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LABORATORY AND CLINICAL INVESTIGATION OF CHRONIC LIVER DISEASE IN HAEMOPHILIA

BY

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A thesis presented to the University of Leicester for the Degree of Doctor of Medicine

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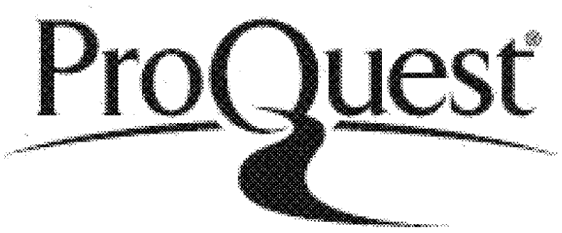

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ABBREVIATIONS USED IN THE TEXT

AFP	Alpha fetoprotein
AHG	Anti-haemophiliac globulin
AIDS	Acquired immunodeficiency syndrome
ALT	Alanine transaminase
bDNA	Branched DNA
cDNA	Complementary DNA
BTH	Breakthrough hepatitis
BTV	Breakthrough viraemia
EIA	Enzyme immunoassay
FVIII	Factor VIII
FIX	Factor IX
HAV	Hepatitis A virus
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HGV	Hepatitis G virus
HIV	Human immunodeficiency virus
HYA	Hyaluronic Acid
IFN	Interferon
NANBH	Non-A, non-B hepatitis
PCR	Polymerase chain reaction
RIA	Radio immunoassay
RIBA	Recombinant immunoblot assay
RT	Reverse transcriptase
VWD	von Willebrands Disease

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TABLE OF CONTENTS

Chapter 1

Introduction

Introduction

Haemophilia A (FVIII deficiency), haemophilia B (FIX deficiency) and von Willebrands Disease (VWD) are the commonest congenital coagulation disorders. The severity of clinical bleeding problems in haemophiliacs is directly related to the baseline factor level in an affected individual. Severely affected haemophiliacs (baseline FVIII or IX level less than 1% of normal) experience recurrent spontaneous bleeding particularly into joints leading to progressive arthropathy and are also at risk of intracranial and other types of life-threatening haemorrhage. Haemophiliacs with higher baseline factor levels usually only bleed after trauma. Prior to the development of modern treatment the average life expectancy for severely affected individuals was less than 30 years (Larsson & Wiechel, 1983).

VWD is a heterogenous disorder due to a quantitative or qualitative deficiency of von Willebrand factor. Clinical bleeding problems range from mucosal and post traumatic bleeding in mild disease to spontaneous haemorrhage in severe VWD.

Treatment of Haemophilia - Historical Aspects

The use of whole blood transfusion in the treatment of haemophiliac bleeding was first described in 1840 (Lane, 1840). In the early part of the 20th century it was established that separated plasma was effective in the treatment of bleeding episodes (Payne & Steen, 1929). This was presumed to be due to the replacement of deficient factors by "Anti-haemophiliac Globulin" (AHG). In the 1950s/1960s considerable advances occurred following the distinction between haemophilia A and haemophilia B (Biggs *et al*, 1952). The process of thawing frozen plasma was found to concentrate much of the AHG activity in the "cryoprecipitate" (Pool & Shannon, 1965). This enabled treatment to be given much more effectively to haemophiliacs.

Subsequently, specific coagulation factor concentrates were manufactured from large pools of donor plasma by fractionation methods. Such concentrates were easy to administer and enabled prompt and effective therapy, often self-administered out of hospital, to halt or prevent bleeding episodes. This led to a marked improvement in quality of life amongst haemophiliacs and subsequent reduction in mortality. In the 1970s the life-expectancy for haemophiliacs in the USA was approaching that of the general population (Aronson, 1988). The clear benefits of the use of coagulation concentrates led to their introduction in many countries in the early 1970s. By 1982, 73% of patients with Haemophilia A treated in the USA had received FVIII concentrates (Johnson *et al*, 1985).

It was recognised from an early stage that the use of coagulation factor concentrates exposed haemophiliacs to plasma obtained from many thousands of individual blood donors. It was well known that blood transfusion was associated with the transmission of agents, presumed to be viruses, which caused jaundice and hepatitis in some recipients (Beeson, 1943). There would, therefore, be a high risk of virus-transmitted disease in haemophiliacs. Steps were taken to reduce the risk of hepatitis transmission in blood products by the introduction of hepatitis B surface antigen (HBsAg) screening of blood donors and later, in some countries, the use of surrogate markers of non-A, non-B hepatitis (NANBH) such as antibody to hepatitis B core antigen and elevated serum alanine transaminase (ALT) (Koziol *et al*, 1986). However the perception in the 1970s was that the clear immediate benefits of coagulation factor concentrates appeared to outweigh any longer term risk of virus associated disease. Some, however, warned of potential problems in the future and urged the manufacturers of concentrates to seek methods to remove contaminating viruses. In 1978 an article in the New England Journal of Medicine stated "It is apparent that a major effort is necessary to develop a "clean" product rapidly for the treatment of the next generation of hemophilic patients" (Spero *et al*, 1978).

The full implications of the failure to develop effective virus-inactivation methods in the manufacture of coagulation factor concentrates were not appreciated until the human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) epidemic in the early 1980s (CDC, 1982). In different countries the risk of HIV seroconversion amongst haemophiliacs was found to be related to HIV seroprevalence in the plasma donor population as well as amount of concentrate used (reviewed by Goedert & Kroner, 1997). Severe haemophiliacs were more commonly infected with HIV (Goedert *et al*, 1989). Prompted by this epidemic much effort has been made to improve the safety of plasma-derived products by the utilisation of various virus-inactivation steps in the manufacturing process such as heat treatment or solvent/detergent.

Some early virus inactivation steps were unsuccessful at preventing the transmission of HIV or NANBH (reviewed by Foster *et al*, 1997). The methods in contemporary use have a good virus safety record (Table 1.2). Most have been evaluated by clinical use in previously untreated patients (PUPs). Such studies involve the close follow-up of patients treated with a new product for evidence of hepatitis or virus transmission.

Table 1.2. Virus inactivation methods used in the manufacture of coagulation factor concentrates

Terminal dry heat
Suspension heating
Moist heating
Pasteurisation
Solvent-detergent treatment
Filtration
β-Propiolactone-ultraviolet treatment

Despite much success in the use of virus inactivation methods since the 1980s there are still reports of virus transmission from concentrates including hepatitis C virus (HCV) infection (Berntorp *et al*, 1990; Shopnick *et al*, 1996) as well as outbreaks of Hepatitis A (Gerritzen *et al*, 1992; Temperley *et al*, 1992; Peerlinck & Vermylen, 1993; Mannucci *et al*, 1994; Kedda *et al*, 1995) and life-threatening parvovirus infection (Yee *et al*, 1995). Non-lipid enveloped viruses are not susceptible to treatment by either solvent/detergent or heating. This has led to a drive for dual inactivation steps to be incorporated into the manufacturing process to increase viral safety further. With the development of molecular techniques and the manufacture of recombinant FVIII and FIX, the possibility of virus transmission to haemophiliacs may be further reduced (see chapter 9).

Chronic liver disease in haemophilia

Over the last decade the problem of chronic liver disease in haemophilia has been overshadowed by HIV and AIDS. Fortunately, the virus inactivation methods which were introduced to combat HIV were also generally effective against hepatitis B virus (HBV) and the agents responsible for NANBH. So, younger haemophiliacs who have been treated since the mid-1980s are generally free from such transfusion transmitted infection. It is now clear that the recipients of concentrates prior to this time were universally exposed to HCV and most have chronic liver disease. A full account of the story of liver disease in haemophilia requires a step back to the 1970s. Following the widespread introduction of coagulation factor concentrates for the treatment of haemophilia in the 1970s, it was noted that the majority of the recipients of such concentrates developed abnormal liver function tests (Kasper & Kipnis, 1972). Serological evidence of previous exposure to HBV was common in haemophiliacs (Lewis, 1970) although chronic HBV replication appeared to be unusual. The presumed

aetiology of the liver function test abnormalities observed in haemophiliacs was a blood-borne virus or multiple viruses giving rise NANBH. Chronic NANBH was characterised by persistently or intermittently raised liver transaminases e.g. ALT and affected individuals were usually asymptomatic (Dienstag, 1983). Early investigators suggested that chronic NANBH in haemophiliacs was usually a benign form of chronic liver disease which seldom progressed to cirrhosis (Mannucci *et al* 1982; Stevens *et al* 1983). Soon thereafter, increasing evidence suggested that NANBH was in fact a much more serious problem than had been considered previously. Liver biopsy studies showed progression of histological changes over a period of time (Hay *et al* 1985) and larger surveys of haemophiliacs demonstrated a significant proportion (15%) with cirrhosis (Aledort *et al* 1985). There was concern about the safety of performing invasive investigations in haemophiliacs due to the perceived risk of bleeding (Aledort *et al*, 1985). In any event, apart from documentation of histological appearances, such investigation seemed pointless in the absence of more understanding of the cause of chronic liver disease or the development of effective treatment.

Hepatitis C virus

In 1989 HCV was identified using sequence subtraction analysis from cDNA libraries derived from serum of individuals with chronic NANBH (Choo *et al*, 1989). Phylogenetic studies have shown that HCV is distantly related to the *flaviviridae.* It is a single stranded RNA virus which contains approximately 9400 nucleotides (Figure 1.1). The RNA strand extends from the 5' non-coding region (a highly conserved region of 300 nucleotides) through a single open reading frame of core, two envelope and four non-structural regions extending to the 3' end.

HCV has subsequently been shown to be the agent responsible for the majority of cases of post-transfusion NANBH (Alter *et al*, 1989). Similarly, in haemophiliacs HCV has been

implicated as the major cause of chronic NANBH (Ludlam *et al,* 1989; Tedder *et al,* 1991). All haemophiliacs treated with non-virus inactivated factor concentrates have anti-HCV antibodies and up to 90% show evidence of persistent viraemia and elevated ALT (Watson *et al,* 1992).

The mechanisms whereby HCV causes persistent infection in the majority of exposed individuals remain unclear. Equally the factors which determine successful virus clearance in the minority are ill-defined. Furthermore, there is no apparent protective immunity to reinfection with HCV even following exposure to the same strain of virus (Lai *et al,* 1994).

There is increasing evidence that HCV circulates a heterogeneous group of variants (termed "quasi-species") which evade host immune responses by continual evolution of new mutants (Enomoto *et al,* 1994). In addition, HCV appears to replicate in extra-hepatic reservoirs such as lymph nodes which may be relatively inaccessible to immune surveillance.

The progressive nature of HCV infection has been confirmed both in non-haemophiliacs (Takahashi *et al,* 1993) and haemophiliacs (Makris *et al,* 1996). The factors which determine the rate of progression remain unclear although older age (Takahashi *et al,* 1993; Telfer *et al,* 1994a) co-infection with HIV (Eyster *et al,* 1993; Telfer *et al,* 1994a) and high alcohol consumption (Cromie *et al,* 1996) appear to accelerate HCV-related liver disease. There is evidence that the incidence of hepatocellular carcinoma is increasing in haemophiliacs (Colombo *et al,* 1991; Preston *et al,* 1995a). There have also been an increasing use of liver transplantation in haemophiliacs since the first reported in 1987 (Bontempo *et al,* 1987).

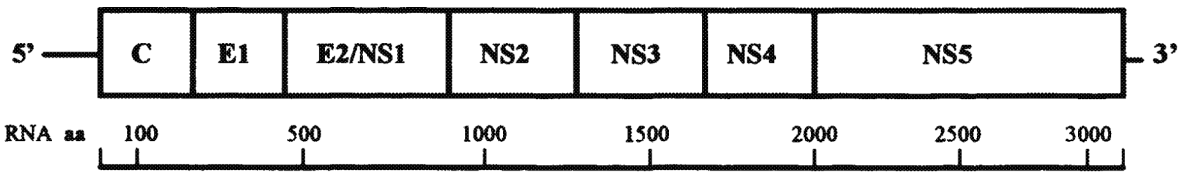
Figure 1.1 **The genomic organisation of HCV and the antigens in first, second and third generation EIA and RIBA assays.**

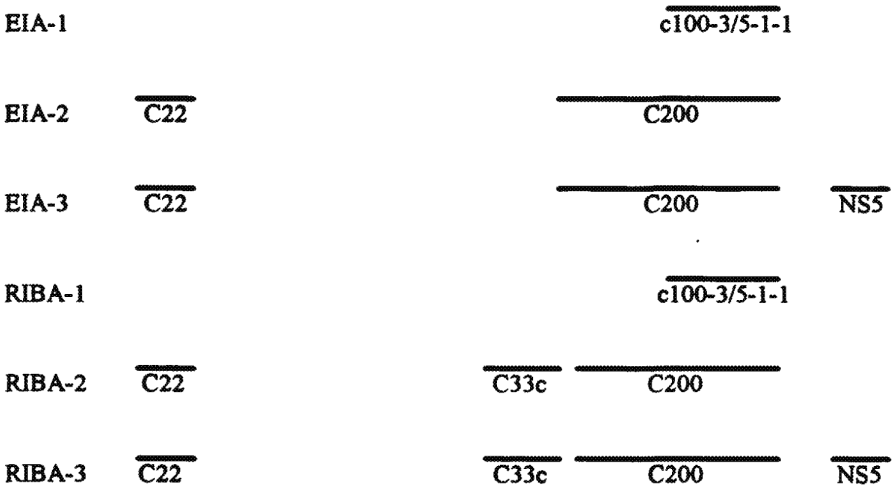
At the 5' end of the genome there is a highly conserved region of 300 nucleotides (the 5' non-coding region (NCR) or untranslated region (UTR). The RNA strand extends through a single open reading frame consisting of core (C), envelope (E1/E2) and four non-structural regions (NS2-NS5) to the 3' end.

EIA: enzyme immunoassay

RIBA: recombinant immunoblot assay

aa: amino acid





Serological tests for HCV antibodies

Enzyme immunoassays (EIA) to detect anti-HCV antibody were developed following the identification of HCV. The first generation EIA used the recombinant antigens from the original 5-1-1 and c100-3 (from the NS3/NS4 region). The test was widely used in the early studies of HCV infection but was associated with both false positive and false negative results. Subsequently, second generation EIAs have improved sensitivity and specificity by using additional antigens from other regions of the HCV genome (Figure 1.1). The routine use of anti-HCV antibody screening in blood donors has led to a marked reduction in post-transfusion hepatitis (Donahue *et al*, 1992). More recently third generation anti-HCV EIA assays have become available with a further improvement in performance.

Confirmatory HCV tests

The relatively poor sensitivity and specificity of first generation anti-HCV antibody EIA led to the requirement for confirmatory assays. The most widely available and used confirmatory test is the recombinant immunoblot assay (RIBA). The recombinant antigens are coated as bands on to a nitrocellulose strip. A third generation assay (RIBA-3) is now available and in routine use as the confirmatory test for EIA positive blood donors in the UK.

Detection of HCV RNA by PCR

The availability of the polymerase chain reaction (PCR) has proved an invaluable tool for the study of HCV infection. HCV RNA is extracted from the test sample. A complementary DNA (cDNA) copy is produced by reverse transcription. Specific PCR primers designed from highly conserved areas of the HCV genome (usually the 5' non-coding region) are used to amplify HCV DNA in a series of PCR reactions. A "nested" PCR technique can be used to further

improve specificity. This involves the use of a second set of PCR primers to amplify target DNA which are internal to the primers used in the first-round PCR reaction.

Although PCR is a useful tool it is important that it is carried out under strict reaction conditions with particular attention to the problem of contamination. There are considerable differences in sensitivity between commercially available PCR kits and also much inter-laboratory variation has been reported (Zaaijer *et al*, 1993).

Quantitative tests for HCV RNA

Quantitation of HCV RNA as a measure of virus load has been found to be clinically useful to monitor the response to antiviral therapy. Several methods for HCV RNA quantitation have been described (reviewed by Gretch *et al*, 1996):

a). Endpoint (or limiting) dilution.

HCV cDNA is serially diluted (e.g. a 10-fold dilution series) and tested by PCR. The endpoint is identified by the last sample yielding a positive result and a calculation of the quantity of HCV RNA in the original sample can be made (this requires identification of the efficiency of the reverse transcription step). This technique should strictly be termed "semi-quantitative" as dilution results in a series of thresholds of detection (e.g. 10^3, 10^4, 10^5 etc). Multiple dilutions may be performed at the end-point for precise quantitation or the isolation of single molecules of HCV RNA (see Chapter 2).

b). Quantitative competitive PCR.

This technique uses a synthetic internal control HCV RNA which has been altered to enable it to be distinguished from wild type HCV RNA. The internal control HCV RNA may be modified by the addition of nucleotides or the introduction of a restriction enzyme site.

Following mixed serial dilution of control and wild-type HCV RNA, PCR product is visualised and the relative quantities present enable a calculation of HCV RNA in the test sample.

c). Quantitative (non-competitive) PCR assays.

Non-competitive PCR assay utilise a biotinylated primer which results in PCR product which can be denatured and attached to a solid phase by specific probes. Quantitation is performed using a microtitre plate reader following detection by an avidin-horseradish peroxidase system.

d). Branched chain DNA (bDNA) assay.

The bDNA assay (Chiron Corporation, Emeryville, CA) uses specific synthetic oligonucleotide capture probes to hybridise target HCV RNA to the solid phase of a microwell. Synthetic bDNA molecules are then hybridised to the immobilised HCV RNA complex. Quantitation of HCV RNA is achieved by measuring light emission following incubation with a chemiluminescent substrate. The bDNA method is rapid and avoids the need for a specific RNA extraction step. However it is much less sensitive than PCR techniques and patients who have undetectable HCV RNA by bDNA should also be assessed by PCR.

HCV genotyping and serotyping

Following the discovery of HCV it was soon apparent that there were considerable variation between isolates from different geographical locations (Davidson *et al*, 1995). The most widely used classification of HCV now identifies 6 major genotypes and over 70 subtypes (Simmonds *et al*, 1993; Simmonds *et al*, 1994). Each genotype differs in nucleotide sequence by at least 30% and are further divided into subtypes which differ in sequence by more than 20%. Within each subtypes variants differ by 10% or less.

There is a striking geographical variation in HCV genotypes with types 1, 2 and 3 most common in Western Europe and North America (Davidson *et al*, 1995). Type 4 is present in the Middle east and North Africa, Type 5 in Southern Africa and Type 6 in South East Asia. It remains unclear whether there are important clinical or pathological differences between HCV genotypes. In practical terms however, there are antigenic differences between genotypes which may be important in the use of screening assays in blood donors from different locations. Haemophiliacs who have received multiple coagulation factor concentrate infusions are a particularly interesting group to study as they often have evidence of infection with multiple HCV genotypes (Jarvis *et al*, 1994).

Several methods to identify HCV genotypes have been described (reviewed by Smith *et al*, 1995):

a). Restriction fragment length polymorphism (RFLP) analysis.

Restriction enzymes are used to digest amplified DNA yielding characteristic patterns which enable the identification of most HCV genotypes.

b). Oligonucleotide hybridization.

Genotype-specific oligonucleotides are hybridised to amplified virus RNA. This technique is used in the line probe assay.

c). Type-specific primers.

HCV RNA is amplified by PCR using primers designed to detect individual genotypes.

d). Direct nucleotide sequencing.

Direct sequencing has been used to define the differences between genotypes but is clearly unsuitable as a routine method for genotype identification.

In addition to the above methods for HCV genotyping, EIA assays to serologically identify HCV genotypes have been developed. This technique is unable to distinguish sub-types or previous from current infection but has the advantage of being easy to perform (Bhattacherjee *et al*, 1995).

Treatment of chronic HCV infection with interferon

Interferons (IFNs) consist of three families of glycoproteins: α, β and γ; the universal classification of IFN is into types I and II; type I is stable at pH 2 (IFN-α and IFN-β), whilst type II is labile at pH 2 (IFN-γ). There are at least fourteen different genes encoding IFN-α and it may be induced in several types of leukocyte (β-lymphocytes, macrophages and null lymphocytes) in response to foreign cells, virus infected cells, tumour cells, bacterial cells and viral envelopes (Weigent *et al*, 1981). In comparison, there is only a single gene encoding IFN-β and IFN-γ; IFN-β is commonly induced in fibroblasts, epithelial cells and macrophages by viral and other foreign proteins. IFN-γ is usually induced in T lymphocytes or NK cells by foreign antigens to which T cells are sensitised.

The human IFN-α genes have been inserted into the plasmids of certain microbes with the use of recombinant genetic engineering techniques and clinically useful quantities of IFN-α proteins produced. There are two varieties of recombinant IFN-α widely available - rIFN-α2a and rIFN-α2b. These differ from each other only by a single amino acid. There is also one purified natural IFN available, lymphoblastoid IFN (IFN-αN3), which consists of 14 subspecies of IFN-α. In addition rIFN-α2c and human leucocyte (IFN-αN1) are licensed in some countries. To avoid confusion with nomenclature commercially available IFNs should be referred to using the International Non-proprietary Name (Finter, 1996).

IFN proteins activate their target cells by first binding to specific receptors on the cell surface to induce the synthesis of effector proteins. These intracellular proteins mediate the varied actions of IFN-α (Pestka *et al*, 1987; Samuel, 1988) Amongst the most widely studied mechanisms of action are those which are antiviral in nature. IFN-α acts primarily indirectly by increasing the intracellular concentrations of a wide range of proteins including protein kinase, 2′5′-oligo A synthetase, 2′5′-phosphodiesterase, Mx protein, HLA antigens, β2 microglobulin, and tumour necrosis factor (TNF). All these substances inhibit different stages of the virus replication cycle, predominantly at the stage of translation of viral mRNA into virus specified proteins. Other antiviral effects of IFN-α are immunoregulatory in origin and include increased MHC expression, macrophage activation, repletion of NK and cytotoxic T cells and cytokine induction.

IFN-α is currently the only licensed treatment for chronic HCV infection in the UK and USA (although IFN-β is approved for use in Japan). Its precise mechanism of action in this disease is unclear. Since its efficacy in the treatment of NANBH was first analysed in 1986 (Hoofnagle *et al*, 1986) many multicentre, randomised trials have been carried out. In non-haemophiliacs the response rate to a standard regimen (3MU thrice weekly for six months) is around 40-50%. However, at the end of treatment, more than half of these responders will relapse giving an overall sustained response rate of approximately 15-25% (Hoofnagle & Di Bisceglie, 1997). There is a subgroup of patients who show an excellent initial response to IFN with subsequent relapse during on-going therapy. This has been termed "breakthrough hepatitis" and is associated with the development of anti-IFN antibodies (Millela *et al*, 1993). The precise significance of such antibodies remains unclear.

Early studies of IFN therapy in haemophiliacs with chronic HCV suggested similar response rates to other HCV infected groups of patients (Makris *et al*, 1991). However more recent

studies have suggested that the response rates in haemophiliacs are inferior to those seen in other groups with only a small number (5-10%) achieving a sustained response to a standard course of IFN (3 mega units administered 3 times per week for 6 months) (Telfer *et al,* 1995). The role of combination therapy using both IFN and other anti-viral drugs (e.g. ribavirin) are currently being evaluated in clinical trials.

Hepatitis G virus infection in haemophiliacs

As HCV accounts for approximately 90% of cases of post-transfusion NANBH there has been considerable interest in the possibility that there may be other transfusion-transmitted agents responsible for the remaining cases. The observation that there are some haemophiliacs with persistent transaminitis without evidence of chronic HCV infection has also led to speculation that there may be other hepatotropic viruses present in the haemophiliac population. Clearly those exposed to pooled coagulation factor concentrates will be exposed to any viruses or other agents present in donated plasma.

Recently two groups reported novel *flaviviridae* termed GB virus-C (GBV-C) (Simons *et al,* 1995; Leary *et al,* 1996) and hepatitis G virus (HGV) (Linnen *et al,* 1996). Both reports implicated these viruses as causes of post-transfusion hepatitis. Further studies have confirmed that the genomes of GBV-C and HGV show substantial similarity suggesting that they are, in fact, closely related variants of the same virus (referred to as HGV in this thesis).

Studies in blood donors have found that HGV viraemia is present in 1-3.2 % (Linnen *et al,* 1996; Jarvis *et al,* 1996a; Alter *et al,* 1997). It is not clear whether this represents common recently acquired infection or a pool of asymptomatic chronic carriers. HGV has also been implicated as a cause of community acquired fulminant hepatitis (Yoshiba *et al,* 1995) although this has not been confirmed by others (Kao *et al,* 1996).

Currently the pathogenic significance of HGV remains unclear. Studies in haemophiliacs may help to more clearly define the clinical significance of HGV infection.

Aims of this thesis

In this thesis I will present the results of studies of several aspects of chronic liver disease in a cohort of haemophiliacs:

1. Assessment of liver disease in haemophiliacs.

I propose to study the cohort of anti-HCV positive haemophiliacs in Edinburgh and assess the extent of liver disease using both non-invasive and invasive methods. In particular I will evaluate the safety and usefulness of laparoscopic inspection of the liver surface and laparoscopic guided liver biopsy. I will also assess the use of serum hyaluronic acid as a non-invasive marker of hepatic fibrosis.

2. Treatment of HCV infection in haemophiliacs.

I propose to study the use of interferon-α2a therapy in haemophiliacs with chronic HCV infection. Response will be monitored by monthly ALT level and HCV RNA quantitation by PCR. I will assess the clinical significance of the development of neutralising antibodies to IFN during treatment.

3. HCV genotype studies

I aim to study the distribution HCV genotypes in coagulation factor concentrates which were not subjected to virus inactivation methods. If particular genotypes are over-represented in the haemophiliacs as compared to the concentrates, this may suggest differences between genotypes in their relative ability to cause persistent infection.

4. Hepatitis G virus infection in haemophiliacs

I will assess patterns of HGV viraemia in haemophiliacs and seek evidence of any association with chronic liver disease.

Chapter 2

Materials and Methods

Patient characteristics

The patients registered at the Edinburgh Haemophilia Centre comprised a heterogenous group of individuals with Haemophilia A and Haemophilia B, von Willebrands disease as well as patients with a variety of rare congenital bleeding disorders. The clinical studies presented in this thesis focus on liver disease in the recipients of pooled coagulation factor concentrates prior to the introduction of effective virus inactivation methods in the mid 1980s. From transfusion records it was possible to identify such patients who had received pooled coagulation factor concentrates. These individuals were screened for anti-HCV antibody when first and second generation anti-HCV tests became available (Watson *et al*, 1992). Serial liver function tests were also available from clinical case records.

The study group referred to in chapter 3 consisted of all patients who were found to be anti-HCV positive by second generation anti-HCV antibody testing in 1991(n=87). A protocol for the assessment of liver disease in these patient was developed and is described in chapter 3. A range of invasive and non-invasive investigations were offered to patients on an individual basis and the value and safety of these investigations was analysed. Following the introduction of PCR testing to detect HCV RNA, investigations were restricted to those with detectable serum HCV RNA or persistently abnormal liver function tests. In chapter 4 the usefulness of serum hyaluronic acid as a non-invasive marker of cirrhosis in 30 haemophiliacs and 33 non-haemophiliacs who underwent liver laparoscopy is described.

All patients with evidence of chronic HCV infection were offered treatment with recombinant interferon-α2a (rIFN-α2a) for six months. In chapter 5 the efficacy of IFN therapy in 31 haemophiliacs is presented.

The patient group described in chapter 6 which discusses the clinical relevance of anti-IFN antibodies includes the 31 patients presented in chapter 5 as well as an additional 8 patients who subsequently received IFN therapy.

Finally, the study cohort in chapter 8 concerning the clinical implications of HGV infection included 68 patients in whom stored serum samples were available from the mid 1980s. This group included many of the 87 patients in the larger study cohort as well as some who died prior to 1991.

Anti-HCV antibody and confirmatory testing

Anti-HCV testing was performed using a second-generation enzyme immunoassay (A-EIA; Abbott, Weisbaden-Dalkenheim, Germany) and confirmatory testing was by a second generation recombinant immunoblot assay (RIBA-2, Chiron Corporation, Emeryville, CA) for antibody to nonstructural proteins 5-1-1 (NS4), c100-3 (NS4), c33c(NS3) and core-associated antigen c22-3.

Laboratory organisation for virus RNA extraction, RT and PCR reactions

In order to avoid potential problems with contamination during the steps in RNA extraction, RT and PCR amplification all laboratory users followed a strict protocol.

All serum and coagulation factor samples were processed in an extraction hood. All lysis buffers and RT or PCR reaction mixtures were prepared on a specific aseptic bench in a designated laboratory. This laboratory was also used for 1^{O} PCR reactions. 1^{O} PCR products were transferred prior to further processing to a separate laboratory on a different floor of the building which was designated for 2^{O} PCR reactions only. Agarose gels were prepared, ethidium bromide stained and examined in a separate laboratory area.

Viral RNA extraction

Coagulation factor concentrates

Each archived concentrate was reconstituted with 10mls of sterile water. 5mls of reconstituted concentrate were made up to 7mL with RPMI medium and ultracentrifuged at 100,000g for 90 minutes at 4^{0}C to pellet virus RNA. A lysis buffer was prepared consisting of 1 mg/ml proteinase K in the presence of 40 µg / ml polyadenylic acid, 0.5% SDS, 0.1 M NaCL, 50mM TRIS HCl (pH 8.0) and 1 mM EDTA. The lysis buffer was incubated at 37^{O}C for 10 minutes to inactivate endogenous RNAses. The pellet was mixed using a vortimixer with 400µl lysis buffer and incubated at 37^{0}C. RNA was initially extracted by addition of 450µl phenol. Samples were thoroughly mixed and centrifuged at 18,000g for 10 minutes. After centrifugation, the supernatant was reextracted successively with phenol-chloroform (1:1) and chloroform-isoamyl alcohol (50:1). Nucleic acid present in the aqueous phase was precipitated by the addition of one-tenth volume of sodium acetate (pH 5.2) and 2 volumes of 100% ethanol followed by overnight storage at -20^{0}C. Nucleic acids were pelleted by centrifugation at 15,000g for 15 minutes. The pellet was washed with 80% ethanol and dried at 42^{0}C. The dried pellet was resuspended in 25µl of diethylpyrocarbonate-treated water. Extraction controls using serially diluted HCV RNA or HGV RNA positive and negative serum as appropriate were included with each batch of samples.

Serum samples

All serum samples studied were separated within 3 hours of collection and stored at -70^{0}C. Virus RNA was extracted from 0.1ml or 0.5ml of stored sera in an identical protocol as described for coagulation factor concentrates.

Primers for RT and nested PCR reactions

The HCV primers used were designed to amplify the 5'non-coding region of the HCV genome (Chan *et al*, 1992). The HGV primers were designed from the 5' non-coding region of the HGV genome (Jarvis *et al*, 1996a).

The following list shows the sequence of individual primers (5' to 3'), the position of the 5' base and the product length:

Hepatitis C virus

1^0 PCR Antisense (position 8).

ATACTCGAGGTGCACGGTCTACGAGACCT (No. 209)

Sense (position 297)

CTGTGAGGAACTACTGTCTT (No. 939)

Product length: 289 base pairs.

2^0 PCR Antisense (position 28)

CACTCTCGAGCACCCTATCAGGCAGT (No. 211)

Sense (position 279)

TTCACGCAGAAAGCGTCTAG (No. 940)

Product length 251 base pairs

Hepatitis G virus

1^0 PCR Antisense (Outer; position 531)

TGCCACCCGCCCTCACCCGAA (No. 4574)

Sense (Outer; position 108)

AGGTGGTGGATGGGTGAT (No. 4571)

Product length: 423 base pairs

2^0 PCR Antisense (Inner; position 476) (No. 4573)

GGRGCTGGGTGGCCYCATGCWT

Sense (Inner; position 134)

TGGTAGGTCGTAAATCCCGGT (No. 4572)

Product length: 342 base pairs

Reverse transcription reaction

Hepatitis C virus

HCV RNA was reverse transcribed to produce cDNA using the following reaction:

2µl 10X RT buffer (containing 50mM Tris-HCl pH8; 5mM MgCl2; 5mMDTT; 50mM KCl and 0.05µg/µl BSA)
3µl 4mM dNTPs (containing 600µM of each dGTP, dATP, dCTP, dTTP)
1µl anti-sense primer
3µl DMSO
4.5µl DEPC H_2O
10 units RNAsin
10 units RT enzyme

According to the number of test samples a reaction mix was prepared and 15µl added to 5µl of test nucleic acid to make a final volume of 20µl for each RT. The RT reaction was performed at 42^0C for 30 minutes in a hot-block.

Hepatitis G virus

The RT reaction was identical to above using the outer, antisense HGV primer.

Nested PCR reaction

Hepatitis C virus

a) Primary PCR reaction

Using 5µl cDNA a final volume of 50µl was prepared using the following reaction mix:

45µl PCR buffer	(containing 10mM Tris-HCl; 1.5mM MgCl2; 50mM KCl; 1mg/ml gelatine, pH8.3)
3µl 3mM dNTPs	(containing 33µM of each dGTP, dATP, dCTP and dTTP)
0.5µM sense primer	
0.5µM antisense	
20 units/ml Taq polymerase	

Twenty five amplification cycles were used, each consisting 0.6 minutes at 94^{O}C, 0.7 minutes at 50^{O}C and 1.5 minutes at 72^{O}C.

b) Secondary PCR reaction

Using 1µl primary PCR product the secondary PCR reaction using inner primers was performed. The final reaction volume was 20µl. The cycling conditions were identical to the primary PCR reaction. PCR product was visualised under UV light on a 2% agarose gel stained with ethidium bromide.

Hepatitis G virus

The PCR reaction was performed in an identical manner using the nested HGV primers listed above. The optimum cycling times were slightly different: 18s at 94^{O}C, 21s at 55^{O}C and 1.5 minutes at 72^{O}C (Jarvis *et al*, 1996a).

HCV RNA quantitation by limiting dilution

The quantity of HCV RNA was assessed both in patient serum samples and coagulation factor concentrates using a limiting dilution analysis of cDNA reverse transcribed from RNA. This method allows a semi-quantitative calculation of HCV RNA levels (see Chapter 1). Centrifugation of 0.5ml of sera provided a level of detection of approximately 800 HCV/ml (see below for details of calculation). To increase the sensitivity of the PCR method, samples which were negative at this level of detection (<800 HCV/ml) were further analysed by centrifugation of 5.0ml of sera, providing a cut off point of approximately 80 HCV/ml. All coagulation factor concentrates were reconstituted in 10mls of sterile water and RNA extraction was performed using a 5ml volume.

Following HCV RNA extraction from a known volume of serum or coagulation factor concentrate, the final quantitation by the limiting dilution method was performed. For example, from a starting volume of 5mls reconstituted coagulation factor concentrate, extracted virus RNA was re-dissolved in 25µl H_2O. 5µl RNA was used to generate 20µl cDNA in the RT reaction, thus 20µl of cDNA would be equivalent to 1000µl of reconstituted concentrate if the RT reaction was 100% efficient. However, the RT reaction has been shown to be approximately 5% efficient (Zhang *et al*, 1991), thus 20µl cDNA was equivalent to 50µl reconstituted concentrate. In the next step, 5µl cDNA (equivalent to 12.5µl reconstituted concentrate) was used in the nested PCR reaction and a limiting dilution (1:10) was performed.

The product of the PCR reaction was detected on an agarose gel and the final quantitation calculated in a limiting dilution series:

1/1	1/10	1/100	1/1000	10,000	HCV RNA (copies/ml)
+	-	-	-	-	$<8 \times 10^1$
+	+	-	-	-	$<8 \times 10^2$
+	+	+	-	-	$<8 \times 10^3$
+	+	+	+	-	$<8 \times 10^4$
+	+	+	+	+	$<8 \times 10^5$

According to the quantity of HCV RNA present in the test sample, the first negative reaction in the dilution series falls below the threshold of detection. However, at this dilution HCV molecules are present in small numbers. For an even more precise calculation of HCV RNA titre, the dilution series may be repeated and, at the first negative dilution, multiple replicates performed. If sufficient replicates are performed at this end-point dilution, each positive replicate is likely to contain a single molecule of HCV RNA. According to the number of positive replicates, a calculation of the quantity of HCV RNA can be made using the Poisson formula, $-(\ln f_0)$ where f_0 is the frequency of negative reactions (Simmonds *et al,* 1990):

$$-\log(\text{natural})\ \frac{\text{number of negative aliquots}}{\text{total number of aliquots}}$$

For example, taking a starting sample of 5mls which was found to yield a negative result at the 1/1000 dilution ($<8 \times 10^3$ HCV RNA copies/ml). If 10 of 50 replicates at this dilution are positive the final HCV RNA quantitation is calculated:

$-\log(\text{natural})\ (40/50) = 0.223 \times 10^4$ HCV RNA copies/ml

HCV genotyping by RFLP

For genotyping, product DNAs were cleaved with restriction enzymes as follows (Davidson *et al*, 1995):

a) HaeIII/RsaI digest:
25µl 2^0 PCR product
1.5µl medium buffer
1.5µl low buffer
1µl (10U) RsaI
1µl (10U) HaeIII

b) MvaI / HinfI
25µl 2^0 PCR product
3µl high buffer
1µl (10U) MvaI
0.25µl (10U) HinfI
0.75µl H_2O

Reaction mixes were incubated at 37°C overnight. Fragments were separated by agarose gel electrophoresis using 4% Metaphor agarose™ (FMC BioProducts, Rockland, ME). Cleavage patterns were examined and a genotype assigned according to the combination of patterns obtained with the two digests. Sub-types 1a and 1b and 2a and 2b were subsequently identified by the cleavage patterns resulting from digestion with BstUI and ScrFI respectively. The expected cleavage patterns from the HaeIII / RsaI and MvaI / HinfI digests are shown diagramatically (Figure 2.1). For example HCV genotype 1 yields pattern "a" (HaeIII / RsaI) in combination with pattern "A" (MvaI / HinfI) whereas HCV genotype 3 yields pattern "f" or "g" (HaeIII / RsaI) in combination with pattern "C" (MvaI / HinfI).

The method of HCV genotype identification is shown in Figure 2.2.

Figure 2.1 **Schematic diagram RFLP digestion patterns with different enzymes used to identify HCV genotypes**

Hae III / Rsa I

a	44	53		114/5	9	26
b	102			114	9	26
c	44	12	46	114	9	26
d	44	12	46	114	9	26
e	56		46	114	9	26
f	33	69		114	9	26
g	33	23	46	114	9	26
h	44	12	46	117	9	26

Mva /Hinf I

A	53	63	41	94
B	53	63	44	94
C	53	56	142/3	
D	53	198		

Figure 2.2 **Combination of RFLP patterns used to identify HCV genotypes 1-6.**

		Mva / Hinf I pattern			
		A	**B**	**C**	**D**
HaeIII / RsaI pattern	**a**	1		4	
	b	1		4	5
	c				2
	d				2
	e				2
	f			3	
	g			3	
	h		6		

Anti-interferon antibody assays

All samples for anti-IFN antibody assays were taken at least 48 hours following the most recent dose of IFN. The timing of blood sampling during IFN treatment is important as anti-IFN antibody titres may fall 1-6 hours post IFN injection and reach pre-injection levels only after 48-72 hours (Von Wussow *et al*, 1987). The transient fall in anti-IFN antibody titre is due to IFN/antibody immune complex formation. Such immune complexes may be demonstrated *in vitro* but no clinical consequences have been reported.

Serum samples were stored at -40^{0}C and sent on dry ice to the laboratory which performed the anti-IFN antibody assays in two batches. No clinical, HCV genotype or response to IFN data was supplied. The following methods have been described in detail (Hennes *et al*, 1987)

All samples were initially screened by an EIA method which detects both binding and neutralising antibodies. Test serum was incubated with rIFNα2a covalently attached to plastic beads. Anti-IFN antibodies present in the test serum bound to the solid phase and were

detected using peroxidase labelled rIFNα2a. Excess rIFNα2a-peroxidase was washed off and a colour produced by addition of a peroxidase substrate mixture (O-Phenylenediamine/H_2O_2). The colour produced was proportional to the anti-IFN antibody concentration in the test serum. The antibody titre was expressed as IFN binding units per ml (IBU/ml).

Samples positive by EIA were then tested using an antiviral neutralisation bioassay (ANB) designed to detect antibodies which neutralise the anti-viral activity of IFN *in vitro*. The ANB method utilised a cell/virus culture system (Madin Darby Bovine Kidney Cells and vesicular stomatitis virus). Dilutions of rIFNα2a and test serum were incubated with cell monolayers and then infected with virus. Following incubation and washing, viable cells were visualised with a crystal violet stain. In the presence of neutralising anti-IFN antibodies, the anti-viral activity of IFN in the test system was reduced. The antibody titre was expressed as IFN neutralising units per ml (INU/ml).

Hyaluronic acid assay

Serum HYA was measured using a radiometric assay (Pharmacia, Uppsala, Sweden). The test is based on the use of specific hyaluronic acid binding proteins (HABP) which are isolated from bovine cartilage. The HYA in the test sample binds to ^{125}I-labelled HABP in solution. The unbound HABP-125I is then quantitiated using HYA covalently coupled to Sepharose particles. The sample is centrifuged to separate followed by decanting. The radioactivity bound to the particles is measured I a gamma counter and the response is inversely proportional to the concentration of HYA in the sample. The HYA normal range is 10-100μg/l. The lower limit of detection with this assay is <5μg/l. For each patient HYA was measured in paired serum samples and the mean HYA level was recorded.

Chapter 3

Investigation of chronic liver disease in haemophiliacs.

Introduction

In 1989 HCV was identified and has subsequently been shown to be the major cause of NANBH in haemophiliacs (Ludlam *et al* 1989; Tedder *et al* 1991). Almost all haemophiliacs treated with non-virus inactivated factor concentrates have anti-HCV antibodies and up to 90% show evidence of persistent viraemia and elevated ALT (Watson *et al* 1992). With the development of the PCR, and its application to the study of HCV, the means to detect and quantify serum HCV RNA and identify the circulating HCV genotype became available.

This chapter describes the study of a cohort of anti-HCV positive haemophiliacs to assess the extent of liver disease using both non-invasive and invasive methods. The safety and usefulness of laparoscopic inspection of the liver surface and laparoscopic guided liver biopsy was assessed. The information gathered from these investigations was used to address the following questions:

1. How serious is liver disease in haemophiliacs who have now been infected with HCV for 10-25 years?

2. Can less invasive methods of assessing liver disease be utilised to reliably assess the extent of liver disease, without resorting to liver biopsy?

3. Is laparoscopic inspection of the liver surface a useful investigation in haemophiliacs and can it be used as an alternative to liver biopsy?

4. Is there a correlation between severity of liver disease and factors related to HCV (e.g. genotype), host factors (e.g. diagnosis, concentrate use, immune system) or co-infection with HIV.

Patients and Methods

This study formed part of an on-going clinical follow-up programme which has evolved with the availability of serological and PCR methods to study HCV. When the first generation anti-HCV antibody tests became available it was not clear whether a positive result indicated current infection or merely past exposure to HCV. It has subsequently become clear that there are only a minority of haemophiliacs who clear HCV RNA.

Patient Characteristics

All individuals (n=113) with bleeding disorders registered at the Edinburgh Haemophilia Centre were screened for anti-HCV antibodies. There were 87 who were anti-HCV positive by second-generation enzyme immunoassay (A-EIA; Abbott, Weisbaden-Dalkenheim, Germany) and also positive on confirmatory testing by recombinant immunoblot assay (RIBA-2, Chiron Corporation, Emeryville, CA). The characteristics of this group are outlined in Table 4.1. Evidence of hepatitis B and HIV infection was sought in all cases. Previous ALT levels were available and the mean ALT was calculated from three measurements over a minimum time of 6 months (in two patients only one ALT and in five patients two ALT values were available). In addition serum immunoglobulins (IgG, IgM and IgA), CD4 counts, platelet counts, serum ferritin, serum albumin and prothrombin times were measured.

A programme of investigation, as described below, was initially offered to all. With the subsequent availability of PCR testing to detect serum HCV RNA invasive investigations were restricted to "PCR positive" individuals. HCV genotyping was performed by restriction length fragment polymorphism (RFLP) as described in chapter 2.

Table 3.1 Patient characteristics.

Diagnosis	Haemophilia A	Mild (FVIII > 5%)	8
		Moderate (1-5%)	19
		Severe (<1%)	35
	Haemophilia B	Mild (FIX > 5%)	9
		Moderate (1-5%)	6
		Severe (<1%)	2
	Von Willebrands Disease		8
		Total	87
Sex		Male	82
		Female	5
Age (years)		Mean	37.7
		Range	12-75
Anti-HCV		Positive	87
HCV RNA		Positive	74
		Negative	13
Anti-HIV		Positive	18
		Negative	69
HBSAg		Positive	2
		Negative	85

Assessment of Liver Disease

Each individual was offered investigations to establish the severity of liver disease. The range of invasive and non-invasive investigations of liver disease available was discussed with each patient. Invasive investigations were not performed on those with FVIII inhibitors (n=5) and individuals with VWD (n=8) were not offered liver biopsy. It was stressed that consent to investigations was not required prior to treatment with interferon (see Chapter 5) but would provide information to counsel the individual as to the extent of their liver disease. No patients

had received interferon therapy prior to the investigations. Following a full discussion regarding the pros and cons of the various investigations, the final numbers of patients who consented to investigations after appropriate factor concentrate replacement were as follows:

1. Abdominal ultrasound scans to assess liver size, echogenicity and the presence of hepatocellular carcinoma (HCC) as well as spleen size were performed on 77 patients.

2. Upper GI (gastro-intestinal) endoscopy was performed in 48 patients to identify the presence or absence of oesophageal varices indicating portal hypertension secondary to cirrhosis. A further two patients with FVIII inhibitors were assessed by barium swallow.

3. Laparoscopic liver inspection was performed in 34 patients. The degree of hepatic inflammation (none, mild or marked), surface fibrosis (none, moderate or pronounced) and presence of cirrhosis and portal hypertension was assessed (Jalan *et al* 1995).

4. A tru-cut liver biopsy was attempted in 23 patients under direct vision during the laparoscopy and histology was assessed.

Histological assessment of Liver Biopsies

All liver biopsy specimens were fixed in formalin and examined in paraffin sections by a single histopathologist. Assessments were made without prior knowledge of the clinical details of the patient. Two methods of assessment were used:

1. A visual scoring system (The Edinburgh Classification) which identifies 7 specific histological features (Lymphoid aggregates, lobulitis, spotty necrosis, bile duct lesions, fatty infiltration, piecemeal necrosis and fibrosis) and scores each feature as absent or present (+ to +++). The overall appearances were then classified as mild, moderate or severe (including cirrhosis).

2. The Sheffield Scoring System (Makris *et al*,1991) which assigns a score of 0 to 3 to each of 5 histological features (steatosis, apoptotic necrosis, piecemeal necrosis, sinusoidal infiltrates and portal infiltrates). The overall score is the sum of the scores for the five features.

Protocol for Investigations

Day 1

Admit to hospital. Full physical examination and baseline investigations including anti factor VIII/IX antibody screen. Informed consent for investigations was obtained.

Day 2 am

Upper GI endoscopy was performed following appropriate coagulation factor replacement. Patients with haemophilia A or von Willebrands disease were treated with factor VIII concentrate before the procedure to achieve post infusion levels of 0.5 iu/ml. Patients with haemophilia B were given factor IX concentrates to achieve post infusion levels of 0.3 iu/ml. Endoscopy was performed using standard techniques. Further treatment with factor concentrates was only given if the procedure had been traumatic or if a biopsy was required. Those patients who were having no further investigations were discharged the same day.

Day 2 pm

Laparoscopic inspection of the liver and biopsy (when indicated) was performed. Patients were given further factor concentrate to attain post-infusion levels of 1.0iu/ml[FVIII] or 0.7iu/ml[FIX]. The infusions were given 2 hours before the procedure to enable post-infusion levels to be measured. The laparoscopy was performed under sedation with diamorphine and diazepam. A small incision was made above and to the right of the umbilicus and two litres of nitrous oxide was inflated into the peritoneal cavity. An appropriately sized trocar was inserted to allow passage of a 5 mm paediatric laparoscope (Olympus) or a 2mm microlaparoscope (Imygen). The systematic inspection of the upper abdomen was undertaken including the falciform ligament

looking at the size of vessels for evidence of portal hypertension and the surface of both lobes of the liver. A permanent record, in the form of a video recording, was made of the liver surface. If appropriate, a biopsy was taken with a trucut needle from the left lobe of the liver. The biopsy was taken under direct vision and if necessary, either pressure or a heater probe was applied to the site of biopsy to arrest any bleeding. The patient was kept in bed for 24 hours after the biopsy. Factor VIII/IX levels were maintained between 0.5-1.0/0.5-0.7 iu/ml respectively for 48 hours. Patients continued to receive factor concentrate infusions twice daily for 4 days after a liver biopsy.

Day 3

A post-biopsy ultrasound scan to detect evidence of bleeding was performed 24 hours after the procedure.

Day 4

Discharge home.

Results

The results of abdominal ultrasound scans, upper GI endoscopy, liver laparoscopy and liver biopsy are summarised in table 3.2.

Abdominal Ultrasound

Of the 77 patients who had an abdominal ultrasound scan there were 11(14%) who were identified as having hepatomegaly, 17(22%) with abnormal liver echogenicity and 13(17%) with splenomegaly. No focal hepatic lesions were identified.

Upper GI Endoscopy

Oesophageal varices were identified in 7(14%) of 50 patients in whom endoscopy or barium swallow was performed. The varices were grade 1 in four, grade 2 in two and grade 3 in one. There were no bleeding complications following endoscopy.

Liver Laparoscopy

The liver was successfully visualised in 33 patients. One procedure failed due to the presence of adhesions from previous abdominal surgery. Of the patients who had an adequate laparoscopy there were 16(49%), 9(27%) and 8(24%) with none, mild or pronounced fibrosis respectively. In 5 of the 8 with pronounced fibrosis the appearances were those of established cirrhosis. Inflammation was present in all patients being mild in 21(64%) and marked in 12(36%). Appearances consistent with portal hypertension were present in 4(12%) patients.

Liver biopsy

Of the 87 patients in the group, 22 underwent successful liver biopsy. Of the remaining 65 patients liver biopsy was not performed for the following reasons: patient declined (n=41); technical failure (n=1); von Willebrands Disease (n=8); inhibitor (n=5); serum HCV RNA negative by PCR (n=8); evidence of cirrhosis visible at laparoscopy (n=2).

Of the 23 liver biopsies attempted, 22 were successful and adequate specimens were obtained. In one patient the procedure was abandoned due to inadequate sedation. There were no serious bleeding problems associated with the procedure and haemostasis was secured prior to the withdrawal of the laparoscope.

Safety of invasive investigations

There were no serious bleeding complications associated with endoscopy, laparoscopy or liver biopsy. In one patient following liver biopsy there was a small hepatic haematoma on the 24 hour post biopsy ultrasound scan. This had resolved by 48 hours. A rectus sheath haematoma developed in another patient with severe haemophilia A who was not pre-treated with factor VIII concentrate prior to the removal of a suture on day 7 post laparoscopy. This responded to factor VIII replacement. There was no prolongation of the planned duration of hospital stay in any patient.

Liver biopsy histology

The final histological appearances were classified as mild, moderate or severe (including cirrhosis) in 8, 9 and 5 cases respectively. There was a strong correlation (spearman rank correlation: $r=0.81$, $p<0.001$) between the Edinburgh visual histological classification and the Sheffield score (Figure 3.1). As the Sheffield system does not specifically score fibrosis it is not unexpected that biopsies in the Edinburgh moderate and severe groups have similar Sheffield scores.

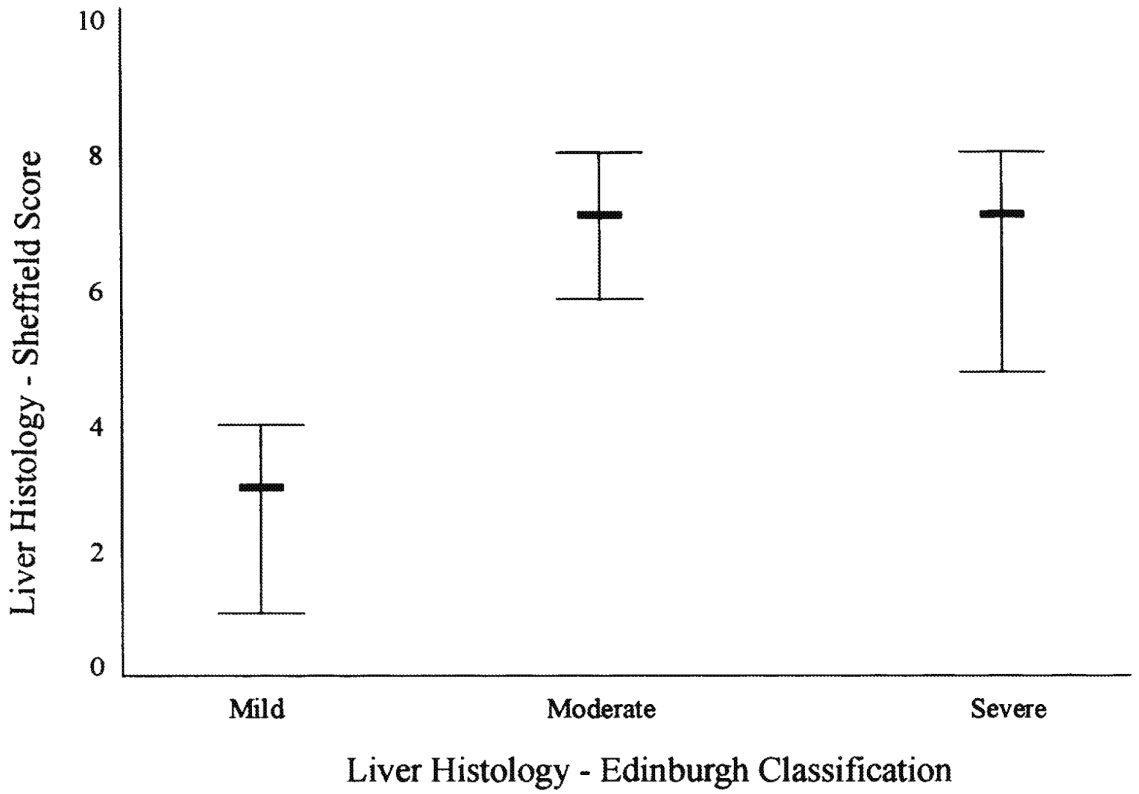
Table 3.2 Summary of investigations to assess liver disease.

			n	%
Ultrasound (n=77)	Hepatomegaly	Present	11	14
		Absent	66	86
	Echogenic Liver	Present	17	22
		Absent	60	78
	Splenomegaly	Present	13	17
		Absent	64	83
Endoscopy* (n=50)	Varices	Present	7	14
		Absent	43	86
Laparoscopy (n=33)	Liver Inflammation	Mild	21	64
		Marked	12	36
	Liver Fibrosis	None	16	49
		Mild	9	27
		Pronounced	8	24
	Portal Hypertension	Present	4	12
		Absent	29	88
Liver Biopsy (n=22)	Histology	Mild	8	36
		Moderate	9	41
		Severe	5	23

* two patients assessed by barium swallow

Figure 3.1 Comparison of Edinburgh histological classification and Sheffield scoring system.

The median Sheffield score is shown by the bold line and the range by the error bars for each histological severity.



Relationship between severity of liver disease andpatient characteristics.

There was a strong relationship between age and more severe liver disease (spearman rank correlation: r=0.58, p=0.004). There was no relationship found between severity of liver disease and diagnosis or concentrate usage.

There was no evidence of any relationship between mean ALT, ferritin, prothrombin time, albumin, IgG, IgA, IgM or CD4 count and severity of liver disease as assessed histology or laparoscopy. There was an inverse correlation between platelet count and severity of liver

histology (Spearman rank correlation: r=-0.48, p=0.022). The results of baseline investigations are summarised in Table 3.3.

Table 3.3 Baseline biochemical, haematological and immunological parameters of the study cohort.

	Median	Range	Normal Range
ALT (U/L)	60	15-479	10-40
Ferritin (μg/l)	52	5-2932	8-300
IgG (g/l)	14.7	7.0-41.8	7.4-16.6
IgA (g/l)	2.9	0.6-12.6	0.8-3.9
IgM (g/l)	2.3	0.6-7.9	0.5-2.0
Albumin (g/l)	44	18-51	36-47
Prothrombin Time (Secs)	12	10-47	10.5-14.5
CD4 Count (10^6/l)	0.580	0.0-1.587	0.5-1.5
Platelet Count (10^9/l)	207	1-387	150-350

Predictive Value of Less Invasive Investigations

Liver histology was available in 22 patients in whom liver biopsies were performed. We assessed if liver histology could have been predicted on the basis of combined information derived from ultrasound (presence or absence of splenomegaly), endoscopy (presence or absence of varices) and laparoscopy (degree of hepatic fibrosis). HCV RNA was detected in 18

and three were anti-HIV positive. Oesophageal varices were identified in seven patients and four of these underwent liver biopsy.

There was agreement between the severity of liver disease from histology when compared with information from other investigations in 18 (86%) of the biopsied patients (Table 3.4). In only one case was the histological appearances more severe than expected. In two cases with established oesophageal varices indicating portal hypertension the liver histology was less severe than expected (moderate in both). Specificity and sensitivity of the predictive value of less invasive investigations was 88% and 80% respectively ("mild" and "moderate" histological categories are combined for this calculation).

Table 3.4 **Relationship between detection of varices by endoscopy, splenomegaly by ultrasound or pronounced fibrosis by liver laparoscopy and the histological appearance of the liver biopsy in 22 haemophiliacs.**

		Liver Histology		
		Mild	**Moderate**	**Severe**
Oesophageal varices				
OR	YES	0	2	4
Splenomegaly				
OR	NO	8	7	1
Pronounced fibrosis				

HIV Infection and Severity of Liver Disease

Of the 18 anti-HIV positive individuals ultrasound scan, laparoscopy, endoscopy and liver biopsy were performed in 15, 3, 10 and 3 respectively. Splenomegaly was detected in 6/15; oesophageal varices were present in 3/10; liver inflammation at laparoscopy was marked in 3/3

and surface fibrosis was pronounced in 1/3 and absent in 2/3. Liver histology was severe in all 3 patients who were biopsied. There was a highly significant association between HIV status and severity of liver histology (Table 3.5). This association is independent of age (median age of HIV positive and HIV negative patients who underwent liver biopsy was 32 and 35 respectively).

Table 3.5 HIV status and severity of liver histology.
Chi-square test for trend: p=0.006

		Liver Histology		
		Mild	**Moderate**	**Severe**
HIV status	**Negative**	8	9	2
	Positive	0	0	3

HCV Genotype and Severity of Liver Disease

The HCV genotype distribution is shown in Table 3.6. Ten of the 13 patients in whom serum HCV RNA was not detected had persistently normal serum ALT levels. In three, however, ALT levels were persistently raised. One of these individuals was HBsAg positive but no definite cause for chronic hepatitis was found in the other two patients.

There was no evidence of any differences in mean ALT between HCV genotypes although genotype 3a had the widest range of values (Figure 3.2). There were no statistically significant differences in laparoscopic appearances or liver histology between genotypes (kruskal-wallis test: p=0.23). Although only small numbers were assessed there was a suggestion that genotype 1b might be associated with more severe histological appearances (Table 3.7).

Table 3.6 HCV genotype distribution in Edinburgh haemophiliacs (n=87)

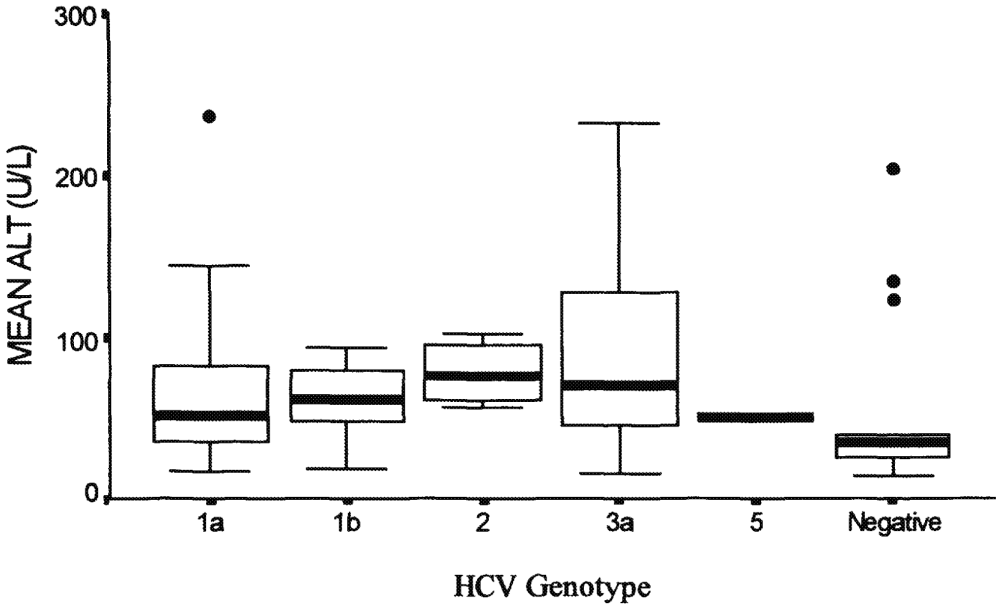
HCV genotype	n	%
1a	32	37
1b	15	17
2a	1	1
2b	3	3
3a	22	25
5	1	1
PCR Negative	13	15
Total	**87**	**100**

Table 3.7 HCV genotype and severity of liver histology.

	Liver histology		
Genotype	**Mild**	**Moderate**	**Severe**
1a	4	2	2
1b	0	0	2
2a	0	1	0
2b	0	1	1
3a	2	3	0

Figure 3.2 Box plot of mean ALT and HCV genotype.

The median ALT is represented by the solid line. For each genotype ALT values between the 25th and 75th centiles are represented in the box. Values less than 1.5 box-lengths outside the 25th or 75th centiles are shown by the error bars. Values more than 1.5 box-lengths from the 25th or 75th centiles are shown as individual dots. There was no significant difference in ALT values between genotypes (kruskal-wallis test: p=0.20).



Discussion

Chronic liver disease due to HCV has emerged as a serious problem for individuals with haemophilia. The majority who received non-virus inactivated concentrates have persistent HCV infection characterised by fluctuating ALT and virus levels (Fletcher *et al,* 1983). Effective elimination of HCV from coagulation concentrates has been achieved by virus-inactivation steps in the manufacturing process which were introduced in the mid-1980s. Even in recent years, however, there have been occasional reports of possible HCV transmission by

concentrates and there is clearly a need for continued close surveillance (Berntorp *et al,* 1990; Schulman *et al*, 1992).

Currently, haemophiliacs with chronic HCV infection have been exposed to the effects of the virus for between 10 and 25 years and the spectrum of liver disease is partly a reflection of the variation in the duration of infection. As more information becomes available concerning the natural history of HCV infection it is clear that complications generally arise only after prolonged infection. This observation may explain why early studies of liver disease in haemophiliacs suggested that chronic NANBH was essentially benign and non-progressive (Stevens *et al*, 1983) whereas later studies, due to the passage of time and progression of the natural history of HCV infection, have demonstrated development of serious liver disease (Hay *et al*, 1985) in a significant and increasing number of patients. The incidence of HCC is also increasing in haemophiliacs (Colombo *et al*, 1991; Preston *et al*, 1995a). Recently the UK Haemophilia Centre Directors Organisation have suggested guidelines for the management of chronic liver disease in haemophilia which attempt to address some of the issues in the practical management of haemophiliacs infected with HCV (Preston *et al*, 1995c).

We have confirmed that a significant number of haemophiliacs have evidence of serious liver disease with direct or indirect evidence of cirrhosis in around 25%. Despite the small number of HIV co-infected individuals in the study group, the observation that HIV co-infection is associated with more severe histological appearances is statistically valid and supports the findings of other studies (Eyster *et al,* 1993; Telfer *et al*, 1994). Concentrate usage was not associated with more severe liver disease suggesting that the immunomodulatory effects of coagulation factor concentrates do not have a major role in the progression of liver disease. In those with established cirrhosis there is clearly the risk of hepatic decompensation or the development of hepatocellular carcinoma. Close surveillance of this group is required and the

most appropriate treatment options, including liver transplantation, should be based on the circumstances of each individual.

In those without established cirrhosis the best approach to treatment and follow-up is less clear. IFN treatment has been used with varying success in haemophiliacs with chronic HCV (Makris *et al*, 1991; Bresters *et al*, 1992; Peerlinck *et al*, 1994; Mauser-Bunschoten *et al*, 1995; Telfer *et al*, 1995; Hanley *et al*, 1996; Rumi *et al*, 1997). The most recent studies suggest the overall sustained response rate is very low (Telfer *et al* 1995; Hanley *et al* 1996; Rumi *et al*, 1997). The value of using IFN for greater than six months, the role of low dose IFN as maintenance therapy and combination therapy with ribavirin remain to be evaluated in haemophiliacs.

It is clear, however, in the absence of more effective therapy for HCV, monitoring for the development of cirrhosis is the most important part of management of liver disease in haemophiliacs. At present it is not clear how many will progress to cirrhosis and there is considerable debate about the best way to monitor for disease progression. In particular the role of liver biopsy remains controversial (Lee, 1997).

Liver biopsy is a well established and widely used diagnostic procedure in non-haemophiliacs. In addition serial biopsies may be useful either to monitor response to treatment or disease progression. What is the current role of liver biopsy in haemophiliacs with chronic liver disease? Several studies of liver biopsy in haemophiliacs have been performed since the first in 1977 (Lesesne *et al*, 1977; Spero *et al*, 1978; Mannucci *et al*, 1978; Hay *et al*, 1985; White *et al*, 1982; Aledort *et al*, 1985; Schimpf, 1986; Makris *et al*, 1993) From a review of the literature, most groups report that the procedure may be performed safely following appropriate coagulation factor replacement. In fact, apart from an anecdotal reference to two deaths in one paper (Aledort *et al* 1985), there are no published reports of mortality associated with liver biopsy in haemophiliacs and in excess of 250

biopsies have now been reported. So, if the procedure is safe, should the clinical indications for performing liver biopsy in haemophiliacs be the same as non-haemophiliacs? Non-haemophiliacs, with persistently abnormal liver function tests, with or without serological and PCR evidence of HCV infection would almost inevitably have a liver biopsy as part of their initial evaluation. The liver biopsy serves a number of purposes including to confirm the diagnosis histologically, exclude co-existing causes of liver pathology, stage the degree of disease and to provide baseline histology for comparison with a repeat biopsy following therapy.

In the context of haemophiliacs, we now know that HCV is responsible for the vast majority of chronic liver disease in this group. Chronic HCV infection may be readily diagnosed by serological testing and persisting viraemia assessed by PCR. In addition response to treatment may be assessed by quantitative PCR to measure levels of circulating HCV RNA. The information gained from a liver biopsy, therefore, may not contribute to the decision to treat most patients - especially as the options for treatment are extremely limited at present.

Is it, however, important to accurately stage the disease? This is necessary in order to provide accurate information when counselling the individual concerning his overall outlook. In addition, it is important to identify those most at risk of developing hepatocellular carcinoma, i.e. those with established cirrhosis. In this study we have shown that it is possible to accurately stage liver disease without resorting to liver biopsy in the majority of patients. Using the information gained from a combination of investigations, it is possible to predict the severity of liver disease. Laparoscopic inspection of the liver surface will diagnose cirrhosis accurately and as has been recently reported other findings such as inflammation and fatty change correlate closely with histology (Jalan *et al*, 1995). Although laparoscopy is still invasive, the use of narrow diameter instruments, particularly the new 2mm microlaparoscpe, allow good visualisation with minimal risk. In those with oesophageal varices present on

endoscopy cirrhosis is almost universally present. This study suggests that laparoscopic liver inspection, in conjunction with upper GI endoscopy and abdominal ultrasound, is an extremely useful method to stage and monitor the progression of liver disease in haemophiliacs without subjecting patients to repeated biopsies which, at worst, may have a cumulative morbidity and mortality and at best require a several day hospital stay.

We have not addressed the use of CT scanning as a non-invasive method of assessing liver disease. This method has been reported to be useful in haemophiliacs (Johnson *et al*, 1983; Miller *et al*, 1988). Both CT and MRI scanning require further study in haemophiliacs.

We opted to use the Sheffield Scoring system to evaluate the liver biopsies. This was chosen as it is the only scoring system which has been used specifically in haemophiliacs. A criticism of this system is the lack of a score for fibrosis. In addition an "in-house" visual classification was used and compared with the Sheffield Score. Further evaluation of such visual scoring systems is required.

It is interesting to note in this study that HCV does not account for 100% of chronic hepatitis in haemophiliacs. We identified 3 patients with biochemical evidence of chronic hepatitis despite being "PCR negative" for serum HCV RNA. The PCR method is extremely sensitive and has a threshold of 80 HCV copies/ml. Thus, a low level of HCV replication is unlikely in these patients. It is possible that as yet unidentified hepatitis viruses are present in the haemophiliac population (chapter 8).

In conclusion, this study provides further evidence that liver disease is emerging as a major problem for haemophiliacs. There is a need for close monitoring for the complications of chronic HCV infection and the precise role of laparoscopic liver inspection needs further evaluation. At present we feel that potentially most individuals may be monitored without resorting to liver biopsy as long as the extent of liver disease is accurately staged by the combination of investigations described.

Chapter 4

Hyaluronic acid as a non-invasive marker of hepatic fibrosis in chronic HCV infection

Introduction

From the discussion in chapter 3 it is clear that HCV infection results in progressive liver disease in a significant proportion of individuals with chronic infection. Liver damage appears to be slowly progressive in the majority with the development of cirrhosis in a significant number. Progression of liver disease appears to be accelerated by the presence of certain factors such as HIV co-infection, heavy alcohol intake and particular HCV genotypes. Those with cirrhosis are at risk of developing decompensated liver disease and/or HCC.

In the last chapter a protocol for initial assessment of liver disease in haemophiliacs was described. The arguments in favour of appropriate investigations to accurately assess the degree of liver damage were outlined. A much more difficult question is, once having staged the liver disease, how to monitor for disease progression to cirrhosis and the development of complications such as variceal bleeding, hepatic decompensation and HCC. In non-haemophiliacs repeated liver biopsy is often performed every few years to detect progressive hepatic damage. Although we argue in general that the management of haemophiliacs with chronic liver disease should be the same as non-haemophiliacs, a non-invasive method of detecting progressive hepatic damage would be invaluable. The situation is further complicated if information from staging investigations is unavailable (a proportion of haemophiliacs decline invasive staging investigations and in others such tests are contra-indicated e.g. in those with anti-FVIII inhibitors). In these cases a non-invasive marker to identify individuals with established cirrhosis may be of considerable use in clinical management.

Many studies have investigated potential markers of both hepatic inflammation and fibrosis. Markers of collagen metabolism such as N-terminal propeptide of type III procollagen (PIIINP) are elevated in both inflammatory and fibrotic liver disease and do not reliably differentiate between chronic hepatitis and cirrhosis (Ramadori *et al*, 1991).

Early studies found that serum hyaluronic acid (HYA) was raised in both acute hepatitis and cirrhosis (Frebourg *et al*, 1986) suggesting that both increased synthesis and reduced clearance may lead to an elevated level. However more recent studies using sensitive assay methods have suggested that HYA is a specific marker of hepatic fibrosis and may be useful to identify patients with established cirrhosis (Ramadori *et al*, 1991; Simpson *et al*, 1993).

This chapter discusses the use of serum HYA as a non-invasive marker of cirrhosis in haemophiliacs and non-haemophiliacs with chronic HCV infection.

Serum hyaluronic acid - background

HYA is a high molecular weight polysaccharide which is mainly synthesised by mesenchymal cells in many body tissues (Laurent & Laurent, 1981). In the liver HYA is produced by stellate cells. The normal serum half-life of HYA is approximately 3-5 minutes and clearance occurs via uptake by hepatic sinusoidal endothelial cells after binding to specific high affinity receptors (Eriksson et al, 1983). There is also a minor renal route of excretion accounting for less than 1%. Elevated serum HYA levels may occur either due to increased production or reduced clearance. HYA levels are elevated due to increased production in rheumatoid arthritis, scleroderma and fibrotic lung disease. Some studies have suggested that serum HYA is a useful marker of liver fibrosis mainly as a result of reduced hepatic clearance.

Patients and methods

The study group consisted of 63 patients with chronic HCV infection (haemophiliacs n=30; non-haemophiliacs n=33). All were anti-HCV positive and had detectable serum HCV RNA by PCR. All were HBSAg negative and also negative for anti-smooth muscle and anti-mitochondrial antibody and had normal levels of α-fetoprotein, caeruloplasmin and α-1-antitrypsin.

The characteristics of the 30 haemophiliacs are shown in table 4.1. All had previously received non-virus inactivated coagulation factor concentrates. The 33 non-haemophiliacs had been referred for evaluation of HCV infection (24 with a previous history of intravenous drug use; 2 previous blood transfusion; 7 with no identified risk factors).

All 63 patients underwent laparoscopic liver inspection. The haemophiliacs received appropriate coagulation factor replacement (see chapter 3). The appearances were classified as either chronic hepatitis without fibrosis or hepatic fibrosis without cirrhosis or established cirrhosis. A serum sample taken on the same day as the liver laparoscopy was used to assay HYA (see chapter 2).

Comparisons between two groups of patients with different laparoscopic appearances were made using the Wilcoxon rank sum test. The Kruskall-Wallis one way analysis of variance method was used to make comparisons of HYA levels across groups. The sensitivity of the HYA assay (using a particular cut-off value) as a predictor of cirrhosis was defined as the number of true positives divided by the number of patients with cirrhosis. The specificity was defined as the number of true negatives divided by the total number of patients without cirrhosis.

Table 4.1 Haemophilia patient characteristics

Haemophilia A	Severe	6
	Moderate	9
	Mild	3
Haemophilia B	Severe	1
	Moderate	2
	Mild	6
von Willebrands disease		3
Total		**30**

Results

The liver laparoscopy results are shown in table 4.2 and the HYA levels according to laparoscopic liver appearance in table 4.3. The results in the entire group are presented graphically in figure 4.1. HYA levels were significantly elevated in those with established cirrhosis compared to those with hepatic fibrosis and chronic hepatitis. A HYA value of 100μg/l provided a reasonable cut-off to distinguish cirrhotics from non-cirrhotics (Sensitivity 79%; Specificity 94%). The specificity of the test increased with higher HYA levels (Table 4.4)

Table 4.2 Severity of liver disease in haemophiliacs and non-haemophiliacs

	Haemophiliacs	Non-haemophiliacs	Total
Established cirrhosis	6	8	14
Hepatic fibrosis	1	5	6
Chronic hepatitis	23	20	43

Table 4.3 HYA levels according to severity of liver disease as assessed by laparoscopic liver inspection.

	Mean HYA (μg/l)	95% CI
Established cirrhosis (n=14)	401	209-597
Hepatic fibrosis (n=6)	80	28-131
Chronic hepatitis (n=43)	41	29-53

Figure 4.1 **HYA levels according to liver disease state in the entire study group.** Mean values are highlighted and 95% confidence intervals shown by the error bars.

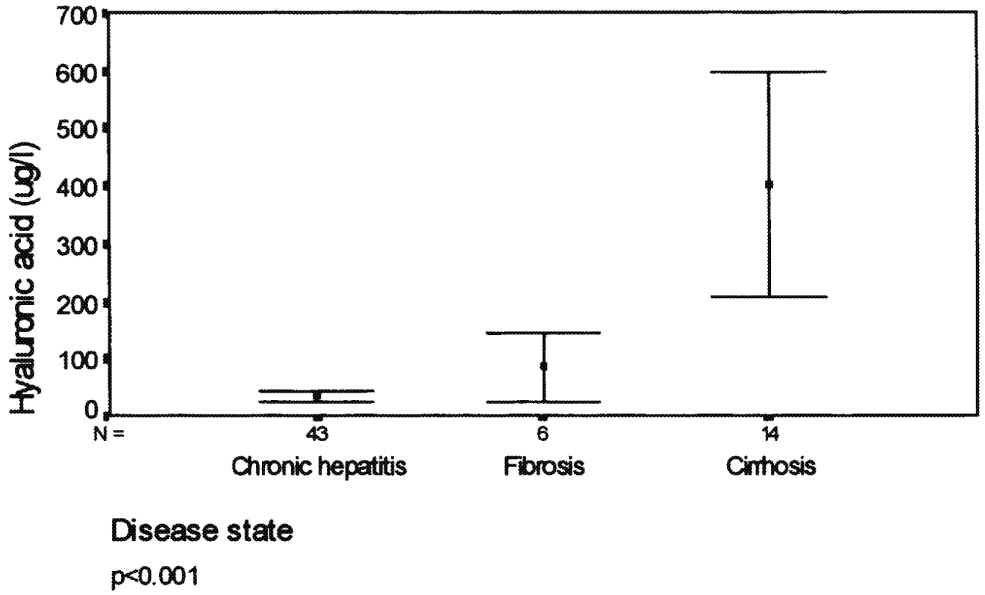


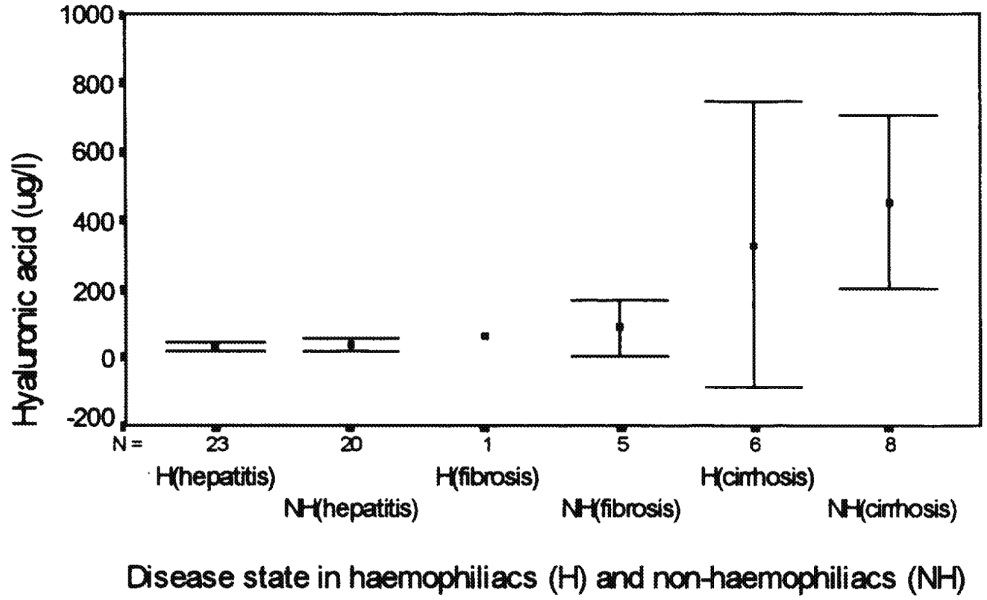
Table 4.4 **Sensitivity and specificity of HYA at different thresholds as a marker of established cirrhosis.**

HYA (μg/l)	Sensitivity	Specificity
100	79%	94%
200	71%	100%
300	50%	100%

Predictive value of HYA in haemophiliacs

In order to specifically assess the value of measuring serum HYA in haemophiliacs the data from the haemophiliac and non-haemophiliac groups were analysed separately. Although there was a clear trend for higher HYA levels in haemophiliacs with cirrhosis, wide confidence intervals were obtained (figure 4.2). This may be a reflection of the relatively small number of haemophiliacs with cirrhosis (n=6). The serum HYA level in one haemophiliac with established cirrhosis was persistently less than 100μg/l when checked on several occasions. Cirrhosis was proven on liver biopsy in this individual and oesophageal varices were present at enoscopy.

Figure 4.2 **HYA levels according to severity of liver disease in the haemophiliac group (n=30) and the non-haemophiliacs (n=33).** Mean values are highlighted and 95% confidence intervals shown by the error bars.



Discussion

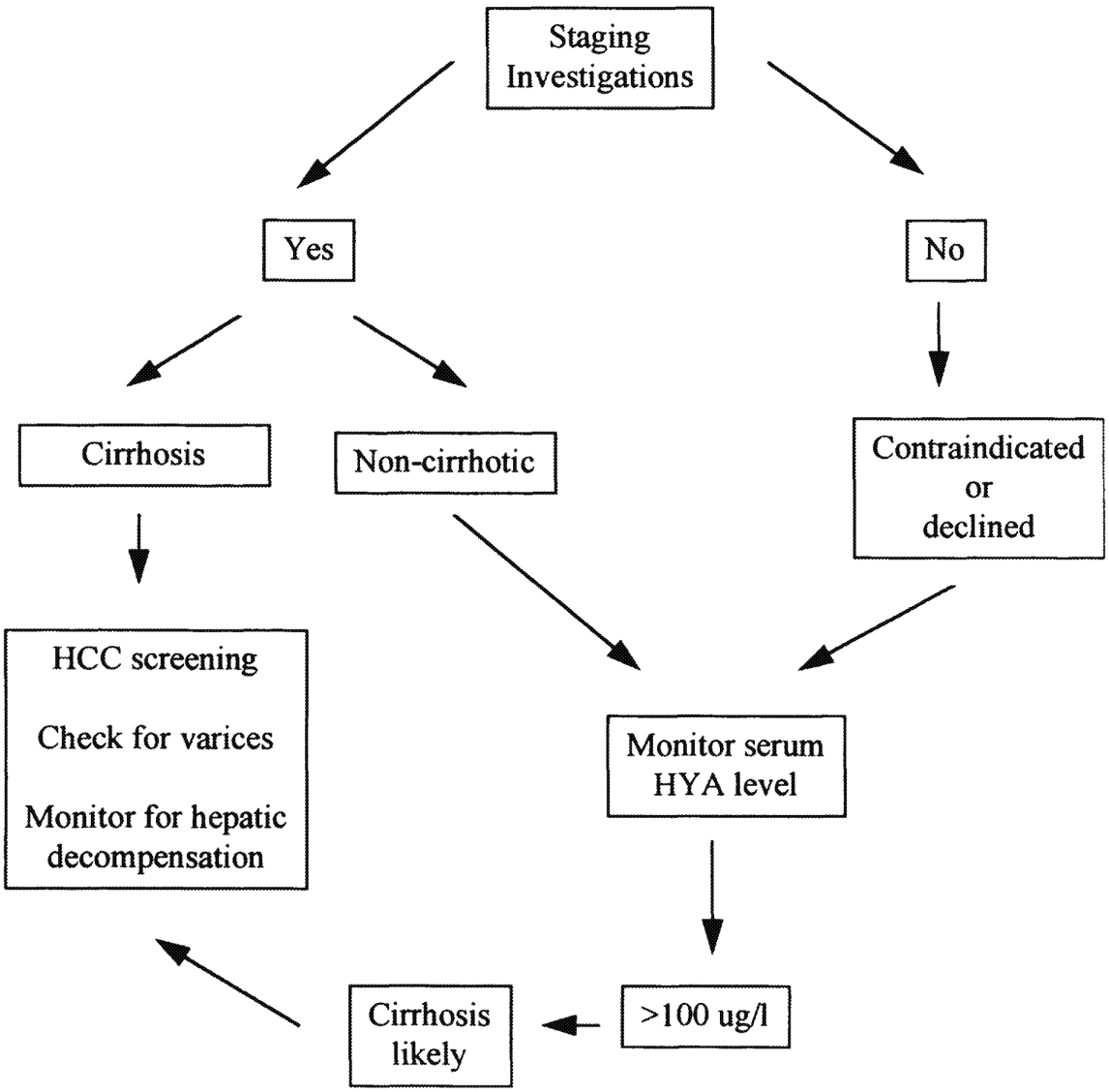
This study suggests that serum HYA may be a useful screening test to distinguish cirrhotics from non-cirrhotics in patients with chronic HCV infection. All individuals with a HYA level of greater than 100μg/l had evidence of established cirrhosis at liver laparoscopy. However there was one haemophiliac with biopsy-proven cirrhosis and oesophageal varices who had a HYA level persistently less than 100μg/l. As a result separate analysis of the haemophiliac group found the predictive value of HYA was less than when the entire group was assessed. It is clearly important to interpret HYA levels less than 100μg/l with the possibility of a false-negative result taken into account. Further studies are required to assess the specificity of HYA in haemophiliacs to ensure false-negative results do not undermine its use as a screening test in this particular population. In large studies in non-haemophiliacs HYA has been found to be a useful discriminator between cirrhotics and non-cirrhotics in liver disease of different aetiologies (Nyberg *et al*, 1988; Simpson *et al*, 1993; Guechot *et al*, 1996).

It remains unclear whether the primary mechanism leading to elevated serum HYA level in cirrhosis is reduced clearance or increased production. It is possible that both mechanisms play a part as cirrhosis develops. *In vitro* studies suggest that proliferation of hepatic stellate cells and an associated increase in HYA is an important component in the mechanisms which lead to fibrogenesis. Thus increased HYA production by stellate cells may be important in early cirrhosis. Also capillarisation of hepatic sinusoids, the site of HYA degradation, is a prominent histological feature of liver cirrhosis leading to impaired HYA clearance with increasing hepatic fibrosis (Takato *et al*, 1993). With the development of portal hypertension there is major disruption of the hepatic circulation which may lead to a further elevation of serum HYA which by-passes its site of degradation.

Whilst all individuals with chronic HCV may benefit from a reliable non-invasive marker of cirrhosis, this may be particularly valuable in haemophiliacs as the detection of a rising HYA

level in keeping with cirrhosis may potentially avoid the need for repeated invasive investigations. Once the HYA level is suggestive of cirrhosis subsequent follow-up should involve screening HCC, endoscopy to detect oesophageal varices and monitoring for signs of liver decompensation (Figure 4.3). Further studies are required to evaluate such an approach.

Figure 4.3 Long term follow-up of haemophiliacs with chronic HCV infection.



Chapter 5

The treatment of chronic HCV infection in haemophiliacs with interferon-α2a.

Introduction

Interferon-α (IFN-α) has both anti-viral and anti-proliferative actions and has been found to be useful in the treatment of a wide range of diseases including chronic viral hepatitis. It is effective treatment of HBV infection and more recently has been used to treat chronic HCV infection. Response to treatment of HCV has been assessed in many studies by serial ALT levels or histological assessment of liver biopsies pre and post IFN therapy. In addition, detection of HCV RNA by PCR has proved a useful method to assess the efficacy of IFN-α treatment. Eradication of viraemia rather than normalisation of ALT is likely to be a pre-requisite for a long term response.

To date there have only been a few small studies on the efficacy of IFN-α for the treatment of HCV infection in haemophiliacs (Makris *et al*, 1991; Bresters *et al*, 1992; Peerlinck *et al*, 1994; Mauser-Bunschoten *et al*, 1995; Telfer *et al*, 1995). Some of these studies have suggested that the response to IFN in haemophiliacs may be lower than in other groups infected with HCV.

This chapter describes the results of IFN therapy in a group of 31 haemophiliacs with chronic HCV infection. The response to IFN was monitored using a sensitive semi-quantitative PCR to detect HCV RNA. In addition, the severity of liver disease present in these patients prior to IFN treatment was investigated using both invasive and non-invasive methods. These investigations were assessed as predictive markers of response to IFN.

Patients and Methods

A total of 31 patients (30 male, 1 female) were treated (Table 5.1). All patients were anti-HCV positive by a second-generation enzyme immunoassay (A-EIA; Abbott, Weisbaden-Dalkenheim, Germany) and also positive on confirmatory testing by second generation recombinant immunoblot assay (RIBA-2, Chiron Corporation, Emeryville, CA) for antibody to nonstructural proteins 5-1-1 (NS4), c100-3 (NS4), c33c(NS3) and core-associated antigen c22-3. The mean age of the patients was 35 years (range 13-67).

Table 5.1 Patient characteristics

		n
Haemophilia A	Severe	11
	Moderate	8
	Mild	1
Haemophilia B	Severe	1
	Moderate	2
	Mild	5
VWD		3
Total		**31**

All had previously received non-virus inactivated factor concentrates and 30/31 had persistently elevated serum ALT levels (ALT levels were measured on at least 3 occasions over the 6 months prior to treatment). Serum ALT level was normal in one individual. The mean pre-treatment ALT was 78U/L (range 37-175U/L). HCV RNA was detected in all patients by PCR as described in chapter 2. The mean pre-treatment HCV RNA titre was

10^6 copies/ml (range 10^3->10^7 copies/ml). In addition data concerning annual factor concentrate consumption and serum immunoglobulins (IgG, IgM and IgA) and serum ferritin were collected.

Six patients were anti-HIV positive (pre-treatment CD4 counts 4, 20, 140, 180, 250 and 360 cells/mm^3 respectively) and all were HBSAg negative. All patients were negative for anti-smooth muscle and anti-mitochondrial antibody and had normal levels of α-fetoprotein, caeruloplasmin and α-1-antitrypsin.

Pre-Treatment Assessment

Informed consent was obtained from all patients participating in the study. Prior to starting IFN treatment a detailed assessment was offered to each patient to evaluate the extent of liver disease (see chapter 3). It was emphasised that the investigations were not essential prior to the administration of IFN but would provide information to counsel the individual as to the severity of their liver disease.

The following investigations were performed after appropriate factor concentrate replacement.

1. Upper GI (gastro-intestinal) endoscopy was performed in 27 patients to identify the presence or absence of esophageal varices.

2. Laparoscopic inspection of the liver was performed in 21 patients. The degree of hepatic inflammation (none, mild or marked), surface fibrosis (none, moderate or pronounced) and presence of cirrhosis and portal hypertension was assessed. In 15 patients a tru-cut biopsy was taken during the laparoscopy and histology was assessed both using the Sheffield scoring system and a visual classification. Using the latter method overall histological appearance was classified as mild, moderate or severe.

3. Abdominal ultrasound scans to assess liver size, echogenicity and the presence of hepatocellular carcinoma (HCC) as well as spleen size were performed on 30 patients.

Drug Dosage and Administration

IFN-α2a (Roche) 3 Mega Units 3 times per week was given subcutaneously. The intention was to treat for 24 weeks.

Assessment of Response to IFN Treatment

Response was assessed on the basis of serial monthly ALT levels (biochemical response) and HCV RNA quantitation (virological response). After 6 months treatment a complete biochemical response was defined as normalization of ALT (<40U/L) sustained for at least 2 months; a partial biocemical response required a >50% reduction in pre-treatment ALT. A complete virological response required HCV RNA to become undetectable in the serum i.e. 'PCR negative' (<80 HCV/ml - see below); a partial virological response required a hundred fold reduction in HCV RNA titre.

Typing and Quantification of HCV RNA

All testing was performed using serum samples separated within 3 hours of collection and stored at -70^{0}C. HCV RNA extraction and quantitation and HCV genotyping was performed as described in chapter 2. HCV genotyping was performed on all patients prior to starting IFN and after 3 and 6 months therapy.

Statistical Analysis

The relationships between biochemical response or virological response and baseline measurements were assessed using Fisher's exact test for nominal baseline variables, chi-square

test for trend for ordered categorical baseline variables, Wilcoxon rank-sum test for non-normally distributed continuous baseline variables and two-sample t-test for normally distributed continuous baseline variables. All significance tests were two-sided.

Results

Of 31 patients 29 completed 6 months treatment with IFN. One stopped after 2 months due to leucopenia and one stopped after 5 months due to a subjective hearing loss which subsequently recovered. The 29 patients who completed 6 months treatment were evaluated.

Normalization of ALT (complete biochemical response, CR) occurred in 8/29 (28%); a >50% reduction in pre-treatment ALT (partial biochemical response, PR) was achieved in 4/29 (14%) and 17/29 (59%) were non-responders (NR). 7/29 (24%) became PCR negative for HCV RNA (complete virological response, CR). In addition 2/29 (7%) achieved at least a 10^2 copies/ml reduction in HCV RNA (partial virological response, PR). In 20/29 (69%) the serum HCV RNA levels were unchanged (non-responders, NR).

There was a statistically significant difference in age between responders (mean age 46 years) and non-responders (mean age 33 years; two-sample t-test: p=0.044). Multivariate analysis, however, showed no independent relationship between age and response after adjusting for the effect of genotype on response (see below).

Response was not associated with HIV status, body weight, severity of haemophilia, duration of infection, annual factor concentrate consumption, serum ferritin or serum immunoglobulins.

Patterns of biochemical and virological response

Normalisation of ALT levels correlated with clearance of HCV RNA (Spearman rank correlation, r= 0.53; p=0.003). Of the 8 patients who normalized ALT, 5 also became HCV RNA negative whereas in 3 there was no corresponding reduction in virus load (Table 5.2). Of 4 patients who achieved a partial biochemical response, only 1 became non-viraemic. 15/17 in whom ALT level was unchanged also had no change in virus load. Only one individual who had no change in ALT became non-viraemic.

Table 5.2 Biochemical and virological response to IFN therapy.

		Virological response			
		CR	PR	NR	Total
Biochemical Response	CR	5	-	3	8
	PR	1	1	2	4
	NR	1	1	15	17
	Total	7	2	20	29

CR: Complete response; PR: Partial response; NR: No response

The median ALT values in patients who became non-viraemic compared to those who failed to clear HCV RNA are shown (Figure 5.1). In one patient only there was a transient HCV RNA clearance at 2 months but at 3 months the HCV RNA titre returned to the pre-treatment level of 10^7 HCV/ml. This was associated with a parallel ALT reduction and subsequent relapse (Figure 5.2). There was no relationship between pre-treatment ALT level and response.

Figure 5.1 **Median ALT levels during IFN treatment in those without (line A, n=22) and with (line B, n=7) an associated virological response.**

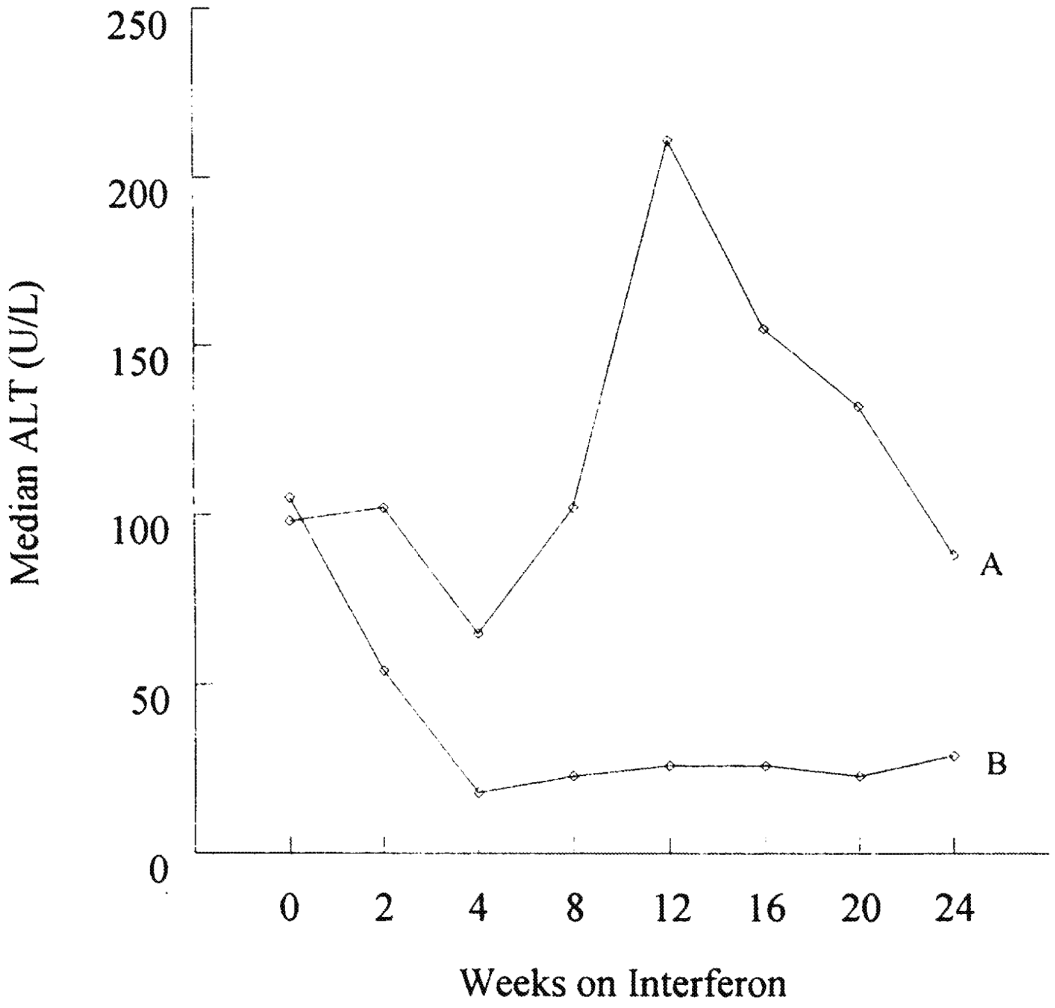
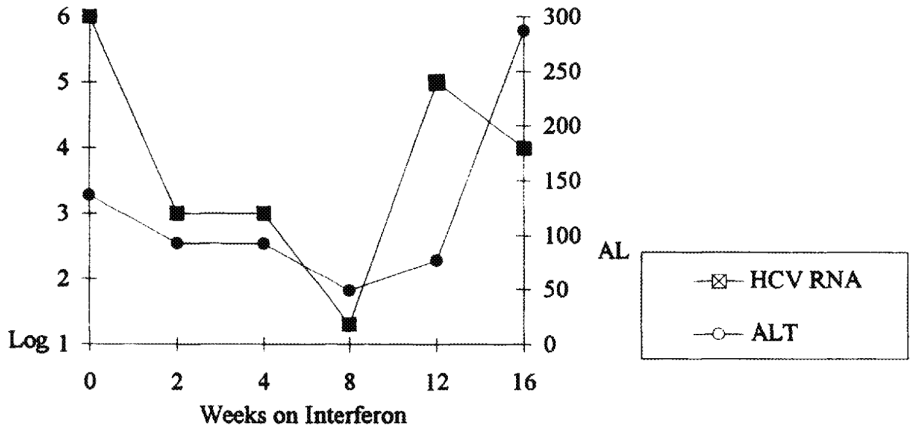


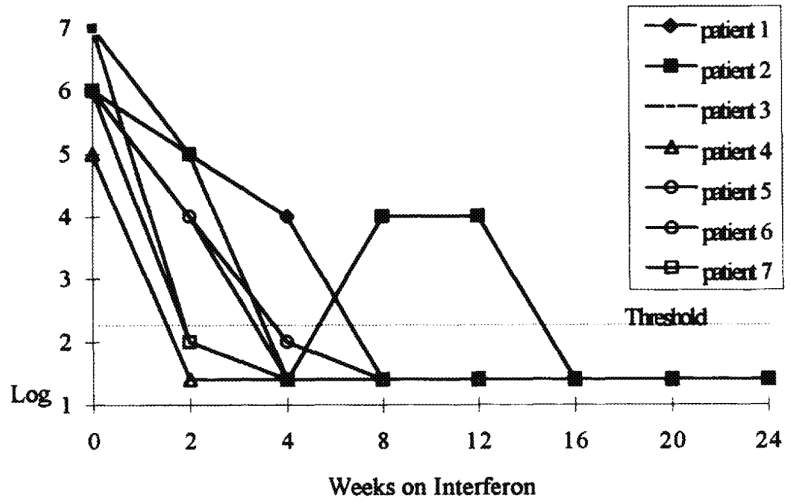
Figure 5.2 Breakthrough hepatitis during interferon therapy occurred in one individual.



Rate of HCV RNA clearance in responders

The rate of HCV RNA clearance in the 7 complete responders was assessed (Figure 5.3). In 6/7 a prompt reduction in HCV RNA occurred to below the limit of detection within 8 weeks of commencing interferon. In one individual (Patient 2, Figure 5.3) initial clearance of HCV RNA at week 4 was followed by a transient rise in virus titre to 10^4 copies/ml at weeks 8 and 12 before sustained clearance between weeks 16 and 24.

Figure 5.3 Rate of HCV RNA clearance in individuals (n=7) who acheived a complete virological response.



Factors for Response to Interferon (Table 5.3)

a)HCV Genotype: There was a striking relationship between HCV genotype and a favourable response to IFN. A total of 6 out of 8 haemophiliacs infected with genotype 3a became non-viremic. In the other 21 patients only one individual (genotype 2b) became HCV RNA negative (Fisher's exact test: p=0.0002). No changes in HCV genotype, as detected by RFLP analysis, were identified in any patients during IFN therapy.

b)Pre-treatment Virus Load: There was no relationship between pre-treatment virus load and either response to IFN (Figure 5.4) or HCV genotype (Figure 5.5).

c)Severity of liver disease: Severity of liver disease was assessed by the methods outlined above. Of 28 abdominal ultrasound scans evaluated 5(18%) demonstrated splenomegaly and 7(25%) showed an abnormal liver (5 with hepatomegaly and 2 with an abnormal liver texture). In 7/25(28%) esophageal varices were demonstrated at endoscopy. Of the 20 laparoscopic liver inspections 10(50%), 4(20%) and 6(30%) showed none, mild or pronounced surface

fibrosis respectively with 5 of the patients with pronounced fibrosis having cirrhosis. Inflammation was identified laparoscopically in all patients being mild in 14(70%) and marked in 6(30%). Evidence of portal hypertension was visible in 5(25%).

Liver histology was evaluated in 14 patients and revealed mild, moderate and severe(including cirrhosis) histological changes in 4(29%), 7(50%) and 3(21%) respectively.

Analysis of response to IFN against laparoscopic liver appearance, liver histology, presence/absence of hepatomegaly, splenomegaly and oesophageal varices showed no evidence that any of these variables were associated with response to IFN.

Table 5.3 Predictive factors for response to IFN.

		Virological response			
		CR	PR/NR	Total	p value
HCV Genotype	1a	0	11	11	
	1b	0	7	7	
	2a	0	1	1	
	2b	1	1	2	
	3	6	2	8	**0.0002***
Anti-HIV	Positive	0	5	5	
	Negative	7	18	25	0.30
Splenomegaly (Ultrasound)	Yes	0	5	5	
	No	7	16	23	0.29
Hepatic Fibrosis (Laparoscopy)	None	3	7	10	
	Mild	1	3	4	
	Pronounced	3	3	6	0.64
Hepatic Inflammation (Laparoscopy)	Mild	5	9	14	
	Marked	2	4	6	1.00
Oesophageal Varices (Endoscopy)	Yes	1	6	7	
	No	6	12	18	0.63
Liver Histology	Mild	1	3	4	
	Moderate	3	4	7	
	Severe	1	2	3	1.00

CR: Complete response PR: Partial response NR: No response

* Fisher's exact test

Figure 5.4 **Pre treatment virus load and response to interferon.**
There was no significant relationship between pre-treatment virus load and response to IFN. (chi-square test for trend p=0.073)
CR: Complete response PR: Partial response NR: No response

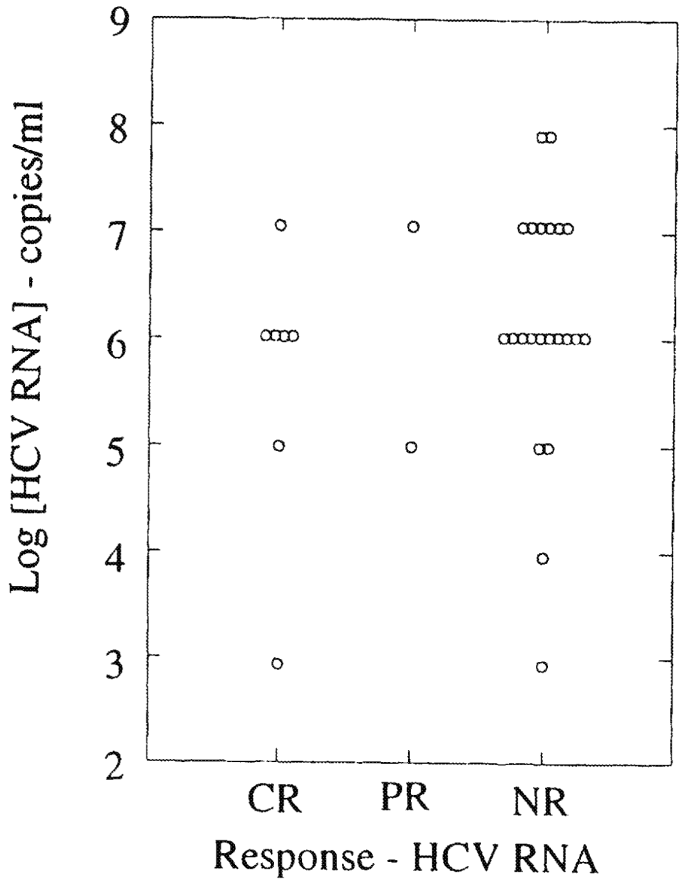
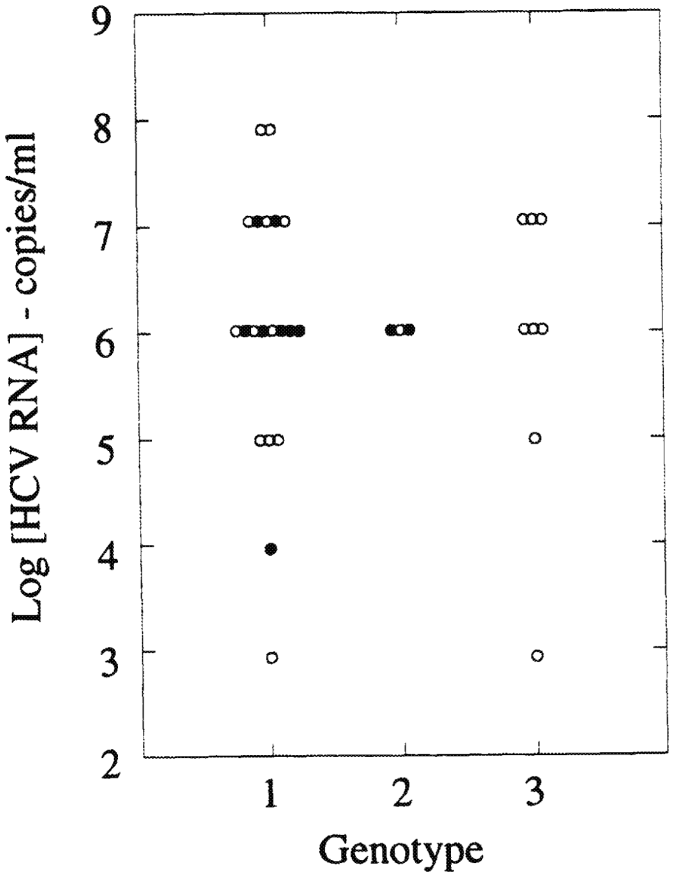


Figure 5.5 **Pre treatment virus load and HCV genotype.**
There was no significant relationship between pre-treatment virus load and HCV genotype (kruskal-wallis test: p=0.77)

o subtype a

• subtype b



Follow-up Information

Within 3 months of discontinuing IFN 5/7 of the patients who became non-viraemic relapsed with reappearance of serum HCV RNA as well as elevated ALT levels. A further individual relapsed months after stoping IFN leaving only one patient with a sustained virological response after prolonged follow-up (more than 24 months). In 2 patients who achieved a partial reduction in virus load, the HCV RNA level returned to pre-treatment values within 1 month of stopping interferon. The 3 patients who normalized ALT levels without a corresponding reduction in HCV RNA developed elevated ALT levels within 2 months of stopping IFN.

Discussion

This study has shown that the response to IFN therapy in haemophiliacs with chronic HCV infection is poor and appears inferior to other groups of infected patients. In this study only 28% normalized serum ALT after six months treatment with IFN and 24% cleared serum HCV RNA. Furthermore, sustained responses were rare (only 1/31 patients). This compares to initial complete biochemical responses of 50% and sustained responses of 20-25% reported in non-hemophiliacs (Tine *et al*, 1991). Early investigators used ALT to assess long term response (Davis *et al*, 1989; Marcellin *et al*, 1991). More recent studies have confirmed that clearance of HCV RNA is a pre-requisite for long term response (Shindo *et al*, 1991).

The importance of HCV quantitation has been increasingly recognised. We describe here a sensitive semi-quantitative PCR method for HCV RNA detection with a lower limit of detection is 80 HCV/ml. This compares with 3.5×10^5 equivalents/ml when the bDNA assay is employed (Bresters et al, 1994; see chapter 1). Using bDNA we would have failed to detect

viraemia in 4(13%) of our patients and incorrectly classified 6(20%) as responders who merely showed a partial reduction in HCV RNA levels. Clearly, sensitive quantitative assays are essential to monitor response to treatment and some studies may have over-estimated response rates by using relatively insensitive methods for RNA quantitation. We found that HCV RNA was cleared within 8 weeks of starting interferon in the 7 patients who responded. In one individual initial HCV RNA clearance was followed by a transient rise in virus titre before further clearance. The reasons for this are not entirely clear. Temporary non-compliance with treatment is a possible explanation or there may be a small number of individuals who achieve a slower reduction in HCV RNA rather than the rapid and sustained reduction observed in the majority of responders. Others have found that loss of serum HCV RNA after only 4 weeks of interferon-α therapy is predictive of a subsequent long-term response to interferon (Orito *et al*, 1995). Thus using sensitive methods of HCV RNA detection and quantitation it is possible to differentiate between responders from non-responders at an early stage of treatment and thus potentially avoid exposure of patients to ineffective therapy. Alternatively, these individuals could be offered a dose-escalation or addition of other anti-viral drugs.

There is usually a good correlation between normalization of ALT and HCV RNA clearance in responders to interferon. Discrepancies between ALT and HCV RNA responses have, however, been described previously (Lau *et al*, 1993). We have identified some individuals who achieved a biochemical response without a corresponding reduction in virus load. Clearly these patients are not "true responders" and biochemical relapse is inevitable once IFN is discontinued. As ALT is not an entirely accurate measure of response, studies using ALT alone to assess response may also overestimate "true responders". Interestingly, there was one individual who cleared HCV RNA without an associated biochemical response response. This raised the possibility of other non-HCV co-existing liver pathology resistant to IFN treatment, but none has been identified. Another individual relapsed during IFN therapy. Such episodes of

'breakthrough hepatitis' are well recognised and may be associated with the development of neutralizing antibodies to IFN (see chapter 6). A change in HCV genotype may also be an explanation for 'breakthrough hepatitis' and a change in the dominant genotype in hemophiliacs treated with IFN has been reported (Devereux *et al*, 1995). In this study, we did not detect any changes in the circulating genotype in any patients during IFN therapy.

Several studies assessing response to interferon have been performed on cohorts of haemophiliacs infected with HCV (Makris *et al*, 1991; Bresters *et al*, 1992; Peerlinck *et al*, 1994; Mauser-Bunschoten *et al*, 1995; Telfer *et al*, 1995; Rumi *et al*, 1997). Some of these studies have suggested that the overall initial response rates are somewhat lower in haemophiliacs than in other groups with HCV. Interestingly, the response rates in the earlier studies are superior to those performed more recently (Table 5.4). This may be a reflection of the relatively small number of patients studied or may have been caused by progression of liver disease in cohorts of haemophiliacs leading to diminished responses to IFN. The current study supports the view that long term responses to IFN are uncommon in haemophiliacs.

Table 5.4 **Results of studies if IFN therapy in haemophiliacs with chronic HCV**
Early reports of a similar response rate in haemohpilacs as those reported in non-haemophiliacs have not been confirmed in subsequent studies. Initial response in the studies listed was assessed at the end of IFN therapy. Sustained responses were measured after a variable period of follow-up.

	n	Initial Response		Sustained Response (%)
		Biochemical (%)	Virological (%)	
Makris *et al*, 1991	16	44	-	25
Breesters *et al*, 1992	8	50	50	50
Peerlinck *et al*, 1994	13	53	53	7
Telfer *et al*, 1995	20	45	25	5
Rumi *et al*, 1997	45	33	27	13

Attempts have been made to identify factors which may predict response to IFN (Davis, 1994). Absence of cirrhosis, younger age, low serum HCV RNA level and genotypes 2 and 3 are all factors associated with a favourable response to IFN. As yet the presence of adverse factors have not been considered sufficient to absolutely exclude some individuals with HCV from IFN therapy. There has been particular concern, in view of the generally poor response to IFN in hemophiliacs, that treatment with IFN is inappropriate in the majority of individuals. Not only are patients exposed to a potentially toxic drug with unpleasant side-effects, but also the cost of a course of treatment is considerable.

This study found that genotype 3a was associated with a favourable response but we failed to identify any other statistically significant independent variables, including pre-treatment virus load and cirrhosis, associated with a poor response to IFN. This may reflect the relatively small study group, however, it is clear that such parameters do not always predict accurately which individuals are likely to respond to IFN and even those with a high pre-treatment virus load and cirrhosis should be offered treatment.

In conclusion, the results of IFN treatment for HCV in haemophiliacs are disappointing. IFN-α2a 3MU 3 times a week for 6 months is unlikely to result in a long term sustained response. Monitoring response with a sensitive semi-quantitative PCR to quantify HCV RNA is extremely useful to identify responders at an early stage of treatment. Those who fail to clear HCV RNA should discontinue IFN. Dose escalation is unlikely to benefit many non-responders but may be attempted in selected individuals. The role of prolonged courses of IFN or combination therapy with additional anti-viral drugs remains to be evaluated.

Chapter 6

Development of anti-interferon antibodies and breakthrough hepatitis during treatment for HCV infection in haemophiliacs.

Introduction

Interferon-alpha (IFN-α) has been widely used to treat chronic HCV infection. Response to therapy may be assessed either by serial measurement of serum ALT or HCV RNA quantitation by PCR. Those who respond to IFN usually show a prompt normalisation of ALT and clearance of serum HCV RNA within 8-12 weeks of treatment. In non-responders serum ALT and HCV RNA levels remain unchanged.

In some individuals "breakthrough hepatitis" (BTH) may occur, i.e. initial response with normalisation of ALT followed by a sustained rise in ALT, during IFN therapy. It has been suggested that such episodes of BTH may be caused by anti-IFN antibodies (Milella *et al,* 1993). This chapter describes a study on the development of anti-IFN antibodies in a group of 39 haemophiliacs receiving IFN-α2a for chronic HCV infection.

Patients and Methods

Patient Characteristics

A total of 39 patients (37 male, 2 female) were studied (26 haemophilia A, 9 Haemophilia B and 4 von Willebrand's Disease). These patients included the 31 patients described in chapter 5 plus a further 8 patietnts who subsequently received IFN therapy. The median age was 35.5 years (range 13-67 years). Six patients were anti-HIV positive and all were HBSAg negative.

All patients were anti-HCV positive by Abbott second-generation enzyme immunoassay (A-EIA; Abbott, Weisbaden-Dalkenheim, Germany) and also positive on confirmatory testing by second generation recombinant immunoblot assay (RIBA-2, Chiron Corporation, Emeryville, CA). All had previously received non-virus inactivated coagulation factor concentrates prior to 1985 and 38/39 had persistently elevated serum ALT levels (Normal range 10-40 U/L). HCV

RNA was detected in 38/39 patients by RT-PCR and quantified by a limiting dilution technique HCV genotyping was performed as described in chapter 2.

Drug Dosage and Administration

IFN-α2a (Roche) 3 Mega Units 3 times per week was given subcutaneously. The intention was to treat for 24 weeks. Response was assessed on the basis of serial monthly ALT levels and HCV RNA quantitation.

Measurement of anti-IFN antibodies

Serum samples for anti-IFN antibody assays were carefully stored at -40^{0}C from all patients at 0, 12 and 24 weeks. All samples were tested at the end of treatment. No response or HCV genotype data was provided to the laboratory performing the anti-IFN antibody assays. All samples were tested using an enzyme immunoassay (EIA) which detects binding antibodies (Hennes *et al*, 1987). Those samples which were positive by EIA were also assessed by an antiviral neutralisation bioassay (ANB) which demonstrates the neutralising properties of the antibody (for details of assay methods see chapter 2)

Results

A low titre binding anti-IFN antibody was detected in 1/39 samples prior to treatment. This was undetectable in the 3 and 6 month samples from this patient. Binding anti-IFN antibodies detected by EIA developed during treatment in 5/39 (13%) patients (Table 6.1). Neutralising properties were demonstrated by ANB in 3 of 5.

Table 6.1 Characteristics of the 5 patients who developed anti-IFN antibodies.

Patient	Age	HCV genotype	ALT response	HCV RNA response	Anti-IFN antibodies	
					EIA	ANB
1	16	3a	Yes +BTH	Yes+BTV	Pos	Pos
2	38	3a	Yes+BTH	Yes+BTV	Pos	Neg
3	13	1b	Yes+BTH	No	Pos	Pos
4	67	3a	Yes	Yes	Pos	Pos
5	33	3a	No	Yes	Pos	Neg

The timing of antibody development as well as antibody titre varied (Figure 6.1-6.5). BTH occurred in three patients (No. 1, 2 and 3) who developed antibodies and this was accompanied by breakthrough viraemia in two of these (No. 1 and 2). BTH hepatitis and viraemia occurred in only one additional patient without evidence of anti-IFN antibodies which was thought to be due to intermittent compliance with IFN therapy.

Figure 6.1-6.5

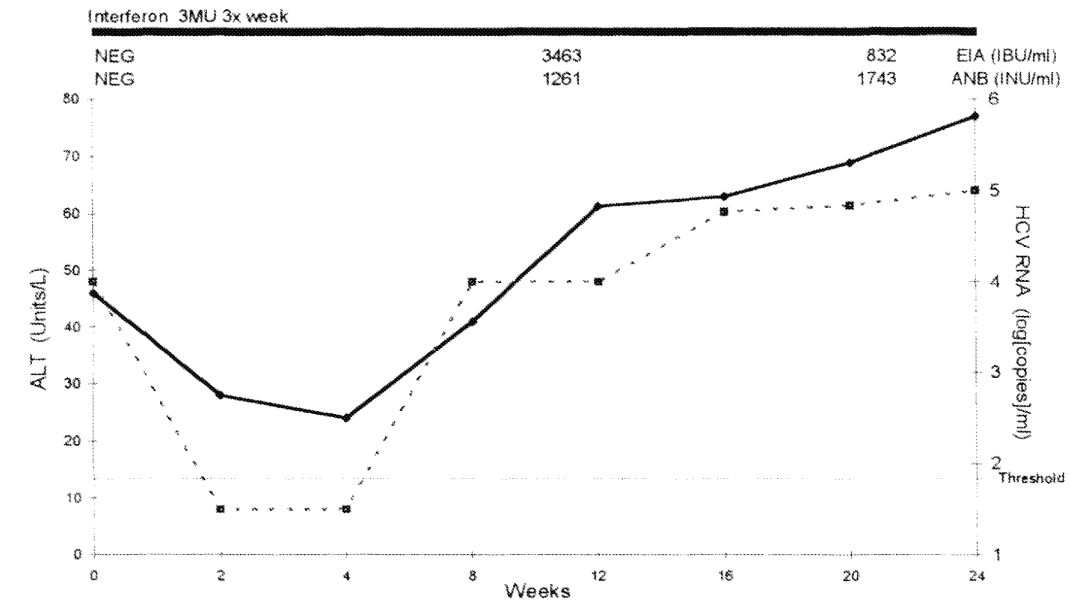
Timing of anti-IFN antibody development in relation to ALT and HCV RNA levels during interferon treatment in patients 1-5.

EIA (IBU/ml): Enzymeimmunoassay (Interferon binding units / ml)

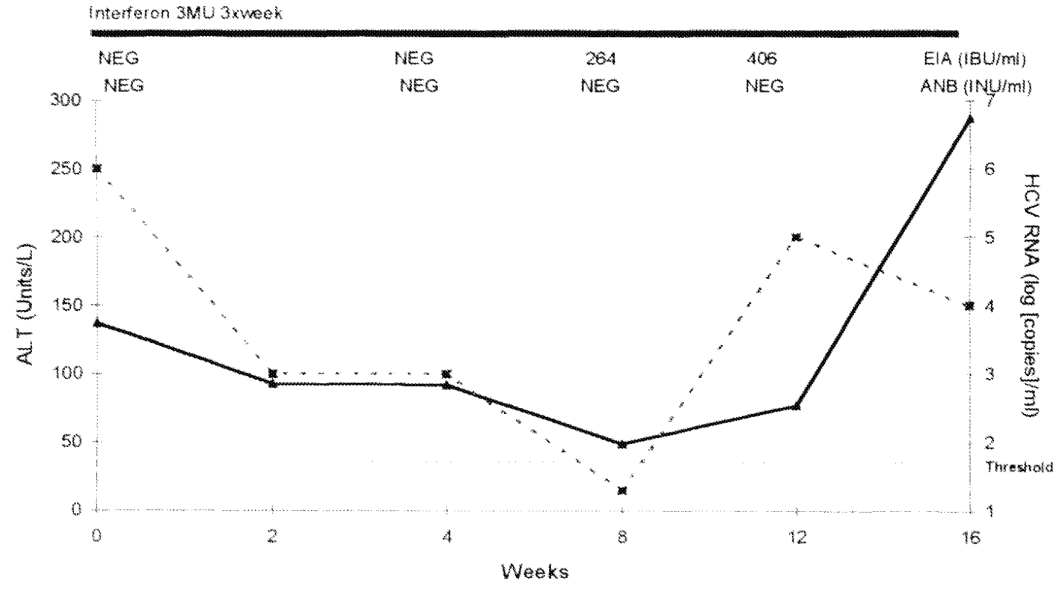
ANB (INU/ml): Anti-viral neutralisation bioassay (Interferon neutralising units / ml)

ALT —— HCVRNA - - -

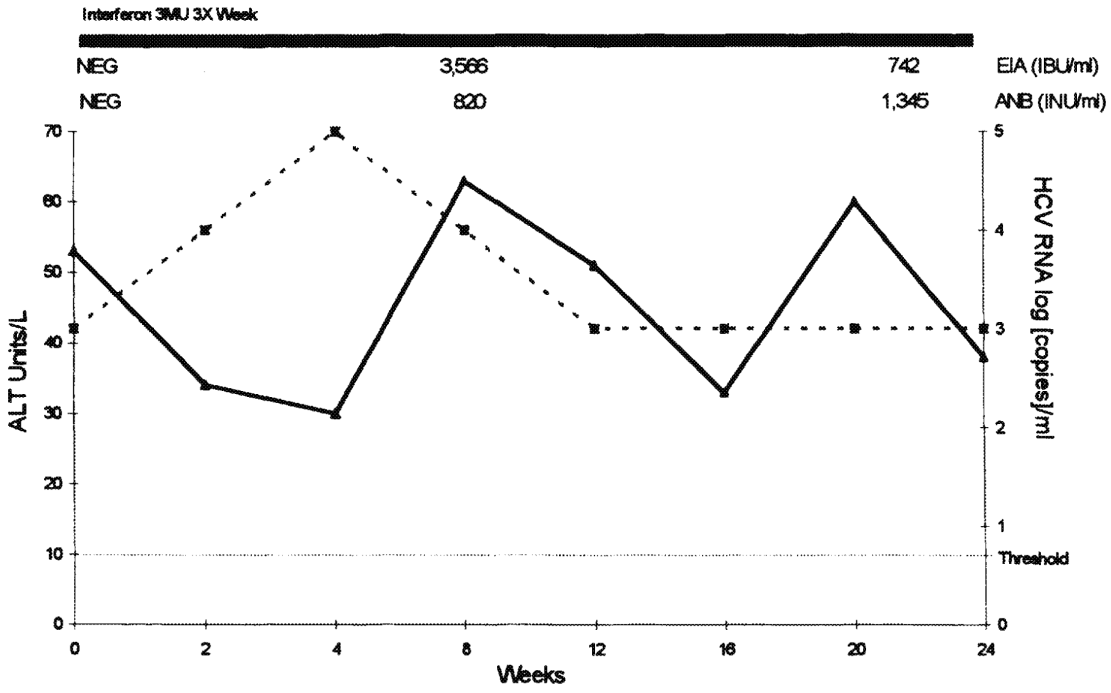
6.1 **Patient 1**



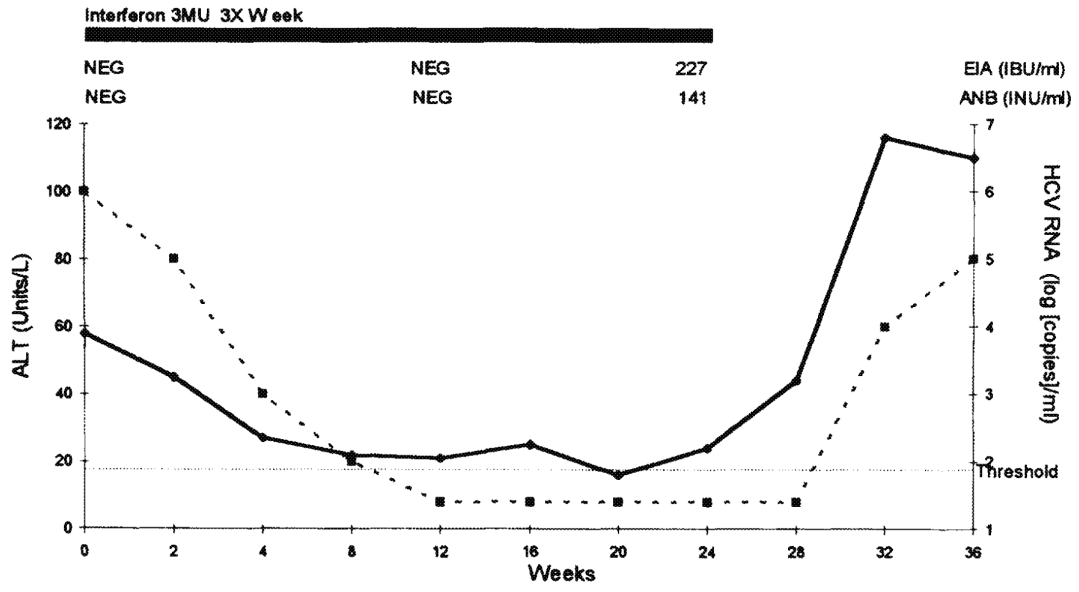
6.2 **Patient 2**



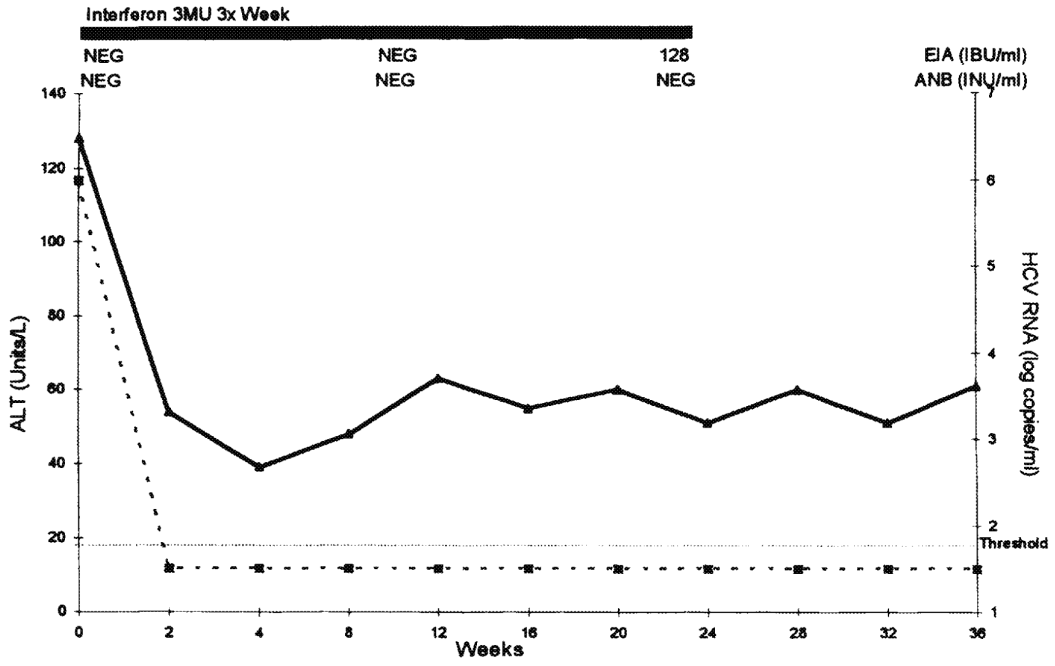
6.3 **Patient 3**



6.4 **Patient 4**



6.5 **Patient 6**



There was a significant association with HCV genotype 3a (chi square test: p=0.01) (Table 6.2). There were no changes in HCV genotype detected in any patients during IFN treatment.

Table 6.2 HCV Genotype and anti-IFN antibody development.

There was a significant association with genotype 3a (p=0.01).

HCV Genotype	Patients (n)	Anti-IFN antibodies (n)
1a	15	0
1b	9	1
2a	1	0
2b	2	0
3a	11	4
PCR negative	1	0
Total	**39**	**5**

Follow-up information

After cessation of IFN therapy, follow-up assays were performed in those individuals who developed antibodies. Within 4-6 months both binding and neutralising antibodies had either reduced to low titre or become undetectable in all cases.

Discussion

Recent studies have suggested that the response to IFN in haemophiliacs with HCV infection is inferior compared with non-haemophiliacs and a standard treatment schedule (3MU 3x per week for 6 months) leads to few sustained responses (Telfer *et al*, 1995; Hanley *et al*, 1996). Factors which influence response to IFN include age, duration of infection, severity of liver disease, pre-treatment virus load and HCV genotype. This study suggests that the development of anti-IFN antibodies leading to BTH may contribute to the poor response to IFN in haemophiliacs.

Naturally occurring anti-IFN antibodies have been reported to occur in autoimmune disease (Panem *et al*, 1982; Bendtzen *et al*, 1994; Thurmond and Reese, 1991), malignant disease (Trown *et al*, 1983; Jones and Itri, 1986), red cell aplasia (Prummer *et al*, 1991) and in healthy volunteers at low titre (Ross *et al*, 1990; Thurmond and Reese, 1991). In this study an apparently naturally occuring anti-IFN antibody was detected in one patient which became undetectable during IFN treatment. The physiological or pathogenic significance of such autoantibodies remains uncertain.

Many studies have suggested a relationship between anti-IFN antibodies and both primary non-response and the loss of therapeutic response to IFN. IFN-inducible proteins, which mediate some of the therapeutic effects of IFN, are reduced in individuals with anti-IFN antibodies (Steis *et al*, 1991; von Wussow *et al*, 1991a). However it should be remembered that both

primary non-response to IFN and treatment failure after initial response may develop for reasons other than the development of anti-IFN antibodies (Einhorn and Grander, 1996) (Table 6.3).

Table 6.3 Factors which may contribute to treatment failure after initial therapeutic response to IFN.

Anti-INF antibodies	Neutralising
	Binding
Non-compliance	
Dose reduction	
Escape mutation	
Down-regulation of IFN cell receptors	

In particular there is some evidence that each HCV genotype circulates as a heterogeneous group of variants (termed "quasi-species") which evade host immune responses by continual evolution of new mutants (Enomoto *et al,* 1994). In theory, when IFN is administered therapeutically, initial response may be overcome by the rapid evolution of quasi-species (even without a change in the major circulating genotype). It has also been reported that the major circulating HCV genotype may change during IFN treatment (Devereux *et al,* 1995). However this study found no evidence of changing HCV genotypes during therapy.

The mere presence of anti-IFN antibodies does not invariably lead to treatment failure (Craxi *et al*, 1988). The timing of antibody development in relation to initial response appears to be particularly important. In this study, antibodies which appeared at the same time as ALT normalisation (No. 1, 2 and 3) or HCV RNA clearance (No. 1 and 2) led to the development of breakthrough hepatitis or viraemia. On the other hand, antibodies which appeared many

weeks after the therapeutic response (No. 4 and 5) were not associated with breakthrough. It is possible, however, that the late appearance of antibody was a factor in the early relapse after stopping IFN seen in patient 4. The importance of timing of antibody development in relation to the response to IFN has been noted by others (Bonetti *et al,* 1994a; Roffi *et al*, 1995). It is likely that the early phase of IFN treatment is the most crucial for virus clearance and that once this has occurred, subsequent anti-IFN antibody development does not result in sufficient neutralisation of IFN to precipitate BTH or BTV.

The association shown in this study between anti-IFN antibody development and infection with HCV genotype 3a has not been previously reported. This observation may be particularly important as genotype 3a is usually associated with a favourable response to IFN. It is also interesting to note that of the five individuals who developed anti-interferon antibodies four were aged less than 40 years. Anti-IFN antibodies are unusual in younger patients (Porres *et al* 1989) and haemophiliacs may be at increased risk of antibody formation due to immunological abnormalities, irrespective of HIV infection (Watson & Ludlam, 1992).

The clinical importance of anti-IFN antibodies is not yet fully understood, however the development of BTH in individuals with factors usually associated with a favourable response to interferon (e.g. young age, genotype 3a, low pre-treatment virus load and absence of cirrhosis) is particularly important to detect. Anti-IFN antibody assays should be performed when breakthrough hepatitis develops during IFN treatment. The recognition of antibody development may lead to the avoidance of treatment failure by a number of strategies. Simply continuing with the same dose and type of IFN may result in antibody disappearance and restoration of response (Steis *et al*, 1991; Ronnblom *et al*, 1992). Alternatively, escalation of IFN dose may result in renewed response in association with falling antibody titres (Steis *et al*, 1988). However, In several other studies dose escalation has proved ineffective whereas restoration of response has been achieved by switching to an alternative IFN preparation. For

example, of 85 HCV patients who responded to rIFN-α2a, 11 developed BTH (neutralising anti-interferon antibodies were found in 6/11 cases). Dose escalation was ineffective but all 11 patients subsequently achieved a response to IFN-αN3 (Mels *et al*, 1993). Similarly, in a study of 191 patients who received rIFN-α2a for HCV infection, 12 of 104 responders developed BTH in association with neutralising (n=6) or binding (n=6) anti-IFN antibodies. Again dose escalation failed to restore response but all 12 responded following a switch to IFN-αN3 (Roffi *et al*, 1995). In antibody-negative patients a successful response to IFN-αN3 has been reported to be less likely (only one of 14 patients) compared with antibody-positive (six of nine patients) (Milella et al, 1995) although there are occasional reports of responses even in antibody-negative individuals (Casato *et al*, 1994).

Patients with chronic myeloid leukaemia (von Wussow *et al*, 1991a), hairy cell leukaemia (von Wussow *et al*, 1991b) , essential thrombocythaemia (Catani *et al*, 1992) and mixed cryoglobulinaemia (Casato *et al*, 1991) who developed treatment failure with rIFN-α2a in association with anti-IFN antibodies have also effectively responded to IFN-αN3, as have patients with carcinoid tumours treated previously with rIFN-α2b (Oberg *et al*, 1989). In patients with CML, haematological response, although not cytogenetic response, may be achieved even in primary non-responders to rIFN-α2a by switching to IFN-αN3 (Russo *et al*, 1996). Although all these studies involve small numbers of patients, there is increasing evidence that it may be worthwhile substituting IFN-αN3 for rIFN if relapse occurs after initial response.

In conclusion, anti-IFN antibodies are implicated as a cause of therapeutic failure in some patients with chronic HCV infection. Those who develop treatment failure in association with the development of anti-IFN antibodies should be switched to an alternative IFN preparation in an attempt to restore response.

Chapter 7

The detection of hepatitis C virus genotypes in coagulation factor concentrates

Introduction

Since the identification of HCV and the subsequent discovery of genotypes and subtypes there has been considerable interest in the possible differences between genotypes and the clinical implications of such differences. There has been speculation that particular HCV genotypes may be more pathogenic than others either in terms of their ability to cause initial infection as well as chronic infection or the associated severity of liver disease. Pathogenic differences between genotypes may also result in differences in sensitivity to anti-viral therapy. From recent studies there has been evidence to suggest that there may, indeed, be such differences in pathogenicity between genotypes. Genotype 1b, for instance, has been reported to be associated with more severe liver disease (Pozzato *et al*, 1995) and the development of hepatocellular carcinoma in the absence of associated cirrhosis (De Mitri *et al*, 1995). Genotypes 1a and 1b respond less well to IFN therapy compared with genotypes 2 and 3a (Brechot, 1996).

In order to test the theory that there may be pathogenic differences between genotypes the study of populations who have been repeatedly exposed to HCV on multiple occasions may be informative. If a particular HCV genotype was more pathogenic in terms of being more capable of establishing chronic infection one would expect, in a population exposed to multiple genotypes over a period of time, that this genotype would become relatively over-represented in the exposed population. This effect may become diminished if the mortality associated with more pathogenic genotypes is increased. Haemophiliacs who received multiple infusions of coagulation factor concentrates prior to the introduction of effective virus inactivation methods represent such a multiply exposed population. Up to several thousand individual plasma donations were pooled in each batch of coagulation factor concentrate and thus any donors with HCV viraemia at the time of plasma donation may result in contamination of the donor pool and if more than one donor is viraemic, there was the potential for multiple HCV genotypes to be present in a

single batch of concentrate. It is also possible that particular HCV genotypes were more susceptible to destruction during the manufacturing process, even prior to the introduction of formal virus inactivation procedures.

In order to test the theory of different pathogenicities between HCV genotypes in the multi-transfused haemophiliac population, it is important to first establish the distribution of HCV genotypes present in the concentrates which they received. In addition, the study of particular batches of concentrate which clearly transmitted HCV to individual previously untreated haemophiliacs may provide the opportunity to study the differences in the ability of particular genotypes to establish initial infection.

In summary, the aims of this study were as follows:

- to detect HCV RNA in non-virus inactivated coagulation factor concentrates
- to identify HCV genotypes present in individual batches of concentrate (using the single molecule method outlined in chapter 2)
- to compare the relative proportions of different HCV genotypes in commercial concentrates with the published genotype distributions in the donor and recipient populations.
- to examine particular batches of SNBTS concentrate known to have transmitted HCV (and compare the HCV genotypes in the concentrate and recipient).

Materials and methods

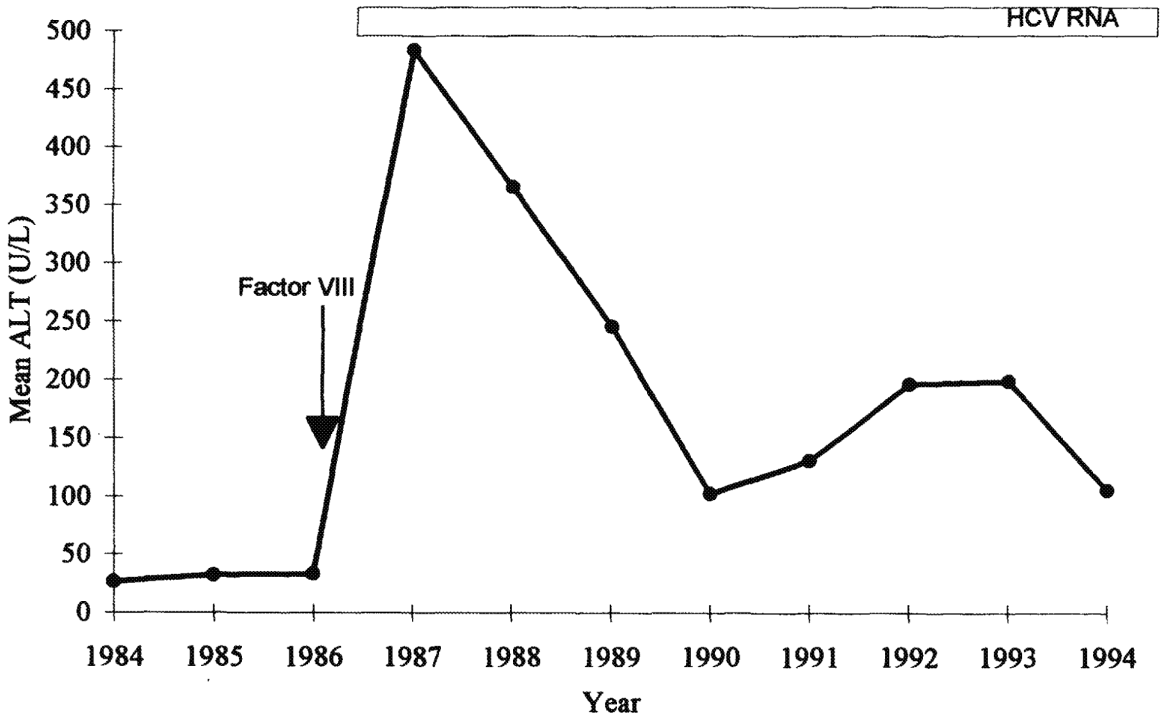
In total 49 archived coagulation factor concentrates were available either from commercial manufacturers (n=32) or SNBTS (n=17). All concentrates were manufactured in the early to mid 1980s and had been freeze-dried and stored at 4^0C. The commercial FVIII concentrates were from a variety of manufacturers who used source plasma derived exclusively from North American blood donors. Some commercial concentrates had been derived from a research stock and were identified only by ID numbers (n=18); the remaining (n=14) were obtained directly from the manufacturer and were identified by their original batch number. None of the commercial concentrates studied had been subjected to a virus-inactivation procedure during the manufacturing process

The SNBTS concentrates (13 FVIII and 4 prothrombin complex concentrates (DEFIX)) concentrates and were manufactured at the Protein Fractionation Centre in Edinburgh from plasma derived from volunteer Scottish blood donors and all were identified by their original batch number. Most SNBTS concentrates studied were manufactured prior to the use of virus inactivation methods. Several concentrates manufactured in 1985 and 1986 had been subjected to early virus inactivation methods. From the beginning of 1985 SNBTS concentrates were heated in solution 68^0 for 24 hours. In mid-1986 dry heat treatment at 75^0 for 72 hours was introduced; after only a few batches this was changed to 80^0 for 72 hours.

From transfusion records it was possible to identify patients who were first treated with SNBTS concentrates in the early to mid 1980s. In several cases there was clear documentation of HCV infection following their first infusion of concentrate with serum samples from these individuals negative for HCV RNA immediately prior to infusion and positive post-infusion. There was also an associated rise in serum ALT level consistent with acute HCV infection. I was particularly interested to study the HCV genotypes in such batches of concentrate which clearly transmitted

HCV to particular individuals. This would enable a comparison between the genotype(s) present in the concentrates and the genotype(s) detected in serum to see if a particular genotype was more capable of establishing acute infection. Unfortunately, despite an extensive search of archived material at the Protein Fractionation Centre in Edinburgh, only one such batch was available for testing (Z8 340150040). This batch had been subjected to heating at 68^0 for 24 hours and was administered to a previously untreated patient in 1986 and transmitted HCV infection (figure 7.1).

Figure 7.1 **Transmission of HCV to a previously untreated patient in 1986.** **HCV RNA was not present in serum samples prior to first treatment with FVIII concentrate and ALT levels were normal. Following FVIII infusion ALT elevation occurs in asociation with the detection of HCV RNA. Viraemia persisted until interferon therapy in 1994.**



RNA extraction

Each concentrate was reconstituted with 10mls of sterile water. 5mls of reconstituted concentrate were made up to 7mL with RPMI medium and ultracentrifuged at 100,000g for 90 minutes at 4^0C to pellet virus RNA. virus RNA was extracted as described in chapter 2.

RT-PCR

Following RNA extraction RT-PCR was performed to detect HCV RNA as described in chapter 2.

HCV genotyping on concentrates

HCV genotyping by RFLP was performed using the PCR product from the neat HCV RNA extraction. Subsequently 17 concentrates single molecules of HCV RNA were isolated from 17 concentrates using limiting dilution. HCV genotyping was performed on individual single molecules of HCV RNA. If insufficient 2^0 PCR product was present for genotyping, a third round of PCR was performed to increase the amount of PCR product available for the RFLP digest.

Results

HCV RNA detection and quantitiation

HCV RNA was detected in 27/32 commercial FVIII concentrates. All 13 SNBTS FVIII concentrates were negative but 2/4 SNBTS prothrombin complex concentrates (DEFIX) were positive for HCV RNA.

HCV genotype analysis

Initial RFLP analysis of the HCV RNA detected in the commercial FVIII concentrates yielded a mixed genotype pattern in 16/27 concentrates, with genotype 1 in 10/27 and genotype 2 in 1/27. Detailed single molecule analysis was performed in 17 concentrates. HCV genotyping was successfully performed in 115 single molecules (Table 7.1). Multiple HCV genotypes were detected in 8/17 concentrates and in a further 8/17 only genotype 1 was detected. Analysis of the remaining concentrate yielded no single molecules. No single molecules were isolated from either of the SNBTS DEFIX concentrates positive for HCV RNA.

Analysis of SNBTS batches implicated in HCV transmission

The SNBTS batch (Z8 340150040) which was implicated in HCV transmission was negative for HCV RNA. Therefore, no genotype analysis was possible.

Table 7.1 **HCV genotypes present in single molecules of HCV RNA isolated from commercial coagulation factor concentrates.**

The table lists the HCV genotypes detected in the 17 commercial concentrates examined. The concentrates were identified by number if derived from the research stock or original batch number if derived direct from the manufactured.

Concentrate	Single Molecules (n)	HCV Genotypes 1	2	3	4	5
FVIII 6	20	13	4	3		
FVIII 8	4	4				
FVIII 13	14	14				
FVIII 16	0					
F VIII 25	15	12	1	3		
FVIII 43	2	2				
FVIII 44	1	1				
V17609	10	5	4			1
V13408	8	3	2	3		
U13408	9	8	1			
800717A007B	1	1				
09M06181	7	6	1			
09MO7676	3	3				
AH 8013A	7	4	2	1		
NC8325	8	4	1	3		
05A0881	1	1				
05A1881	4	4				
Total	**115**	**85**	**16**	**13**	**0**	**1**
%	**100**	**73.9**	**13.9**	**11.3**	**0**	**0.9**

Comparison of HCV genotypes in concentrates, blood donors and haemophiliacs.

Following the identification of the HCV genotype distribution in commercial concentrates, a comparison was made with the published genotype distribution in North American blood donors and UK haemophiliacs who had been treated with such commercial products (table 7.2).

Table 7.2 Relative proportion of HCV genotypes detected in commercial concentrates compared with North American blood donor and English haemophilia populations.

	HCV Genotype (%)				
	1	**2**	**3**	**4**	**5**
Commercial concentrates	73.9	13.9	11.3	-	0.9
English haemophiliacs[1]	63	10	18	4	1
US blood donors[2]	76	17	5	-	2

[1] Preston *et al* 1995c; [2] Davidson *et al*, 1995

Discussion

This study confirms that HCV RNA is detectable in the majority of commercial coagulation factor concentrates manufactured prior to the introduction of virus inactivation methods. In addition, using single molecule analysis it is clear that multiple HCV genotypes may be present in a single batch of concentrate. Thus, those haemophiliacs who received regular infusions will have been exposed to multiple HCV genotypes on numerous occasions.

Longitudinal studies have shown that the major circulating HCV genotype found in serum may change over time in individual haemophiliacs (Jarvis *et al*, 1994). This may be due to

superinfection with a different genotype as a result of the lack of protective immunity conferred from previous exposure to HCV (Lai *et al*, 1994). In addition, the major circulating HCV genotype was found to change in haemophiliacs even in the years following the introduction of virus-inactivated concentrates in the mid-1980s (Jarvis *et al*, 1994). This suggests that haemophiliacs are infected with multiple HCV genotypes which may circulate at low levels in the blood or emerge from tissue reservoirs to become the major circulating genotype. These observations suggested the possibility that in a population of multiply transfused haemophiliacs, particular HCV genotype(s) with a greater ability to establish chronic infection may become relatively over-represented over time. In fact, there is little evidence that this is the case as other studies comparing HCV genotype distribution in haemophiliacs and the blood donor population from which concentrates were derived which have shown a remarkably consistent relative proportion of genotypes. Comparison between the Edinburgh haemophiliacs studied in chapter 3 who have been treated exclusively with Scottish plasma showed a similar genotype distribution to Scottish blood donors (Jarvis *et al*, 1996b).

The current study is in broad agreement with the conclusion that HCV genotypes are equally likely to establish chronic infection in haemophiliacs as the relative proportion of each genotype detected in the concentrates is similar to those present in North American blood donors from whom plasma for commercial concentrates was obtained as well as the English haemophiliac recipients. The only exception may be genotype 3 which is relatively over-represented in UK haemophiliacs (18%) compared to concentrates (11%) and North American blood donors (5%) from whom the source plasma was derived (however these differences are not statistically significant). There is no comprehensive survey of HCV genotypes in North American Blood donors from different geographical areas. The study quoted is derived from blood donors in Pittsburgh which may not be representative of the entire country. However

studies of patients from North America with chronic HCV confirm that genotype 1 (75-90%) predominates with type 2 (10-15%) in a minority and types 3 (5%) and 4 (1%) in a small proportion of individuals (Mahaney *et al*, 1994; Lau *et al*, 1995; Zein *et al*, 1996).

It has been suggested that the detection of particular HCV genotypes in the recipients of commercial concentrates may identify the geographical origin of plasma used to manufacture concentrate (Preston *et al*, 1995c; Hormann, 1995). "Imported" HCV genotypes have been found in Japanese haemophiliacs (Kinoshita *et al*, 1993). Although the geographical distribution of HCV genotypes is relatively well defined there are likely to be low levels of unusual genotypes in migrant or traveled populations. We found evidence of HCV genotype 5 in one commercial concentrate. This particular HCV genotype is geographically restricted to Southern Africa. There is clearly no reason why the plasma donor infected with genotype 5 may not have either traveled to or moved from Southern Africa and subsequently donated blood in North America. Genotype 5 has been found in studies of Canadian blood donors (Murphy *et al*, 1996). Thus, genotyping studies are unlikely to yield useful information concerning the geographical location of source plasma used in the manufacture of commercial concentrates.

It is important to note that HCV RNA was not detected in some batches of both commercial and SNBTS concentrate. In particular HCV RNA was not detected in any SNBTS FVIII concentrates even those clearly responsible for HCV transmission. This contrasts with other studies which have demonstrated a correlation between the detection of HCV RNA by PCR and infectivity (Garson *et al*, 1990; Makris *et al*, 1993). There are several possible explanations for the absence of HCV RNA in the SNBTS FVIII batches tested:

1. Variable distribution of HCV RNA between the bottles in any particular batch.
2. HCV RNA may decay over time during storage of concentrate.
3. HCV RNA is present but below the threshold of detection by PCR method.

4. PCR method was optimised for the amplification of HCV RNA derived from serum. Different reaction conditions may be required to amplify HCV RNA derived from concentrates.
5. Successful HCV RNA extraction may have been inhibited by excess proteins/nucleases present in the concentrates.
6. In those concentrates subjected to early virus inactivation methods, HCV RNA titre may be reduced below level of detection by PCR but not sufficient to prevent transmission.

It is difficult to be certain which are the most important reasons for the lack of detectable HCV RNA in these batches. I would speculate that an original low level of HCV RNA has decayed during storage and fallen below the level of detection by even a sesitive PCR method. The use of PCR to test coagulation factor concentrates to detect potentially infectious virus contamination prior to release for clinical use has been suggested ("end-product testing"). PCR used in this way will act as a final safety check to ensure adequate virus inactivation which may fail either as a result of an intrinsic failure of the inactivation procedure or a breakdown of manufacturing practice. There are potential problems with this approach which may lead to either false-negative or false-positive results. If the PCR method is not sensitive enough end-product testing may fail to detect potentially infectious virus (Hilfenhaus, 1996). Equally it is clear that PCR positivity may merely detect non-infectious fragments of HCV RNA and such batches may be disgaurded unnecessarily (Berntorp *et al*, 1995). The results of the current study suggest that even using an extremely sensitive PCR method for the detection of HCV RNA, that "PCR negative" batches may still transmit HCV.

The continued virus safety of plasma-derived coagulation factor relies on the effective exclusion of infectious donors (by identifying high-risk donors as well as screening for markers of virus infection) and the use of validated methods of virus inactivation. There may

be a role for PCR screening of plasma pools but the use of PCR for end-product testing appears limited.

In summary, this study has demonstrated that multiple HCV genotypes were present in coagulation concentrates prior to the introduction of virus inactivation procedures. The relative distribution of HCV genotypes detected in concentrates was broadly similar to those present both in the donor and recipient populations. This adds support to the view that there is no major pathogenic differences between HCV genotypes.

Chapter 8

Patterns of hepatitis G viraemia and liver disease in haemophiliacs

Introduction

Chronic liver disease has emerged as a serious problem in haemophiliacs who received treatment with coagulation factor concentrates prior to the widespread introduction of virus inactivation procedures in the mid-1980s. HCV has proved to be the agent responsible for almost all chronic liver disease in the haemophiliac population (Tedder *et al*, 1991; Watson *et al*, 1992). All individuals who received such concentrates are anti-HCV antibody positive and the majority have persistent HCV viraemia as assessed by the detection of HCV RNA in serum using PCR. There is a wide spectrum of liver disease present amongst haemophiliacs ranging from mild chronic hepatitis to established cirrhosis (Ahmed *et al*, 1996; Makris *et al*, 1996)

There has been considerable interest in the role of other bloodbourne viruses which may also be contributing to chronic liver disease in haemophiliacs. Recently a novel flavivirus (GB virus-C (GBV-C) (Simons *et al*, 1995; Leary *et al*, 1996) or hepatitis G virus (HGV) (Linnen *et al*, 1996) has been implicated as a cause of post-transfusion hepatitis. The genomes of GBV-C and HGV show substantial similarity suggesting that they are, in fact, closely related variants of the same virus (referred to as HGV in the current study).

HGV RNA is readily detected in coagulation factor concentrates manufactured prior to the use of virus inactivation methods (Jarvis *et al*, 1996), so individuals who received such concentrates will have been exposed to HGV, often on multiple occasions. We have performed a longitudinal study to assess patterns of HGV viraemia in haemophiliacs and to investigate the severity of liver disease present in individuals with and without HGV viraemia.

Methods

Patient characteristics

Serum samples from the mid 1980s (stored at -70°C) were available in 68 individuals with haemophilia A, haemophilia B and von Willebrands disease. The samples were from a long-term serum store and represented an unselected group of patients who had received non-virus inactivated coagulation factor concentrates in the past. The median age of the group at the time of sampling was 23.5 years (range 2-64 years). From clinical records the date of initial exposure to concentrate was documented in all cases. All 68 patients were anti-HCV positive by second-generation enzyme immunoassay (A-EIA; Abbott, Weisbaden-Dalkenheim, Germany) and also positive on confirmatory testing by recombinant immunoblot assay (RIBA-2, Chiron Corporation, Emeryville, CA). HCV RNA was detected by PCR as previously described (Jarvis *et al*, 1994) in 58/68 with 10/68 persistently negative for HCV RNA. Co-infection with HIV was present in 16/68. Only 1 individual had evidence of on-going hepatitis B infection (HBsAg positive).

Detection of HGV RNA

RNA was extracted from 0.1ml of stored serum followed by reverse transcription. HGV RNA was detected by PCR using nested primers designed from the 5' non-coding region as described in chapter 2. In those in whom HGV RNA was detected, further samples from multiple timepoints in the 1980s and 1990s were tested in order to assess patterns of viraemia over time. Non-viraemic patients were assessed at two timepoints.

Assessment of liver disease

Evidence of liver disease in association with HGV viraemia was sought. In those individuals who cleared HGV (n=7), median ALT levels before and after clearance were compared. In addition, the results of laparoscopic liver inspection were available in 26 patients (liver biopsy was performed in 18/26). These investigations were performed in 1993/94 as part of the study of chronic liver disease in haemophiliacs described in chapter 3. Laparoscopic liver inspection has been shown to be a reliable method for staging liver disease (chapter 3) and information derived from these investigations enabled classification of liver disease into three categories (chronic hepatitis without fibrosis, hepatic fibrosis without cirrhosis or established cirrhosis).

Statistical methods

χ^2 tests or Fisher's exact tests were used to test for associations between categorical variables. χ^2 test for trend was used to test for a relationship between severity of haemophilia and HGV viraemia. When estimating the duration of HGV viraemia it was assumed that patients became viraemic on their first exposure to coagulation factor concentrate, except for one patient who was viraemic prior to first treatment with concentrate. He had received a blood transfusion in 1978 which was taken as the date of exposure to HGV. Several survival distributions were fitted to the duration of HGV viraemia data from all 68 patients including the Weibull, log-normal and log-logistic distributions. The Wilcoxon Signed Rank test was used to compare the median of the annual mean ALT value before and after HGV clearance in the those who cleared HGV RNA.

Results

HGV viraemia in the mid-1980s

HGV RNA was detected in 17/68 (25%) samples from the mid-1980s (table 8.1). The relationship between HGV viraemia in the mid-1980s and HCV or HIV infection is shown in tables 8.2 and 8.3. Of the 17 individuals with HGV viraemia, 15 were co-infected with HCV (including 3 patients who were HIV positive). There was no association between HIV infection and HGV viraemia (Fisher's exact test, p=0.74). There was also no association between HGV viraemia and HCV genotype (Fisher's exact test, p=0.62). There was, however, an association between severity of haemophilia and HGV viraemia. HGV RNA was found in only 3/33 severe compared to 9/16 mild and 5/19 moderate haemophiliacs (χ^2 test for trend, p=0.0004).

Table 8.1 Patient characteristics and HGV viraemia in the mid-1980s.

			HGV RNA	
		n	Pos	Neg
Haemophilia A	Mild	6	3	3
	Moderate	13	5	8
	Severe	30	3	27
Haemophilia B	Mild	7	5	2
	Moderate	6	0	6
	Severe	2	0	2
von Willebrands Disease		4	1	3
Total		**68**	**17**	**51**

Table 8.2 **Patterns of HGV and HCV viraemia.**
There was no association between HGV and HCV viraemia (Fisher's exact test p=1.0).

		HGV RNA		
		Pos	**Neg**	**Total**
HCV RNA	**Pos**	15	43	**58**
	Neg	2	8	**10**
	Total	**17**	**51**	**68**

Table 8.3 **HGV viraemia in the mid 1980s and HIV infection.**
There was no association between HIV infection and HGV viraemia (Fisher's exact test, p=0.74).

		HGV RNA		
		Pos	**Neg**	**Total**
Anti-HIV	**Pos**	3	12	**15**
	Neg	14	39	**53**
	Total	**17**	**51**	**68**

Patterns of HGV viraemia and duration since first exposure to coagulation factor concentrates

A summary of the relationship between first exposure to coagulation factor concentrates and subsequent patterns of HGV viraemia in the study group is shown in figure 8.1. Stored serum samples were tested from multiple timepoints in the 1980s and 1990s in 15/17 viraemic individuals. Samples were not available in 2/17 patients (both died of AIDS in 1987).

Persistent viraemia was detected in 8/15 and in the remaining 7, HGV RNA became undetectable over a variable period of time. There was no significant difference (t-test, p=0.18) in age between those patients in whom HGV viraemia persisted (mean 16.6 years, se 3.37) and those who cleared HGV RNA (mean 26.3 years, se 6.71).

The Weibull model provided the best fit to the duration of HGV viraemia data from all 68 patients in the study. The proportion of patients with persistent viraemia is plotted against time (Figure 8.2).

Legends to figures 8.1 and 8.2 on subsequent pages

Figure 8.1 Patterns of HGV viraemia in relation to first exposure to coagulation concentrate in the 68 study patients.

The diagnosis (A: haemophilia A; B: haemophilia B; V: von Willebrands disease), disease severity (Sev: severe; Mod: moderate and Mild) and HIV status (+ or -) is shown for each individual. The start of each line (|) is the date of first exposure to coagulation factor concentrate. The circles represent serum samples tested for HGV RNA (positive ●; negative O). Patients who died during the study period are designated †. HGV RNA was detected in patient 1 prior to first treatment with concentrate (see figure 8.3).

Figure 8.2 Weibull model of duration of HGV viraemia.

There were few samples available from most patients in the first years following exposure to coagulation factor concentrates; this part of the curve (– – –) was therefore extrapolated from the rest of the model. This model estimates the median duration of HGV viraemia to be 2.2 years. The 75th and 90th percentiles for the duration of HGV viraemia were estimated to be 8.7 years (95% confidence interval 4.8-15.7) and 23.6 years (95% confidence interval 11.8-47.1) respectively.

Figure 8.1

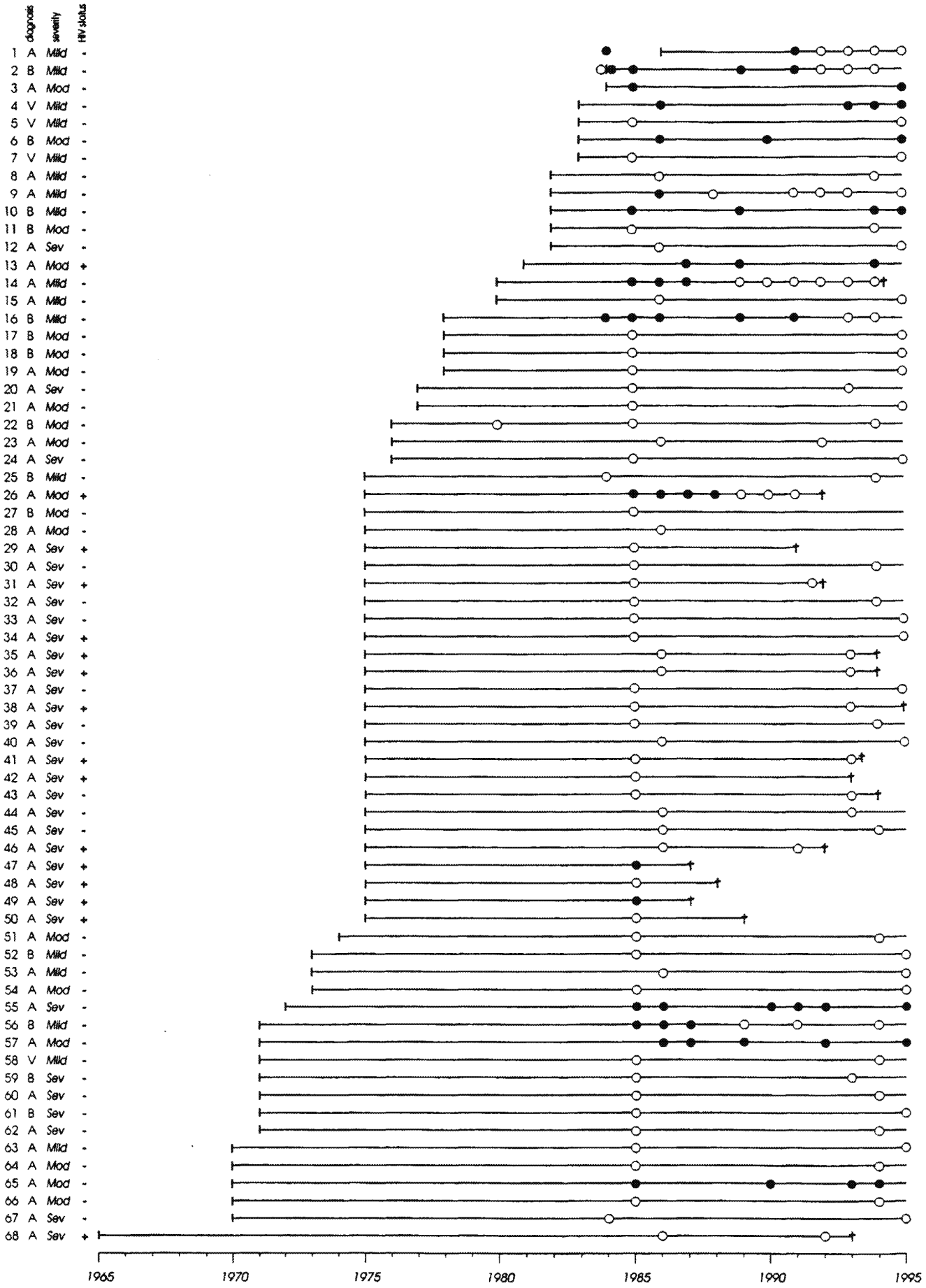
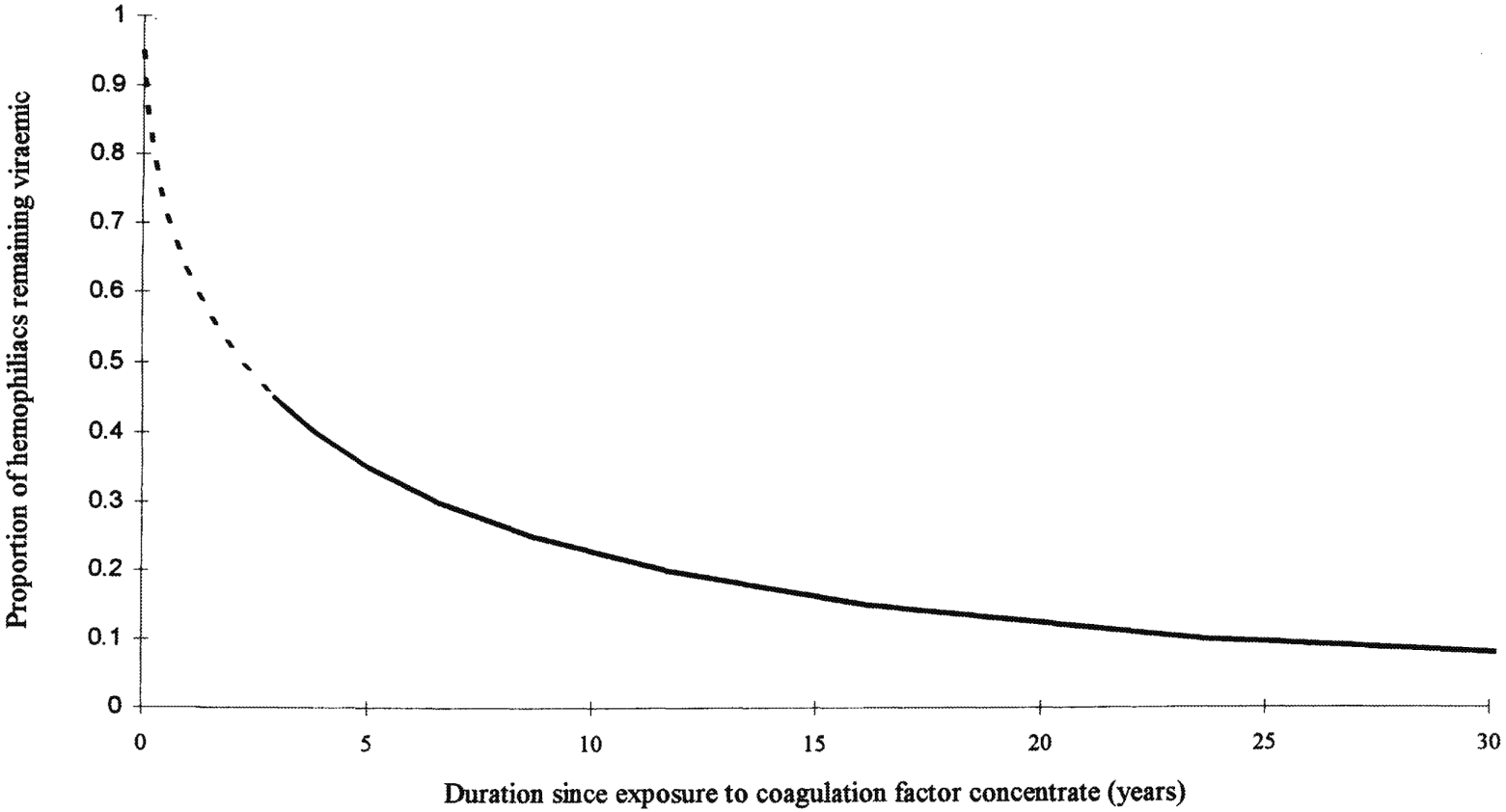

diagnosis
severity
HIV status
1 A Mild -
2 B Mild -
3 A Mod -
4 V Mild -
5 V Mild -
6 B Mod -
7 V Mild -
8 A Mild -
9 A Mild -
10 B Mild -
11 B Mod -
12 A Sev -
13 A Mod +
14 A Mild -
15 A Mild -
16 B Mild -
17 B Mod -
18 B Mod -
19 A Mod -
20 A Sev -
21 A Mod -
22 B Mod -
23 A Mod -
24 A Sev -
25 B Mild -
26 A Mod +
27 B Mod -
28 A Mod -
29 A Sev +
30 A Sev -
31 A Sev +
32 A Sev -
33 A Sev -
34 A Sev +
35 A Sev +
36 A Sev +
37 A Sev -
38 A Sev +
39 A Sev -
40 A Sev -
41 A Sev +
42 A Sev +
43 A Sev -
44 A Sev -
45 A Sev -
46 A Sev +
47 A Sev +
48 A Sev +
49 A Sev +
50 A Sev +
51 A Mod -
52 B Mild -
53 A Mild -
54 A Mod -
55 A Sev -
56 B Mild -
57 A Mod -
58 V Mild -
59 B Sev -
60 A Sev -
61 B Sev -
62 A Sev -
63 A Mild -
64 A Mod -
65 A Mod -
66 A Mod -
67 A Sev -
68 A Sev +
1965
1970
1975
1980
1985
1990
1995

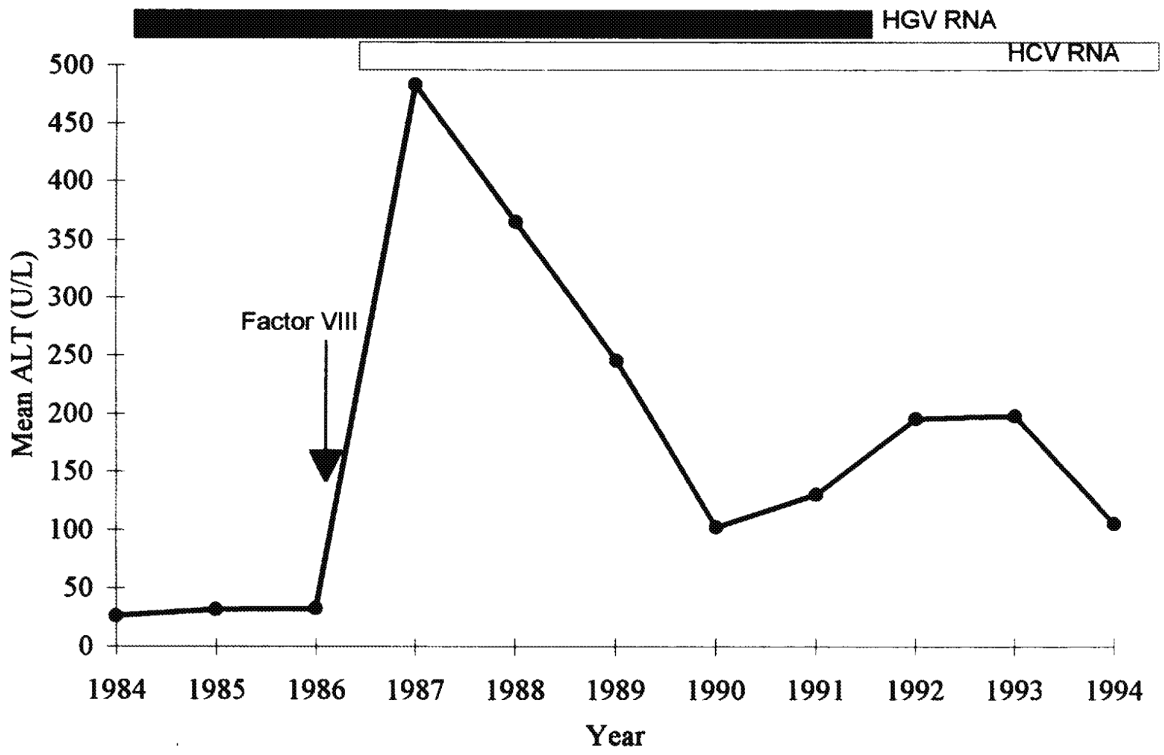
Figure 8.2


Proportion of hemophiliacs remaining viraemic
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
Duration since exposure to coagulation factor concentrate (years)

HGV viraemia and severity of liver disease

Evidence for an association between HGV and liver disease was sought. In the 7 individuals who cleared HGV RNA the median of the annual mean ALT was significantly higher in the years prior to clearance of HGV RNA (Wilcoxon Signed Rank test, p=0.02). However, this analysis included one individual (patient 1, figure 8.1) who had HGV viraemia prior to infusion of concentrate (he had received a blood transfusion in 1978). Serum ALT was normal prior to 1986 when he acquired HCV infection (Figure 8.3).

Figure 8.3 **ALT levels and HGV/HCV viraemia in patient 1, figure 1.**
HGV RNA was detected prior to first infusion of FVIII in early 1986 (he received a blood transfusion in 1978 when he may have acquired HGV). Serum ALT first became elevated when he acquired HCV infection in 1986. HGV RNA cleared spontaneously in 1991.



Serum ALT was persistently elevated in one of the two individuals with HGV viraemia without concurrent HCV infection. Chronic hepatitis without fibrosis was present on liver biopsy in this patient.

ALT levels were persistently normal in 6/8 who were negative for both HGV and HCV RNA The ALT elevation in the remaining two was due to HBV infection in one (HBsAg positive) and drug induced (imipramine) in the other.

Laparoscopic liver inspection and liver biopsy was performed in 25/68 patients (4 with persistent HGV viraemia; 4 with previous HGV viraemia and 17 non-viraemic). There was no association between severity of liver disease and either previous or persistent HGV viraemia (Fisher's exact test, p=0.43) with chronic hepatitis without fibrosis present in the 9/9 individuals with either persistent or previous HGV viraemia. In contrast, 5/16 with no associated HGV viraemia had established cirrhosis (Table 8.4).

Table 8.4 **Relationship between patterns of HGV viraemia and severity of liver disease.**
There was no association between severity of liver disease and either previous or persistent HGV viraemia (Fisher's exact test, p=0.43)

		Severity of liver disease assessed by liver laparoscopy or biopsy		
	n	Chronic hepatitis	Hepatic fibrosis	Established cirrhosis
Persistent HGV viraemia	4	4	0	0
Previous HGV viraemia	5	5	0	0
Absent HGV viraemia	17	11	1	5

Discussion

This study provides further data concerning the natural history of HGV infection. We have shown that 25% of haemophiliacs previously treated with non-virus inactivated concentrates had detectable serum HGV RNA in the mid-1980s falling to 12% by the mid-1990s. This compares to recent studies which have shown the recent prevalence of HGV viraemia in haemophiliacs to be 18% (Linnen *et al*, 1996) and 14% (Jarvis *et al*, 1996) and 6% (Tagariello *et al*, 1996). The differences in prevalence of HGV found in these studies may either reflect in the sensitivity of PCR primers used or geographical variation.

As HGV RNA is readily detected in non-virus inactivated coagulation factor concentrates (Jarvis *et al*, 1996) we have assumed that all 68 haemophiliacs in this study were exposed to HGV and became viraemic on first treatment with such concentrate. As serum samples were available only from the mid-1980s onwards, when 25% had detectable HGV RNA, the remaining 75% had already successfully cleared HGV presumably due to the development of neutralizing antibody and subsequent protective immunity. In view of the lack of available samples prior to the 1980s , the precise duration of HGV viraemia in the 75% non-viraemic by the mid-1980s is uncertain. Serological tests for anti-HGV antibody have now been described (Tacke *et al*, 1997) and a recent study of haemophiliacs found evidence of previous exposure to HGV in 79% (61% anti-HGV positive and 18% with persistent HGV viraemia) (Karayiannis *et al*, 1997) which supports our assumption that the majority of haemophiliacs have been previously exposed to HGV. With the development of more sensitive anti-HGV antibody assays it is possible that evidence for past exposure to HGV in the recipients of non-virus inactivated coagulation factor concentrates will approach 100%.

We have constructed a Weibull model to estimate the duration of HGV viraemia in the entire group. The resulting plot of proportion of patients with persisting viraemia over time was extrapolated to include the first few years post-exposure to coagulation factor concentrate

when there were few samples available for analysis, therefore the estimates may be unreliable over this time. Despite the possible limitations of this model, it suggests that the median duration of HGV viraemia in this cohort of haemophiliacs is approximately 2.2 years. From our data it appears that most haemophiliacs clear HGV RNA after a relatively short period of time (2-3 years) but in a significant number viraemia may persist for many years as observed in studies of non-haemophiliacs (Wang *et al*, 1996). It seems likely that a proportion have persistent viraemia and probably represent chronic carriers of HGV.

The factors which determine successful clearance of HGV remain obscure. We found no relationship between HIV infection or co-infection with a particular HCV genotype and persistent HGV viraemia. There was, however, an association between severity of haemophilia and HGV viraemia with non-severe haemophiliacs more commonly infected with HGV. This observation is difficult to explain and needs to be studied further in other cohorts of haemophiliacs. A highly speculatuive reason may relate to less repetative exposure to HGV being less likely to be associated with an effective immune response and successful virus clearance.

In addition, we have attempted to address the question as to whether HGV is a significant cause of chronic liver disease in haemophiliacs by analysis of ALT levels as well as invasive investigations. As HCV co-infection is common in this group it may be difficult to distinguish between HCV-related hepatitis from liver disease due to HGV. The group of individuals who cleared HGV RNA did have significantly higher ALT levels prior to clearance of HGV. This analysis, however, included episodes of acute HCV infection with markedly elevated ALT levels which may have influenced the results. In one patient, for example, HGV viraemia was present with normal ALT prior to first infusion of FVIII. ALT elevation occurred only following co-infection with HCV. In contrast there was only one individual with persistent HGV viraemia without HCV co-infection in whom serum ALT was elevated (chronic

hepatitis was present on liver biopsy). This was the only individual in the entire group in whom HGV was implicated as the sole possible cause of chronic liver disease in the entire group. Transaminitis alone, however, is not a true indicator of the severity of liver disease. It is well known that there is a poor correlation between histology of liver biopsy and ALT level. We therefore assessed the relationship between HGV and severity of liver disease by laparoscopic liver inspection and liver biopsy and we found no association between more severe liver disease and HGV viraemia. We conclude that there is little evidence to implicate HGV as a major cause of chronic liver disease in haemophiliacs. The interaction between HGV and HCV needs further study. Individuals with persisting HGV viraemia require careful follow-up to further establish the natural history of HGV infection as well as monitoring for any progression of liver disease or the emergence of any other diseases which may be caused by HGV. Further studies of non-haemophiliacs (e.g. blood donors/recipients) who have HGV viraemia in isolation are required to establish whether or not it is, in fact, a definite cause of chronic liver disease.

Chapter 9

Discussion

Introduction

There have been enormous benefits of modern treatment with coagulation factor concentrates for individuals with haemophilia. Previously haemophiliacs suffered chronic morbidity and high age-related mortality. The availability of coagulation factor concentrates to treat bleeding episodes promptly and effectively has not only reduced mortality due to serious bleeding but has also enabled haemophiliacs to lead increasingly normal lives. Unfortunately, all these benefits have been overshadowed by the consequences of transfusion transmitted viruses.

The HIV epidemic in the 1980s affected haemophiliacs in a dramatic and devastating manner with a sharp increase in mortality (Darby *et al*, 1995). In contrast the full impact of HCV related chronic liver disease is only slowly becoming more clear after many haemophiliacs have been infected for some time. The reasons for this are partly related to the slowly progressive natural history of HCV infection and the relatively asymptomatic nature of both acute and chronic infection. In addition HCV was only identified as the agent responsible for the majority of chronic liver disease in haemophiliacs a few years ago and as a result the tools with which to study HCV have only been available for a short period of time.

Natural history of chronic HCV infection in haemophiliacs

Since the identification of HCV in 1989 there has been a rapid increase in information concerning its molecular biology, transmission, epidemiology and diagnosis. However the precise natural history of chronic HCV infection remains unclear. There is considerable evidence that HCV causes slowly progressive liver disease leading to cirrhosis in a proportion of individuals. However it remains uncertain if progression is an inevitable consequence of HCV infection or whether some patients have infection which is truly non-progressive and benign.

Studies of those who acquired HCV infection following blood transfusion have provided contrasting information concerning the natural history of the disease. These studies provide important data as the date of initial infection is usually well documented. In a retrospective analysis of 131 patients who developed post-transfusion hepatitis due to HCV, cirrhosis and HCC developed in 60 and 14 patients after a mean interval of 20.6 and 28.3 years respectively (Tong *et al*, 1995). However a prospective study of patients who acquired HCV following blood transfusion found no excess in overall mortality compared to controls after an average follow-up of 18 years. There was a small increase in deaths due to liver disease (Seeff *et al*, 1992). It is possible that the proportion of individuals with cirrhosis and HCC will increase in prospective studies as the duration of follow-up lengthens. A recent study of disease progression in those with established cirrhosis due to HCV suggests that the development of hepatic decompensation, HCC and death occurs gradually with 79% still alive at 10 years (Fattovich *et al*, 1997).

The factors which determine the rate of disease progression are becoming more well defined. These include older age at the time of initial infection, duration of infection, heavy alcohol intake, HCV genotype (more rapid with genotype 1b), HIV co-infection and possibly high levels of HCV RNA (see chapter 1). We have confirmed that HIV co-infection is associated with more severe liver disease in haemophiliacs along with several other groups (Eyster *et al* 1994; Telfer *et al*, 1994b). This is thought to be related to increased HCV replication as HIV-related immunosuppression progresses.

Several studies concerning the natural history of HCV infection in haemophiliacs have been performed. Recent reports in UK haemophiliacs have found that a significant number have already developed serious liver disease or died of hepatic failure (Telfer *et al*, 1994; Makris *et al*, 1996). In the largest study of 128 patients (median age 38 years) after a duration of 22 years since first exposure to coagulation concentrates 19% had established cirrhosis and 9%

had developed liver failure (Makris *et al*, 1996). Liver histology was available in 63 patients and was abnormal in every case. Both advanced age at time of initial infection and increased duration of infection were associated with more severe liver disease. In contrast other liver biopsy studies of UK haemophiliacs have found a much lower incidence of cirrhosis. One centre reported 35 biopsies in a cohort of 55 haemophiliacs (median age 33; median duration of infection 20 years). In seven cases the liver histology was considered entirely normal and there were no individuals with established cirrhosis (Wong *et al*, 1997). Another report found established cirrhosis in only two of 50 liver biopsies haemophiliacs (median duration of infection 16 years) (Ahmed *et al*, 1996). It is possible that the surprisingly low rates of cirrhosis reported in these studies is a reflection of the relatively young age of those biopsied and a somewhat shorter duration of infection. The findings in this thesis are more in keeping with the data from Makris *et al* with 25% of Edinburgh haemophiliacs with evidence of cirrhosis after a duration of infection between 10 and 25 years. Studies of mortality in UK haemophiliacs also strongly suggest that deaths due to liver disease are increasing even in HIV negative haemophiliacs (Darby *et al*, 1995). The next decade will reveal the true natural history of HCV infection in haemophiliacs.

The significance of HCV genotypes in haemophiliacs

There has been considerable debate as to whether the natural history of HCV infection is influenced by HCV genotype. There is evidence that genotype 1 is associated with more severe liver disease and genotypes 3a and 2b respond more readily to IFN therapy (Pozzato *et al*, 1995; chapter 6). Although many studies have identified genotype as an independent prognostic variable, it may be difficult to separate the influence of genotype from other factors such as duration of infection (Pol *et al*, 1995). The relationship between genotype and HCV RNA titre is influenced by assay method used for virus quantitation (Hawkins *et al*, 1997).

We have confirmed that the distribution of HCV genotypes in haemophiliacs reflects the genotype distribution in the coagulation factor concentrates used in their treatment (chapter 7). There is no over-representation of a particular genotype in haemophiliacs suggesting there are no major pathogenic differences between genotypes. This suggests that there are few intrinsic pathogenic differences between HCV genotypes.

Assessment of chronic liver disease in haemophiliacs using invasive methods

There has been an understandable reluctance to perform invasive procedures such as liver biopsy in haemophiliacs due to the perceived risk of bleeding complications. However with modern treatment methods the risk of liver biopsy in haemophiliacs does not appear to be greater compared with non-haemophiliacs. In excess of 400 liver biopsies in haemophiliacs have been reported in the literature since the first in 1977 (Lesesne *et al* 1977; Spero *et al* 1978; Mannucci *et al* 1978; Bamber *et al*, 1981; White *et al* 1982; Mannucci *et al* 1982; Stevens *et al*, 1983; Hay *et al* 1985; Aledort *et al* 1985; Schimpf, 1986; Miller *et al* 1988; Makris *et al* 1991; Hanley *et al*, 1996a; Ahmed *et al*, 1996; Wong *et al*, 1997). The only fatalities associated with liver biopsy were referred to in a study of liver disease in haemophiliacs (Aledort *et al*, 1985). More recently one of these deaths was reported in more detail (Lee 1997). In this case, at laparotomy, bleeding was found to be due to a torn capsule and despite intensive blood product support, hepatic artery ligation and subsequent right hemihepatectomy, bleeding continued and the patient died 8 days post-biopsy. The report illustrates that there are occasional fatal complications of liver biopsy. However this applies to haemophiliacs and non-haemophiliacs alike as serious bleeding following liver biopsy may occur in individuals without demonstrable abnormalities of the coagulation system (McVoy & Toy, 1990). Following appropriate coagulation factor replacement the haemostatic defect in

haemophilia is effectively corrected and there is no reason why liver biopsy is intrinsically more hazardous in haemophiliacs.

Contemporary role of liver biopsy in haemophiliacs

Despite the safety of liver biopsy in haemophiliacs there is a need to ensure that the indications for undertaking invasive investigations are kept under review. In the past liver biopsy studies in haemophiliacs were important to demonstrate the progressive nature of chronic non-A, non-B hepatitis (Hay *et al*, 1985). As HCV is now known to be responsible for almost all chronic liver disease in haemophiliacs and reliable non-invasive diagnostic tests are available to demonstrate chronic infection e.g. detection HCV RNA by PCR, liver biopsy is seldom required for diagnostic purposes. In fact, liver biopsy is often of limited diagnostic value in the assessment of liver disease in general as even in non-haemophiliacs most causes of chronic liver disease are identified by non-invasive tests prior to biopsy (Gilmore *et al*, 1995).

Currently the main indication for liver biopsy in haemophiliacs with chronic HCV infection is to accurately stage the disease in order to identify those with significant hepatic fibrosis or cirrhosis who are at particular risk of hepatocellular carcinoma, variceal bleeding or hepatic decompensation. This information is important in order to counsel the individual as to the severity of their liver disease and appropriate long-term follow up.

As far as further improvements to the safety of liver biopsy are concerned, there is some evidence that contemporary techniques may be associated with fewer complications than previously. The use of ultrasound guidance (Caturelli *et al*, 1996), transjugular approach (McAfee *et al*, 1992) or laparoscopic biopsy methods are associated with fewer bleeding complications than the traditional blind biopsy approach. Laparoscopic techniques also have the advantage of direct visualisation of the liver surface which may actually reduce the need for liver biopsy. We have found that laparoscopic liver inspection is a safe and effective method

for the assessment of chronic liver disease in haemophiliacs (chapter 4) which has also been confirmed in larger studies in non-haemophiliacs (Herrera *et al*, 1989; Jalan *et al*, 1995). The information derived from histological examination of liver biopsy specimens did not improve the laparoscopic assessment. Although liver laparoscopy is still an invasive technique the risk of serious complications are less than liver biopsy (Orlando *et al*, 1990). In our experience it a well tolerated procedure in haemophiliacs and in conjunction with information derived from upper GI endoscopy and ultrasound provides a reliable method of staging liver disease. In the UK laparoscopic liver inspection and laparoscopic liver biopsy are not commonly used techniques. It has been advocated that training in liver laparoscopy should be encouraged so this method may become more widely available in the future (Haydon & Hayes, 1997).

Long-term follow-up of haemophiliacs with chronic HCV infection

From accumulating data concerning the natural history of HCV infection discussed above it appears likely that a growing number of haemophiliacs are likely to develop cirrhosis. In the absence of more effective therapy (see below) the most important aspect of their clinical management is long-term follow-up to identify those who develop cirrhosis, hepatic decompensation or HCC. The UK Haemophilia Centre Directors Organisation have recently produced guidelines which recommend a follow-up strategy for haemophiliacs with HCV. These include four monthly clinical assessments and liver function tests; upper GI endoscopy to detect oesophageal varices every 5 years and liver ultrasound and alpha fetoprotein levels every at 4-monthly intervals in those with cirrhosis (Preston *et al*, 1995c).

In non-haemophiliacs with chronic HCV infection periodic liver biopsy is often performed to assess disease progression. Although in general we argue that haemophiliacs should be managed in the same manner as non-haemophiliacs, there is a significant cost associated with

performing repeated biopsies in haemophiliacs and there may be a cumulative morbidity and mortality.

A non-invasive method of monitoring disease progression would be particularly valuable in haemophiliacs. Many serum markers of hepatic fibrosis as well as non-invasive imaging techniques have been found to be poor predictors of liver histology. However several studies have found that serum hyaluronic acid is a potentially useful test to distinguish cirrhotics from non-cirrhotics. We have found that serum hyaluronic acid is a useful screening test to identify individuals with cirrhosis (chapter 4). We have incorporated serial measurement of hyaluronic acid into the follow-up of haemophiliacs with HCV to examine its usefulness in a prospective study.

HCC screening in haemophiliacs

The role of screening programmes to detect HCC remains controversial. HCC is a potentially treatable condition if detected early and screening at-risk groups have been advocated. Cirrhosis is the major risk factor for the development of HCC and it is common practice to screen individuals with established cirrhosis of any aetiology. It has been suggested that HCC screening should be extended as it may develop even in the absence of cirrhosis in patients infected with HCV (De Mitri *et al*, 1995; El-Refaie *et al*, 1996). An additional factor in selecting haemophiliacs for screening is that staging investigations may be contraindicated or declined so the presence or absence of cirrhosis is uncertain in some individuals.

The most widely used HCC screening tests are hepatic ultrasound scans and serial measurement of serum AFP. Unfortunately the sensitivity and specificity of ultrasound or AFP are not high although improved when used together (Oka *et al*, 1994). In non-haemophiliacs such screening has not definitely improved overall survival in HCC patients. The only prospective evaluation of HCC screening in haemophiliacs recently showed that annual

ultrasound scans were too infrequent to effectively detect treatable HCC (Tradati *et al*, 1997). Scans at least every six months and possibly every four months are required. There is a need for further prospective studies to define the precise role of HCC screening in haemophiliacs. The current policy in Edinburgh is to offer screening for HCC (four monthly ultrasound and AFP) to all patients aged over 40 years; all known to be cirrhotic and all in whom staging investigations are contraindicated or have been declined.

Therapy of chronic HCV infection in haemophiliacs

Current role of IFN treatment in haemophiliacs

IFN is the only therapy which has been widely used for the treatment of chronic HCV infection. Studies of IFN treatment in haemophiliacs initially suggested that the response rates were similar to those seen in other HCV infected groups (Makris *et al*, 1991). However our results suggest that sustained responses to six months therapy with IFN (3MU thrice weekly) are uncommon. This is in agreement with other recent studies in haemophiliacs (Telfer *et al* 1995; Rumi *et al* 1997).

Despite the poor response to IFN in haemophiliacs no studies have identified consistent pre-treatment predictors of response. In addition there have been several studies in non-haemophiliacs which have suggested that IFN may protect against the subsequent development of HCC (Nishiguchi *et al*, 1995; Mazzella *et al*, 1996). So despite the generally poor response to IFN treatment in haemophiliacs, in the absence of more effective therapy, at present it is still reasonable to offer haemophiliacs treatment with IFN.

We have shown that failure to clear HCV RNA within the first 8-12 weeks of treatment is associated with non-response and therapy should be discontinued. In those who successfully clear HCV RNA the optimum dose and duration of IFN therapy is uncertain. Several studies

suggest that sustained virological responses are improved by a increasing the duration of treatment to 12-18 months (Reichard *et al*, 1994; Chemello *et al*, 1995). In addition there is increasing evidence to suggest that combined treatment with IFN and ribavirin leads to improved responses (Schalm *et al*, 1997). Ribavirin is currently unlicensed in the UK for the treatment of chronic HCV infection but is available on a named patient basis. As ribavirin is now available there is a need for prospective evaluation in three groups of haemophiliacs (i) previous responders to IFN who relapsed on stopping therapy; (ii) previous non-responders to IFN and (iii) previously untreated with IFN alone.

Immunogenicity of IFN preparations

Anti-IFN antibodies develop in a minority of patients treated with IFN. The development of such antibodies may contribute to the poor response to IFN in haemophiliacs (chapter 6). There is considerable variation in the reported frequency of anti-IFN antibody development between commercially available IFN preparations perhaps indicating inherent differences in immunogenicity. Some commercial manufacturers of IFNs suggest that these differences are sufficiently important to warrant the use of "less immunogenic" IFN as first choice treatment in HCV infection.

Several studies have reported a higher incidence of anti-IFN antibodies in patients treated with rIFN-α2a compared to rIFN-α2b. For example, in a study of a group of patients with viral hepatitis, neutralising anti-IFN antibodies were found in 15/74(20.2%), 10/144 (6.9%) and 1/78 (1.2%) in patients treated with rIFN-α2a, rIFN-α2b and IFN-αN1 respectively (Antonelli *et al*, 1991). A generally low incidence of anti-IFN antibody production with IFN-αN1 and IFN-αN3 has been confirmed by others (Osterborg *et al*, 1991; Liao *et al*, 1992; Camps *et al*, 1994).

There are several possible explanations for the observed differences in immunogenicity between IFN preparations. For example, it has been suggested that the low incidence in anti-

IFN antibody development with rIFN-α2b (Spiegel *et al*, 1986) could be attributed to poor sensitivity of some detection methods (Itri *et al*, 1987) although this was subsequently refuted (Spiegel *et al*, 1989) More recently the manufacturers of rIFN-α2a have claimed that the formulation and storage conditions previously recommended may be responsible for higher anti-IFN antibody development. In support of this claim, neutralising anti-IFN antibodies were found in only 7/81 (9%) patients who received treatment for HCV using a new human serum albumin free liquid formulation of rIFN-α2a (Carreno & Wolfe, 1995) which is certainly lower than most previous reported studies of rIFN-α2a.

The two widely available recombinant α-IFNs, rIFN-α2a and rIFN-α2b as well as rIFN-α2c (not widely available for clinical use) differ in structure by only one amino acid (Table 9.1) and it has been suggested that this single minor amino acid difference may alter the immunogenicity of the IFN molecule (von Gabain *et al*, 1990). In a single study rIFN-α2c has been reported to be less immunogenic than either IFN-α2a or IFN-α2b (Steinmann *et al*, 1992).

Alternatively, differences in glycosylation between IFN preparations may contribute to immunogenic variation (Adolf *et al*, 1991) and, as natural IFNs contain many IFN subtypes, the immunogenic potential of each may be reduced compared with rIFNs which consist of a single IFN subtype.

Table 9.1 Amino acid differences between rIFN-α preparations.

	Amino acid at position	
	23	34
IFNα2a	Lys	His
IFNα2b	Arg	His
IFNα2c	Arg	Arg

Host factors may also influence anti-IFN antibody development. IFN-α alleles which are present at low frequency in certain ethnic groups which may increase the risk of anti-IFN antibody formation with particular IFN preparation (Hosoi *et al*, 1992). However, in a study of 16 Chinese patients, the allele for IFN-α2a was absent in all cases, and anti-IFN antibodies developed during treatment with rIFN-α2a in only 50% (Crowe *et al*, 1993).

The precise clinical implications of differences in immunogenicity between IFN preparations remains unclear. The indications for switching to an alternative IFN in the event of the development of breakthrough hepatitis are discussed in chapter 4. The more difficult question as to whether the reported differences between IFNs should alter the initial prescription. At present, on the basis of the evidence available, no firm recommendation can be made as to whether the immunogenic differences between IFN-α preparations should alter the initial choice of first-line IFN-α therapy.

Indications for liver transplantation in haemophiliacs

There have been an increasing number of reports of the successful use of liver transplantation in haemophiliacs with either HCV induced cirrhosis or HCC (McCarthy *et al*, 1996). Occasional patients co-infected with HIV have also undergone successful transplantation (Hanley *et al*, 1996b). If the progressive nature of HCV infection is confirmed in the future it is likely that liver transplantation will be required in an increasing number of haemophiliacs. Liver transplantation in haemophilia carries the added bonus of phenotypic cure of the bleeding disorder as FVIII/IX is produced by the new liver.

Generally the criteria applied to the selection of candidates for transplantation are the same as those applied to non-haemophiliacs. These include hepatic decompensation with ascites and recurrent variceal haemorrhage. It may be that in the future there may be a need to refine the transplant criteria for haemophiliacs if there is an unacceptable mortality associated with

variceal bleeding. In addition cost/benefit analysis should include the saving of subsequent coagulation factor concentrates which are no longer required (Lee, 1996).

HGV infection in haemophiliacs

There has been much interest in the possibility that there may be other hepatitis viruses transmitted by blood products. As HCV does not account for 100% of chronic liver disease present in haemophiliacs it is likely that such viruses will be detectable in the haemophiliac population. Recently a novel flavivirus which was termed GB virus-C (GBV-C) (Simons *et al*, 1995; Leary *et al*, 1996) or hepatitis G virus (HGV) (Linnen *et al*, 1996) has been implicated as a cause of post-transfusion hepatitis.

HGV viraemia is common in blood donors with 1-3.2% with detectable HGV RNA by PCR. (Linnen *et al*, 1996; Jarvis *et al*, 1996a). There is currently conflicting evidence as to whether HGV is a true hepatitis virus. It has been shown that HGV is transmitted by blood transfusion but is not associated with biochemical hepatitis in the majority of cases (Alter *et al*, 1997). Studies in patients with community acquired hepatitis suggest a minor aetiological role for HGV (Alter *et al* 1997). In addition initial reports implicating HGV as a cause of acute fulminant (Yoshiba *et al*, 1995) hepatitis have not been confirmed by others (Kao *et al*, 1996; Haydon *et al* 1997) although it has been suggested that specific HGV strains may be responsible (Heringlake *et al*, 1996).

Studies in haemophiliacs have reported the recent prevalence of HGV viraemia to be 6-18% (Linnen *et al*, 1996; Jarvis *et al*, 1996a; Tagariello *et al*, 1996). Most of these patients are co-infected with HCV and none of these studies have found more severe liver disease associated with HGV. We have found considerable variations in the patterns of HGV viraemia in

haemophiliacs (chapter 8). However, persistent HGV viraemia was not associated with more severe liver disease.

The precise significance of HGV infection remains to be determined. It is possible that it is a non-pathogenic virus which is common in the general population. Studies in individuals infected with HGV alone are required to assess any association with liver disease. Haemophiliacs with chronic HGV infection require further follow-up to determine if there are any disease associations.

Concluding remarks

There has been much progress in the prevention of transfusion-transmitted viruses in haemophiliacs. Plasma donors are selected and screened; virus inactivation methods are utilised in the production of concentrates and recipients are vaccinated against some viruses. All these factors have contributed to the viral safety of contemporary coagulation factor concentrates.

However the problem of virus transmission to haemophiliacs has not been solved as there are occasional reports of possible HCV transmission and it is widely accepted that some viruses such as parvovirus are not successfully inactivated by currently used methods. There remains anxiety that novel pathogenic viruses may enter the blood supply and potentially infect the recipients of plasma derived products.

The search for improved virus safety has been the main impetus behind the development of recombinant coagulation factors using DNA technology. Recombinant FVIII is now in use in many countries and has been shown to be well tolerated and effective. Recombinant FIX is undergoing clinical trials and recombinant FVIIa is used to treat haemophiliacs with inhibitors. Whilst these products should carry a smaller viral risk than plasma-derived concentrates there is still a theoretical risk of infection as human albumin is used in some preparations and animal

proteins are added to cell culture systems. The promise of gene therapy may provide a further option for the treatment of haemophiliacs in the future.

In the meantime the legacy of HIV and HCV infection in haemophiliacs requires continued care of those infected and continued vigilance to prevent further infections.

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Appendix

Reprints of published work

Interferon Treatment for Chronic Hepatitis C Infection in Hemophiliacs—Influence of Virus Load, Genotype, and Liver Pathology on Response

By John P. Hanley, Lisa M. Jarvis, Janet Andrews, Rosemary Dennis, Peter C. Hayes, Juan Piris, Robert Lee, Peter Simmonds, and Christopher A. Ludlam

In this study, we assessed the effectiveness of interferon treatment in 31 hemophiliacs with chronic hepatitis C virus (HCV) infection. Interferon alfa-2a (3 MU three times weekly) was administered for 6 months. Response was assessed by both serial alanine transaminase (ALT) and HCV RNA levels measured by a sensitive semiquantitative polymerase chain reaction (PCR) method. HCV genotype was determined by restriction fragment length polymorphism (RFLP), and evidence of changing genotypes during interferon therapy was sought. Severity of liver disease was assessed by both noninvasive and invasive methods, including laparoscopic liver inspection and biopsy. Sustained normalization of ALT levels occurred in eight patients (28%), and seven (24%) became nonviremic as assessed by PCR (<80 HCV/mL). Responders universally cleared HCV RNA within 2 months of starting interferon. Genotype 3a was associated with a favorable response to interferon. No evidence was found for a change in circulating genotype in patients who failed to respond to interferon or who relapsed. This study confirms that response rates to interferon are low in hemophiliacs as compared with other groups with chronic HCV infection. We have also demonstrated that virus load measurement over the first 8 to 12 weeks of treatment is an extremely useful method to identify responders at an early stage.

ALMOST ALL HEMOPHILIACS who received clotting factor concentrates before the introduction of effective viral-inactivation techniques were infected with hepatitis C virus (HCV).[1,2] The majority of individuals exposed to the virus have become chronic carriers, characterized by both persistent fluctuating viremia and variable abnormalities in liver function tests. There is a wide spectrum of liver disease in these patients, ranging from only minor histologic evidence of chronic hepatitis to cirrhosis[3] and hepatocellular carcinoma.[4]

Interferon has been used to treat chronic HCV in both nonhemophiliacs and hemophiliacs. Response to treatment has been assessed by serial serum alanine transaminase (ALT) levels, clearance of viremia by polymerase chain reaction (PCR), and direct assessment of liver histology. Clearance of viremia is likely to be a prerequisite for a long-term response rather than normalization of ALT. To date, there have only been a few small studies on the efficacy of interferon alfa for the treatment of HCV infection in individuals with hemophilia.[5-9] Some of these studies have suggested that the response to interferon in hemophiliacs may be lower than in other groups infected with HCV. We report the results of interferon therapy in a group of 31 hemophiliacs with chronic HCV infection. We have monitored response to interferon using a sensitive semiquantitative PCR to detect HCV RNA. In addition, we have assessed the extent of liver disease in these patients using both invasive and noninvasive methods, and have critically evaluated whether these investigations are useful in predicting the response to interferon.

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PATIENTS AND METHODS

Patient characteristics. A total of 31 patients (30 male and one female) were treated (Table 1). All patients were anti-HCV–positive by Abbott second-generation enzyme immunoassay (A-EIA; Abbott, Weisbaden-Dalkenheim, Germany) and were also positive on confirmatory testing by second-generation recombinant immunoblot assay (RIBA-2; Chiron Corp, Emeryville, CA) for antibody to nonstructural proteins 5-1-1 (NS4), c100-3 (NS4), c33c (NS3), and core-associated antigen c22-3.

All had previously received non–virus-inactivated factor concentrates, and 30 of 31 had persistently elevated serum ALT levels (ALT levels were measured on ≥3 occasions over the 6 months before treatment). Serum ALT level was normal in one individual. HCV RNA was detected in all patients by PCR as described below. Six patients were anti-HIV–positive (pretreatment CD4 counts, 4, 20, 140, 180, 250, and 360 cells/mm^3, respectively), and all were HBSAg-negative. In addition, all patients were negative for anti–smooth muscle and antimitochondrial antibody and had normal levels of α-fetoprotein, ceruloplasmin, and α1-antitrypsin.

Pretreatment assessment. Informed consent was obtained from all patients participating in the study. Before starting interferon treatment, a detailed assessment was undertaken of the extent of liver disease in each patient, with particular reference to the presence or absence of cirrhosis, using both noninvasive and invasive methods. The role of invasive investigations in the assessment of liver disease was discussed with all patients. Those with anti-FVIII or anti-FIX inhibitors were not offered invasive investigations, and individuals with von Willebrands disease were not offered liver biopsy. It was emphasized that the investigations were not essential for administration of interferon but would provide information to counsel the individual as to the extent of liver disease.

The following investigations were performed after appropriate factor concentrate replacement. (1) Upper gastrointestinal endoscopy was performed in 27 patients to identify the presence or absence of esophageal varices. (2) Laparoscopy was performed under sedation using a 5-mm pediatric laparoscope (Olympus, Tokyo, Japan) or microlaparoscope (Imagyn, Laguna Niguel, CA) in 21 patients. The degree of hepatic inflammation (none, mild, or marked), degree of surface fibrosis (none, moderate, or pronounced), and presence of cirrhosis and portal hypertension were assessed.[10,11] In 15 patients, a tru-cut biopsy was taken during the laparoscopy, and histology was assessed using both the Sheffield scoring system[5] (data not

Table 1. Patient Characteristics

Parameter	No. of Subjects	Median	Range
Hemophilia A			
Severe	11		
Moderate	8		
Mild	1		
Hemophilia B			
Severe	1		
Moderate	2		
Mild	5		
von Willebrand disease	3		
Total	31		
Age (yr)		35.0	13-67
Pretreatment ALT (U/L)		78	37-175
Pretreatment HCV RNA (copies/mL)		10^6	10^3->10^7

shown) and a visual classification. Using the latter method, overall histologic appearance was classified as mild, moderate, or severe. (3) Abdominal ultrasound scans to assess liver size, echogenicity, and presence of hepatocellular carcinoma, as well as spleen size, were performed on 30 patients. Baseline measurements are listed in Table 1. In addition, data concerning annual factor concentrate consumption and serum Igs (IgG, IgM, and IgA) and serum ferritin were collected.

Drug dosage and administration. Interferon alfa-2a (Roche, Welwyn Garden City, UK) 3 MU three times per week was given subcutaneously. The intention was to treat for 24 weeks.

Assessment of response to interferon treatment. Response was assessed on the basis of serial monthly ALT levels and HCV RNA quantitation. After 6 months' treatment, a complete ALT response (CR) was defined as normalization of ALT (<40 U/L) sustained for at least 2 months; a partial ALT response (PR) required a more than 50% reduction in pretreatment ALT. A HCV RNA CR required the virus to be undetectable in the serum, ie, PCR-negative (<80 HCV/mL, see below); a PR required a 100-fold reduction in HCV RNA titer.

Typing and quantification of HCV RNA. All testing was performed using serum samples separated within 3 hours of collection and stored at −70°C. Virus RNA was extracted from 0.5 mL stored sera after pelleting of virus by centrifugation at 100,000*g* for 90 minutes at 4°C and incubation at 37°C for 2 hours with 1 mg/mL proteinase K in the presence of 40 μg/mL polyadenylic acid, 0.5% sodium dodecyl sulfate, 0.1 mol/L NaCl, 50 mmol/L Tris hydrochloride (pH 8.0), and 1 mmol/L EDTA. RNA was extracted with phenol, and after centrifugation the supernatant was reextracted successively with phenol/chloroform (1:1) and chloroform/isoamylalcohol (50:1). Nucleic acid was precipitated by the addition of .10 vol sodium acetate (pH 5.2) and 2 vol ethanol. The dried pellet was resuspended in 25 μL diethylpyrocarbonate-treated water.[12] RNA was reverse-transcribed by nested PCR using 5′ noncoding region–specific primers 939, 209, 940, and 211.[13] For genotyping, product DNAs were cleaved with restriction enzymes HaeIII/RsaI and MvaI/HinfI as described previously,[14] and the fragments were separated by agarose gel electrophoresis using 4% Metaphor agarose (FMC BioProducts, Rockland, ME). Subtypes 1a and 1b and 2a and 2b were identified by the cleavage patterns resulting from digestion with BstUI and ScrFI, respectively.[15] Genotyping was performed on all patients before starting interferon and after 3 and 6 months' therapy.

Virus levels were measured semiquantitatively by limiting-dilution analysis of cDNA reverse-transcribed from RNA.[16,17] Centrifugation of 0.5 mL sera provided a level of detection of approximately 800 HCV/mL. To increase sensitivity of the PCR method, samples that were negative at this level of detection (<800 HCV/mL) were further analyzed by centrifugation of 5.0 mL sera, providing a cutoff point of approximately 80 HCV/mL.

Statistical analysis. The relationships between ALT response or HCV RNA response and baseline measurements were assessed using Fisher's exact test for nominal baseline variables, chi-square test for trend for ordered categorical baseline variables, Wilcoxon rank-sum test for non–normally distributed continuous baseline variables, and two-sample *t*-test for normally distributed continuous baseline variables. All significance tests were two-sided.

RESULTS

Of 31 patients, 29 completed 6 months' treatment with interferon. One stopped after 2 months due to leukopenia, and one stopped after 5 months due to a subjective hearing loss that subsequently recovered. The 29 patients who completed 6 months of treatment were evaluated. Normalization of ALT (CR) occurred in eight of 29 (28%); a more than 50% reduction in pretreatment ALT (PR) was achieved in four of 29 (14%), and 17 of 29 (59%) were nonresponders (NR). Seven of 29 (24%) became PCR-negative for HCV RNA (CR). In addition two of 29 (7%) achieved at least a 10^2-copies/mL reduction in HCV RNA (PR). In 20 of 29 (69%), serum HCV RNA levels were unchanged (NR).

There was a statistically significant difference in age between responders (mean age, 46 years) and NR (mean age, 33 years; two-sample *t*-test, $P = .044$). However, multivariate analysis showed no independent relationship between age and response after adjusting for the effect of genotype on response (see below).

Response was not associated with HIV status, body weight, severity of hemophilia, duration of infection, annual factor concentrate consumption, serum ferritin, or serum IgS (data not shown).

Patterns of ALT and HCV RNA responses. Normalization of ALT levels correlated with clearance of viremia as measured by PCR (Spearman rank correlation, $r = .53$, $P = .003$; Table 2). Of eight patients who had a normalized ALT, five also became HCV RNA–negative, whereas in three there was no corresponding reduction in virus load. Of four patients who achieved a partial reduction in ALT, only one became nonviremic. Fifteen of 17 in whom ALT level was unchanged also had no change in virus load. Only one individual who had no change in ALT became nonviremic. Median ALT values in patients who became nonviremic versus those who failed to clear HCV RNA are shown in Fig 1. In one patient only, there was a transient HCV RNA clearance at 2 months, but at 3 months the HCV RNA titer returned to the pretreatment level of 10^7 HCV/mL. This was associ-

Table 2. ALT Response and HCV RNA Response

	HCV RNA Response			
ALT Response	CR	PR	NR	Total
CR	5	—	3	8
PR	1	1	2	4
NR	1	1	15	17
Total	7	2	20	29

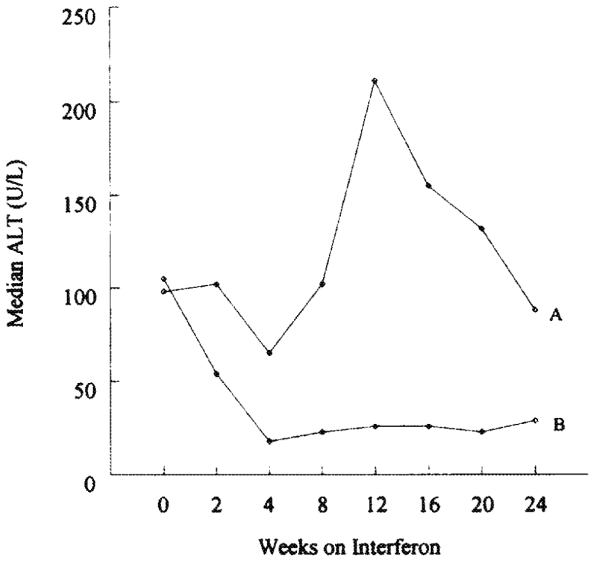

Fig 1. Association of virus clearance with normalization of ALT. Patients with a partial or no reduction in virus load (line A, n = 22) showed no corresponding reduction in ALT, whereas patients who became nonviremic (line B, n = 7) achieved a reduction in ALT during treatment with interferon.

ated with a parallel ALT reduction and subsequent relapse (Fig 2). There was no relationship between pretreatment ALT level and response.

Rate of HCV RNA clearance in responders. The rate of HCV RNA clearance in seven CR was assessed (Fig 3). In six of seven, a prompt reduction in HCV RNA occurred to less than the limit of detection within 8 weeks of commencing interferon. In one individual (patient no. 2, Fig 3), initial clearance of HCV RNA at week 4 was followed by a transient increase in virus titer to 10^4 copies/mL at weeks 8 and 12 before sustained clearance between weeks 16 and 24.

Predictive factors for response to interferon. There was a striking relationship between HCV genotype and a favorable response to interferon. A total of six of eight hemophiliacs infected with genotype 3a became nonviremic. In the other 21 patients, only one individual (genotype 2b) became HCV RNA–negative (Fisher's exact test, $P = .0002$). No changes in HCV genotype, as detected by RFLP analysis, were identified in any patients during interferon therapy (Table 3).

There was no relationship between pretreatment virus load and either response to interferon (Fig 4) or HCV genotype (Fig 5).

Severity of liver disease was assessed by methods outlined earlier. Of 28 abdominal ultrasound scans evaluated, five (18%) demonstrated splenomegaly and seven (25%) showed an abnormal liver (five with hepatomegaly and two with an abnormal liver texture). In seven of 25 (28%), esophageal varices were demonstrated at endoscopy. Of 20 laparoscopic liver inspections, 10 (50%), four (20%), and six (30%) showed none, mild, or pronounced surface fibrosis, respectively, with five patients with pronounced fibrosis having cirrhosis. Inflammation was identified laparoscopically in all patients, being mild in 14 (70%) and marked in six (30%). Evidence of portal hypertension was visible in five (25%).

Liver histology was evaluated in 14 patients and showed mild, moderate, and severe (including cirrhosis) histologic changes in four (29%), seven (50%), and three (21%), respectively.

Analysis of response to interferon against laparoscopic liver appearance, liver histology, presence/absence of hepatomegaly, splenomegaly, and esophageal varices showed no evidence that any of these variables were associated with response to interferon.

Follow-up information. Within 3 months of discontinuing interferon, five of seven patients who became nonviremic have relapsed, with reappearance of serum HCV RNA and elevated ALT levels. In two patients who achieved a partial reduction in virus load, HCV RNA levels returned to pretreatment values within 1 month of stopping interferon. Only two patients remain HCV RNA–negative with normal ALT. The three patients who showed normalized ALT levels without a corresponding reduction in HCV RNA developed elevated ALT levels within 2 months of stopping interferon.

DISCUSSION

We have shown that the response to interferon therapy in hemophiliacs with chronic HCV infection is poor and appears inferior to that of other groups of infected patients. In this study, only 28% had a normalized serum ALT after 6 months' treatment with interferon, and 24% became PCR-negative for HCV RNA. Furthermore, sustained responses were uncommon. This compares with initial ALT CR of 50% and sustained responses of 20% to 25% reported in nonhemophiliacs.[18] Early investigators used ALT to assess long-term response.[19,20] More recent studies have confirmed that clearance of HCV RNA is a prerequisite for long-term response.[21]

The importance of HCV quantitation has been increasingly recognized. We describe herein a sensitive semiquantitative PCR method for HCV RNA detection with a lower

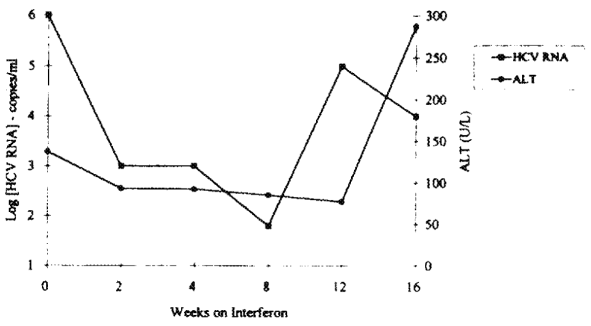

Fig 2. Breakthrough hepatitis during interferon therapy. This patient achieved an initial response to interferon with clearance of HCV RNA by week 8 and corresponding reduction in ALT level. As interferon was continued, viremia recurred by week 12 and there was an associated increase in ALT. Such episodes of breakthrough hepatitis during interferon treatment may be associated with the development of neutralizing antibodies to interferon.

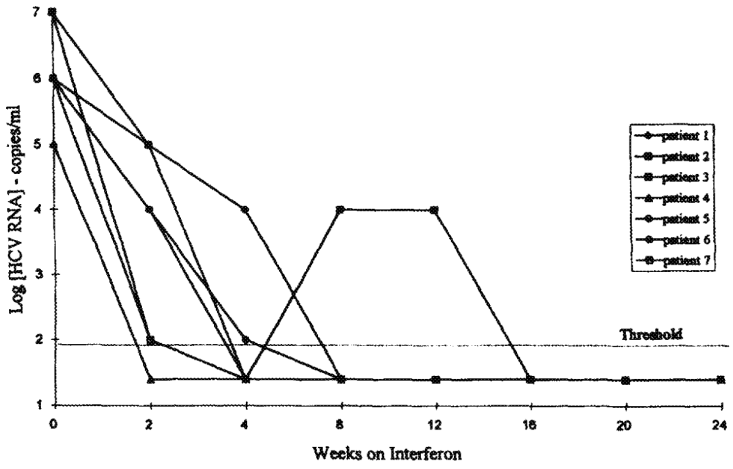

Fig 3. Rate of virus clearance in HCV RNA responders (n = 7). In 6 of 7 patients who became nonviremic, there was a prompt reduction in HCV RNA to < the limit of detection within 8 weeks of commencing interferon. In one individual (patient no. 2), initial clearance of HCV RNA at week 4 was followed by a transient increase in virus titer to 10^4 copies/mL at weeks 8 and 12, before sustained clearance between weeks 16 and 24.

limit of detection of 80 HCV/mL. This compares with 3.5 × 10^5 equivalents/mL when the branched DNA (bDNA) assay is used.[22] Using bDNA, we would have failed to detect viremia in four (13%) of our patients and incorrectly classified six (20%) as responders who merely showed a partial reduction in HCV RNA levels. Clearly, sensitive quantitative assays are essential to monitor response to treatment, and some studies may have overestimated response rates by using relatively insensitive methods for RNA quantitation. We found that HCV RNA was cleared within 8 weeks of starting interferon in seven patients who responded. Using this method, it would therefore be possible to differentiate responders from nonresponders at an early stage of treatment and discontinue interferon in nonresponders. Alternatively, these individuals could be offered a dose-escalation, although there are few data available in hemophiliacs concern-

Table 3. Predictive Factors for Response to Interferon

Factor	HCV RNA			*P*
	CR	PR/NR	Total	
Genotype				
1a	0	11	11	
1b	0	7	7	
2a	0	1	1	
2b	1	1	2	
3	6	2	8	.0002*
Anti-HIV				
Positive	0	5	5	
Negative	7	18	25	.30
Splenomegaly (ultrasound)				
Yes	0	5	5	
No	7	16	23	.29
Hepatic fibrosis (laparoscopy)				
None	3	7	10	
Mild	1	3	4	
Pronounced	3	3	6	.64
Hepatic inflammation (laparoscopy)				
Mild	5	9	14	
Marked	2	4	6	1.00
Esophageal varices (endoscopy)				
Yes	1	6	7	
No	6	12	18	.63
Liver histology				
Mild	1	3	4	
Moderate	3	4	7	
Severe	1	2	3	1.00

* Fisher's exact test.

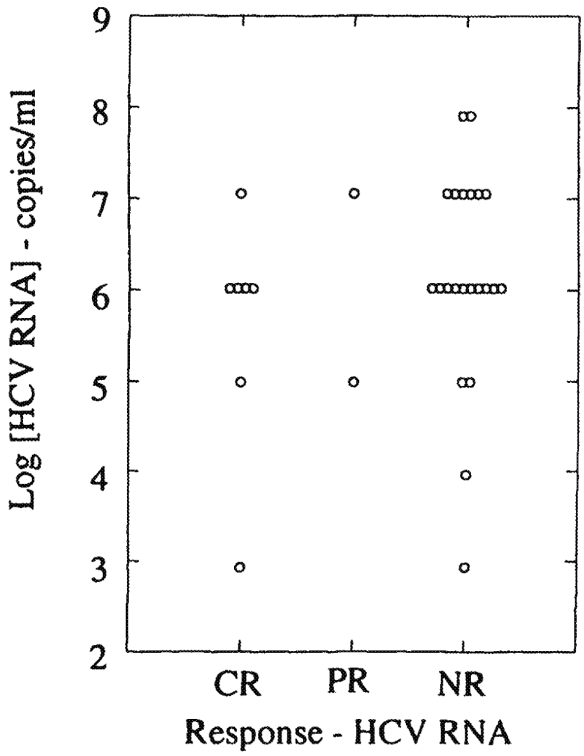

Fig 4. Pretreatment virus load and response to interferon. There was no significant relationship between pretreatment virus load and response to interferon (χ^2 test for trend, $P = .073$).

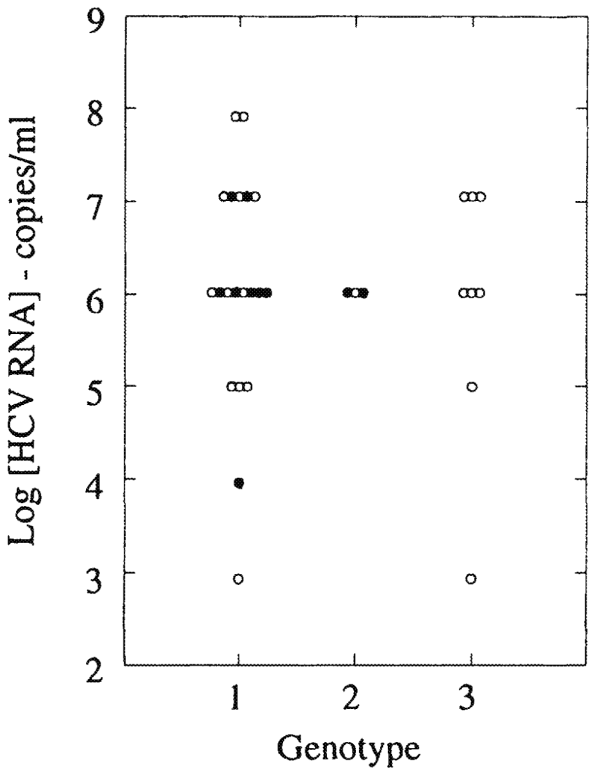

Fig 5. Pretreatment virus load and HCV genotype. There was no significant relationship between pretreatment virus load and HCV genotype (Kruskal-Wallis test, $P = .77$). (○) subtype a; (●) subtype b.

ing the value of this to increase response rates. In view of the generally poor overall responses to interferon in hemophiliacs, we believe this approach is unlikely to benefit a significant number of patients.

In one individual, initial clearance was followed by a transient increase in virus titer before further clearance. The reasons for this are not entirely clear. Temporary noncompliance with treatment is a possible explanation, or there may be a small number of individuals who achieve a slower reduction in HCV RNA rather than the rapid and sustained reduction observed in the majority of responders.

There is usually a good correlation between normalization of ALT and HCV RNA clearance in responders to interferon. However, discrepancies between ALT and HCV RNA responses have been described previously.[23] We have identified some individuals who achieved a biochemical response without a corresponding reduction in virus load. Clearly, these patients are not true responders, and biochemical relapse is inevitable once interferon is discontinued. Since ALT is not an entirely accurate measure of response, studies using ALT alone to assess response may also overestimate true responders. Interestingly, there was one individual who cleared HCV RNA without an associated ALT response. This raised the possibility of other non-HCV coexisting liver pathology resistant to interferon treatment, but none has been identified. Another individual relapsed during interferon therapy. Such episodes of "breakthrough hepatitis" are well recognized and may be associated with the development of neutralizing antibodies to interferon.[24] A change in HCV genotype may also be an explanation for breakthrough hepatitis, and a change in the dominant genotype in hemophiliacs treated with interferon has been reported.[25] In this study, we did not detect any changes in the circulating genotype in any patients during interferon therapy. However, it is recognized that the RFLP method will not detect co-infecting genotypes circulating at low frequencies. In addition, interferon NR may be associated with a change in variants of the same genotype (termed quasi-species). Analysis of variants within an individual before and after interferon treatment is in progress.

Several studies assessing response to interferon have been performed on cohorts of hemophiliacs infected with HCV.[5-9] Some of these studies have suggested that the overall initial response rates are somewhat lower in hemophiliacs than in other groups with HCV. Interestingly, response rates in the earlier studies are superior to those performed more recently. This may be a reflection of the relatively small number of patients studied, or may have been caused by progression of liver disease in cohorts of hemophiliacs, leading to diminished responses to interferon. In addition, although the data available provide conflicting results, long-term response appears unusual. In the largest study,[9] only one of 20 (5%) achieved a sustained response to interferon. In the only trial that assessed response by liver biopsy,[5] four of seven responders had a long-term response. Our study, which contains the largest treated group of hemophiliacs with HCV reported to date, supports the view that long-term responses to interferon are uncommon in hemophiliacs. Sustained response rates may be improved by longer courses of interferon.

Attempts have been made to identify factors that may predict response to interferon.[26] Absence of cirrhosis, younger age, low serum HCV RNA level, and genotypes 2 and 3 are all factors associated with a favorable response to interferon. As yet, the presence of adverse factors has not been considered sufficient to absolutely exclude some individuals with HCV from interferon therapy. There has been particular concern, in view of the generally poor response to interferon in hemophiliacs, that treatment with interferon is inappropriate in the majority of individuals. Not only are patients exposed to a potentially toxic drug with unpleasant side effects, but the cost of a course of interferon is considerable.

We have tried to identify parameters that may predict response to interferon in hemophiliacs with HCV. Assessment of liver disease has included both invasive and noninvasive methods. We have shown that genotype 3a is associated with a favorable response, but we failed to identify any other statistically significant independent variables, including pretreatment virus load, associated with a poor response to interferon. In view of this, despite the apparent poor response to interferon in hemophiliacs, it is not possible to predict accurately which individuals are likely to respond.

In conclusion, the results of interferon treatment for HCV in hemophiliacs are disappointing. Interferon alfa-2a 3 MU

three times per week for 6 months is unlikely to result in a long-term sustained response. Monitoring response with a sensitive semiquantitative PCR to quantify HCV RNA is extremely useful to identify responders at an early stage of treatment. Those who fail to clear HCV RNA should discontinue interferon. Dose-escalation is unlikely to benefit many patients, but may be attempted in selected individuals.

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Investigation of chronic hepatitis C infection in individuals with haemophilia: assessment of invasive and non-invasive methods

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Summary. Hepatitis C virus (HCV) infection is the major cause of chronic liver disease in individuals with haemophilia. A wide spectrum of disease severity is found in this group, ranging from mild hepatitis to cirrhosis. We have studied a cohort of 87 anti-HCV positive haemophiliacs who have been infected with HCV for 10–25 years and assessed the relative value of invasive and non-invasive methods of evaluating liver disease. The severity of liver disease was assessed using ultrasound scan ($n = 77$), upper GI endoscopy ($n = 50$), laparoscopic liver inspection ($n = 33$) and liver biopsy ($n = 22$). Invasive investigations were performed without any significant bleeding complications.

Evidence of severe liver disease was found in approximately 25% of patients. There was agreement between the severity of liver histology and the information derived from the laparoscopic liver inspection, endoscopy and ultrasound in 86%. Co-infection with HIV was significantly associated with more severe liver disease ($P = 0{\cdot}006$).

This study provides further evidence that liver disease is emerging as a major complication in haemophiliacs and severe liver disease is more common in those co-infected with HIV. We have shown the potential value of laparoscopic liver inspection, in combination with endoscopy and ultrasound, in staging the extent of liver disease, and suggest that most patients may be managed without resorting to liver biopsy.

Keywords: haemophilia, HCV, HIV, liver biopsy, laparoscopy.

Following the widespread introduction around 1970 of coagulation factor concentrates for the treatment of haemophilia, it was noted that the majority of the recipients of such concentrates developed abnormal liver function tests. A small number of patients were found to have hepatitis B virus (HBV) infection. In the majority, however, the presumed aetiology was an uncharacterized virus giving rise to non-A, non-B hepatitis (NANBH). Chronic NANBH was characterized by persistently, or intermittently, raised liver transaminases, e.g. alanine transaminase (ALT), and affected individuals were usually asymptomatic. Early investigators suggested that chronic NANBH in haemophiliacs was usually a benign form of chronic liver disease which seldom progressed to cirrhosis (Mannucci *et al*, 1982; Stevens *et al*, 1983). Soon after, increasing evidence suggested that NANBH was a much more serious problem than had been previously considered. Liver biopsy studies showed progression of histological changes over a period of time (Hay *et al*, 1985) and larger surveys of haemophiliacs demonstrated a significant proportion with cirrhosis (Aledort *et al*, 1985). Debate continued, however, concerning the best way to investigate and monitor chronic liver disease in those with haemophilia.

In 1989 hepatitis C virus (HCV) was identified and has subsequently been shown to be the major cause of NANBH in haemophiliacs (Ludlam *et al*, 1989; Tedder *et al*, 1991). Almost all haemophiliacs treated with non-virus-inactivated factor concentrates have anti-HCV antibodies and up to 90% show evidence of persistent viraemia and elevated ALT (Watson *et al*, 1992). With the development of the polymerase chain reaction (PCR), and its application to the study of HCV, the means to detect and quantify serum HCV RNA and identify the circulating HCV genotype became available.

We have studied a cohort of anti-HCV-positive haemophiliacs and assessed the extent of liver disease using both non-invasive and invasive methods. We have particularly evaluated the safety and usefulness of laparoscopic inspection of the liver surface and laparoscopic-guided liver biopsy.

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We have used the information gathered from these investigations to address the following questions: (1) How serious is liver disease in haemophiliacs who have now been infected with HCV for 10–25 years? (2) Can less invasive methods of assessing liver disease be utilized to reliably assess the extent of liver disease, without resorting to liver biopsy? (3) Is laparoscopic inspection of the liver surface a useful investigation in haemophiliacs and can it be used as an alternative to liver biopsy? (4) Is there a correlation between severity of liver disease and factors related to HCV (e.g. genotype), host factors (e.g. diagnosis, concentrate use, immune system) or co-infection with HIV.

PATIENTS AND METHODS

This study formed part of an on-going clinical follow-up programme which has evolved with the availability of serological and PCR methods to study HCV. When the first-generation anti-HCV antibody tests became available it was not clear whether a positive result indicated current infection or merely past exposure to HCV. It has subsequently become clear that there are only a minority of haemophiliacs who clear HCV RNA.

Patient characteristics. All individuals with bleeding disorders registered at the Edinburgh Haemophilia Centre were screened for anti-HCV antibodies. There were 87 who were anti-HCV positive by second-generation enzyme immunoassay (A-EIA; Abbott, Weisbaden-Dalkenheim, Germany) and also positive on confirmatory testing by recombinant immunoblot assay (RIBA-2, Chiron Corporation, Emeryville, Calif.). The characteristics of this group are outlined in Table I(a). Evidence of hepatitis B and HIV infection was sought in all cases. Previous ALT levels were available and the mean ALT was calculated from three measurements over a minimum of 6 months (in two patients only one ALT and in five patients two ALT values were available). In addition serum immunoglobulins (IgG, IgM and IgA), CD4 counts, platelet counts, serum ferritin, serum albumin and prothrombin times were measured (Table Ib).

A programme of investigation, as described below, was initially offered to all. With the subsequent availability of PCR testing to detect serum HCV RNA, invasive investigations were restricted to 'PCR positive' individuals.

HCV genotyping was performed by restriction length fragment polymorphism (RFLP) as previously described (Hanley *et al.* 1996).

Assessment of liver disease. We took the view that each individual should be offered investigations to establish the extent of liver disease. The range of invasive and non-invasive investigations of liver disease was discussed with each patient. Invasive investigations were not performed on those with factor VIII (FVIII) inhibitors ($n = 5$) and individuals with von Willebrand's disease (VWD) ($n = 8$) were not offered liver biopsy. It was stressed that consent to investigations was not required prior to treatment with interferon (Hanley *et al.* 1996) but would provide information to counsel the individual as to the extent of their liver disease. No patients had received interferon therapy prior to the investigations. Following a full discussion regarding the pros and cons of the various investigations, the final numbers of patients who consented to investigations after appropriate factor concentrate replacement were as follows: (1) Abdominal ultrasound scans to assess liver size, echogenicity and the presence of hepatocellular carcinoma (HCC) as well as spleen size were performed on 77 patients. (2) Upper GI (gastro-intestinal) endoscopy was performed in 48 patients to identify the presence or absence of oesophageal varices indicating portal hypertension secondary to cirrhosis. A further two patients with FVIII inhibitors were assessed by barium swallow. (3) Laparoscopic liver inspection was performed in 34 patients. The degree of hepatic inflammation (none, mild or marked), surface fibrosis (none, moderate or pronounced) and presence of cirrhosis and portal hypertension was assessed (Jalan *et al.* 1995). (4) A tru-cut liver biopsy was attempted in 23 patients under direct vision during the laparoscopy, and histology was assessed.

Histological assessment of liver biopsies. All liver biopsy specimens were fixed in formalin and examined in paraffin

Table I(a). Patient characteristics.

Diagnosis	Haemophilia A	Mild (FVIII > 5%)	8
		Moderate (1–5%)	19
		Severe (< 1%)	35
	Haemophilia B	Mild (FIX > 5%)	9
		Moderate (1–5%)	6
		Severe (< 1%)	2
	Von Willebrand's disease		8
	Total		87
Sex		Male	82
		Female	5
Age (years)		Mean	37·7
		Range	12–75
Anti-HCV		Positive	87
HCV RNA		Positive	74
		Negative	13
Anti-HIV		Positive	18
		Negative	69
HBSAg		Positive	2
		Negative	85

Table I(b). Baseline investigations.

	Median	Range	Normal range
ALT (U/l)	60	15–479	10–40
Ferritin (μg/l)	52	5–2932	8–300
IgG (g/l)	14·7	7·0–41·8	7·4–16·6
IgA (g/l)	2·9	0·6–12·6	0·8–3·9
IgM (g/l)	2·3	0·6—7·9	0·5–2·0
Albumin (g/l)	44	18–51	36–47
Prothrombin time (s)	12	10–47	10·5–14·5
CD4 count (10^6/l)	0·580	0·0–1·587	0·5–1·5
Platelet count (10^9/l)	207	1–387	150–350

sections by a single histopathologist. Assessments were made without prior knowledge of the clinical details of the patient. Two methods of assessment were used: (1) A visual scoring system (The Edinburgh Classification) which identifies seven specific histological features (lymphoid aggregates, lobulitis, spotty necrosis, bile duct lesions, fatty infiltration, piecemeal necrosis and fibrosis) and scores each feature as absent or present (+ to + + +). The overall appearances were then classified as mild, moderate or severe (including cirrhosis). (2) The Sheffield Scoring System (Makris *et al*, 1991) which assigns a score of 0 to 3 to each of five histological features (steatosis, apoptotic necrosis, piecemeal necrosis, sinusoidal infiltrates and portal infiltrates). The overall score is the sum of the scores for the five features.

Protocol for investigations

Day 1. Admit to hospital. Full physical examination and baseline investigations including anti factor VIII/IX antibody screen. Informed consent for investigations was obtained.

Day 2, a.m. Endoscopy was performed following appropriate coagulation factor replacement. Patients with haemophilia A or von Willebrand's disease were treated with factor VIII concentrate before the procedure to achieve post-infusion levels of 0·5 iu/ml. Patients with haemophilia B were given factor IX concentrates to achieve post-infusion levels of 0·3 iu/ml. Endoscopy was performed using standard techniques. Further treatment with factor concentrates was only given if the procedure had been traumatic or if a biopsy was required. Those patients who were having no further investigations were discharged the same day.

Day 2, p.m. Laparoscopic inspection of the liver and biopsy (when indicated) was performed. Patients were given further factor concentrate to attain post-infusion levels of 1·0 iu/ml (FVIII) or 0·7 iu/ml (FIX). The infusions were given 2 h before the procedure to enable post-infusion levels to be measured. The laparoscopy was performed under sedation with diamorphine and diazepam. A small incision was made above and to the right of the umbilicus and 2 litres of nitrous oxide was inflated into the peritoneal cavity. An appropriately sized trocar was inserted to allow passage of a 5 mm paediatric laparoscope (Olympus) or a 2 mm microlaparoscope (Imygen). The systematic inspection of the upper abdomen was undertaken, including the falciform ligament, looking at the size of vessels for evidence of portal hypertension and the surface of both lobes of the liver. A permanent record, in the form of a video recording, was made of the liver surface. If appropriate, a biopsy was taken with a tru-cut needle from the left lobe of the liver. The biopsy was taken under direct vision and, if necessary, either pressure or a heater probe was applied to the site of biopsy to arrest any bleeding. The patient was confined in bed for 24 h after the biopsy. Factor VIII/IX levels were maintained between 0·5–1·0/0·5–0·7 iu/ml respectively for 48 h. Patients continued to receive factor concentrate infusions for 4 d after liver biopsy.

Day 3. A post-biopsy ultrasound scan to detect evidence of bleeding was performed 24 h after the procedure.

Day 4. Discharge home.

RESULTS

There was no evidence of any relationship between mean ALT, ferritin, prothrombin time, albumin, IgG, IgA, IgM or CD4 count and severity of liver disease as assessed histology or laparoscopy. There was an inverse correlation between platelet count and severity of liver histology (Spearman rank correlation: $r = -0{\cdot}48$, $P = 0{\cdot}022$).

The results of investigations are summarized in Table II.

Table II. Summary of investigations to assess liver disease.

Investigation			*n*	%
Ultrasound ($n = 77$)	Hepatomegaly	Present	11	14
		Absent	66	86
	Echogenic liver	Present	17	22
		Absent	60	78
	Splenomegaly	Present	13	17
		Absent	64	83
Endoscopy* ($n = 50$)	Varices	Present	7	14
		Absent	43	86
Laparoscopy ($n = 33$)	Liver inflammation	Mild	21	64
		Marked	12	36
	Liver fibrosis	None	16	49
		Mild	9	27
		Pronounced	8	24
	Portal hypertension	Present	4	12
		Absent	29	88
Liver biopsy ($n = 22$)	Histology	Mild	8	36
		Moderate	9	41
		Severe	5	23

*Two patients assessed by barium swallow.

Abdominal ultrasound

Of the 77 patients who had an abdominal ultrasound scan there were 11 (14%) who were identified as having hepatomegaly, 17 (22%) with abnormal liver echogenicity, and 13 (17%) with splenomegaly. No focal hepatic lesions were identified.

Upper GI endoscopy

Oesophageal varices were identified in seven (14%) of 50 patients in whom endoscopy or barium swallow was performed. The varices were grade 1 in four, grade 2 in two, and grade 3 in one. There were no bleeding complications following endoscopy.

Liver laparoscopy

The liver was successfully visualized in 33 patients. One procedure failed due to the presence of adhesions from previous abdominal surgery. Of the patients who had an adequate laparoscopy there were 16 (49%), nine (27%) and eight (24%) with none, mild or pronounced fibrosis respectively. In five of the eight with pronounced fibrosis the appearances were those of established cirrhosis. Inflammation

was present in all patients being mild in 21 (64%) and marked in 12 (36%). Appearances consistent with portal hypertension were present in four (12%) patients.

Liver biopsy

Of the 87 patients in the group, 22 underwent successful liver biopsy. Of the remaining 65 patients, liver biopsy was not performed for the following reasons: patient declined ($n = 41$); technical failure ($n = 1$); von Willebrand's disease ($n = 8$); inhibitor ($n = 5$); serum HCV RNA negative by PCR ($n = 8$); evidence of cirrhosis visible at laparoscopy ($n = 2$).

Of the 23 liver biopsies attempted, 22 were successful and adequate specimens were obtained. In one patient the procedure was abandoned due to inadequate sedation. There were no serious bleeding problems associated with the procedure and haemostasis was secured prior to the withdrawal of the laparoscope.

The final histological appearances were classified as mild, moderate or severe (including cirrhosis) in eight, nine and five cases respectively. There was a strong correlation (Spearman rank correlation: $r = 0{\cdot}81$, $P < 0{\cdot}001$) between the Edinburgh visual histological classification and the Sheffield score (Fig 1). As the Sheffield system does not specifically score fibrosis it is not unexpected that biopsies in the Edinburgh moderate and severe groups have similar Sheffield scores.

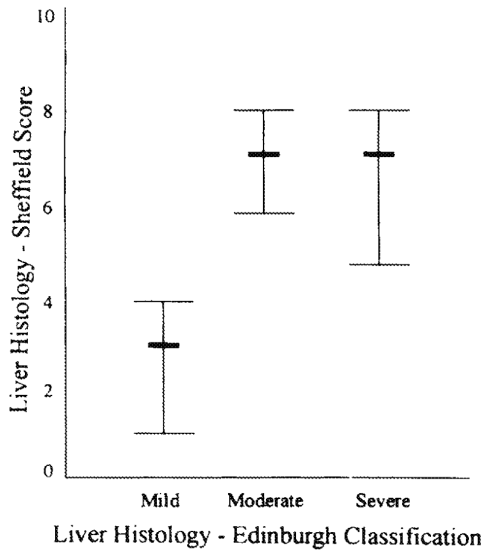

Fig 1. Comparison of Edinburgh histological classification and Sheffield scoring system. The median Sheffield score is shown by the bold line and the range by the error bars for each histological severity.

There were no serious bleeding complications associated with endoscopy, laparoscopy or liver biopsy. In one patient following liver biopsy there was a small hepatic haematoma on the 24 h post-biopsy ultrasound scan; this had resolved by 48 h. A rectus sheath haematoma developed in another patient with severe haemophilia A who was not pretreated with factor VIII concentrate prior to the removal of a suture on day 7 post laparoscopy. This responded to factor VIII replacement. There was no prolongation of the planned duration of hospital stay in any patient.

Predictive value of less invasive investigations

Liver histology was available in 22 patients in whom liver biopsies were performed. We assessed if liver histology could have been predicted on the basis of combined information derived from ultrasound (presence or absence of splenomegaly), endoscopy (presence or absence of varices) and laparoscopy (degree of hepatic fibrosis). HCV RNA was detected in 18 and three were anti-HIV positive. Oesophageal varices were identified in seven patients and four of these underwent liver biopsy.

There was agreement between the severity of liver disease from histology when compared with information from other investigations in 18 (86%) of the biopsied patients (Table III). In only one case was the histological appearance more severe than expected. In two cases with established oesophageal varices indicating portal hypertension, the liver histology was less severe than expected (moderate in both). Specificity and sensitivity of the predictive value of less invasive investigations is 88% and 80% respectively ('mild' and 'moderate' histological categories are combined for this calculation).

Table III. Relationship between liver histology and other investigations of liver disease.

		Liver histology		
		Mild	Moderate	Severe
Varices at endoscopy *or* splenomegaly at ultrasound *or* pronounced fibrosis at laparoscopy	Yes	0	2	4
	No	8	7	1

HIV infection and severity of liver disease

Of the 18 anti-HIV positive individuals, ultrasound scan, laparoscopy, endoscopy and liver biopsy were performed in 15, three, 10 and three respectively. Splenomegaly was detected in 6/15; oesophageal varices were present in 3/10; liver inflammation at laparoscopy was marked in 3/3 and surface fibrosis was pronounced in 1/3 and absent in 2/3.

Table IV. HIV status and severity of liver histology.

		Severity of liver histology		
		Mild	Moderate	Severe
HIV status	Negative	8	9	2
	Positive	0	0	3

Chi-square test for trend: $P = 0{\cdot}006$.

Liver histology was severe in all three patients who were biopsied. There was a highly significant association between HIV status and severity of liver histology (Table IV). This association is independent of age (median age of HIV-positive and HIV-negative patients who underwent liver biopsy was 32 and 35 respectively).

HCV genotype and severity of liver disease

The HCV genotype distribution is shown in Table V. 10/13 patients in whom serum HCV RNA was not detected had persistently normal serum ALT levels. In three, however, ALT levels were persistently raised. One of these individuals was HBsAg positive, but no definite cause for chronic hepatitis was found in the other two patients.

Table V. HCV genotype distribution.

HCV genotype	*n*	%
1a	32	37
1b	15	17
2a	1	1
2b	3	3
3a	22	25
5	1	1
PCR negative	13	15
Total	87	100

There was no evidence of any differences in mean ALT between HCV genotypes, although genotype 3a had the widest range of values (Fig 2). There were no statistically significant differences in laparoscopic appearances or liver histology between genotypes (Kruskal-Wallis test: $P = 0{\cdot}23$). Although only small numbers were assessed, there was a suggestion that genotype 1b might be associated with more severe histological appearances (Table VI).

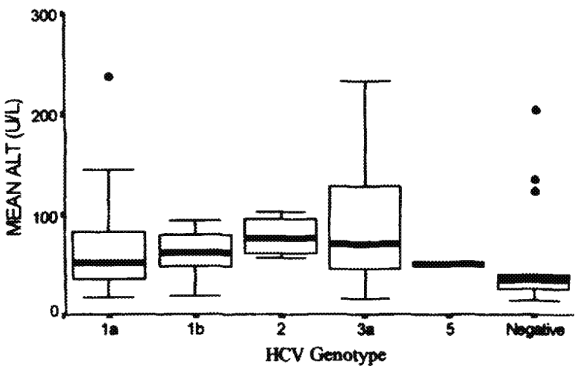

Fig 2. Box plot of mean ALT and HCV genotype. The median ALT is represented by the solid line. For each genotype ALT values between the 25th and 75th centiles are represented in the box. Values $<1{\cdot}5$ box-lengths outside the 25th or 75th centiles are shown by the error bars. Values $>1{\cdot}5$ box-lengths from the 25th or 75th centiles are shown as individual dots. There was no significant difference in ALT values between genotypes (Kruskal-Wallis test: $P = 0{\cdot}20$).

Table VI. HCV genotype and liver histology.

	Severity of liver histology		
Genotype	Mild	Moderate	Severe
1a	4	2	2
1b	0	0	2
2a	0	1	0
2b	0	1	1
3a	2	3	0

DISCUSSION

Chronic liver disease due to HCV has emerged as a serious problem for individuals with haemophilia. The majority who received non-virus-inactivated concentrates have persistent HCV infection characterized by fluctuating ALT and virus levels (Fletcher *et al*, 1983). Effective elimination of HCV from coagulation concentrates has been achieved by virus-inactivation steps in the manufacturing process which were introduced in the mid-1980s. Even in recent years, however, there have been occasional reports of possible HCV transmission by concentrates, and there is clearly a need for continued close surveillance (Berntorp *et al*, 1990; Schulman *et al*, 1992).

Currently, haemophiliacs with chronic HCV infection have been exposed to the effects of the virus for between 10 and 25 years, and the spectrum of liver disease is partly a reflection of the variation in the duration of infection. As more information becomes available concerning the natural history of HCV infection, it is clear that complications generally arise only after prolonged infection. This observation may explain why early studies of liver disease in haemophiliacs suggested that chronic NANBH was essentially benign and non-progressive (Stevens *et al*, 1983), whereas later studies, due to the passage of time and progression of the natural history of HCV infection, have demonstrated development of serious liver disease (Hay *et al*, 1985) in a significant and increasing number of patients. The incidence of hepatocellular carcinoma is also increasing in haemophiliacs (Colombo *et al*, 1991; Preston *et al*, 1995a). Recently the U.K. Haemophilia Centre Directors Organization have suggested guidelines for the management of chronic liver disease in haemophilia which attempt to address some of the issues in the practical management of haemophiliacs infected with HCV (Preston *et al*, 1995b).

We have confirmed that a significant number of haemophiliacs have evidence of serious liver disease with direct or indirect evidence of cirrhosis in around 25%. HIV co-infection is associated with more severe histological appearances suggesting that HIV accelerates progression of HCV-related liver disease in haemophiliacs (Eyster *et al*, 1993; Telfer *et al*, 1994). In those with established cirrhosis there is clearly the risk of hepatic decompensation or the development of hepatocellular carcinoma. Close surveillance of this group is required, and the most appropriate treatment options, including liver transplantation, should be based on the circumstances of each individual.

In those without established cirrhosis the best approach to treatment and follow-up is less clear. Interferon treatment has been used with varying success in haemophiliacs with chronic HCV (Makris *et al.* 1991; Bresters *et al.* 1992; Peerlinck *et al.* 1994; Mauser-Bunschoten *et al.* 1995; Telfer *et al.* 1995; Hanley *et al.* 1996). The most recent studies suggest the overall sustained response rate is very low (Telfer *et al.* 1995; Hanley *et al.* 1996). The value of using interferon for longer than 6 months as well as the role of low-dose maintenance therapy remain to be evaluated in haemophiliacs.

It is clear, however, in the absence of more effective therapy for HCV, monitoring for the development of cirrhosis is the most important part of management of liver disease in haemophiliacs. At present it is not clear how many will progress to cirrhosis, and there is considerable debate about the best way to monitor these cases. In particular, the role of liver biopsy remains controversial.

Liver biopsy is a well-established and widely-used diagnostic procedure in non-haemophiliacs. In addition, serial biopsies may be useful either to monitor response to treatment or disease progression. What is the current role of liver biopsy in haemophiliacs with chronic liver disease? Several studies of liver biopsy in haemophiliacs have been performed since the first in 1977 (Lesesne *et al.* 1977; Spero *et al.* 1978; Mannucci *et al.* 1978; Hay *et al.* 1985; White *et al.* 1982; Aledort *et al.* 1985; Schimpf, 1986; Makris *et al.* 1991). From a review of the literature, most groups report that the procedure may be performed safely following appropriate coagulation factor replacement. In fact, apart from an anecdotal reference in one paper to two deaths (Aledort *et al.* 1985), there are no published reports of mortality associated with liver biopsy in haemophiliacs, and in excess of 250 biopsies have now been reported. So, if the procedure is safe, should the clinical indications for performing liver biopsy in haemophiliacs be the same as non-haemophiliacs? Non-haemophiliacs, with persistently abnormal liver function tests, with or without serological and PCR evidence of HCV infection, would almost inevitably have a liver biopsy as part of their initial evaluation. The liver biopsy serves a number of purposes including to confirm the diagnosis histologically, exclude co-existing causes of liver pathology, stage the degree of disease, and to provide baseline histology for comparison with a repeat biopsy following therapy.

In the context of haemophiliacs, we now know that HCV is responsible for the vast majority of chronic liver disease in this group. Chronic HCV infection may be readily diagnosed by serological testing and persisting viraemia assessed by PCR. In addition response to treatment may be assessed by quantitative PCR to measure levels of circulating HCV RNA. The information gained from a liver biopsy, therefore, may not contribute to the decision to treat most patients – especially as the options for treatment are extremely limited at present.

Is it, however, important to accurately stage the disease? This is necessary in order to provide accurate information when counselling the individual concerning his overall outlook. In addition, it is important to identify those most at risk of developing hepatocellular carcinoma, i.e. those with established cirrhosis. In this study we have shown that it is possible to accurately stage liver disease without resorting to liver biopsy in the majority of patients. Using the information gained from a combination of investigations, it is possible to predict the severity of liver disease. Laparoscopic inspection of the liver surface will diagnose cirrhosis accurately and, as has recently been reported, other findings such as inflammation and fatty change correlate closely with histology (Jalan *et al.* 1995). Although laparoscopy is still invasive, the use of narrow-diameter instruments, particularly the new 2 mm microlaparoscpe, enable good visualization with minimal risk. In those with oesophageal varices present on endoscopy, cirrhosis is almost universally present. This study suggests that laparoscopic liver inspection, in conjunction with upper GI endoscopy and abdominal ultrasound, is an extremely useful method to stage and monitor the progression of liver disease in haemophiliacs without subjecting patients to repeated biopsies which, at worst, may have a cumulative morbidity and mortality and at best require a several-day hospital stay.

We have not addressed the use of CT scanning as a non-invasive method of assessing liver disease. This method has been reported to be useful in haemophiliacs (Johnson *et al.* 1983; Miller *et al.* 1988). Both CT and MRI scanning require further study in haemophiliacs.

It is interesting to note in this study that HCV does not account for 100% of chronic hepatitis in haemophiliacs. We identified three patients with biochemical evidence of chronic hepatitis despite being 'PCR negative' for serum HCV RNA. The PCR method is extremely sensitive and has a threshold of 80 HCV copies/ml. Thus, a low level of HCV replication is unlikely in these patients. It is possible that as-yet-unidentified hepatitis viruses are present in the haemophiliac population and the role of the recently described GB agents (Simons *et al.* 1995) needs further evaluation.

In conclusion, this study provides further evidence that liver disease is emerging as a major problem for haemophiliacs, in particular those co-infected with HIV. There is a need for close monitoring for the complications of chronic HCV infection and the precise role of laparoscopic liver inspection needs further evaluation. It is important to stress the importance of adequate training of those performing laparoscopic liver inspection and biopsy. At present we feel that potentially most individuals may be monitored without resorting to liver biopsy as long as the extent of liver disease is accurately staged by the combination of investigations described.

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SHORT REPORT

Development of anti-interferon antibodies and breakthrough hepatitis during treatment for HCV infection in haemophiliacs

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Summary. The development of anti-interferon antibodies may lead to treatment failure during interferon therapy. We have studied the development of such antibodies in a group of 39 haemophiliacs receiving interferon-α2a for chronic hepatitis C virus (HCV) infection.

Anti-interferon antibodies developed in five (13%) patients and were associated with 'breakthrough hepatitis' in three cases. There was an association between the development of anti-interferon antibodies and infection with HCV genotype 3a ($P=0{\cdot}01$). This study suggests that the development of anti-interferon antibodies may lead to treatment failure in a proportion of haemophiliacs with HCV infection. The association with genotype 3a has not previously been reported. Monitoring for the development of breakthrough hepatitis due to anti-interferon antibodies may provide the opportunity to develop strategies to overcome their effects.

Keywords: haemophilia, hepatitis C virus, genotype, interferon, anti-interferon antibodies.

Alpha interferon is used to treat hepatitis C virus (HCV) infection and response may be assessed both by serial measurement of serum alanine transaminase (ALT) or HCV RNA quantitation by polymerase chain reaction (PCR). Those who respond to interferon usually show a prompt normalization of ALT and clearance of serum HCV RNA within 8–12 weeks of treatment (Hanley *et al*, 1996). In non-responders ALT and HCV RNA remain unchanged.

In some individuals 'breakthrough hepatitis' may occur, i.e. initial response with normalization of ALT followed by a sustained rise in ALT, during interferon therapy. It has been suggested that such episodes of 'breakthrough hepatitis' may be caused by anti-interferon antibodies (Milella *et al*, 1993). Therefore we investigated the development of anti-interferon antibodies in a group of 39 haemophiliacs receiving interferon-α2a for chronic HCV infection.

PATIENTS AND METHODS

Patient characteristics. A total of 39 patients (37 male, two female) were studied (26 haemophilia A, nine haemophilia B, four von Willebrand's disease). The median age was 35·5 years (range 13–67 years). Six patients were anti-HIV positive and all were HBSAg negative.

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All patients were anti-HCV positive by Abbott second-generation enzyme immunoassay (A-EIA; Abbott, Weisbaden-Dalkenheim, Germany) and also positive on confirmatory testing by second-generation recombinant immunoblot assay (RIBA-2, Chiron Corporation, Emeryville, Calif.). All had previously received non-virus-inactivated coagulation factor concentrates prior to 1985 and 38/39 had persistently elevated serum ALT levels (normal range 10–40 U/l). HCV RNA was detected in 38/39 patients by RT-PCR (Jarvis *et al*, 1994) and quantified by a limiting dilution technique (Simmonds *et al*, 1990). The lower limit of detection using this assay was 80 HCV copies/ml. HCV genotyping was performed as described previously (Davidson *et al*, 1995).

Drug dosage and administration. Interferon-α2a (Roche) 3 Mega Units three times per week was given subcutaneously. The intention was to treat for 24 weeks. Response was assessed on the basis of serial monthly ALT levels and HCV RNA quantitation.

Measurement of anti-interferon antibodies. Serum samples for anti-interferon antibody assays were taken and stored from all patients at 0, 12 and 24 weeks. All samples were tested at the end of treatment. No response or genotype data was provided to the laboratory performing the anti-interferon antibody assays. All samples were tested using an enzyme immunoassay (EIA) which detects binding antibodies (Hennes *et al*, 1987). Those samples which were positive by EIA were also assessed by an antiviral

Table I(a). Characteristics of the five patients who developed anti-interferon antibodies.

Patient	Age (yr)	HCV genotype	ALT response	HCV RNA response	Anti-interferon antibody: EIA	Anti-interferon antibody: ANB	Duration of antibody persistence (months)
1	16	3a	Yes + BTH	Yes + BTV	Pos	Pos	6
2	38	3a	Yes + BTH	Yes + BTV	Pos	Neg	4
3	13	1b	Yes + BTH	No	Pos	Pos	4
4	67	3a	Yes	Yes	Pos	Pos	5
5	33	3a	No	Yes	Pos	Neg	6

BTH: breakthrough hepatitis; BTV: breakthrough viraemia.

Table I(b). HCV genotype and anti-interferon antibody development showing a significant association with genotype 3a ($P = 0.01$).

Genotype	Anti-interferon antibodies	Total
1a	0	15
1b	1	9
2a	0	1
2b	0	2
3a	4	11

neutralization bioassay (ANB) which demonstrates the neutralizing properties of the antibody (Hennes *et al.* 1987).

RESULTS

Binding anti-interferon antibodies were detected by EIA in five (13%) patients (Table Ia). Neutralizing properties were demonstrated by ANB in 3/5 patients. The timing of antibody development and antibody titre varied (Fig. 1). Breakthrough hepatitis occurred in three patients (nos. 1–3) who developed antibodies and this was accompanied by breakthrough viraemia in two of these (nos. 1 and 2).

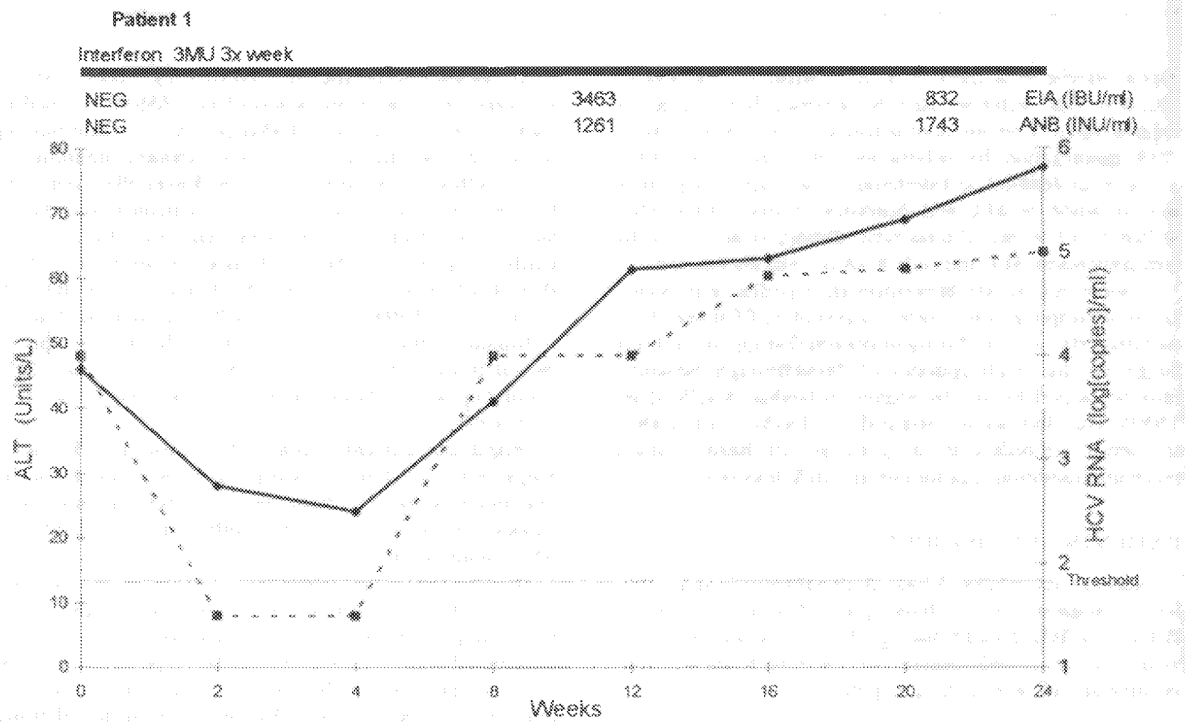

Fig 1. Timing of anti-interferon antibody development in relation to ALT (——) and HCV RNA (— — —) levels during interferon treatment in patients 1–5. EIA (IBU/ml): enzyme immunoassay (interferon binding units/ml); ANB (INU/ml): anti-viral neutralization bioassay (interferon neutralizing units/ml).

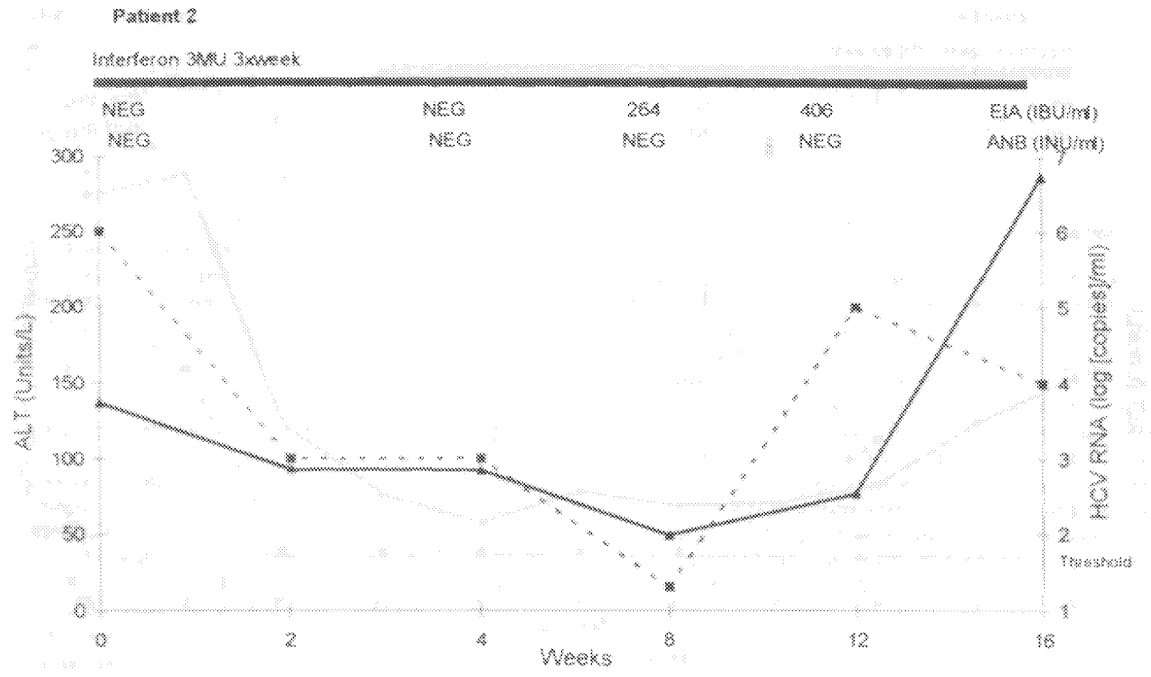

Fig 1 (continued) Patient 2.

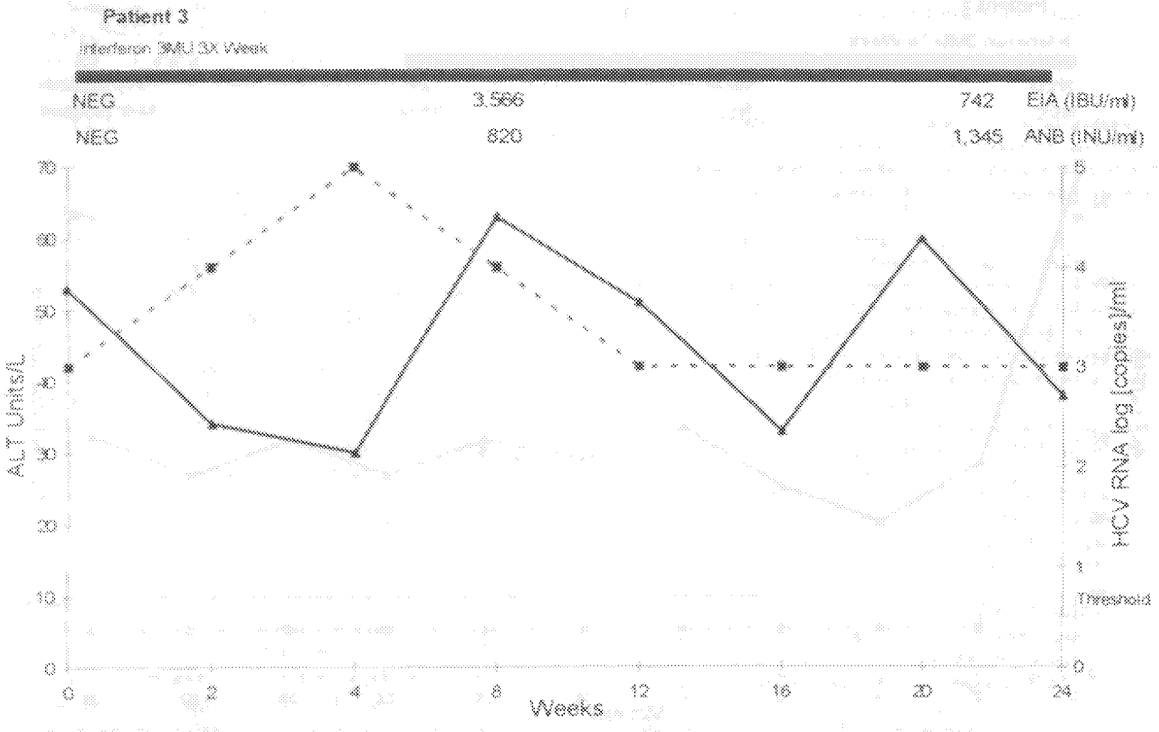

Fig 1 (continued). Patient 3.

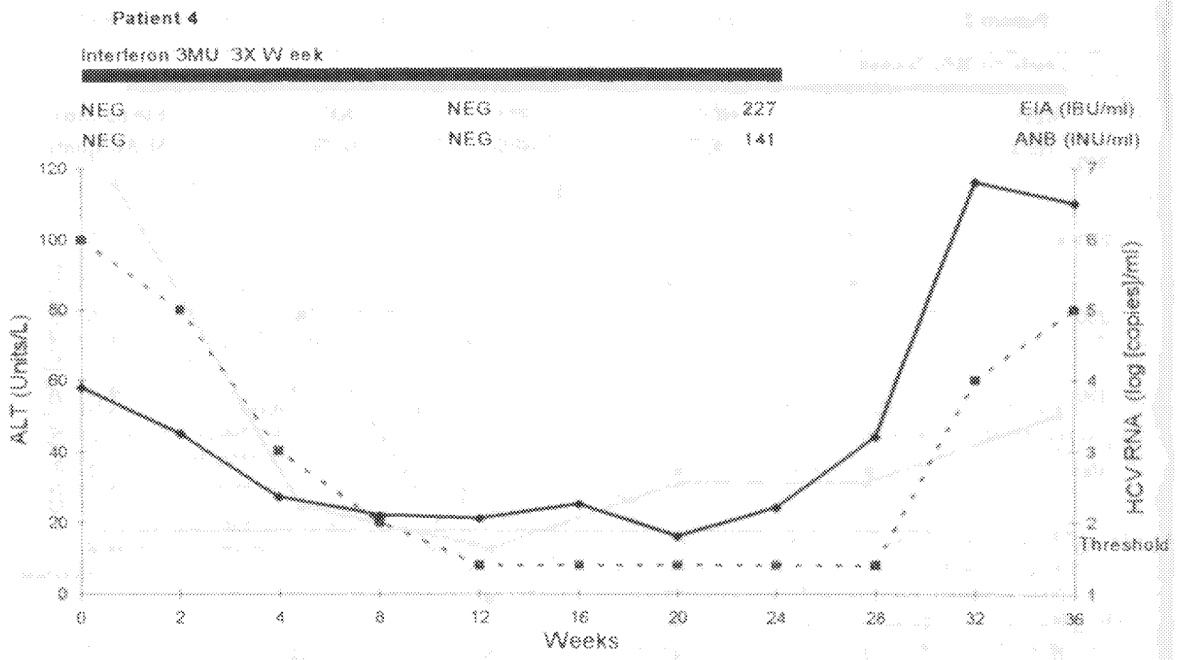

Fig 1 (*continued*). Patient 4.

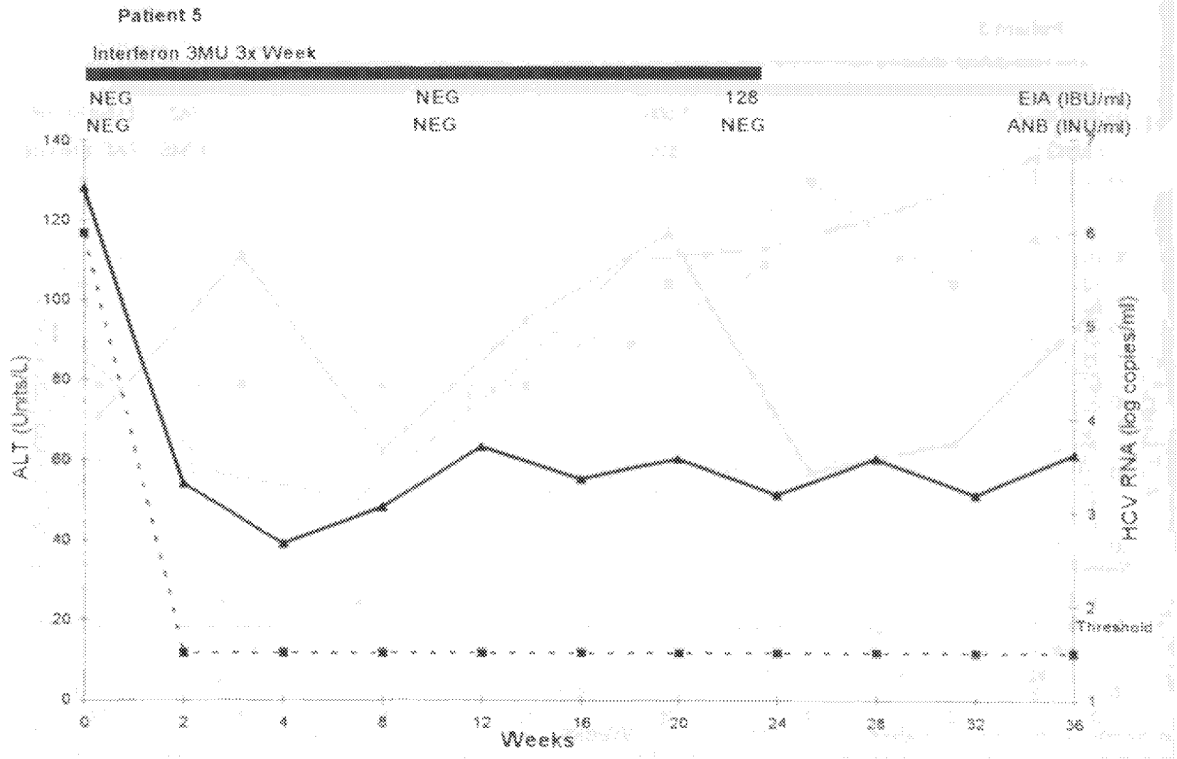

Fig 1 (*continued*). Patient 5.

Breakthrough hepatitis and viraemia occurred in only one additional patient without evidence of anti-interferon antibodies which was thought to be due to intermittent compliance with interferon therapy (data not shown).

There was a significant association with HCV genotype 3a (chi-square test: $P = 0{\cdot}01$) (Table Ib). There were no changes in HCV genotype detected in any patients during interferon treatment.

Follow-up information. After cessation of interferon therapy, follow-up assays were performed in those individuals who developed antibodies. Within 4–6 months, both binding and neutralizing antibodies had either reduced to low titre or become undetectable (Table I).

DISCUSSION

Interferon therapy for HCV infection in non-haemophiliacs leads to a sustained response in approximately 25% of individuals. Recent studies have shown that the response to interferon in haemophiliacs with HCV infection is inferior and a standard treatment schedule (3 MU three times per week for 6 months) leads to few sustained responses (Telfer *et al*, 1995; Hanley *et al*, 1996). Factors which influence response to interferon include age, duration of infection, severity of liver disease, pre-treatment virus load and HCV genotype. Evaluation of treatment protocols using higher doses or more prolonged courses of interferon in combination with other anti-viral drugs have yet to be reported in haemophiliacs.

We have shown that the development of anti-interferon antibodies leading to breakthrough hepatitis may contribute to the poor response to interferon in haemophiliacs. The importance of timing of antibody development in relation to the response to interferon has been noted by others (Bonetti *et al*, 1994). In our study, antibodies which appeared at the same time as ALT normalization (nos. 1–3) or HCV RNA clearance (nos. 1 and 2) led to the development of breakthrough hepatitis or viraemia. On the other hand, antibodies which appeared many weeks after the therapeutic response (nos. 4 and 5) were not associated with breakthrough. It is possible, however, that the late appearance of antibody was a factor in the early relapse seen in patient 4 after stopping interferon.

The association shown in this study between anti-interferon antibody development and infection with HCV genotype 3a has not previously been reported. This observation may be particularly important, because genotype 3a is usually associated with a favourable response to interferon. It is also interesting to note that of the five individuals who developed anti-interferon antibodies, four were aged <40 years. Anti-interferon antibodies are unusual in younger patients (Porres *et al*, 1989) and haemophiliacs may be at increased risk of antibody formation due to immunological abnormalities, irrespective of HIV infection (Watson & Ludlam, 1992).

It is important to recognize that breakthrough hepatitis may occur for reasons other than the development of anti-interferon antibodies, such as does reduction necessitated by side-effects or due to poor compliance. In addition, there is some evidence that each HCV genotype circulates as a heterogenous group of variants (terms 'quasi-species') which evade host immune responses by continual evolution of new mutants (Enomoto *et al*, 1994). In theory, when interferon is given, initial response may be overcome by the rapid evolution of quasi-species (without a change in the major circulating genotype). It has also been reported that the major circulating genotype may change during interferon treatment (Devereux *et al*, 1995). We, however, found no evidence of changing genotypes in this study.

The clinical importance of anti-interferon antibodies is not yet fully understood; however, the development of breakthrough hepatitis in individuals with factors usually associated with a favourable response to interferon (e.g. young age, genotype 3a, low pre-treatment virus load, and absence of cirrhosis) is particularly important to detect. Anti-interferon antibody assays should be performed when breakthrough hepatitis develops during interferon treatment. The recognition of antibody development may lead to the avoidance of treatment failure either by dose escalation or switching to lymphoblastoid interferon which may not cross react with the antibody. The effectiveness of such approaches is yet to be evaluated.

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