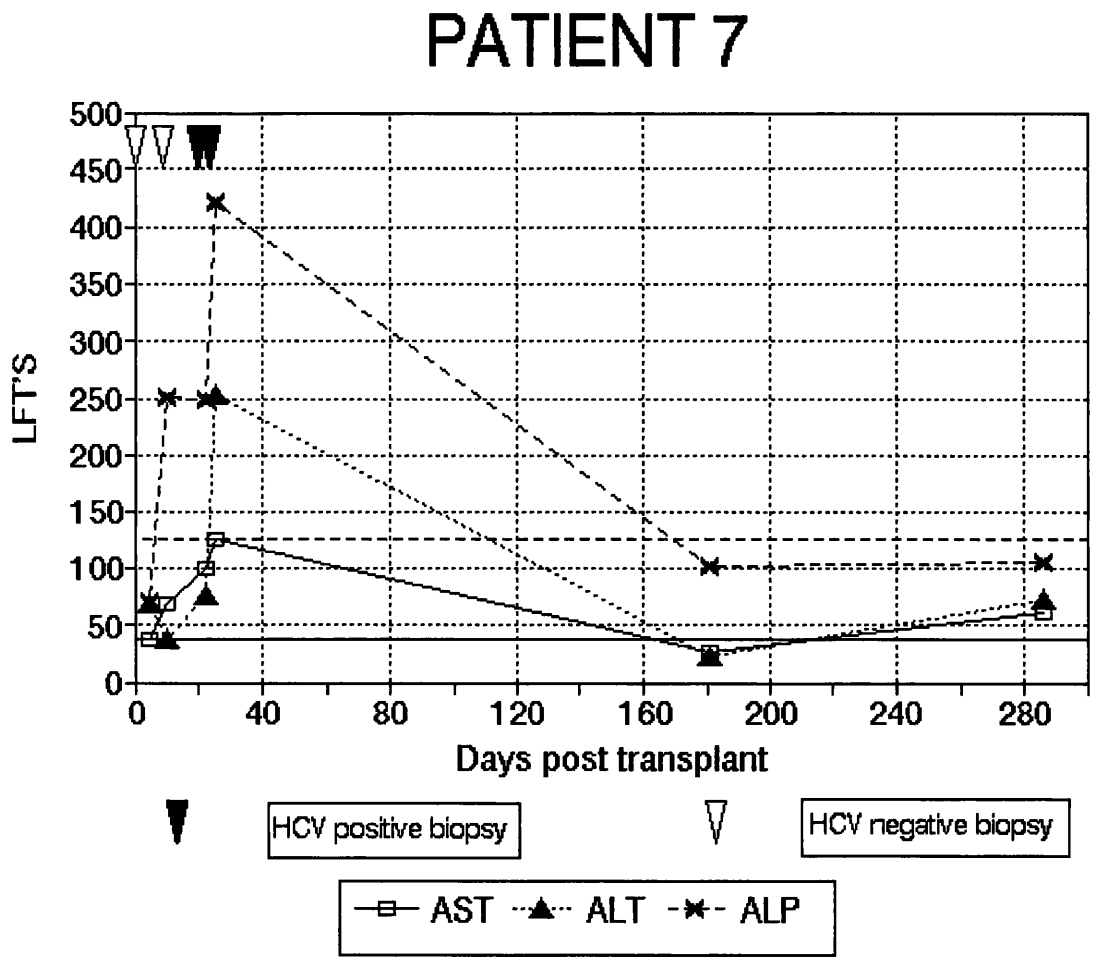


Figure 5.16. LFT s against time post transplant in relation to HCV-RNA results on liver biopsies for patient 8

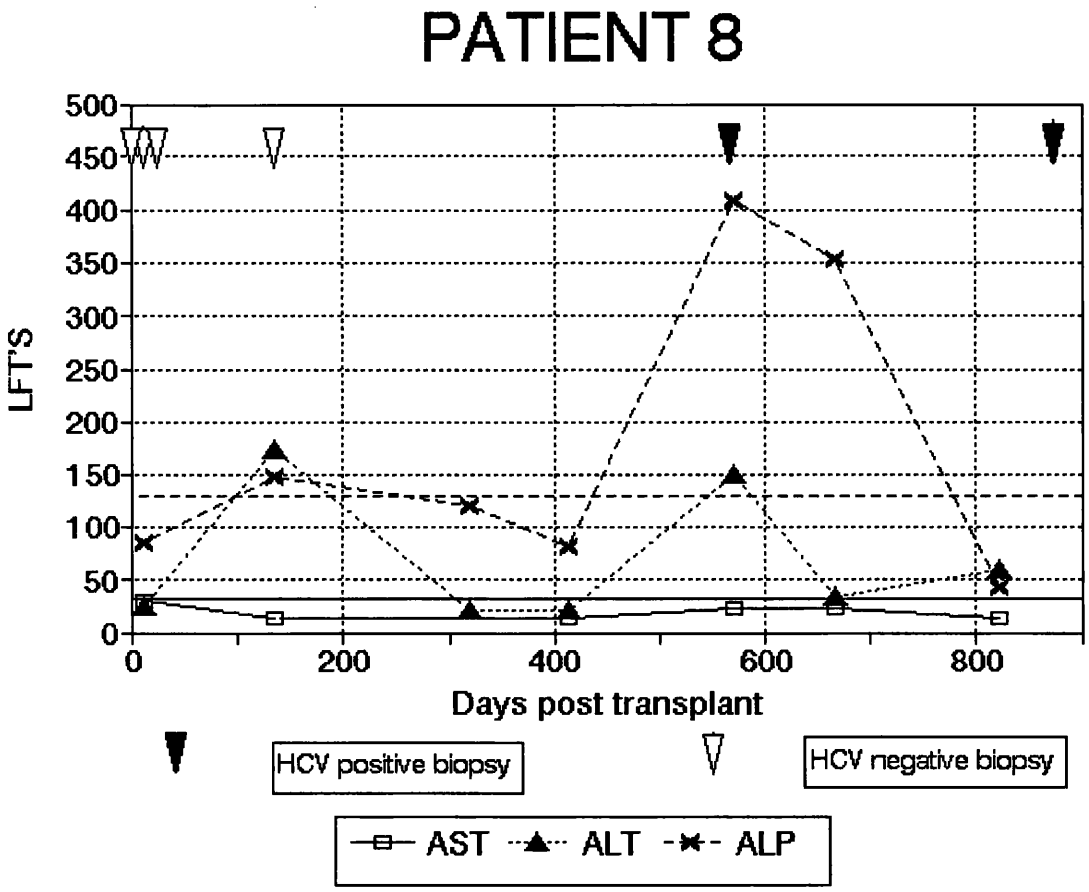


Figure 5.17. LFT s against time post transplant in relation to HCV-RNA results on liver biopsies for patient 9

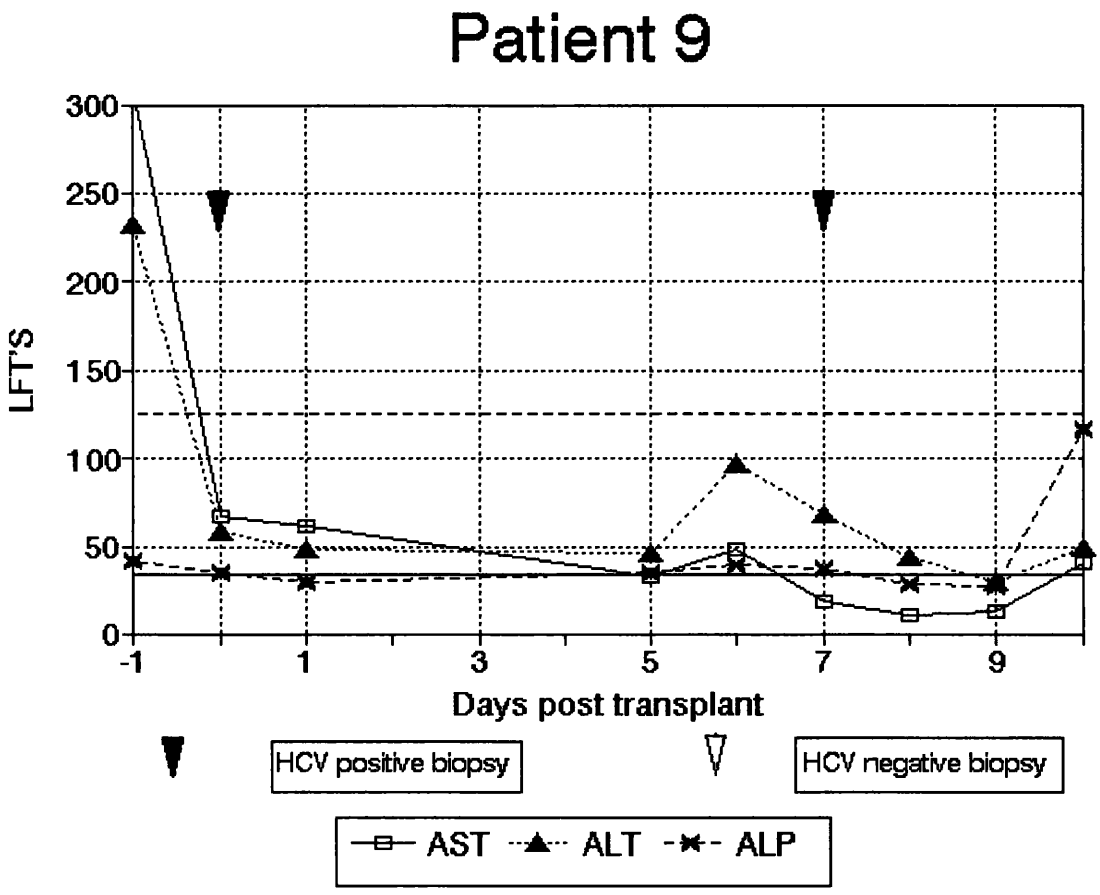
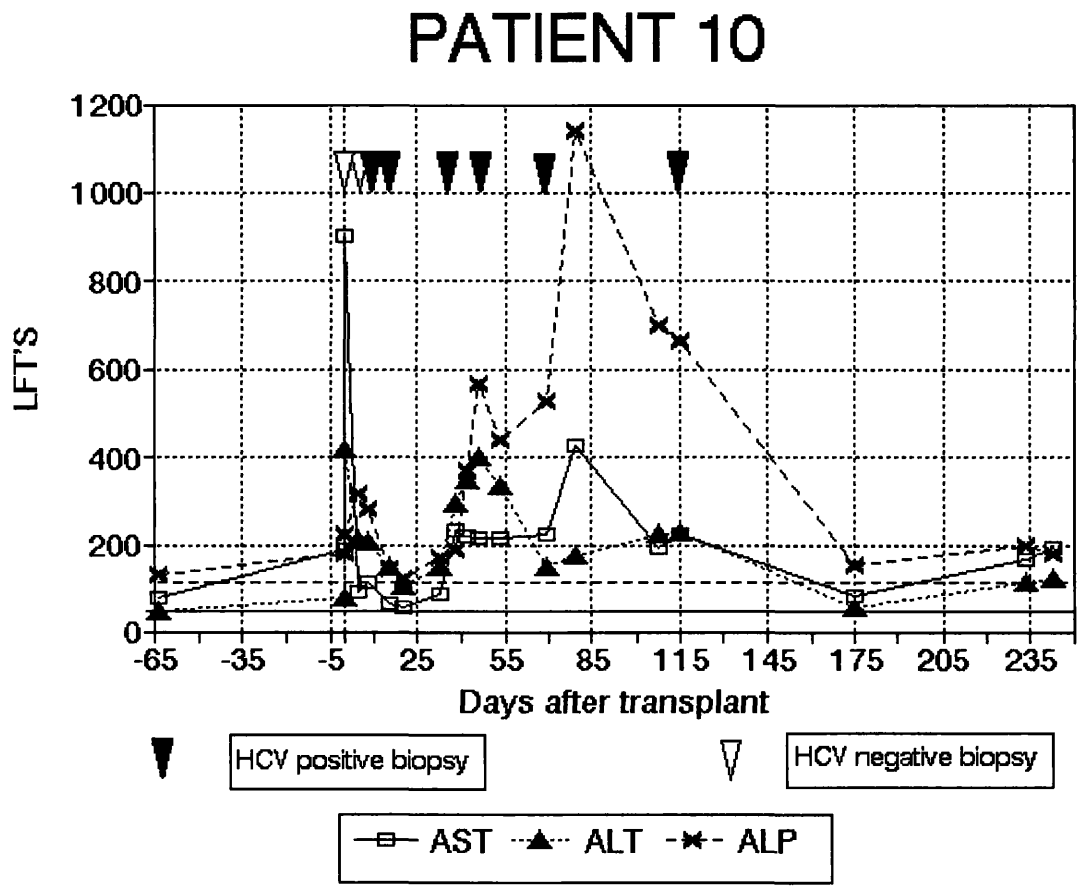


Figure 5.18. LFT s against time post transplant in relation to HCV-RNA results on liver biopsies for patient 10



Discussion

Detection of HCV-RNA by PCR.

This study showed that, given sufficient follow up, recurrence of hepatitis C virus was 100% in this group of patients. This finding is similar to those of other studies using PCR methodology (Sallie *et al.*, 1994; Feray *et al.*, 1994; Konig *et al.*, 1992), in which recurrence rates of 90%, 87% and 87% respectively were reported; but at variance with earlier studies using only anti-HCV testing (Martin *et al.*, 1991; Read *et al.*, 1991), which reported histological recurrence rates of 40% and 31% respectively.

Patients were clearly divided into two groups; those in whom HCV recurrence was detected early post transplant (less than or equal to 22 days (mean 10 days); 7/10 patients), and those in whom HCV was not detected until a later stage post transplant (greater than or equal to 80 days post transplant (mean 256 days); 3/10 patients). In the group showing early recurrence of HCV (group 1), 2/7 patients (29%) developed severe fibrosis or cirrhosis and died within three years of transplant; four of the remaining five patients (57%) developed mild to moderate chronic hepatitis consistent with HCV infection. The fifth patient only had 43 days of histological follow up available; this was probably not long enough for histological features of hepatitis to develop, as the other patients developed PTH at a range of 72 - 286 days post transplant (mean 131 days). In total, 86% of patients in this group had symptomatic recurrence of HCV infection, ranging from mild chronic hepatitis to cirrhosis.

In the group showing later recurrence of HCV (group 2), only 1/3 patients (33%) showed histological evidence of viral hepatitis (patient 3; acute hepatitis

d117); no other biopsies from this patient showed evidence of HCV infection, and at day 306, features suggestive of CMV infection predominated (see table 5.4). None of these patients developed chronic hepatitis post transplant after follow up ranging from 306 - 1195 days (mean 870 days).

It is interesting that the patient from group 2 showing acute hepatitis (patient 3) was one of only two patients whose post transplant liver biopsies were not consistently positive after first detection of HCV-RNA in the liver graft (patients 3 and 4; see figures 5.11 and 5.12). In patient 4, HCV-RNA was first detected in the biopsy taken at day 5 post transplant. However, the biopsy at day 8 was HCV-RNA negative. The remaining 8 biopsies taken (between day 14 and day 549) were all HCV-RNA positive. This discrepant result was probably due to sampling error within the liver at a time when there were low levels of virus soon after reinfection. Work on antigen detection within HCV positive livers has shown marked lobule to lobule variance in expression of HCV antigen (Krawczynski *et al.*, 1992a; Di Bisceglie *et al.*, 1993; Nouri-Aria *et al.*, 1995). It is possible that this reflects a similar variation in viral infected cells, increasing the chances of false negative results owing to sampling error.

Patient 3 showed a different pattern of positive and negative biopsies after transplant (see figure 5.11). The biopsy taken at day 109 was HCV-RNA negative, whereas that taken at day 117 was positive. The following two biopsies, taken at day 122 and day 141 were HCV-RNA negative; the final biopsy tested, taken at day 206, was HCV-RNA positive. At this time, this patient was negative for HCV-RNA in serum by PCR. Whilst this result could also be due to biopsy sampling error, as discussed above, the data implies that in this patient, virus levels were low, towards

the limit of detection of PCR. Levels of HCV-RNA have been shown to be higher in liver than in serum from HCV infected patients (Yatsuhashi *et al.*, 1995), so it is not surprising that an infection with low copy numbers of virus could be detected in liver and not in serum. It was also noted that in one patient (pt 6), from whom sequential serum samples were available, HCV-RNA was detected in the liver before it was detectable in the serum (see figure 5.2). Both Sallie *et al* (1994) and Marzano *et al* (1994) have shown that HCV-RNA can be detected in liver before it is detected in serum in post transplant patients: Sallie *et al* (1994) also had two patients who were HCV-RNA positive in the liver but remained negative in serum at one year after transplantation.

It can therefore be seen that these two groups showed marked differences in outcome according to whether they showed early or late recurrence of HCV-RNA in the graft. Whilst this work is only on a small number of patients, the findings are supported by those of Konig *et al* (1992), who noted that early recurrence of HCV-RNA was more likely to be associated with histological injury. In addition, Gane *et al* (1996) have recently described the long term outcome of HCV post transplant in a large group of patients, and found both that a small percentage of patients had no evidence of chronic hepatitis up to five years after transplant, and that some patients had a greatly accelerated course of disease (Gane *et al*, 1996).

It is not clear what could be causing these different patterns of HCV recurrence in different patients. It could be that levels of HCV-RNA pre-transplant are an important factor in speed of recurrence post transplant. However, several studies which measured pre- and post- transplant levels of HCV-RNA have found that levels of virus pre-transplant did not predict recurrence (Feray *et al.*, 1994) or

degree of hepatic damage (Chazouilleres *et al.*, 1994). Interestingly, a recent study measuring levels of HCV-RNA post transplant found that whilst levels of HCV-RNA at 1 month post transplant and later did not correlate with histological damage, patients who subsequently developed chronic active hepatitis (CAH) had significantly higher levels of HCV-RNA at 3, 7 and 14 days post transplant (Gretch *et al.*, 1995a).

Another factor affecting speed and severity of HCV recurrence could be the genotype of the virus. Different genotypes of HCV have been associated with differing response to interferon (Garson *et al.*, 1995), and with different severity of disease (Dusheiko *et al.*, 1994; Pistello *et al.*, 1994). Genotype could also influence the natural history of HCV infection in the post transplant situation. Gretch *et al* (1995a) noted that whilst there was no significant association between genotype and development of CAH during the first year, 4/5 patients with genotype 1b developed CAH in the first year compared with 3/8 with type 1a, 1/3 with type 2b, and 1/2 with mixed types. Indeed, Gane *et al* (1996) found that there was a significant correlation between infection with genotype 1b and incidence of severe graft damage.

It would also be interesting to assess in more detail the immunological status of these patients. Cyclosporin, which is part of the standard immunosuppressive regime used at RFH, is known to suppress T cell activity (Starzl *et al.*, 1982). If cytotoxic T cell responses are important in the control of HCV infection, as discussed in chapter 3, it may be that the precise nature of an individual's response to cyclosporin and the other immunosuppresive drugs determines whether they can maintain immune pressure on the virus, and perhaps limit viral replication, whilst

still avoiding rejection of the graft. The patients showing late recurrence of HCV each had between 0 and 1 (mean 0.6) episodes of histological proven rejection requiring additional immunosuppresion, whereas the patients with early recurrence had between 1 and 2 (mean 1.3) episodes of histologically proven rejection. Indeed, the patient who developed cirrhosis post transplant was given OKT3 for steroid resistant rejection. Whilst it is difficult to judge the significance of this finding on only 10 patients, Sheiner *et al* (1995) have demonstrated that patients with multiple or severe rejection episodes, and consequent increase in immunosuppression, have earlier histological recurrence of HCV. Loinaz *et al* (1995) noted a higher rate of chronic rejection in HCV infected recipients, as well as a higher rate of post transplant hepatitis. The possible reasons for a causative link between HCV infection and higher rates of rejection are i) increased expression of class I HLA molecules as proposed for CMV (O'Grady *et al.*, 1988): ii) stimulation of the immune system through high HCV antigen expression: iii) a co-incidental link to higher rates of rejection caused by factors such as a tendency to use of lower doses of immunosuppresion in recipients with viral infection: iv) underdiagnosis of acute rejection (Loinaz *et al.*, 1995). However, this possible link between histological recurrence of HCV and episodes of acute or chronic rejection has recently been contradicted by the findings of Gane *et al* (1996). These workers found no significant difference in the histological findings between HCV-infected transplant patients treated for acute rejection and those not. In addition, they did not find an increase in chronic rejection in HCV positive transplant patients when compared to HCV negative recipients (Gane *et al*, 1996).

It should be noted that the two patients with severe disease after transplant

had moderate to severe inflammation with lobular activity and lymphoid aggregates. This inflammation did not appear affected by immunosuppression. Indeed, most patients (86%) with early reinfection of the graft showed inflammation characteristic of HCV outside the transplant situation.

In two patients, HCV-RNA was undetected in serum at a time when it was detectable in the liver allograft. In one of these patients, serum was still HCV-RNA negative a considerable time after transplantation (>206 days). In the other patient, HCV-RNA was detected in the liver at day 15 post transplant, but was not detected in serum until after day 35. Early detection of HCV-RNA in serum could be compromised by the number of transfusions a patient has received during the operation, either through diluting the amount of HCV in the blood, or by inhibiting PCR with heparin or other substances (Higuchi, 1989). As already stated, HCV-RNA is present at a higher concentration in the liver than in the serum of an infected individual (Yatsuhashi *et al.*, 1995). This phenomenon could contribute to a low level of HCV-RNA in serum immediately after transplantation, when the main reservoir of replicating virus, the liver, has been removed, and is no longer shedding viral particles into the circulation. Whilst it has been reported that HCV can replicate in circulating mononuclear cells (Muller *et al.*, 1993), this data raises the questions: where did the HCV which reinfected the graft come from? and are there other extrahepatic sites of HCV replication, which harbour pools of replicating virus?

In one patient, the donor liver biopsy was HCV-RNA positive. This biopsy was taken during the operation after reperfusion of the graft. The donor was anti-HCV negative, and the biopsy did not show histological evidence of liver disease.

This could represent very early graft reinfection, or could be due to recent HCV infection of the donor; Marzano *et al* (1994) described this in 4 out of 16 donors.

HCV antigen detection.

Anti-HCV staining of serial post transplant biopsies from patient 10 yielded several interesting findings. It clearly demonstrated that recurrent HCV-RNA in the graft is a true infection of the new liver, and that the virus is actively expressing protein within hepatocytes. As translation is directly linked to replication in HCV, this also shows that the virus is actively replicating (van Doorn, 1994). Konig *et al* (1992) noted that detection of HCV-RNA in liver biopsies by tissue PCR does not prove that the virus is within liver cells; the demonstration of HCV antigen within hepatocytes does exactly this.

Unsurprisingly, HCV-RNA could be detected in the new liver whilst anti-HCV^{FITC} staining was still negative (see table 5.2). Viral antigen is usually present in relatively low amounts in infected individuals, and PCR is a very sensitive technique, with the ability to detect very low copy numbers of virus. This demonstrates again the importance of using a battery of tests to detect HCV infection in a particular patient at a particular time.

The amount of HCV antigen expressed within the liver appeared to be greatly increased post transplant. Whilst immunocytochemical detection of HCV antigen with this method is not directly quantitative, more hepatocytes were stained, and to a higher intensity, in the biopsies taken at day 33, 42 and 69 days post transplant, than were seen stained in the patient's explanted liver (see table 5.2, figures 5.3 - 5.8). Other workers have shown that levels of HCV-RNA are much higher in immunosuppressed post transplant patients than in immunocompetent

individuals (Feray *et al.*, 1994; Chazouilleres *et al.*, 1994). This indicates that HCV replication and expression of antigen is controlled by the immune system in immunocompetent individuals, and that this control is reduced when patients are immunosuppressed after transplantation. Martell *et al* (1994) showed that the numbers of viral quasispecies circulating in these patients are decreased after transplant; while the reasons for this are complex, lack of immunological selection pressure is a likely cause. Gretch *et al* (1995a) found that intensity of staining for NS4 antigen was higher for post transplant biopsies showing CAH than those without active hepatitis; our patient 10, who showed a high level of antigen expression, developed chronic hepatitis within 10 months of transplant.

Histopathology of post transplant biopsies.

The direct demonstration of HCV-RNA in individual post transplant biopsies allowed the histology of these biopsies to be reassessed in the light of PCR results in an attempt, retrospectively, to identify features which might indicate recurrent HCV. 90% of the routine reports on the first HCV-RNA positive biopsy from each patient described rejection of some degree - only one patient had a diagnosis of acute hepatitis. This of course does not mean that the original diagnosis was incorrect - the two conditions could co-exist. The retrospective analysis of these biopsies suggested that a predominance of lobular activity in a patient with previous HCV infection should raise the question of recurrent HCV. 70% of patients showed lobular inflammation in their first HCV-RNA positive biopsy; 60% had zone 3 (centrilobular) damage. This zone 3 damage has previously been reported as a feature which may be associated with HCV recurrence (Dhillon *et al.*, 1992). However, HCV recurrence can only be determined definitively by tissue PCR of the

biopsy.

Biochemical liver function tests.

Review of biochemical liver function tests revealed that whilst 5/10 patients had a rise in transaminases around the time that HCV-RNA was first detected in the allograft, a concomitant rise in alkaline phosphatase levels was also seen. The typical pattern of LFT s often seen in viral infection outside the transplant situation are ALT > AST, with no derangement of alkaline phosphatase (McIntyre and Rosalki, 1991); however this was not seen in these patients. All patients showed either a chronic elevation of transaminases, or fluctuating levels which sometimes dipped below the upper limits of normal. These findings agree with those of Henley *et al* (1992), who showed that LFT s were not helpful in the first 180 days of transplantation in distinguishing patients with normal histology from those with hepatitis or rejection. These were in contrast, however, to the findings of Shiffman *et al* (1994), who found that in a large proportion of patients (38%), ALT levels returned to normal after transplantation, and showed a peak lasting 7-10 days some time before post transplant hepatitis was observed histologically. Patterns similar to the ones seen in our patients were noted in 29% of their cohort; the remaining 14% of patients had consistently normal ALT. Our own data, as well as that of Henley *et al* (1992) shows that monitoring of LFT s after transplantation is important as an indicator of problems for the patient, and of when to biopsy, but may not reveal the cause of any liver problems, and is not always helpful in distinguishing rejection from hepatitis, and may not always reflect the histological state of the liver. In addition, interpretation of post transplant LFT s is made more difficult because of the effect of drugs the patient may be taking, and of any post transplant complications

such as sepsis, ischaemia or biliary problems. This emphasises that in the post transplant situation, the taking of protocol biopsies at designated times after transplantation, as well as biopsy of patients with abnormal liver function tests, is essential.

Conclusions

HCV recurrence in liver allografts was universal in this study. This recurrence was related to some degree of hepatic damage in 60% of patients; two patients (20%) progressed to severe fibrosis or cirrhosis within 3 years of transplant. HCV appeared to have a direct cytopathic effect in these patients, since HCV related immune reactions should have been ameliorated by immunosuppressive therapy. In these two patients the natural course of chronic HCV infection was greatly accelerated, and the long term outcome for the remaining patients has yet to be determined.

Although chronic HCV infection in liver allografts shows "characteristic" histological features, those seen at the time of first detection of recurrent HCV are not specific and a typical acute hepatitic pattern is uncommon. PCR for detection of HCV-RNA in post transplant liver biopsies is an valuable tool for detecting time of HCV recurrence in these patients, which may be an important prognostic indicator.

The increased levels of HCV antigen seen in these immunosuppressed patients (see also chapter 3, patients 11 and 12) supports the theory that the immune system exerts a control on the expression and replication of hepatitis C virus. Levels of immunosuppresive therapy could be very important in determining the timing and severity of HCV recurrence post transplant. This suggests a great paradox in the

pathology of HCV-induced liver disease; in the immunocompetent individual, it may be the host response to viral infection which causes liver damage and disease, but in immunosuppressed patients, the virus may replicate out of control and cause direct damage to the liver.

In summary, in agreement with other reports, these results show that whilst in the majority of patients, HCV post transplant is relatively indolent and the medium term survival of these patients is good, some patients have severe disease after transplantation. The long term prognosis for the patient population as a whole is not yet clear.

CHAPTER 6

DETECTION OF EXTRAHEPATIC HEPATITIS C VIRUS

Introduction

There has been some controversy over the means of transmission of hepatitis C virus. Only about 40% of cases of HCV infection worldwide are associated with known parenteral risk (Alter, 1993). To elucidate how HCV is transmitted in these "sporadic" or "community acquired" cases of HCV, many groups have looked in body fluids other than blood, such as saliva, semen, sweat, urine, vaginal discharge and breast milk for the presence of HCV-RNA (Kurauchi *et al.*, 1993; Numata *et al.*, 1993; Liou *et al.*, 1992; Hsu *et al.*, 1991; Fried *et al.*, 1992). However, such studies have produced contradictory results. Fried *et al.* (1992) and Hsu *et al.* (1991) reported that HCV-RNA was not detectable within the saliva or semen of HCV viraemic patients, whereas other workers reported detection of HCV-RNA in 35-48% of saliva samples (Numata *et al.*, 1993; Liou *et al.*, 1992) and 24% of semen samples (Liou *et al.*, 1992). Likewise, Hsu *et al.* (1991) reported that vaginal secretions and urine were negative for HCV-RNA, whilst Numata *et al.* (1993) found 56.5% of urine samples from viraemic patients positive for HCV-RNA, and other workers found HCV-RNA positive vaginal discharge samples in 50% of anti-HCV positive mothers (Kurauchi *et al.*, 1993).

The evidence for vertical, sexual and intrafamilial transmission is also controversial. Marcellin *et al.* (1993) reported that none of the children of 26 HCV positive, HIV negative mothers were HCV-RNA positive, whereas other workers have related rates varying from 5.6% (Ohto *et al.*, 1994) to 33% (Kuroki *et al.*, 1993) HCV-RNA positive. Similarly, Bresters and others (Bresters *et al.*, 1993; Brettler *et al.*, 1992; Hallam *et al.*, 1993) reported a low or zero rate of transmission of HCV between infected haemophiliacs and their partners, whereas

other groups found a significantly increased rate of anti-HCV of 24-28% in spouses of index patients compared to the general community (Oshita *et al.*, 1993; Chang *et al.*, 1994). These workers also found an increased incidence of anti-HCV amongst family members of index cases, which was refuted by the findings of Camarero *et al.* (1993), who found no such link.

Some of the strongest evidence for vertical, sexual and familial transmission comes from Japan and Taiwan, where workers have used sequence analysis to study the genetic variation between isolates of HCV from different individuals. As already discussed, quasispecies of HCV evolve within an individual during the course of an infection and consensus sequences can show significant variation between individuals even when the genotype of the infecting virus is the same. This variation in sequence can be used to judge the 'relatedness' of virus isolates from different individuals - ie where a patient's infection may have come from (Bukh *et al.*, 1995).

Honda *et al.* (1993) found that 18% of family members of index patients were HCV-RNA positive, and that the concensus sequences from index patients and their family members were significantly more homologous than those from unrelated pairs of HCV infected patients. They demonstrated 97.7% homology between a child and mother, and 98.1% homology between a husband and wife, although they did not speculate on the specific means of transmission between the patients. Likewise Inoue *et al.* (1992) investigated HCV sequences within an infected family and found that the concensus sequence from a baby was identical to that of her mother, and closer to those of her grandmother and uncle than to other unrelated HCV positive patients from the same area, demonstrating possible vertical

transmission. Kao and coworkers (1992) found that 5.4% of family members, including 21% of spouses, were HCV positive. Among spouses, 3/4 sequence pairs were identical at the nucleotide level, with the remaining pair showing 96% homology. This was thought to demonstrate sexual transmission, although at a low efficiency.

The aim of this section of work was to investigate possible alternative routes of HCV transmission (other than overt parenteral contact) and to identify any extrahepatic sites where HCV can be found, and hence to attempt to clarify some of these contradictory findings. RT-PCR was used to detect HCV-RNA in postmortem samples of various organs from patients with HCV infection. A selection of these organs were then tested for the presence of HCV antigen using the human polyclonal anti-HCV^{FITC}.

Materials and Methods

Liver tissue samples from eleven patients positive for anti-HCV by 2nd generation ELISA (Abbott Laboratories) who came to post mortem were tested for HCV-RNA. Patients who were positive for HCV-RNA in liver were then tested for the presence of HCV-RNA in other tissues, using the RT-PCR technique already described. All testing was performed in triplicate, using appropriate positive and negative controls. All tissues were tested in a blind fashion, with samples from anti-HCV positive and anti-HCV negative patients tested randomly in mixed batches.

In the first instance, formalin fixed, paraffin embedded (FFPE) tissues collected at routine post mortem from 3 anti-HCV positive and 2 anti-HCV negative patients were tested retrospectively by RT-PCR for the presence of HCV-RNA. HCV-RNA was detected in the liver tissue of all three anti-HCV positive patients, but in neither of the anti-HCV negative patients. A range of other tissues, including thyroid, salivary gland, spleen, kidney, adrenal, trachea, heart, ovary, lung, pancreas, testis and seminal vesicle were also tested. A selected range of tissues from a further five cases positive for HCV-RNA in liver were then tested. From three of these cases, tissue was prospectively collected; both FFPE and frozen material was taken. Samples from various organs, including liver, salivary gland, thyroid, kidney, seminal vesicle, ovary and cervix, were embedded in OCT (RA Lamb) on cork squares and frozen, via isopentane (BDH), in liquid nitrogen (BOC). Samples were stored at -70^{0}C until use. FFPE and frozen tissue was also collected from a further four anti-HCV negative cases. These cases were tested by tissue PCR to confirm the specificity of the HCV RT-PCR technique in this material.

Southern blotting and hybridisation was performed on PCR products from

liver, kidney, thyroid, seminal vesicle, pancreas and salivary gland from one patient (whose liver was HCV-RNA positive), to ensure that any PCR product detected was amplified from HCV-RNA, and did not represent non-specific amplification of an endogenous RNA. An agarose gel of the products was run and blotted as described in appendix 2.4. Hybridisation was performed using the oligonucleotide probe ALX89 (see figure 2.5), labelled with digoxigenin (Boehringer Mannheim; see appendix 6.1). Binding of the probe was detected using an alkaline phosphatase conjugated anti-digoxigenin antibody (Boehringer Mannheim), with nitroblue tetrazolium (NBT; Boehringer Mannheim) as chromogen (see appendix 6.2).

Frozen tissue samples of selected organs from 3 HCV positive and 3 HCV negative cases were tested for presence of HCV antigen using the human anti-HCV^{FITC} antibody. Cryostat sections (5μm) were cut from tissue blocks, air dried for 20 minutes, then fixed in chloroform for 5 minutes. After air drying, sections were incubated with polyclonal human anti-HCV^{FITC}, diluted 1/300 in normal human serum, overnight at 4^0C. Sections were then washed in PBS (Oxoid, pH 7.2) for 3 x 7 minutes, mounted in citifluor, and observed using a DVC 250 confocal microscope (Biorad Ltd.). Photomicrographs were taken using a Nikon F-601M camera, with Fujichrome 1600 slide film. Films were developed by 1 stop push processing, then transparencies were transferred onto a photo CD (Kodak). They were then imported onto a Dan multimedia PC and printed onto HQ gloss paper (Epson) using an Epson stylus colour printer.

Post mortem histology results were reviewed, and tissue sections were checked for state of preservation. In addition, HCV positive tissues were reviewed for signs of chronic inflammation or any other abnormalities.

Results

Eight of eleven anti-HCV positive cases (73%) had HCV-RNA detectable in liver tissue by tissue PCR. PCR results for each case were compared with the time taken between patient death and performing the post mortem; and with the state of preservation of the liver (see table 6.1).

Testing of samples from other tissues was carried out on the eight cases positive for HCV-RNA in liver.

In the first three cases (2M, 1F), HCV-RNA was detected in liver (3/3 cases), thyroid (2/3 cases), salivary gland (2/2 cases), pancreas (2/2 cases) and seminal vesicle (1/1). It was not detected in spleen, kidney, adrenal, heart, trachea, stomach, lung, ovary or testis samples from any of the cases. In the other five cases positive for HCV-RNA in liver (4M, 1F), a smaller range of tissues was tested, including thyroid, salivary gland, pancreas, kidney, ovary and seminal vesicle. Not all tissues were available from each case. HCV-RNA was detected in thyroid in 2/3 cases, in salivary gland in 2/3 cases, and in seminal vesicle in 2/2 cases. It was not detected in pancreas (3 cases), kidney (5 cases) or ovary (1 case). The results of the tests are summarised in Table 6.2. All tissues tested from the six HCV-RNA negative cases (5M, 1F) were negative for HCV-RNA by tissue PCR. These results are summarised in Table 6.2.

PCR products from all tissues tested from one patient were blotted and hybridized with an HCV oligoprobe ALX89 to confirm specific HCV amplification. The products from liver, thyroid, seminal vesicle, pancreas and salivary gland were confirmed as true HCV amplimers, whilst the kidney tissue was confirmed as HCV negative (see figures 6.1 and 6.2).

Table 6.1 HCV-RNA results versus post mortem preservation for anti-HCV positive cases

PM No.	Anti-HCV status	HCV-RNA in liver by tissue PCR	Time between death and performing post mortem (days)	State of histological preservation of liver tissue
1	+	+	2	Moderate
2	+	+	3	Moderate
3	+	+	4	Poor
4	+	+	2	Moderate
5	+	+	1	Good
6	+	+	0	Good
7	+	+	4	Moderate
8	+	+	4	Moderate
9	+	-	1	Poor
10	+	-	5	Moderate
11	+	-	1	Poor

Table 6.2. Results of HCV-RNA detection in post mortem tissues.
Summary of tissue PCR results from eight patients who were anti-HCV positive and positive for HCV-RNA in liver, and six patients who were anti-HCV negative and negative for HCV-RNA in liver.

	HCV-RNA POSITIVE THYROID	HCV-RNA POSITIVE SALIVARY GLAND	HCV-RNA POSITIVE PANCREAS	HCV-RNA POSITIVE KIDNEY	HCV-RNA POSITIVE OVARY	HCV-RNA POSITIVE SEMINAL VESICLE
ANTI-HCV POSITIVE CASES	4/6 (66%)	4/5 (80%)	2/5 (40%)	0/8 (0%)	0/2 (0%)	3/3 (100%)
ANTI-HCV NEGATIVE CASES	0/6 (0%)	0/5 (0%)	0/5 (0%)	0/6 (0%)	0/2 (0%)	0/2 (0%)

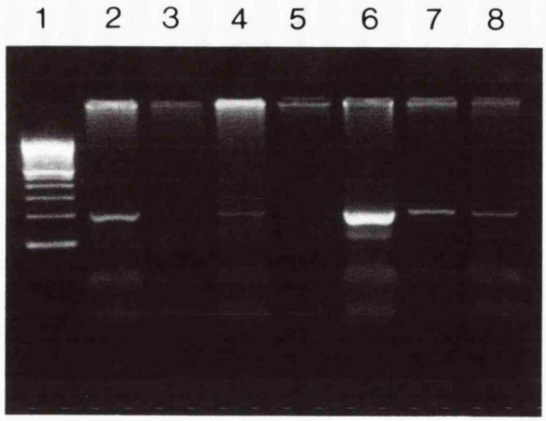


Figure 6.1. Ethidium bromide stained agarose gel of PCR products from various tissues from a single autopsy.

Lane 1 100bp ladder
Lane 2 HCV-RNA positive post mortem liver, showing band at 200bp
Lane 3 PCR product from kidney of same patient, without any bands.
Lane 4 PCR product from thyroid, showing band at 200bp
Lane 5 Negative control
Lane 6 PCR product from seminal vesicle.
Lane 7 PCR product from pancreas
Lane 8 PCR product from salivary gland

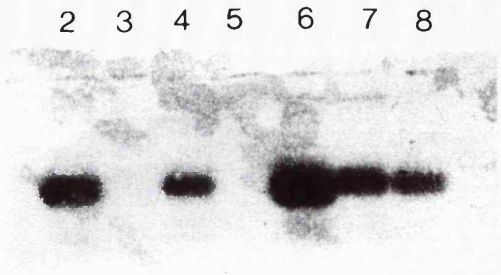


Figure 6.2. Corresponding Southern blot hybridised with digoxigenin labelled ALX89 probe.

Showing binding of probe to samples in lanes 2, 4, 6 7 and 8, but not to lanes 3 and 5. This confirms that liver, thyroid, pancreas, seminal vesicle and salivary gland contained specific HCV PCR products, and that kidney tissue from the same patient did not.

Livers from 3 HCV-RNA positive (2M,1F) and 3 HCV-RNA negative (3M) patients were tested using human anti-HCV^{FITC}.

Two out of three HCV-RNA positive patients had detectable HCV antigen within the liver. Both the livers showed intense staining of more than 50% of hepatocytes (see figures 6.3 and 6.4). None of the three HCV negative patients had detectable HCV antigen within liver tissue.

Thyroid and salivary gland samples from the same patients were tested for HCV antigen. Two out of three thyroid and 2/3 salivary gland samples from HCV-RNA positive cases showed staining with anti-HCV^{FITC}. In the salivary glands, the glandular epithelium was stained by anti-HCV^{FITC}, whilst in the thyroid, the follicular lining cells were positive (see figures 6.5 - 6.8). Samples from HCV negative cases were negative.

These results were the same as the PCR results for the same tissue (see table 6.3). One of two seminal vesicle samples from HCV positive patients showed some staining with anti-HCV^{FITC} (when compared to the HCV negative control tissue) but the staining was not convincing.

Table 6.3 Tissue PCR results versus α-HCV staining in patients from whom frozen tissue was available (2M, 1F).

	Anti-HCV positive cases		Anti-HCV negative cases	
	HCV-RNA positive	α-HCV^{FITC} positive	HCV-RNA positive	α-HCV^{FITC} positive
Liver	3/3	2/3	0/3	0/3
Thyroid	2/3	2/3	0/3	0/3
Salivary gland	2/3	2/3	0/3	0/3
Kidney	0/3	0/1	0/3	0/1
Seminal vesicle	2/2	1/2	0/3	0/2

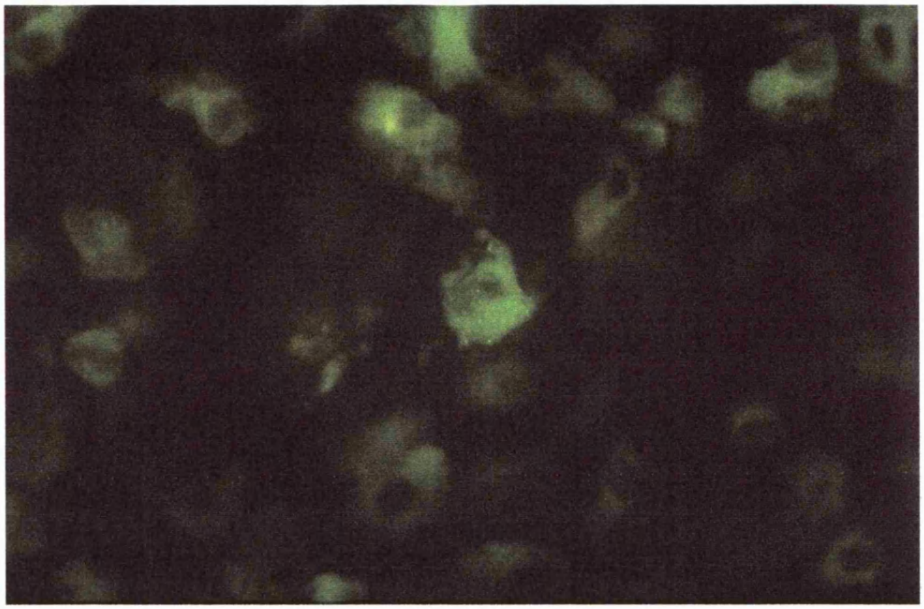


Figure 6.3 Post mortem liver sample stained with anti-HCV. Strong staining of hepatocyte cytoplasm is seen. Mag x660.

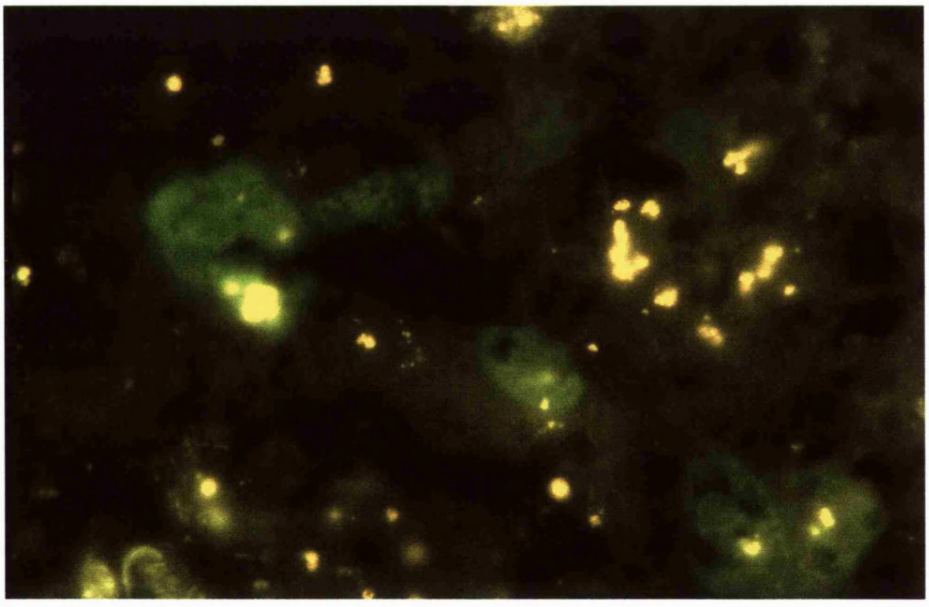


Figure 6.4 Post mortem liver sample from the second post mortem showing positive staining of liver tissue. Intense staining of some hepatocytes is seen, together with some lipofuscin. Mag x660.

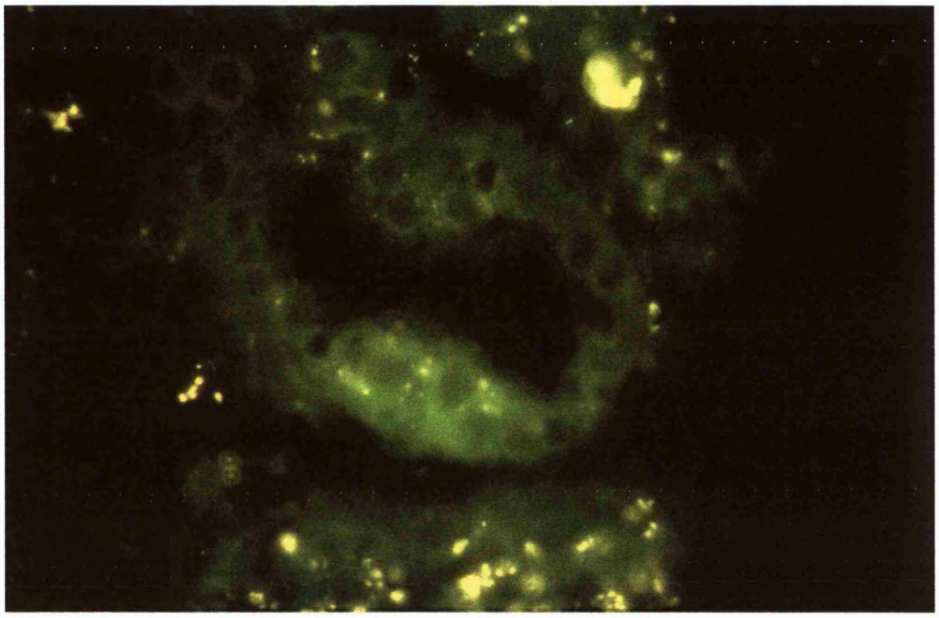


Figure 6.5 Salivary gland from HCV positive patient stained with anti-HCV antibody. Glandular epithelium showed positive staining for HCV. Mag x660.

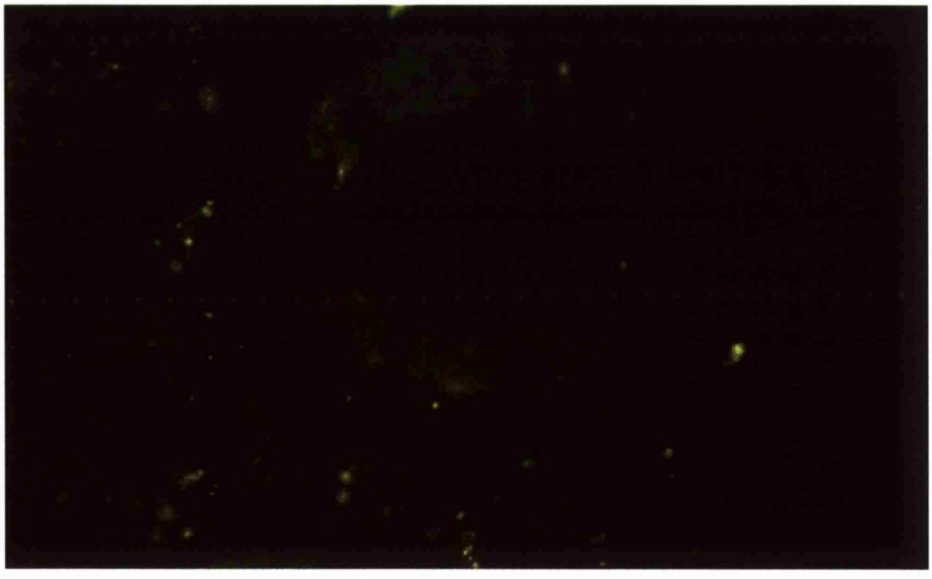


Figure 6.6 Salivary gland from HCV negative patient stained with the fluorescent anti-HCV antibody. No labelling of any tissue element was seen. Mag x660.

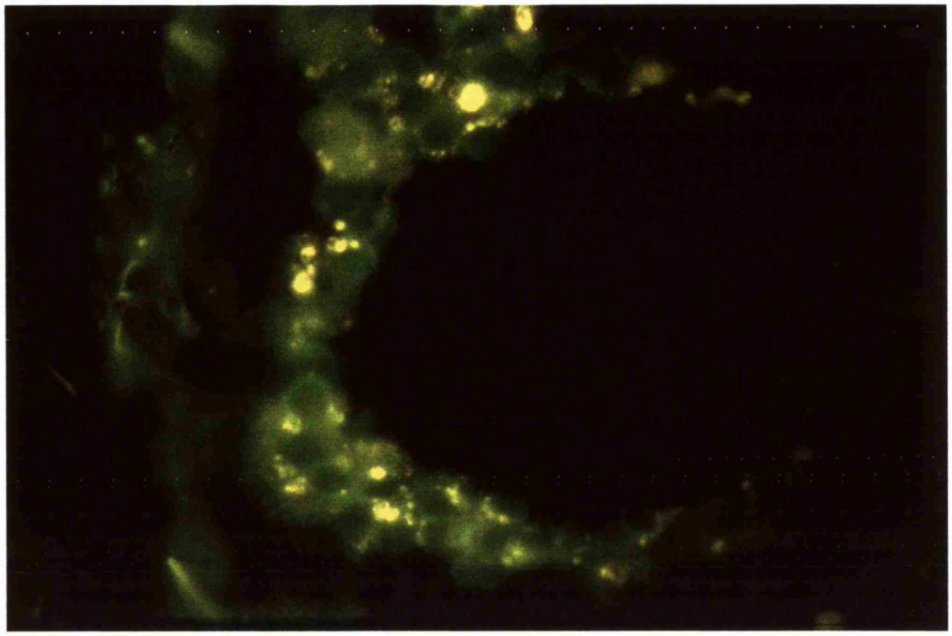


Figure 6.7 Thyroid from HCV positive patient stained with anti-HCV. Follicular cells showed positive staining with the antibody. Mag x660.

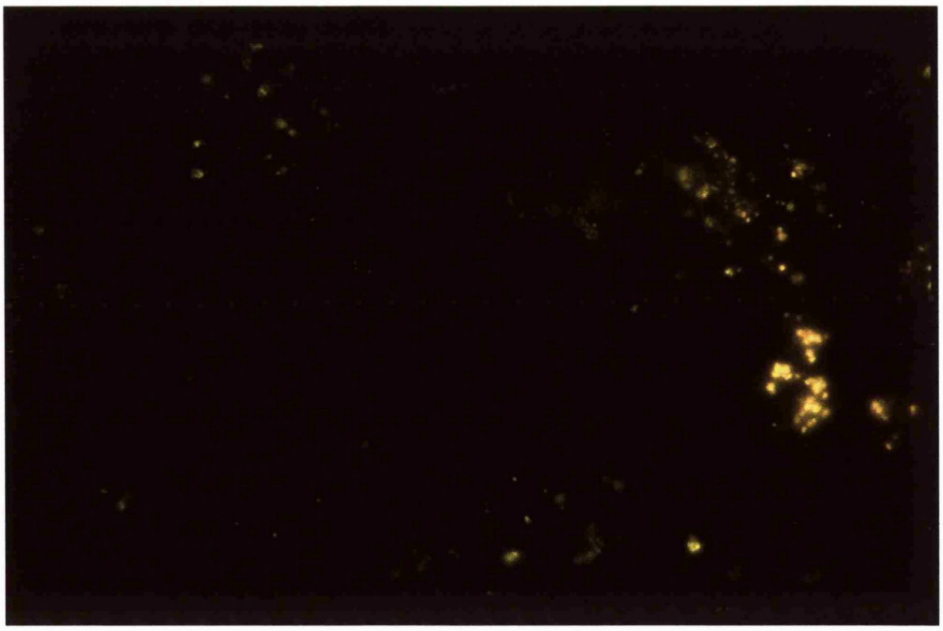


Figure 6.8 Thyroid from HCV negative patient stained with anti-HCV antibody. No positive staining of any cellular element is seen. Mag x660.

Discussion

This study addresses the question of which tissues, other than liver, contain HCV-RNA as detected by tissue PCR. Firstly, it was shown that HCV-RNA can be detected in post mortem liver tissue. Eight of eleven anti-HCV positive cases (73%) had HCV-RNA detectable in post mortem liver samples by tissue PCR. This result is comparable to that obtained on FFPE liver biopsies which have been stored for less than 1 year (see chapter 2).

This work has shown that HCV-RNA can also be detected in post mortem tissue samples from thyroid (4/6 cases), salivary gland (4/5 cases), pancreas (2/5 cases) and seminal vesicle (3/3 cases), but not in kidney (0/8 cases) or a range of other tissues. These data support the findings of other workers, who have detected HCV-RNA in saliva and semen,(Numata *et al.*, 1993; Liou *et al.*, 1992; Couzigou *et al.*, 1993; Wang *et al.*, 1992; Young *et al.*, 1993) and imply that the virus in these secretions comes not from the blood, but from a reservoir of virus within the tissue. Liou *et al.*(1992) detected HCV-RNA in 48% of saliva and 24% of seminal fluid samples from patients with chronic hepatitis C; Couzigou *et al.* (1993) found 62% of chronic hepatitis C patients had HCV-RNA detectable in saliva, which correlates with the results here, with detection of HCV-RNA in 80% of salivary glands.

Whilst these results do not prove that HCV is transmitted by saliva or semen, they support previous epidemiological studies reporting the transmission of hepatitis C by human bite, (Figueiredo *et al.*, 1994; Dusheiko *et al.*, 1990) by saliva, (Abe *et al.*, 1987; Abe and Inchauspe, 1991) and by sexual and household contact (Chang *et al.*, 1994; Pol *et al.*, 1993). Kao *et al.* (1992) have suggested that

interspousal sexual transmission, although of low efficiency, may be the most important route of intrafamilial spread. The detection of HCV-RNA in seminal vesicle but not in female gonadal samples is very interesting in view of the findings of Pipan *et al.* (1994) who found a much higher transmission of HCV from husbands to wives than vice versa, with a reported incidence of anti-HCV of 24.2% in female partners of male index cases, but only 7.1% in male partners of female index cases.

The detection of HCV-RNA within the thyroid is also of interest. Many groups have reported thyroid abnormalities in HCV-positive patients, as well as a high rate of anti-HCV positivity amongst patients with thyroid disease (Tran *et al.*, 1992). Whilst some of these abnormalities are induced by interferon treatment (Baudin *et al.*, 1993; Chung and Shong, 1993), thyroid autoantibodies have been detected in 12% of HCV positive patients in one study (Nagayama *et al.*, 1994), and in 31% of women with chronic hepatitis C before interferon therapy in another (Tran *et al.*, 1993). This study provides the first evidence that HCV within the thyroid could be causing these abnormalities, although the data does not indicate whether the virus has a direct cytopathic effect or triggers an autoimmune type response.

HCV-RNA was also detected in a proportion of pancreatic tissue samples (2/5). A recent report by Allison *et al.* (1994) found that there was a link between HCV related cirrhosis and diabetes mellitus. Whilst diabetes mellitus is a recognised complication of cirrhosis, this group demonstrated that there was significantly more diabetes mellitus in patients with HCV related cirrhosis than in patients with cirrhosis due to other causes (50% cf 9%; $p<0.0001$). Current opinion holds that

type I diabetes mellitus can be triggered by viral infection (Cotran *et al.*, 1989); on this evidence, HCV could be a trigger of this disease.

Detection of extrahepatic HCV-RNA is of importance in relation to the reinfection of liver allografts following transplantation for HCV disease. These areas of infected tissue provide sites from which the virus can re-infect the new liver. The results also have important implication for pathologists and mortuary technicians. Detection of HCV-RNA and HCV antigen within liver and other tissues demonstrates that viable virus may survive within these patients and pose an infectious hazard for those in contact with the body. Indeed, a recent survey of infections within laboratories showed that post mortem technicians had a higher rate of NANB infections than other laboratory and medical staff (Grist and Emslie, 1994).

Tissue PCR results of this nature could be difficult to interpret, as it could be argued that the positive results come from blood or PBMC's trapped within the tissue and not from the solid tissue cells. However, we have recently carried out a study (appendix 6.3) which shows that HCV-RNA is not detected in formalin fixed clotted blood samples from serum PCR positive patients using this tissue extraction procedure, even though liver biopsies from the same patients are positive using the same protocol. This is partly caused by the volumes tested. Five 3μm sections from a 1cm^2 block of tissue (or, in this experiment, fixed blood) are equivalent to a volume of only 1.5μl; a volume of 100μl of serum is usually used for detection of HCV (Brown *et al.*, 1992a), and low amounts of target DNA can be associated with failure to amplify (An and Fleming, 1991). Moreover, most of the volume a tissue sample consists of endogenous cells, with blood contributing only a small proportion

of this volume. In addition, the observation that most tissues (including organs with a greater proportion of blood than those which tested positive) were negative in patients 1-3; and that certain tissues were consistently positive, and others consistently negative, in the other patients, supports the conclusion that the HCV-RNA comes from the tissue cells and not from any blood within the block.

The direct detection of HCV antigen within liver, salivary gland and thyroid tissue from two patients lends support to this statement. This suggests that HCV is directly infecting these tissues rather than simply 'pooling' there.

The original aim of this section of work was to investigate possible alternative routes of HCV transmission. However, since the work began it has become clear that HCV infection is linked to a number of extrahepatic immunological disorders of uncertain pathogenesis. Early after the identification of the virus, it was noticed that patients with Sjögrens syndrome had a high incidence of anti-HCV antibodies (Marson *et al.*, 1991); a further study showed a high incidence of lesions resembling those of Sjögrens syndrome in the salivary glands of HCV positive patients (Haddad *et al.*, 1992). In a prospective study of chronic hepatitis C patients, Pawlotsky *et al.* (1994) found salivary gland lesions in 49% of patients. The results presented here suggest that these abnormalities are linked to a direct infection of the salivary gland by HCV, as evidenced not only by the presence of HCV-RNA, but by the detection of HCV antigen within the gland. Similarly, a link has been noted between HCV infection and Hashimoto's thyroiditis (Tran *et al.*, 1992); thyroid abnormalities have also been documented in a proportion of HCV positive patients both before and after interferon treatment (Tran *et al.*, 1993; Marcellin *et al.*, 1992).

These observations are the first demonstration of a possible role for HCV in the pathogenesis of salivary gland lesions and thyroid abnormalities. It is not apparent however if the virus is directly cytopathic, or if any damage is mediated by the immune system. Both thyroid abnormalities and Sjögrens syndrome are associated with the production of a variety of autoantibodies; it could be that HCV infection of these organs triggers this autoantibody production in some patients, and it is these which are pathogenic. None of the patients described here with detectable HCV-RNA and HCV antigen in their salivary gland and/or thyroid had any histological evidence of lymphocytic sialadenitis or of chronic inflammation of the thyroid. This suggests that the interaction between HCV and the host immune system is important in the pathogenesis of these lesions.

HCV infection has more recently been identified as a major cause of essential mixed cryoglobulinaemia (Levey *et al.*, 1994), membranoproliferative glomerulonephritis and other vasculitic diseases (Koff and Dienstag, 1995). HCV-RNA was not detected in the kidney of any of these HCV positive patients; nor was HCV antigen. Whilst none of these patients showed structural abnormalities in the kidney, this finding suggests that membranoproliferative glomerulonephritis is probably not caused by a direct infection of the kidney. This evidence supports the conclusions of Pawlotsky *et al.* (1994); that the immunological abnormalities associated with HCV infection can be grouped into immune-complex mediated disease, such as membranoproliferative glomerulonephritis and essential mixed cryoglobulinaemia; and autoimmune diseases, in which HCV may trigger an abberant immune response. The evidence presented here suggests that salivary gland and thyroid abnormalities seen in these patients is related to a direct infection of the

tissue by HCV, whilst vasculitic diseases are related to the effects of immune complex deposition. The pathogenesis of salivary gland and thyroid damage may either be a direct effect of the virus, or indirect effect via an antiviral immune response or an abberant autoimmune response triggered by HCV infection.

CHAPTER 7

GENERAL DISCUSSION

The aim of this thesis was to explore the pathogenesis of HCV infection. The experiments described herein have provided some interesting insights into how hepatitis C virus causes disease, but have also left many questions unanswered.

This body of work has established that tissue PCR for detection of hepatitis C virus is reproducible, reliable, a sensitive technique, and is a useful addition to the battery of tests available for HCV testing. It provides the invaluable knowledge of which tissue samples contain hepatitis C virus. Whilst detection of the virus in a particular sample does not prove that the virus is causing any disease or abnormality within the tissue, it does allow the differentiation of samples into those which do and those which do not contain virus, and the collection of observations and correlation of differences between the two groups. This was particularly useful in respect to the study on autoimmune hepatitis, where the differentiation of samples positive for HCV from those without detectable virus allowed the assessment of features which help distinguish true type I AIH from hepatitis C virus infection associated with autoantibody production. Such differentiation was not possible using serological techniques alone, as samples were not available from all patients, and one patient was negative in serum, but positive in tissue. This study showed that HCV infection can be detected in some patients with high levels of autoantibodies, who do not respond to steroid therapy. It did not show whether HCV infection induced autoantibody production in these patients, or if their liver disease was solely caused by the virus or was due to a combination of the two factors. Other groups who have studied these patients have found that they do not respond well to steroid treatment, particularly in the long term, and that they may show an adverse reaction to interferon therapy. Likewise, in the post transplant series, tissue PCR allowed

identification of biopsies containing HCV RNA, and revealed that when HCV was first detectable after transplant, in most cases, there were no histological features in the biopsy which indicated reinfection of the graft. These features only became apparent at a later stage, and were similar to the features of chronic hepatitis C infection outside the transplant situation, ie lobular hepatitis and presence of lymphoid aggregates in particular.

HCV antigen detection showed that the topography of infected cells does not appear to relate to these histological features of HCV infection. No direct relationship was seen between interface hepatitis and presence of lymphoid aggregates and the position of HCV antigen expressing cells. This suggests that aggregates may not be a direct response to HCV infected cells but could represent an autoimmune reaction triggered by infection (Khakoo *et al.*, 1995). However, a direct relationship was seen between total HCV staining and the amount of inflammation, as assessed by Knodell scoring. Also, cytotoxic T cells appear to be important in the control of HCV antigen expression, as numbers of CTL were generally lower in cases with intensely stained hepatocytes. Antigen expression was also much higher in immunosupressed post transplant patients than in non-immunosuppressed patients (see figures 3.4 - 3.9; figures 5.3 - 5.8), which supports the theory that the immune system controls antigen expression in some way. This complex link between the immune status of the infected individual and the course of HCV-induced disease was emphasised in another aspect of the post transplant work. A proportion of the patients who received a liver transplant for end stage HCV-associated cirrhosis, and were subsequently under immunosuppressive treatment, had a more severe and accelerated course of disease post transplant than is seen

outwith the transplant situation. This could reflect a direct cytopathic effect of the virus when relieved of immune control. However, these patients also showed the characteristic picture of inflammation associated with HCV infection, ie lymphoid aggregates, lobular inflammation and interface hepatitis. This inflammation appeared unaffected by the immunosuppressive therapy the patients were under. It could be argued that this response was ameliorated by immunosuppresive therapy, as levels of HCV-RNA and HCV antigen are demonstrably higher in post transplant patients, whereas the inflammation did not appear correspondingly increased. However, chronic inflammation was seen eventually in the majority of patients with an early recurrence of HCV; this response did not prevent or control reinfection of the liver. This could indirectly support the theory that lymphoid aggregates are not a direct antiviral response but represent an autoimmune response triggered by viral infection. Tissue damage could be mediated via this response while the more effective, possibly CTL mediated antiviral response is more severely limited by immunosuppression, allowing additional cytopathic effects of the virus to come into play.

As already stated, liver transplant patients at RFH receive a standard immunosuppresive regime post transplant, which includes corticosteroids, cyclosporine and azathioprine. All are non-specific immunosuppressants, which lower the activity of the immune system regardless of the antigen (Hutchinson, 1993). Steroids have general anti-inflammatory properties and suppress activated macrophages; they also interfere with antigen presenting cell function and reduce expression of MHC antigens. Cyclosporine suppresses lymphokine production by T_H cells and reduces expression of receptors for IL-2 on lymphocytes undergoing

activation, whilst azathioprine is an anti-proliferative drug. Cytotoxic T cells generally act by recognizing viral antigen when presented by the infected cell in the context of MHC class I molecules. They then release perforin which causes apoptosis of the target cell by producing transmembrane channels. However this response is only triggered after exposure to high levels of IL-2. As steroids inhibit MHC expression, cyclosporine interleukin expression and azathioprine cell proliferation, CTL response is hit with a "triple whammy" and may be thus disproportionately affected by combined immunosuppresive therapy. This is not undesirable in the context of rejection of the transplant as cytotoxic T cells are thought to be one of the two effector cell types in tissue rejection (Krams *et al*, 1993). However, if cytotoxic T cells provide the first line of defence against hepatitis C virus, then lack of an effective CTL response in immunosuppressed patients could result in a higher level of virus and antigen expression in these patients, and an accelerated course of disease in some. This effect could be exacerbated by the fact that by the time a patient comes to transplant, the infecting virus is already a chronic insult, and may no longer be recognised by either cellular or humoral defences.

It was also noted that patients with an early reinfection of the graft had a higher later incidence of chronic hepatitis and cirrhosis than those in whom reinfection was not detected until a later stage ($\geq$80 days) post transplant. Reasons for this later reinfection in some patients was not clear; however, patients with an early re-infection also appeared to have more episodes of rejection, and consequently may have had greater immunosuppression. This could have allowed faster reinfection of the graft in these patients. In addition, the work presented here

has shown the difficulties in differentiating the portal inflammation of rejection from hepatitis. A mistaken diagnosis of rejection could lead to more immunosuppression, resulting in worse hepatitis and a diagnosis of "steroid resistant rejection".

The post mortem work presented here represents the first evidence that HCV may infect organs other than the liver. This has very important implications, both in the inapparent and non-parenteral transmission of hepatitis C, possibly by saliva and semen; and in relation to noted extrahepatic effects of HCV infection. Transmission of HCV via saliva, containing virus secreted from the salivary glands, could represent one of the inapparent sources of HCV infection. Pooling of virus within seminal vesicle could represent a source of HCV which is shed into semen, so providing a route for sexual transmission. The evidence presented here does not give a direct measure of the amount of HCV in these tissues: if present in only a low amount, this could explain the low frequency of transmission via these routes. Indeed, the relatively low level of staining with anti-HCV^{FITC} seen in all the extrahepatic tissues suggests that viral load is low; Francesconi *et al* (1995) have suggested that antigen staining is directly related to the amount of virus present within the tissue.

The evidence presented here suggests that as proposed by other workers, HCV associated extrahepatic disease falls into two categories; that mediated via immune complex deposition, and that caused by either a direct effect of the virus, or by immune response to infection. Failure to detect HCV-RNA in any renal samples suggests that glomerulonephritis observed in a proportion of HCV positive patients may be mediated via immune complex deposition rather than direct viral infection. The thyroid abnormalities observed in these patients are associated with

the production of anti-thyroid antibodies; the demonstration of HCV-RNA and HCV antigen within thyroid is additional evidence that HCV infection can trigger production of autoantibodies. Detection of HCV-RNA within pancreatic tissue from a proportion of these patients provides an explanation for the increased incidence of diabetes seen in HCV positive cirrhotics (Allison *et al.*, 1994): this may represent either a direct effect of the virus or may be viral triggered autoimmune disease. Infection of salivary and pancreatic tissue by a hepatotropic virus may not be that surprising. These organs share a common developmental origin, ie the endoderm, and therefore may share common cell surface proteins and receptors recognised by the virus. This does not explain the infection of the other organs observed.

Viral load in these extrahepatic sites of HCV infection could influence the speed of reinfection of the liver graft in the post transplant situation, and hence the long term prognosis for the patient.

Overall, the work suggests that in some circumstances HCV can be directly cytopathic when the patient is immunosuppressed. The immune response to infection is complicated. It is possible that infection triggers in the first instance a cytotoxic T cell response, but this immune pressure causes selection of a different quasispecies of virus expressing an antigenically different protein not recognised by cytotoxic T cells. This mechanism also allows the virus to elude any humoral response and cause chronic infection. HCV may also trigger an autoimmune response, either through cross-reactivity with a host epitope or through exposing autoantigens normally sequestered within the cell and not encountered by the immune system. The association of HCV infection with autoantibodies against liver, thyroid and possibly salivary gland and pancreatic antigens could be a reflection of this type of

immune response.

Future work.

A prime aim of future work on HCV would be to continue the development of the human anti-HCV antibody for detection of antigen within paraffin embedded tissue samples. This could include labelling the antibody with alternative reporter molecules, such as digoxigenin, which may provide a more sensitive antibody detection system. The failure of non-human poly- and mono- clonal antibodies raised against synthetic antigens to detect HCV infected samples in this study suggests that the tertiary structure of the antigen is important in eliciting specific antibody response. It may be possible to produce purified 'natural' HCV antigen using the human polyclonal antibody. This antigen could then be used to immunise animals with a view to producing a more sensitive, specific and versatile antibody, which could allow much more detailed studies of the relation of HCV infected cells to inflammation, apoptosis and fatty change within biopsies. An improved antibody could also allow studies at the electron microscopic level, and allow visualisation of the bright spots of staining seen in some biopsies, as well as the virus itself.

These post mortem studies should be furthered by increasing the number of cases tested, possibly by collaboration with other centres. This is particularly important to substantiate evidence that salivary gland and thyroid are directly infected. Further development of the anti-HCV antibody would be useful in this respect. Detection of replicative intermediates of HCV within these tissues would support the evidence already presented of direct infection, and quantification of viral load would help clarify the relative risks of transmission via body fluids.

The future investigations of HCV positive patients post OLT requires

increased numbers to substantiate the finding of a relationship between early and late infection of the graft, and differences in outcome between the two groups. Staining of sequential biopsies with anti-HCV will elucidate the course of infection post transplant. It has recently been suggested that the level of HCV antigen expression in the first month post transplant is an important prognostic indicator of graft survival/success. Quantification of hepatic viral load may also be important in outcome, and HCV genotyping may be an important factor in outcome. A more detailed investigation of the precise effect of immunosuppression on the different subsets of lymphocytes and the relation of this to speed of reinfection of the graft and long term outcome would greatly clarify the interactions between the immune system and the virus, and would improve the understanding of the pathogenesis of liver damage in HCV infection generally.

In addition to studying the topographical relationship between HCV infected cells and inflammation, the expression of MHC class I and II on infected hepatocytes could be studied, with a view to elucidating the important mechanisms in the anti-HCV immune response. In particular, any change in expression of histocompatibility antigens and important cell signalling molecules such as IL-2 and ICAM-1, in relation to stage of disease, and amount and type of inflammation, could be very revealing both in the post transplant situation and in chronic HCV patients.

There are many questions still to be answered in relation to HCV infection - how is the virus transmitted apart from the parenteral route, what proportion of people infected develop chronic hepatitis and cirrhosis and why, and what factors affect progression of liver disease? All the work presented here demonstrates that

interactions between the immune system of the infected individual and HCV infected cells are fundamental to our understanding of the pathogenesis of HCV disease, but accentuate how little we know of such interactions. This is probably the most important area for future work on the pathogenesis of HCV-related liver disease.

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APPENDICES

APPENDIX 2.1

Routine processing schedule used for all specimens

1) Buffered formalin	2 hours at 40°C
2) 70% Alcohol	1 hour at 40°C
3) 90% Alcohol	1 hour at 40°C
4) Absolute alcohol	1 hour at 40°C
5) Absolute alcohol	1 hour at 40°C
6) Absolute alcohol	1 hour at 40°C
7) Xylene	1 hour at 40°C
8) Xylene	1 hour at 40°C
9) Xylene	1 hour at 40°C
10) Xylene	1 hour at 40°C
11) Wax	rinse at 60°C
12) Wax	1 hour at 60°C
13) Wax	1 hour at 60°C
14) Wax	1 hour at 60°C

All solutions were pumped in and aspirated with a pressure/vacuum cycle.

APPENDIX 2.2

PCR FOR HEPATITIS C VIRUS

1) Once reverse transcribed, samples can be PCR'd using primers to the 5' end of the HCV genome (see table 2.1 for sequences). All primers used in this work were synthesised by the Oswell DNA Service, Dept of Chemistry, University of Edinburgh. The conditions described for 1st round PCR were used both for HCV 1st round and for albumin PCR.

1st round PCR mix

10 x buffer	(Gibco/BRL)	10μl
dNTP's (5mM)	(Pharmacia)	4μl
$MgCl_2$ (50mM)	(Gibco/BRL)	3μl
Sterile H_2O		75.5μl
Primer i (50 pmol)		1μl
Primer ii (50 pmol)		1μl
Taq DNA polymerase (2.5units)	(Gibco/BRL)	0.5μl
		95μl

A 'master mix' for PCR is made up inside a laminar air flow cabinet, to minimise contamination. The recommendations of Kwok and Higuchi (1989) were strictly adhered to. The amount of each constituent is scaled up to give enough mixture for each sample being PCR'd, plus a negative control, in which water is added instead of sample. The first four constituents are combined in a sterile tube, and treated with U.V. light for 5 minutes (to reduce contamination). The primers and Taq DNA polymerase are then

added, the mixture is aliquoted (inside the cabinet) into sterile tubes, and overlaid with sterile mineral oil.

2) For 1st round amplification, and albumin PCR, 5μl of reverse transcribed sample is added to the 95μl of PCR mix. .Samples are then amplified on a programmable thermal cycler (Techne), according to the programme detailed in figure 2.1.

3) 2μl of first round amplified HCV product is added to 48μl of 2nd round PCR mix, which is made as before, in the laminar air flow cabinet.

2nd round PCR mix

10 x buffer	(Gibco/BRL)	5μl
dNTP's (5mM)	(Pharmacia)	2μl
$MgCl_2$		1.5μl
Sterile H_2O		37μl
Primer iii (50 pmol)		1μl
Primer iv (50 pmol)		1μl
Taq DNA polymerase (2.5 units)		0.5μl
		48μl

The samples are then amplified on the thermal cycler according to the programme detailed in figure 2.2.

10) Results are analysed by agarose gel electrophoresis (see appendix 2.3).

APPENDIX 2.3

AGAROSE GEL ELECTROPHORESIS

Amplified samples are run on an agarose gel to visualise the final PCR product.

1) Make a 2% agarose gel by suspending 0.5g of electrophoresis agarose (Pharmacia) in 25ml of TBE (see below) buffer. The suspension is boiled and shaken gently to dissolve the agarose. Allow the solution to cool for 2-3 mins. Add 0.4μl of ethidium bromide (10 mg/ml, Sigma) and shake to disperse it throughout the molten agarose. N.B. Ethidium bromide is mutagenic and must only be handled whilst wearing gloves. The molten agarose is now poured into the gel mould, and the comb for the wells placed before the gel sets.

2) While the gel is setting, the samples are prepared. For each sample, 1μl of loading buffer (see below) is put into a tube. To this is added 5μl of PCR product. The negative controls are also run on the gel, along with a 100bp DNA ladder (Gibco/BRL; 0.5μg ladder + 4μl H_2O + 1μl loading buffer), which allows products to be sized.

3) Once the gel has set, the comb and dams are removed and the electrophoresis chamber filled with enough running buffer (TBE) to just cover the gel. Samples are then loaded carefully into the wells. The gel is run at 100 volts for 15-20 mins - the loading dye travels slightly faster than

the PCR products and can be used to monitor the running.

4) The results are viewed by looking at the gel with a U.V. transilluminator. The ethidium bromide makes the DNA fluoresce orange in U.V. light. HCV PCR products (using primers detailed; see table 2.1) should be 200 base pairs in length (size can be estimated by comparing with the DNA ladder); albumin PCR products should be 270bp long. Results were photographed using a polaroid land camera with polaroid film. A 10 second exposure was used, and the picture allowed to develop for 30 seconds (see figure 2.4).

TBE buffer:	Working solution, 0.5X:	0.045M Tris-borate
		0.001M EDTA
	Stock solution, 5X:	54g Tris base
	($1dm^3$)	27.5g boric acid
		20ml 0.5M EDTA (pH 8)

10X loading buffer:	50% glycerol
	100mM EDTA
	1% SDS
	0.1% Bromophenol blue

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APPENDIX 2.4

SOUTHERN BLOTTING

1. After electrophoresis and photography, any unused areas of the gel are trimmed off with a scalpel.

2. Denature the DNA by soaking the gel for 45 minutes in several volumes of 1.5M NaCl, 0.5N NaOH with constant, gentle agitation.

3. The gel is rinsed briefly in deionised water, and neutralised by soaking for 30 minutes in several volumes of a solution of 1M Tris (pH 7.4), 1.5M NaCl at room temperature with constant, gentle agitation. Change the neutralisation solution and soak for a further 15 minutes.

4. While the gel is in the neutralisation solution, a piece of blotting paper (Sigma) is wrapped around a stack of large glass slides to make a support longer and wider than the gel. This support is put into a plastic container, which is then filled with 10xSSC (1.5M NaCl, 0.15M NaCitrate) almost to the top of the support. The blotting paper is left to soak, then any bubbles smoothed out with a glass rod.

5. A charge modified nylon hybridisation membrane (Sigma) is prepared by floating it on a dish of deionised water to soak it, then transferring to a dish of 10xSSC for at least 5 minutes.

6. The denatured gel is inverted and put onto the support, with the underside uppermost, and centred on the blotting paper. The gel is then surrounded by cling film, which will stop 'short circuiting' of the buffer into the towels (see later).

7. The wet membrane is put on top of the gel, and any air bubbles excluded. Two pieces of blotting paper the same size as the gel are soaked in 2xSSC and put on top of the membrane.

8. A stack of paper towels (5-8cm high) is cut, just smaller than the gel. These are put on top of the blotting paper, covered with a glass plate, and weighed down with a 500g weight. This is now left overnight so that the buffer passes through the gel into the towels, whilst at the same time transferring the DNA from the gel onto the membrane.

9. The next day, the towels are removed, and with a soft pencil, the positions of the gel slots marked on the membrane. The gel is then peeled from the membrane and discarded, and the membrane washed in 6xSSC. After draining for 30 minutes, the membrane is baked at 80^0C for 30 minutes, to fix the DNA to the membrane. It is now ready for hybridisation (see appendix 2.6), and can be probed immediately or stored at room temperature for up to two months.

APPENDIX 2.5

LABELLING OF PROBE FOR SOUTHERN HYBRIDISATION

1) The oligoprobe ALX89 was end labelled with ^{32}P-ATP (Amersham International) by incubating at 37°C for 1 hour with T4 polynucleotide kinase (Boehringer Mannheim).

10 x kinase buffer	2μl
ALX89 (30 pmol)	1μl
gamma ^{32}P-ATP (100 pmol)	10μl
T4 polynucleotide kinase (20u)	2μl
H_2O	5μl
	20μl

2) The reaction was stopped by heating the mixture to 95°C for 3 minutes.

3) Unincorporated nucleotides are separated from radiolabelled probe by passing down a sephadex G50 (Pharmacia) column. This is made by plugging the end of a glass pasteur pipette with glass wool. The glass wool is tamped down gently, then the pipette is clamped in a vertical position, and filled with Sephadex G50 suspended in TE buffer (see below).

4) The column is washed with 2ml of TE buffer, then the labelling mixture added to the column. The eluent is collected in sterile eppendorf tubes (7 - 10 drops per tube), whilst keeping the column topped up with TE to wash down the probe.

5) Twelve to sixteen aliquots of eluent are collected. Using a geiger counter, the radioactivity of the tubes is compared. There should be a peak of activity starting at aliquot 4 or 5 (representing labelled ALX89 probe), and another peak at aliquot 9 or 10, representing the unincorporated nucleotides. The hot aliquots from the first peak are combined, and all other tubes discarded.

6) The activity of the probe is measured by scintillation counting. 2μl of the eluent is added to 5ml of scintillant (Cocktail T, BDH) and counted on a liquid scintillation counter (LKB).

7) The probe is added to the filter at an activity of 10^6 counts/ml of hybridisation buffer (see appendix 2.6).

TE buffer (pH 8.0): 10mM Tris.Cl (pH 8.0)
1mM EDTA (pH 8.0)

APPENDIX 2.6

SOUTHERN HYBRIDISATION

1) The prepared membrane (see appendix 2.4) is put into a plastic bag just larger than itself. Hybridisation buffer (HB) is added to the bag, allowing approximately 0.2ml of buffer per cm^2 of membrane. Excluding as much air as possible, the open end of the bag is sealed with a heat sealer. The bag is then incubated at 42°C in a shaking waterbath for at least two hours.

Hybridisation buffer :-

6 X SSC (see below)

0.1% SDS (Sigma)

50mM Na Phosphate, dibasic (pH 7.4) (BDH)

10 X Denhardts (see below)

100μg/ml yeast tRNA (Sigma)

2) After 2 hours, the labelled probe is added to the hybridisation buffer in the bag. ^{32}P - labelled A89 oligoprobe was added at a concentration of 2 x 10^6 counts per ml of HB. Again, as much air as possible is excluded from the bag before sealing. The filter is incubated overnight at 42°C in the shaking waterbath.

3) After hybridisation, the filter is washed in 6 X SSC, 0.1% SDS as follows :-

2 x 5 minutes at room temperature

2 x 10 minutes at 55°C

4) The membrane is drained and wrapped in cling film, then put into a film exposure cassette (Amersham International) with intensifying screens. In the dark room, an X-ray film (Amersham International) is put on top of the filter, and the cassette sealed. The film is exposed at -70°C for 4 hours, then developed using an automatic X-ray developing machine. Dark bands should be visible on the autoradiograph where the probe has hybridised.

Denhardts solution (50X):

Ficoll (Sigma)	5g
Polyvinylpyrrolidone (Sigma)	5g
Bovine serum albumin (Sigma)	5g
Sterile H_2O	up to 500ml

20X SSC (pH 7.0):

NaCl (BDH)	175.3g
NaCitrate (BDH)	88.2g
H_2O	up to $1dm^3$

APPENDIX 2.7

EXTRACTION OF NUCLEIC ACIDS FROM FORMALIN FIXED, PARAFFIN EMBEDDED MATERIAL.

1) 5 x 3μm sections are cut from the routine block, using a fresh piece of blade for each block. For each batch of samples, the microtome is cleaned between each block, and the positive control block is cut last.

2) Sections are put into a sterile microcentrifuge tube, with sterile forceps. 200μl of digestion buffer (100mM NaCl, 10mM Tris-Cl, 2.5mM EDTA, 0.5% SDS, pH 8.4) containing 0.2mg/ml proteinase K (Sigma) is added.

3) The tube is vortexed vigorously, and incubated at 42^{0}C for 3 days.

4) After incubation, proteins and the remaining paraffin wax are removed from the digestion mix by phenol/chloroform extraction. 200μl of phenol/chloroform/isoamyl alcohol (25:24:1 ; Tris buffered phenol, pH7.9, Sigma; Chloroform, BDH; Isoamyl alcohol, Sigma) is added to the digestion mix. The solution is vortexed vigorously, then spun for 5 mins at 12,000g in a microcentrifuge (MSE).

5) The upper, aqueous layer is removed using a sterile, filtered tip (Labsystems), into a fresh, sterile microcentrifuge tube, and 200μl of

chloroform/isoamyl alcohol (24:1) added. The tube is mixed and spun for 5 mins at 12,000g.

6) Step 5 is repeated.

7) The aqueous layer is removed to a sterile tube. 60μl of 3M Na Acetate and 400μl of high grade ethanol (BDH) are added to each sample, and all are left to precipitate at -70°C for at least 2 hours or overnight.

8) The samples are spun at 12000g for 15 mins to pellet the nucleic acid. The ethanol is poured off, and the pellets dried in vacuo. Each pellet is resuspended in 10μl H_2O containing 25u RNAase inhibitor (Amersham International).

9) Samples can be reverse transcribed and amplified at this stage or stored at -20°C.

APPENDIX 3.1

Optimised immunocytochemistry (ICC) technique used for rabbit polyclonal anti-HCV antibodies.

All buffers and solutions for ICC are made using double distilled water.

1. Slides are dewaxed in xylene (Chemix) for 2 x 5 minutes.
   100% alcohol (Chemix) for 2 x 30s
2. Rinse in double distilled H_2O for 5 mins.
3. Endogenous peroxidase activity in the tissue is blocked by incubating the slides in 0.5% H_2O_2 for 30 minutes.
4. Slides are rinsed in double distilled H_2O for 5 mins.
5. Slides are rinsed in PBS, pH 7.2 (Oxoid).
6. Non-specific binding of secondary antibody is blocked by incubating slides in normal swine serum (Dako), 50% in PBS for 30 mins.
7. Slides are drained and optimally diluted antibody (1/1000 in PBS with 0.1% bovine serum albumin (BSA)) is added to cover the section.
8. Slides are incubated in a humidity chamber overnight at 4^0C.
9. Antibody is rinsed off with PBS for 5 mins.
10. Slides are drained and swine anti-rabbit antibody (Dako), diluted 1/100 in PBS, is added to cover the sections. Slides are incubated at room temperature for 30 mins.
11. Antibody is rinsed off with PBS for 5 mins.
12. Slides are drained and rabbit PAP (Dako), diluted 1/500 in PBS is added for 30 mins.

13. Rinse off antibody with PBS, 5 mins.

14. Incubate with swine anti-rabbit, 1/100 in PBS for 15 mins.

15. Rinse with PBS, 5 mins.

16. Incubate with rabbit PAP, 1/500 in PBS for 15 mins.

17. Rinse in PBS, 5 mins.

18. Incubate sections with 0.06% 3,3 diaminobenzidine tetrachloride (DAB) in 0.05M Tris (pH 7.6) containing 0.04% H_2O_2 for 3 to 5 minutes.

19. Rinse of the DAB with PBS, then wash slides in distilled H_2O for 5 minutes.

20. Counterstain in Harris's haematoxylin (Stevens, 1982) for 3 to 5 minutes.

21. Differentiate in 1% acid alcohol, 3-5 secs.

22. Blue in 0.5% sodium tetraborate, 20 secs.

23. Rinse in double distilled water.

22. Dehydrate slides in alcohol for 2 x 30 secs, clear in xylene, then mount coverslip with DPX (BDH).

APPENDIX 3.2

Optimised immunocytochemistry (ICC) technique used for mouse monoclonal anti-HCV antibodies.

All buffers and solutions for ICC are made using double distilled water.

1. Slides are dewaxed in xylene (Chemix) for 2 x 5 minutes.
   100% alcohol (Chemix) for 2 x 30s
2. Rinse in double distilled H_2O for 5 mins.
3. Endogenous peroxidase activity in the tissue is blocked by incubating the slides in 0.3% H_2O_2 for 12 minutes.
4. Slides are rinsed in double distilled H_2O for 5 mins.
5. Slides are rinsed in TBS.
6. Non-specific binding of secondary antibody is blocked by incubating slides in normal rabbit serum (Dako), 10% in TBS for 15 mins.
7. Slides are drained and optimally diluted antibody (1/10 in TBS/neat) is added to cover the section.
8. Slides are incubated in a humidity chamber overnight at 4^0C.
9. Antibody is rinsed off with TBS for 5 mins.
10. Slides are drained and biotinylated rabbit anti mouse (Dako), diluted 1/200 in TBS, is added to cover the sections. Slides are incubated at room temperature for 30 mins.
11. Antibody is rinsed off with TBS for 5 mins.
12. Slides are drained and ABC (Dako), made up according to the manufacturers instructions, is added for 30 minutes.

13. Rinse off antibody with TBS, 5 mins.
14. Incubate sections with 0.06% 3,3 diaminobenzidine tetrachloride (DAB) in 0.05M Tris (pH 7.6) containing 0.04% H_2O_2 for 3 to 5 minutes.
15. Rinse of the DAB with TBS, then wash slides in distilled H_2O for 5 minutes.
16. Counterstain in Harris's haematoxylin (Stevens, 1982) for 3 to 5 minutes.
17. Differentiate in 1% acid alcohol, 3-5 secs.
18. Blue in 0.5% sodium tetraborate, 20 secs.
19. Rinse in double distilled water.
20. Dehydrate slides in alcohol for 2 x 30 secs, clear in xylene, then mount coverslip with DPX (BDH).

APPENDIX 3.3

Double staining with anti-HCV^{FITC} and anti-TIA antibody.

All buffers and solutions for ICC are made using double distilled water.

1. Cut frozen sections (5μm) and pick up onto PLL (Sigma) coated slides.
2. Air dry for 20 minutes.
3. Fix in chloroform for 5 minutes.
4. Air dry for 5 mins.
5. Sections are incubated with anti-HCV^{FITC}, diluted 1/300 in normal human serum.
6. Rinse for 3 x 7 minutes in phosphate buffered saline (PBS; pH 7.2, Oxoid).
7. Cover sections with anti-TIA (Coulter Clone) 1/1000 in PBS, incubate for 1 hour at room temperature (RT).
8. Rinse in PBS for 5 minutes.
9. Apply rabbit anti-mouse, rhodamine conjugated (Dako), diluted 1/80 in PBS.
10. Rinse in PBS for 5 minutes.
11. Coverslip slides in PBS/glycerol (Citifluor).

APPENDIX 3.4

SCORE SHEET FOR KNODELL SYSTEM

(See footnotes for definitions).

I. Periportal +/- bridging necrosis		SCORE
A:	None	0
B:	Mild piecemeal necrosis (interface hepatitis)	1
C:	Moderate piecemeal necrosis (involves less than 50% of the circumference of most portal tracts).	3
D:	Marked piecemeal necrosis (involves more than 50% of the circumference of most portal tracts).	4
E:	Moderate piecemeal necrosis **plus** bridging necrosis	5
F:	Marked piecemeal necrosis **plus** bridging necrosis	6
G:	Multilobular necrosis	10

II. Intralobular degeneration and focal necrosis		
A:	None.	0
B:	Mild (acidophilic bodies, ballooning degeneration and/or scattered foci of hepatocellular necrosis in $< 1/3$ of lobules or nodules).	1
C:	Moderate (involvement of 1/3 - 2/3 of lobules or nodules).	3
D:	Marked (involvement of $> 2/3$ of lobules or nodules).	4

III. Portal inflammation

A:	No portal inflammation.	0
B:	Mild (sprinkling of inflammatory cells in $<1/3$ of portal tracts).	1
C:	Moderate (increased inflammatory cells in 1/3 - 2/3 of portal tracts).	3
D:	Marked (dense packing of inflammatory cells in $>2/3$ of portal tracts).	4

IV Fibrosis.

A:	No fibrosis.	0
B:	Fibrous portal expansion.	1
C:	Bridging fibrosis (portal - portal or portal - central linkage).	3
D:	Cirrhosis.	4

Footnotes

a) Degeneration - acidophilic bodies, ballooning, focal necrosis - scattered foci of hepatocellular necrosis.

b) Cirrhosis - loss of normal hepatic lobular architecture with fibrous septae separating and surrounding nodules.

c) Bridging is defined as 2 or more bridges in the liver biopsy specimen; no distinction is made between portal - portal and portal - central linkage.

d) Multilobular necrosis - 2 or more contiguous lobules with panlobular necrosis.

APPENDIX 6.1

LABELLING OF PROBE WITH DIGOXIGENIN FOR SOUTHERN HYBRIDISATION

1) The oligoprobe ALX89 was end labelled with digoxigenin (Boehringer Mannheim) by combining the following ingredients and incubating at 37°C for 15 minutes.

5 x buffer (Boehringer Mannheim)	4μl
ALX89 (120 pmol)	4μl
DigdUTP (1mM)	1μl
Tris-dATP (10mM)	1μl
Sterile H_2O	7μl
TdT (50u/μl)	3μl
	20μl

2) The reaction was stopped by placing the mixture on ice, and adding 1ul of 200mM EDTA .

3) To pellet the labelled probe, 2.5μl of LiCl (5M) and 75μl of prechilled ethanol are added to the tube. The mix is then incubated at -70^{0}C for 30 minutes.

4) Spin for 15 minutes at 12,000g in a microcentrifuge (MSE), then pour off the ethanol. The pellet is then dried *in vacuuo* for 15 minutes.

5) The probe is resuspended in 120μl of water, giving a final concentration of 1pmol/μl, which for ALX89 is approximately equal to 10 ng/μl.

6) The probe was then used to detect HCV PCR products by Southern

hybridisation. Hybridisation was carried out as described in appendix 2.6. Probe was added at a concentration of 1ng per ml of hybridisation buffer, and any binding was detected as described in appendix 6.2.

APPENDIX 6.2

DETECTION OF BOUND DIGOXIGENIN PROBE.

All volumes given are sufficient for a 100cm^2 membrane. All incubations are carried out at room temperature with shaking unless stated to the contrary.

1. After overnight hybridisation with the digoxigenin probe (see appendix 2.6), the membrane is washed in 6 X SSC, 0.1% SDS as follows :-

   2 x 5 minutes at room temperature (250ml buffer each).

   2 x 10 minutes at 55°C (250ml buffer each).

2. The membrane is then rinsed for 5 minutes in washing buffer (see below).
3. Membrane is incubated for 30 minutes in buffer 2 (see below).
4. Incubate for 30 minutes in 20ml of anti-digoxigenin antibody, alkaline phosphatase conjugated (Boehringer Mannheim), diluted 1/5000 in buffer 2.
5. Wash 2 x 15 minutes in 100 ml washing buffer.
6. Equilibrate membrane for 2 mins with 20ml buffer 3 (see below).
7. Incubate membrane with 10ml freshly prepared colour solution (see below) in the dark until colour develops. This usually takes about 1 hour but may take up to 24 hours.
8. Stop reaction by washing membrane for 5 minutes with 50ml of buffer 4 (see below).
9. Document results by photographing or photocopying the wet filter.
10. Membrane may be dried at room temperature or baked at 80°C for 20 mins and stored.
11. Colour wil fade on drying but may be restored by wetting in buffer 4.

REAGEANTS (Need to be prepared in advance).

Buffer 1	Maleic acid (100 mM)	11.607g	
	NaCl (150 mM)	8.766g	
	double distilled H_2O	1l	
	Adjust to pH 7.5 with solid NaOH. Autoclave.		

Blocking stock solution (500ml)

10% blocking reagent (Boehringer Mannheim) in buffer 1.

Autoclave and store at 4^0C.

Washing solution

3% Tween 20 (Sigma) in buffer 1.

Buffer 2 (100ml)

Blocking stock solution 1/10 in buffer 1. Final concentration = 1%

Buffer 3 (100ml)

Tris (100 mM)	1.211g	}
NaCl (100 mM)	0.5844g	} Adjust pH to 9.5
MgCl (50 mM)	1.02g	}

Buffer 4 (1l)

Tris (10 mM)	1.211g	}
		} Adjust pH to 8.0
EDTA (1mM)	0.2922g	}

Colour Solution (10ml, freshly prepared)

NBT solution (75mg/ml)	45μl
X-phosphate (50mg/ml)	35μl
Buffer 3	10ml

APPENDIX 6.3

HCV PCR positivity does not result from serum or blood in the tissue block.

Introduction

There are limitations associated with the interpretation of PCR results on the detection of HCV-RNA in tissues, in that HCV-RNA in serum or PBMCs in the blood contained within organs could lead to tissue positivity, given that PCR is such a sensitive procedure. This study was undertaken, in collaboration with Dr Mohamed El-Batanony, to examine this question.

Materials and methods

Routine diagnostic blood samples from six consecutive patients with chronic hepatitis C were studied. Each sample was divided into two portions. One portion was fixed in formalin for one day after being allowed to clot. A piece of the fixed clot (1cm^2 cut surface area x 0.3cm thick) was then processed routinely and embedded in paraffin. Serum was separated from the other portion of the sample and stored at -70°C. The processed clot and stored serum were tested for HCV-RNA by PCR using a tissue and a serum protocol respectively. The two protocols differed only in the extraction procedure. In addition, liver biopsies were available from five of the patients. These were also tested by tissue PCR. In the tissue protocol nucleic acids were extracted from the fixed blood and from the liver biopsies as described in appendix 2.7. In the serum protocol, nucleic acids were extracted and purified using an RNaid kit (Bio101, Inc., P.O. Box 2284, La Jolla, CA 92038, USA) according to the manufacturers instructions. This was performed in the department of Medicine, RFH. In short, 100μl 4M guanidium thiocyanate, 20μl 3M sodium acetate and 123μg yeast tRNA were added to 100μl of serum in an

RNAase-free microcentrifuge tube. The mixture was vortexed and incubated at room temperature for five minutes, then extracted with phenol/chloroform. The aqueous phase was separated and an equal volume of RNA binding salt was added. Five μl of RNAMATRIX was added to the mixture, which was then incubated at room temperature for five minutes and centrifuged for one minute. The supernatant was discarded and the pellet was washed twice and resuspended in 20μl of RNAase free water. The suspension was incubated at 45-55^{0}C for five minutes then centrifuged for two minutes. The supernatant, now containing any RNA, was transferred into another tube and stored at -70 C until further processed.

Ten μl of nucleic acid suspension resulting from each protocol was reverse transcribed as described in chapter 2. The cDNA generated was amplified by nested PCR as described in appendix 2.2, with appropriate positive and negative controls, and results analysed as described in appendix 2.3.

Results

Five out of the six blood samples were positive for HCV-RNA using the serum PCR protocol. All of the six fixed blood samples were negative for HCV-RNA using the tissue PCR protocol. Three of the five liver biopsies were positive by tissue PCR (see table 1). Positive controls were successfully amplified with both protocols.

Table 1. Results of serum PCR vs tissue PCR on fixed blood and liver tissue.

Patient	Serum	Fixed blood	Liver biopsy
1	Pos	Neg	Pos
2	Neg	Neg	Neg
3	Pos	Neg	NA
4	Pos	Neg	Neg
5	Pos	Neg	Pos
6	Pos	Neg	Pos

Discussion

Although PCR is an extremely sensitive technique, there is a lower practical limit to the amount of DNA that is necessary to start with to obtain a detectable PCR amplification product. An and Fleming (1991) have shown that less than 50 ng of target DNA can be associated with failure to amplify. The volume of tissue contained in 5 x 3μm thick paraffin sections cut from a 1cm^3 paraffin-embedded tissue-block was estimated to be 1.5μl; this was the equivalent volume of the blood tested using the tissue protocol. This is 66 times smaller than the 100μl serum usually used for the serum protocol and yet smaller volumes of liver biopsies are reliably HCV-RNA positive (El-Batanony *et al.*, 1994). This difference in volume alone could explain the discrepant results of the two protocols. In addition, the numbers of circulating viral copies in HCV infection are relatively few, from 10^2 to $5x10^7$ (Ulrich *et al.*, 1990). Recently, the level of HCV-RNA in serum and liver tissue has been quantified using a bDNA amplification technique; between 27 and 80 times higher concentrations of HCV-RNA were found in 1g of liver tissue than in 1ml of serum, with that difference being larger in mild than in severe hepatitis (Idrovo *et al,* 1993). Moreover, most of the volume of a biopsy sample consists of

solid tissue (e.g. liver cells). Serum or blood contributes to only a small portion of this volume. The very small amount of serum or blood in the tissue, together with the markedly lower level of HCV-RNA in serum (than in liver tissue), means that likliehood of tissue PCR positivity being due to serum or blood within the tissue is very small.

Whole blood is a potent inhibitor of PCR. The porphyrin compounds present in haemoglobin may be important in this regard (Higuchi, 1989). It may be possible that in the serum protocol this inhibition has been removed by further purification of the extracted RNA using the RNA kits. In the tissue protocol, however, RNA kits were not used and any red blood cells there could act as an additional inhibitory factor.

Formalin fixation induces extensive cross-linking between nucleic acids and proteins leading to reduction of PCR sensitivity on formalin-fixed tissue (Ben-Ezra *et al.*, 1991). Bresters *et al.* (1992) found a higher concentration of HCV-RNA in fresh-frozen than in formalin-fixed liver biopsies of comparable sizes. Therefore, in the tissue protocol, formalin fixation is more likely to produce a negative PCR result when only marginal amounts of viral copies are present, as in serum or blood within fixed tissue blocks. It is concluded that tissue HCV-RNA positivity comes from the viral RNA inside parenchymal cells and not from blood or serum contained in the tissue at the time of fixation.