OBSERVATION OF TREATMENT RESPONSES TO INTERFERON-α
BY QUANTITATIVE ANALYSES OF HEPATITIS C VIRUS RNA

by

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We accept this thesis as conforming
to the required standard

UNIVERSITY OF BRITISH COLUMBIA
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ABSTRACT

Non-A, non-B (NANB) hepatitis accounts for 90% of cases of transfusion associated hepatitis and 20-40% of sporadic cases. Most of them become chronic and develop severe consequences such as cirrhosis and primary hepatocellular carcinoma. Recently, *hepatitis C virus* (HCV), an RNA virus, was identified as the major etiological agent of NANB hepatitis. Interferon-α (IFNα) has been shown to be beneficial in treatment of patients with chronic hepatitis C by decreasing alanine amino-transferase (ALT) levels and improving liver histology, since IFNα has a wide spectrum of antiviral activities. Unfortunately there is little information about the state of HCV replication during or after IFNα treatment. It is not clear whether therapy eradicates the presence of the virus or merely suppresses viral replication. Therefore, the relationship between response to IFNα treatment and suppression of viral activity during therapy requires further study.

We hypothesized that: 1) IFNα could inhibit the replication of HCV in the patient with chronic hepatitis C. 2) HCV-RNA would decrease or disappear in both serum and liver cells in the responding patients. 3) the changes of HCV-RNA in both serum and liver cell could be measured by the quantitative RT-PCR detection.
The overall objective of this study was to evaluate therapeutic efficacy of IFNα for the treatment of chronic hepatitis C. The following specific aims were: 1) to reveal the state of HCV replication, as quantitatively measured by serum and liver HCV-RNA, in patients with chronic hepatitis C before and after treatment with αIFN. 2) to compare quantitative analysis results of HCV-RNA with serum AST levels as a marker of hepatic necroinflammatory changes. 3) to determine whether apparently favourable changes in disease activity are correlated with suppression of viral replication.

A quantitative reverse transcription polymerase chain reaction (RT-PCR) assay was established for the detection of HCV-RNA. Sensitivity of the RT-PCR assay was quantitatively analyzed by a piece of recombinant HCV-RNA which was in vitro transcribed from a transcription vector HCV-cDNA. Specificity of the RT-PCR assay was verified by Southern Blot and DNA typing methods.

Serum HCV-RNA was quantitatively detected by the established RT-PCR assay on 12 patients with chronic hepatitis C and treated with interferon-α. Liver HCV-RNA was also measured in 4 of 12 patients. The therapeutic efficacy of interferon was assessed by the correlation between clinical responses and quantitative results for HCV-RNA.

The results showed that 7 of 12 (58%) patients had a beneficial response to
IFNα treatment at 3-6 months of therapy as reflected by a decreased titer of HCV-RNA. Four of 7 (57%) initial responders relapsed 3 months later. Three (25%) patients’ sera became negative for HCV-RNA after more than 12 months in treatment. Serum HCV-RNA titers had parallel changes with liver function in 7 (58%) patients. There was a positive correlation between patients with lower serum HCV-RNA titers prior to initiation of treatment and better therapeutic responses.

Although similar trials need to be carried out on large sample size of patients, the following conclusions could be drawn from preliminary results: interferon-α has a beneficial effect on more than half the patients with chronic hepatitis C by inhibiting viral replication and improving the liver function within the first 6 months of treatment; long term IFNα treatment (6-12 months or more) may increase the probability of eradicating HCV; the results of quantitative analysis on HCV-RNA may be used as a valuable mean to predict and monitor the therapeutic response of IFNα.

Topics that further studies should focus on are to monitor the therapeutic response by using other means such as measurement of 2’,5’-oligoadenylate synthetase activity, to reveal the possible reason for poor response such as genotype variation and anti-IFN antibody, to try other approaches to IFN administration, and to develop other antiviral agents.
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<tr>
<td>2-5A</td>
<td>2',5'-oligoadenylate synthetase</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine amino-transferase</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary deoxyribonucleic acid</td>
</tr>
<tr>
<td>C-100</td>
<td>Recombinant antigen for ELISA</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>IFNα</td>
<td>Interferon-α</td>
</tr>
<tr>
<td>NANB</td>
<td>Non-A, non-B</td>
</tr>
<tr>
<td>NS</td>
<td>Non structural</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
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CHAPTER 1

INTRODUCTION

Non-A, non-B (NANB) hepatitis accounts for 90% of cases of transfusion associated hepatitis and 20-40% of sporadic cases of viral hepatitis. Furthermore, post transfusion NANB hepatitis becomes chronic in as many as 60% of cases and at least 20% of patients with chronic hepatitis will eventually develop severe consequences such as cirrhosis and primary hepatocellular carcinoma. Recently, the major etiological agent of NANB hepatitis was identified as an RNA virus, the *hepatitis C virus* (HCV). Based on the result from first generation ELISA testing for anti-HCV antibody, HCV is the cause of approximately 80% of parenterally acquired NANB hepatitis.

Interferon-α (IFNα) has been shown to be beneficial in the treatment of patients with chronic hepatitis C by decreasing alanine amino-transferase (ALT) levels and improving liver histology, since IFNα has a wide spectrum of antiviral activities. Unfortunately there is little information about the state of HCV replication during or after IFNα treatment. It is not clear whether therapy eradicates the virus or merely suppresses viral replication. Therefore, more studies are needed to clarify the relationship between response to αIFN treatment and suppression of viral activities during the therapy.

Reverse transcription polymerase chain reaction (RT-PCR), a valuable technique which enables the direct detection of HCV-RNA in serum and liver, has provided a viral
replication marker for the observation of chronic hepatitis C infection. Quantitative detection by the RT-PCR is capable of detecting the amount of HCV-RNA in serum from the patients treated with αIFN. If the serum concentration of HCV-RNA is correlated with the response to IFN therapy, HCV-RNA detection could play an important role in monitoring the antiviral effects of IFNα.

We hypothesized that: 1) IFNα could inhibit the replication of HCV in the patient with chronic hepatitis C. 2) HCV-RNA would decrease or disappear in both serum and liver cells in the responding patients. 3) the changes of HCV-RNA in both serum and liver cell could be measured by the quantitative RT-PCR detection.

The overall objective of this retrospective study was to determine the utility of RT-PCR for monitoring therapeutic efficacy of IFNα for treatment of chronic hepatitis C. The following specific aims were: 1) to determine the degree of HCV replication by quantitative detection of serum HCV-RNA and both genomic and replicative forms of liver HCV-RNA in patients with chronic hepatitis C infection before, during and after treatment with IFNα. 2) to compare quantitative analysis results of HCV-RNA with serum ALT levels as a marker of hepatic necroinflammatory changes. 3) to determine whether apparently favourable changes in disease activity correlated with suppression of viral replication.

The research plan for this research project was: 1) to collect serum and liver biopsy specimens from chronic hepatitis C patients who were treated with IFNα2B. 2) to extract HCV-RNA from these serum and hepatic tissues. 3) to establish a reverse transcription-polymerase chain reaction (RT-PCR) assay for the detection of HCV-RNA.
4) to quantify the sensitivity of this RT-PCR detection system. 5) to quantitatively detect HCV-RNA from serum and liver by using the established RT-PCR method. 6) to assess the therapeutic efficacy of interferon by the correlation between clinical responses and quantitative analysis results of HCV-RNA.

Interferon is a natural choice as a possible therapeutic agent for chronic hepatitis C due to its wide spectrum of antiviral activities. The treatment responses have been widely studied. Some clinical trials have being carried out to observe the therapeutic effects of IFNα on the chronic hepatitis C while we were performing this research. In order to evaluate the treatment responses, results from several other reports are reviewed in the thesis. It appears that there are some dissimilarities in the results obtained from different groups. Overall, IFN treatment still needs more studies to evaluate efficacy. From this study, we believe: 1) the method of serial quantitative analyses of HCV-RNA should be very useful for evaluation and prediction of IFNα treatment responses. 2) the results would lead to a deeper understanding of the therapeutic functions of IFNα. 3) the conclusion could make useful recommendations for viral monitoring in further clinical trials in larger groups of patients. 4) the questions raised will provide a basis for further studies to clarify the true treatment effects of IFNα in the future.
2.1 FROM NON-A, NON-B HEPATITIS TO HEPATITIS C

Our knowledge of non-A, non-B (NANB) hepatitis has increased dramatically since the historical description of the infectious agent which may contribute to most of the disease. Although many scientists had made efforts to prove their existence as infectious entities from clinical, epidemiologic, and experimental investigations, the putative agents for NANB hepatitis remained unknown until the exciting discovery in 1989. In this year, investigators from the Chiron Corporation in California, USA, reported that the genome of an RNA virus was successfully cloned from the plasma of a chimpanzee infected with material from a patient with non-A, non-B hepatitis, and designated as hepatitis C virus (HCV) (Choo QL, 1989). It has become evident from following studies that the hepatitis C virus is responsible for the majority of NANB hepatitis.

2.2 EPIDEMIOLOGY OF HEPATITIS C VIRUS INFECTION

As soon as the isolation and cloning of HCV was accomplished, an enzyme-linked immunosorbent assay (ELISA) was developed to detect anti-HCV antibody by using recombinant C100-3 antigen, a translation product of the non-structural (NS) region in HCV genome (Kuo G, 1989), which resulted in worldwide studies on the prevalence of
HCV infection. The preliminary evaluation based on the anti-HCV test results showed that HCV was responsible for the majority of cases of post-transfusion and sporadic NANB hepatitis, as well as most cases of unidentified chronic liver diseases throughout the world (Esteban R, 1993). The results of worldwide studies on HCV prevalence in different risk groups are reviewed in Table 1 (page 6).

2.3 THE GENOMIC STRUCTURE OF HCV

Using sophisticated molecular biological approaches the new termed hepatitis C virus was identified as a positive-sense, single-stranded, linear RNA virus with a genomic size of approximately 10Kb. The sequence of a major portion of the viral genome has been reported, and the virus has been related to members of the flavi- and pestiviruses (Houghton M, 1991).

The HCV-RNA genome, comprising approximately 9,400 nucleotides (nt) (Choo QL, 1991; Kato N, 1990), contains a single, large translational open-reading frame (ORF) that spans almost the entire genome and encodes a large viral peptide of either 3,011 (Choo QL, 1991.) or 3,010 (Kato N, 1990; Takamizawa A, 1991) amino acids. As shown on the Fig. 1, the HCV genome is composed of 7 regions: C, E1, E2/NS1, NS2, NS3, NS4, and NS5, each of which encodes different products (Houghton M, 1991; Okamoto H, 1992; Hayashi N, 1993).
### Table 1. Prevalence of HCV infection in different risk groups*

<table>
<thead>
<tr>
<th>Transmission Type</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parenteral Transmission</td>
<td></td>
</tr>
<tr>
<td>Transfusion-associated hepatitis</td>
<td></td>
</tr>
<tr>
<td><em>Cellular blood products</em></td>
<td>50-70%</td>
</tr>
<tr>
<td><em>Plasma products</em></td>
<td>50-90%</td>
</tr>
<tr>
<td>Intravenous drug users</td>
<td>70-92%</td>
</tr>
<tr>
<td>Haemodialysis and renal transplantation</td>
<td>9-25%</td>
</tr>
<tr>
<td>Nosocomial and occupational exposure</td>
<td>1-3%</td>
</tr>
<tr>
<td>Non-Parenteral Transmission</td>
<td></td>
</tr>
<tr>
<td>Perinatal Transmission</td>
<td>50%</td>
</tr>
<tr>
<td>Sexual and household transmission</td>
<td>5-23%</td>
</tr>
<tr>
<td>Other Liver Diseases</td>
<td></td>
</tr>
<tr>
<td>Chronic hepatitis B</td>
<td>17-40%</td>
</tr>
<tr>
<td>Alcoholic liver disease</td>
<td>25-52%</td>
</tr>
<tr>
<td>Cryptogenic hepatitis</td>
<td>68-80%</td>
</tr>
<tr>
<td>Autoimmune hepatitis</td>
<td>40-80%</td>
</tr>
<tr>
<td>Hepatocarcinoma</td>
<td>34-75%</td>
</tr>
</tbody>
</table>

* See Esteban R, 1993 for references.
It should be emphasized that the 5' terminal region of HCV genome represents the most highly conserved sequence among different viral isolates (Takamizawa A, 1991; Okamoto H, 1991), this suggests that it may play a very important regulatory role during viral replication, perhaps at the level of translation. Meanwhile, this is also important for the design of oligonucleotide primers to develop general PCR for HCV-RNA detection, in order to exclude the possibility of missing viraemia due to sequence heterogeneity (Okamoto H, 1991; Han JH, 1991; Garson JA, 1990).
2.4 DIVERSITY OF HCV GENOME

A great deal of information is now appearing in the literature about the nucleotide sequence of different HCV isolates. Comparative sequence analysis of all complete and partial HCV sequences published to date indicates that they can be broadly subdivided into at least four basic groups (Table 2).

Table 2. Type classification of HCV*

<table>
<thead>
<tr>
<th>TYPE-I</th>
<th>TYPE-II</th>
<th>TYPE-III</th>
<th>TYPE-IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV-1</td>
<td>HCV-J</td>
<td>HC-J6</td>
<td>HC-J8</td>
</tr>
<tr>
<td>HCV-H</td>
<td>HCV-BK</td>
<td>HC-J5</td>
<td>HC-J7</td>
</tr>
<tr>
<td>HCT18,23,27.</td>
<td>HC-J4</td>
<td>HCV-K2a</td>
<td>HCV-K2b</td>
</tr>
<tr>
<td>HCV-E1</td>
<td>HC-J2</td>
<td></td>
<td>clone A</td>
</tr>
<tr>
<td>EC1,10.</td>
<td>HCV-K1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM1,2.</td>
<td>HCV-JH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCV-J1</td>
<td>HCV-T3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCVpt-1</td>
<td>C8-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HC-J1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Based on the comparison of sequence in different HCV isolates, it is revealed that they are different in both nucleotide and polypeptide sequences in NS3, NS4, NS5 and envelope regions (Hayashi N, 1993; Choo Q-L, 1991; Takeuchi K, 1990). Among these, the protein containing an N-terminal hypervariable region of about 30 amino acids shows large variation between nearly all isolates (Weiner AJ. et al., 1991). The hypervariation may be a consequence of a strong selection pressure on a protective B cell or T cell epitope(s).

The significance of HCV gene diversity is:

1. Heterogeneity observed between different HCV isolates deserves close attention with respect to virus/host interactions, evolution of chronicity and vaccine development (Houghton M, 1991).

2. Multiple infections are caused by different HCV agents, which is proposed by the important clinical aspects investigated among the patients in United States, Japan and Europe (Weiner AJ, 1991; Simmonds P, 1990; Tsukiyama-Kohara K, 1992).

3. The diversity of genome should be considered for the detection of viral RNA as the primers are designed for PCR. Some research results show it is possible to increase the sensitivity of PCR detection by using primers designed from both the most conserved 5' terminal region and the diverse region (Hideki H, 1992; Jenes B, 1992).
2.5 DETECTION OF ANTI-HCV ANTIBODY AND HCV-RNA

2.5.1 Detection of HCV Antibody

Initial development of ELISA by using recombinant C100-3 as antigen (first generation) has resulted in a large number of reports on the prevalence of HCV infection and significance of detectable antibodies against HCV. Meanwhile, the unsatisfactory sensitivity of C100-3 ELISA is being reported by more and more investigators (John G, 1992; McFarlane IG, 1990). Because heterogeneity of the HCV genome was further understood by nucleotide and peptide sequence analyses later, the reason for the poor sensitivity of C100-3 ELISA has been realized. C100-3 recombinant antigen is the expression product of nearly all of the NS4 region of pro-isolate HCV-1. However, the NS4 region is one of the diverse areas among different HCV genome groups (Choo QL, 1991; Juo G, 1989). It means that c100-3 ELISA is not capable of detecting antibodies against all genotypes of HCV. Therefore, scientists are trying to find antigens expressed from more conserved regions or combinations of different antigens for a more sensitive HCV antibody test. Now many antigens from different regions are artificially synthesized or recombinantly expressed in E.coli for the ELISA and Recombinant Immunoblot Assay (RIBA) to monitor the antibody. The antigens and encoded genomic regions are shown in Figure 2. (Prohaska W, 1992; Hosein B, 1991; Nasoff MS, 1991; Okamoto H, 1990).

Using the different recombinant antigens above, the following improved methods are established for HCV antibody detection to increase the sensitivity (Table 3):
Figure 2. HCV recombinant antigens and genomic location

Genomic location of Recombinant antigens

![Genomic location of Recombinant antigens](image)

Table 3. Methods for anti HCV antibody detection

<table>
<thead>
<tr>
<th>Methods</th>
<th>Antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ELISA:</strong></td>
<td></td>
</tr>
<tr>
<td>1. First Generation:</td>
<td>C100-3, or 5-1-1.</td>
</tr>
<tr>
<td>2. Second Generation:</td>
<td>C100-3, 5-1-1, C22c, C33c.</td>
</tr>
<tr>
<td>3. Third Generation:</td>
<td>C100, C22c, C200.</td>
</tr>
<tr>
<td><strong>RIBA:</strong></td>
<td></td>
</tr>
<tr>
<td>1. First Generation:</td>
<td>C100-3, or 5-1-1.</td>
</tr>
<tr>
<td>2. Second Generation:</td>
<td>C100-3, 5-1-1, C22-3, C33c.</td>
</tr>
</tbody>
</table>
Among these methods, the second generation assay (John G, 1992), and putative core protein ELISA (Okamoto H, 1992) showed improved sensitivity and specificity over the first generation C100-3 assay as 10-30% additional cases of anti-HCV positive individuals were detected in high-risk groups and a larger number of patients with acute hepatitis were found positive during the acute phase (Bonino F, 1993). The significantly lower sensitivity of the first-generation assay could be attributed to a prevalence of anti-C100 lower than anti-C22 and anti-C33. The second generation RIBA is considered as the sensitive confirmatory test for antibody detection (Chaudhary RK, 1993; Vander Poel CL, 1991).

Although recent developments of anti-HCV antibody detection have markedly improved the sensitivity and specificity of serological tests, it still has several drawbacks:

a. Delayed seroconversion in acute infection (around 6-8 weeks on average);
b. HCV infection in seronegative individuals;
c. Difficulties in interpreting some indeterminate results;
d. Absence of tests detecting HCV antigens and HCV multiplication.

These problems can largely circumvented by the development of methods for direct detection of HCV-RNA.

2.5.2 Detection of HCV-RNA

Direct detection of HCV RNA aids the diagnosis of HCV infection in many ways. After the whole HCV genome had been identified, the method of Reverse Transcription Polymerase Chain Reaction (RT-PCR) was widely used to amplify the HCV-RNA in the serum and tissue biopsy specimens from the high risk group patients. Because the result
of PCR is to show the existence of viral RNA, it is more valuable than antibody testing to indicate the active HCV infection. The PCR assay has provided valuable information concerning the viraemia status and thus may be a powerful tool to monitor therapeutic efficacy.

The critical step of PCR assay for HCV-RNA detection is the correct design of the primers. In order to perform general PCR assays, it is now clear that primers specific for the 5'-terminus region should be used, since this region is highly conserved among all HCV isolates studied to date. This therefore excludes the possibility of missing viraemia because of sequence heterogeneity (as reviewed above). On the other hand, it is advisable to design either the PCR primers or the hybridization probes in regions that are not highly conserved to avoid potential misdiagnosis and detect the specific isolates (Jene B, 1992).

Several reports have described the RT-PCR methods on the serum, liver tissue and mononuclear blood cell specimens to detect the HCV-RNA by careful performance in the process of RNA extraction, reverse transcription, and PCR itself (Zaaijer HL, 1993; Brechot C, 1993; Hideki H, 1992). The main clinical applications of RT-PCR detection are indicated as:

a. Diagnosis of acute HCV infection and anti-HCV(-) patient with chronic hepatitis;
b. Evaluation of HCV viraemia in asymptomatic blood donors with normal ALT levels;
c. Demonstration of reinfection of liver graft after transplantation;
d. Analysis of mother to child or sexual HCV transmission;
e. Follow-up antiviral therapy.
2.6 INTERFERON-α TREATMENT ON PATIENTS WITH CHRONIC HEPATITIS C

Unfortunately, a large proportion (at least half) of the patients with acute NANB hepatitis progress to chronic liver disease despite the relatively mild nature of acute disease (Koretz RL, 1985). Many cases of chronic hepatitis C have severe consequences such as cirrhosis, portal hypertension, and primary hepatocellular carcinoma (Alter HJ, 1988). At present, only recombinant interferon is considered standard therapy for the treatment of chronic hepatitis C (Hoofnagle JH, 1993; Hideki H, 1992).

Interferons have been widely used in medicine during the past decade for their wide variety of actions including antitumour, immunoregulatory, cytostatic, and above all, antiviral activity. Although IFN was discovered as an antiviral agent more than 30 years ago, the biochemical mechanisms leading to the antiviral activity are still not completely understood. In fact, IFN induces the production of several cellular proteins, but only a few have been clearly correlated with the antiviral action. IFN acts by binding to a cell surface receptor which induces 3 proteins that prevent the translation of viral mRNA without affecting the translation of cellular mRNA (Levinson WE, 1992):

a. a protein kinase that phosphorylates an initiation factor for cell protein synthesis, thereby inactivating it;

b. a 2,5-oligonucleotide synthetase that synthesizes an adenine trinucleotide;

c. an endonuclease that is activated by the adenine trinucleotide and that degrades viral but not cellular mRNAs.
As a natural choice of a possible therapeutic agent for viral infections due to its wide spectrum of antiviral activities, IFN has been used to treat many acute and chronic viral diseases. This agent has already been shown to inhibit replication of several human hepatitis viruses, including hepatitis A virus (Vallbracht A, 1984), hepatitis B virus (Sherlock S, 1985), and hepatitis D virus (Hoofnagle JH, 1985). Several investigators have reported that recombinant alpha interferon therapy is effective in decreasing serum ALT levels and improving liver histological findings in chronic hepatitis C (Hideki H, 1992; Michiko S, 1991; Gary L, 1989; Davis GL, 1989; Di Bisceglie AM, 1989).

2.7. RESULTS AND PROBLEMS IN THE PREVIOUS STUDIES

2.7.1 Predictors of Treatment Response

Previous studies show only 50% of patients with chronic hepatitis C respond to interferon-α therapy. It would be helpful if there were clinical or serologic features that would predict which patients were likely to respond to therapy and which were not. Unfortunately, retrospective analyses from the controlled trials of interferon have failed to identify any clinical, serum biochemical, serological or histological feature of disease that reliably predicted a response to treatment (Hoofnagle JH, 1993; Saracco G, 1990). Importantly, the presence or titers of anti-HCV in serum did not identify patients who were likely to have a response to interferon (Shindo M, 1991). These findings were in contrast to hepatitis B, where the decrease of serum aminotransferases and level of HBV-DNA identified those who were likely to benefit from treatment (Perrillo RP, 1990). In some studies, patients who lacked anti-HCV and who had cirrhosis histologically were less likely to benefit from therapy (Marcellin P, 1991).
2.7.2 Treatment Response and Suppression of HCV Replication

The relationship between response to interferon therapy and suppression of HCV replication during the treatment of hepatitis C has not been carefully studied. Whether this is due to the inhibition of HCV or to improved immunologic responses must await the availability of direct tests for the virus. Hagiwara, H. et al. (Hagiwara H, 1993) evaluated HCV RNA by an RT-PCR assay in serial serum samples during interferon therapy and found that HCV RNA disappeared before ALT levels became normal in cases responding to interferon and this viral marker reappeared before the rise of ALT in patients who relapsed. In nonresponders, HCV RNA did not disappear. However, the problem in their study is the insufficiently low detection limit of the RT-PCR assay on the serum samples.

2.7.3 Limitation of Serum HCV-RNA Detection

The detection limit of RT-PCR assay is ideally equivalent to a single copy of HCV-RNA per sample. However, some loss and degradation of RNA fragments are likely in the steps of serum preparation, RNA extraction and precipitation, as well as the reverse transcriptase reaction. Furthermore, interferon may shut down HCV RNA replication but not lead to complete clearance of HCV genome in all liver cells. Therefore, to detect HCV RNA in liver tissue may be more sensitive than serum in characterizing the correlation between interferon therapy and viral activities, and predicting the chance of relapse.
All of these factors encouraged us to do further studies to investigate the therapeutic functions of interferon by directly testing for the presence and quantity of viral RNA in both serum and liver tissue, and revealing the replication status of HCV during the chronic infection treated with interferon. In particular, the serial quantitative analyses of HCV-RNA should be very useful for retrospective evaluation and prospective prediction of interferon treatment. We hope results from this study will lead to a deeper understanding of the therapeutic functions of interferon by the observation of treatment responses, and make some recommendation for further clinical trials in large group of patients.
CHAPTER 3

MATERIALS AND METHODS

3.1 COLLECTION AND PREPARATION OF SPECIMENS

All of the specimens were collected from the patients clinically diagnosed with chronic hepatitis C, subcutaneously administered with IFNα 2b 3 MU 3 times weekly. All of these were supervised by Dr. Frank H. Anderson in the division of gastroenterology, department of medicine, UBC, and Vancouver General Hospital.

3.1.1 Collection of Specimens

3.1.1.1 Serum

According to protocol for the clinical trial, blood samples were taken from patients at the time treatment was started and 1, 3, 6, 12, and 24 month later. After centrifugation, serum of each specimen was divided to three parts. Two of them were for liver function and anti-HCV antibody tests, and the rest was frozen in -30°C for HCV-RNA detection.

3.1.1.2 Liver

Liver tissue samples were collected by needle biopsy before, and after the end of treatment. Liver tissues were routinely formalin-fixed, paraffin-embedded, and sliced for pathological diagnosis. The rest of each specimen was chosen for HCV-RNA detection.

3.1.2 Preparation of Specimens

3.1.2.1 Serum

In order to do the quantitative PCR detection, each of the serum specimens was serially 10 fold diluted from $10^0$ (undiluted serum) to $10^{10}$ before the reverse transcription.

Briefly, 100μl of
serum were mixed with 900µl of 0.1% diethyl pyrocarbonate (DEPC) ddH₂O and vortexed for 15 sec. The same steps were repeated for further dilutions on previously diluted serum up to the lowest concentration.

3.1.2.2 Liver

Paraffin-embedded liver tissues were sliced and dewaxed before the HCV RNA extraction. Briefly, 5 pieces of tissues were mixed with 500µl of xylene in 1.5 ml Ependorf tubes at 55°C for 15 min. After centrifugation at 12000 rpm for 5 min., xylene was discarded, tissue pellets were washed with 70% ethanol and dried at room temperature for HCV-RNA extraction.

3.2 TEST OF LIVER FUNCTION

Aspartate aminotransferase (AST) was tested on each of collected serum specimens at reference laboratories for the liver function evaluation. Normal value of AST ranged between 19-38 IU/liter.

3.3 TEST OF ANTI-HEPATITIS C VIRUS ANTIBODY

Anti-HCV antibody was also detected in different reference laboratories by first or second generation ELISA assay. The results were recorded as negative or positive.

3.4 DETECTION OF HEPATITIS C VIRUS RNA

HCV-RNA from both serum and liver was detected by reverse transcription polymerase chain reaction (RT-PCR) assay, which included the following steps: extraction of HCV-RNA, reverse transcription, first and nested-PCR amplification.
3.4.1 Extraction of HCV-RNA

3.4.1.1 HCV-RNA extraction from serum specimens

In order to select the most sensitive and practical technique to obtain HCV-RNA for reverse transcription, two major methods, extraction and non-extracted, were developed. Sensitivities were compared between the two methods on same set of serially diluted serum specimens.

Procedure:
1. Extraction method (Abe K, 1992):
   1) 100μl of serum specimens were mixed with 400μl of buffer A including 4.2M guanidinium thiocyanate, 25 Mm Tris-HCl (pH 8.0), 0.5% sarcosyl, and buffer B including 0.1M Tris-HCl, 10 mM EDTA, and 1% SDS.
   2) After thorough mixing, 500μl of phenol/chloroform (1:1) were added, then the samples were heated at 65°C, 30 min. with agitation.
   3) The samples were centrifuged at 13000 rpm for 5 min., then the aqueous phase was transferred to a fresh tube. The organic phase was extracted with phenol/chloroform once again.
   4) The two aqueous phases were mixed together, then extracted with chloroform alone.
   5) Two volumes of isopropanol and 1/10 volume 3M sodium acetate were added, and resulting mixture at -70°C over 2 h.
   6) The samples were centrifuged at 15000 rpm for 20 min., then pellets were washed with 70% ethanol and air-dried.
   7) Finally the extracted RNA was dissolved in 20μl of 0.1%DEPC ddH₂O.
MATERIALS AND METHODS

2. Non-extraction method (Ravaggi A, 1992):

1) 5µl of serum or diluted serum was added to the bottom of 0.5 ml Ependorf tube.
2) The tube was heated at 92° C, for 45 sec., then stored at 4° C for reverse transcription.

Finally, the non-extraction method was used for the detection of HCV-RNA on serum specimens from αIFN treated patients.

3.4.1.2 HCV-RNA extraction from liver tissue

HCV-RNA was extracted from the liver tissue according to the acid guanidinium thiocyanate-phenol/chloroform extraction method with modification (Stanta 1991; Chomczynski 1987).

Procedure:

1) Dewaxed liver tissue was minced and homogenized with 500 µl of lysing solution including 4M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, 0.1M 2-mercaptoethanol, then left at room temperature for 24 h.
2) Sequentially, 50µl of 2M sodium acetate (pH 4.0), 1 ml of phenol/chloroform/isoamyl alcohol (25:24:1) were added to the homogenate, with thorough mixing by inversion after the addition of each reagent.
3) After centrifugation at 13000 rpm for 10 min., the aqueous phase was transferred to a fresh tube, mixed with 1 ml of isopropanol, and then placed at -20°C over night to precipitate RNA.
4) Centrifugation at 13000 rpm for 20 min. was again performed and the pellet was washed with 70% ethnol and air-dried.
5) Finally the extracted RNA was dissolved in 20 µl of 0.1%DEPC ddH₂O. The
MATERIALS AND METHODS

concentration of extracted nucleotide was determined by spectrophotometer O.D. reading.

3.4.2 Reverse Transcription

Using heated serum and extracted HCV-RNA from liver tissue as the template, HCV-cDNA was synthesized by reverse transcription assay.

Procedure:
1) 5μl of serum or extracted HCV-RNA was heated at 92°C, 45 sec. for denaturation.
2) The template RNA was incubated at 37°C, 60 min. with 32μl of RT-Mixture containing 400U Moloney Murine Leukaemia Virus (M-MLV) Reverse Transcriptase (BRL, MA), 1 mM (each) dNTP (dATP, dCTP, dGTP and dTTP), 10 mM dithiothreitol, 400 ng B.S.A., 40U RNase, 1 mM outer anti-sense primer (for genomic form HCV-RNA detection) or outer sense primer (for replicative form HCV-RNA detection), 3.7μl 10X Vent Buffer (100 mM KCl, 100 mM [NH₄]₂SO₄, 200 mM Tris-HCl, 20 mM MgSO₄, 1.0% Triton X-100).
3) After heating the tubes at 95°C, 5 min., synthesized HCV-cDNA was ready for PCR.

3.4.3 Polymerase Chain Reaction

3.4.3.1 Design of Primers

Based on sequence analysis of the HCV genome, the most conserved 5' non-coding region was chosen for PCR amplification. The primer sequence, genome location and product size are shown on Table 4 and Figure 3. The ratio of GC is 58.8%.
MATERIALS AND METHODS

Table 4. HCV primer sequence, location and size of PCR products

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Location</th>
<th>Size</th>
</tr>
</thead>
</table>
| Outer   | 5' CTG TGA GGA ACT ACT GTC        | pos. 33  | 221bp.
| 1.      |                                   |          |       |
| 2.      | 5' AAC ACT ACT CGG CTA GCA        | " 253   |       |
| Inner   | 5' TTC ACG CAG AAA GCG TCT        | " 51    | 145bp.|
| 1.      |                                   |          |       |
| 2.      | 5' GTT GAT CCA AGA AAG GAC        | " 195   |       |

Figure 3. Genomic location of PCR products

Genomic location of PCR Primers

5' C E1 E2/NS1 NS2 NS3 NS4 NS5 3'

33 51 195 253

Hep1 Hep2 Hep3 Hep4

145 bp 221 bp
MATERIALS AND METHODS

As the internal control, a region of the β-actin gene was detected on extracted nucleotide from liver tissue. The primer sequence, genome location and product size are also shown on Table 5. The ratio of GC is 58.7%.

Table 5. β-actin primer sequence, location and size of PCR products

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Location</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer</td>
<td>5' GGG AAA TCG TGC GTG ACA</td>
<td>pos. 658</td>
<td>477bp.</td>
</tr>
<tr>
<td>1</td>
<td>&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5' ACT CGT CAT CCT GCT TGC T</td>
<td>&quot; 1113</td>
<td></td>
</tr>
<tr>
<td>Inner</td>
<td>5' GTG TGA CGT GGS CST CCG CAA</td>
<td>&quot; 893</td>
<td>208bp.</td>
</tr>
<tr>
<td>1</td>
<td>&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5' CTG GAA GGT GGA CAG CGA GGC</td>
<td>&quot; 1080</td>
<td></td>
</tr>
</tbody>
</table>

3.4.3.2 PCR amplification

After RT, PCR was performed according to the procedure below:

Procedure:

1) 37μl of RT solution was added with 3 μl PCR mixture containing 1 mM outer sense primer, 2U Vent(exo-) DNA Polymerase (BioLabs, MA) in the final reaction condition, then each tube was topped with 2 drops of mineral oil.

2) 30 cycles were run as follows: 98º C, 1 min. for denaturation, 45º C, 1 min. for annealing, 72º C, 1 min. for extension, followed by 72º C, 7 min. for final extension.
3.4.3.3 Nested-PCR amplification

After first PCR amplification, the products were not visible on electrophoresis gel. Therefore, nested-PCR was carried out on each specimen following first PCR to increase the sensitivity.

Procedure:
1) 2 µl first PCR products was added with 38 µl of nested-PCR mixture using the same conditions as the first PCR except 1mM each of sense and anti-sense inner primers.
2) 30 cycles were run as follows:
   - 98°C, 1 min. for denaturation,
   - 45°C, 1 min. for annealing,
   - 72°C, 1 min. for extension,
followed by 72°C, 7 min. for final extension.

The products were ready for electrophoresis and endonuclease digestion.

3.4.4 Specificity of RT-PCR System

3.4.4.1 Electrophoresis

Nested-PCR products were analyzed by electrophoresis assay.

Procedure:
1) 8 µl of nested-PCR products mixed with 2 µl of tracking dye were loaded in the wells of 3% NewSieve agarose containing 0.025% ethidium bromide.
2) After running at 120V. for 40 min., the gel was viewed and photographed on the long wave ultraviolet light box.
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long wave ultraviolet light box.

3.4.4.2 DNA typing

According to genomic analysis, the nested-PCR product was incubated with Rsal to confirm the PCR results by distinguishing the pattern of digested products on the gel (Figure 4).

Procedure:
1) 10 μl of Nested-PCR products was mixed with 1 μl of Rsal, 2 μl of 10x buffer, and 7 μl of dH2O, then incubated at 37°C for 60 min.
2) Digested DNA fragments were analyzed by electrophoresis.

Figure 4. Rsal digested nested-PCR products

Rsal digested nested-PCR products
3.4.4.3 Southern blot

The nested-PCR results were confirmed by Southern Blot assay using cloned HCV-cDNA as probe.

Procedure:

1) PCR products were transferred to nylon membrane.
   a) After electrophoresis, DNA was denatured by soaking the gel in several volumes of 1.5M NaCl, 0.5N NaOH solution with constant, gentle agitation for 45 min..
   b) Neutralization was performed by soaking the gel in several volumes of 1M Tris (pH 7.4), 1.5M NaCl at room temperature for 30 min. with constant, gentle agitation, then repeated once by changing the neutralization solution.
   c) The gel was placed on the support in a large baking dish filled with transfer buffer (10x SSC), then put a nylon membrane, Whatman 3MM paper, paper towels, glass plate and 500 g of weight were placed on the top (Figure 5.).
   d) The transfer of DNA was allowed to proceed over night.
   e) The paper towels and 3MM papers were removed. The membrane was peeled off and placed flat on a paper towel to dry for 30 min..
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Figure 5. Capillary transfer of DNA from gel*

Capillary transfer of DNA from agarose gels. Buffer is drawn from a reservoir and passes through the gel into a stack of paper towels. The DNA is eluted from the gel by the moving stream of buffer and is deposited on a nitrocellulose filter or nylon membrane. A weight applied to the top of the paper towels helps to ensure a tight connection between the layers of material used in the transfer system.

* from Sambrook 1989.

2) Nick translation for radioactive labelling probe:

To get the template, *Bluescript HCV CDNA* was linearized by incubation with restriction enzyme *Xba I* and *Hinc II* resulting in a 372bp long HCV cDNA segment of which was located in the HCV genome nt.7-378. The radioactive labelling probe was made by using a *Nick Translation Kit* (BRL).

a) Five µl of each Solution A2, C, (α-32P)dCTP, 35 µl of dH2O, and 1 µg template DNA were added in the 50 µl of final reaction solution. There were 20 µM of each dATP, dGTP, dTTP and (α-32P)dCTP, 2 units of *DNA polymerase I*, 200 pg of *DNase I* in the reaction.

b) The mixture was incubated for 1 h. in 15°C water bath, then 5 µl of stop buffer was added.

c) The probe was purified by passing it through a Spun-column. The probe was then ready to use.
3) Hybridization of radiolabeled probes to immobilized PCR products:

a) Wet membrane was put in heat-sealable bag. For each cm² of membrane, 0.2 ml of prehybridization solution including 6xSSC, 5x Denhardt's reagent, 0.5% SDS, 50% formamide was added. The sealed bag was submerged at the 45° and incubated for 2 h. b) The bag was opened and added with 50 µl of denatured probe. After being resealed, the bag was incubated at 45°C over night with agitation.

c) The membrane was removed from the bag and washed 2 times with each of 2x SSC and 0.1% SDS, 0.1x SSC and 0.5% SDS. The membrane was placed on a flat paper towel to dry for 30 min.

d) The membrane was covered with a sheet of wrap, and exposed to X-ray film at -20°C for 24 h. After development, the film is ready for analysis.

3.4.4.4 Positive, negative and internal controls

Both positive and negative control samples were tested during the process of RT-PCR detection.

1) Positive control: a) synthesized HCV-RNA.
   b) previously HCV-RNA positive serum.

2) Negative control: a) extracted RNA from biopsy liver with non-HCV infection.
   b) previously HCV-RNA negative serum.
   c) salmon sperm DNA.

3) Internal control:

   The β-actin gene, a "house-keeping" gene, was amplified at the same time with the detection of extracted HCV-RNA from liver tissue.
3.4.5 Sensitivity of RT-PCR System

The goal of this thesis is focused on the study of interferon treatment response through the observation of HCV-RNA viral replication as detected by quantitative RT-PCR, so quantification of the testing system is a very important step in the project. The sensitivity of the RT-PCR method was determined by the detection of serially diluted synthesized HCV-RNA followed by the correlation between genomic numbers of template RNA and cycles of PCR amplification. The following processes were carried out to quantitate the system. The cell transformation, sequencing, and in vitro transcription were performed in Dr. S. Gillam's laboratory in Research Centre of U.B.C. with generous instruction and help of Z.Y. Qiu and D.C. Yang.

3.4.5.1 Multiplication of HCV-cDNA

* pBluescript-HCV-cDNA which contains part of HCV-cDNA in pBluescript vector, was generously provided by Dr. G. Inchauspe in the New York Blood Center. The cloned HCV-cDNA was multiplied by cell transformation.

**Steps:**
1) Transformation of the recombinant plasmid into E.coli strain DH5α.
2) Multiplication of E.coli by bacterial culture.
3) Isolation and purification of plasmid DNA from transformant.

3.4.5.2 Sequencing cloned HCV-cDNA

Based on the results of PCR detection, the cloned HCV-cDNA was found to be a
relevant portion which the PCR could amplify. However, the border of inserted HCV-cDNA was not known, so the sequence of cloned HCV-cDNA was identified by the dideoxy sequencing method (Sanger F. 1981) on double strand DNA.

Steps:

1) Annealing of primer to double-stranded template DNA.
2) Sequencing reactions.
3) Electrophoresis.
4) Autoradiography and analysis.

3.4.5.3 In Vitro transcription

After the sequence of cloned HCV-cDNA was identified, negative strand was used as the template to synthesize the positive strand HCV RNA by in vitro transcription.

Procedure:

1) 2μg of plasmid DNA was linearized by XbaI.

2) Template DNA was incubated with 40U of T7 RNA polymerase for 90 min. at 37°C in the presence of 1mM (each) ribonucleoside triphosphates (GTP, ATP, UTP, and CTP), 100U of RNasin, 10 mM dithiothreitol, 40 mM Tris-HCl (pH 7.5), 6 mM MgCl2, 2 nM spermidine, and 10 mM NaCl in a total reaction volume of 40μl.

3) After the transcription reaction, the DNA template was degraded by two rounds of digestion with RNase-free DNase I (BRL) for 15 min. at 37°C with 10U and 5U enzyme respectively.
MATERIALS AND METHODS

4) The HCV RNA transcripts were purified by two time phenol/chloroform extraction, precipitated with isopropanol, washed sequentially with 70% and 95% ethanol, and then analyzed by denatured agarose gel electrophoresis to assess its integrity.

5) Finally, HCV RNA was determined spectrophotometrically by UV A260.

3.4.5.4 Quantitative analysis of HCV-RNA

As soon as the synthesized HCV-RNA was obtained, it was quantitatively analyzed.

Steps:

1) The synthesized HCV-RNA was serially 10 fold diluted.

2) Component analysis on the RNA: According to the sequence and the O.D. reading, the ribonucleic acid composition and molecular weight of the RNA were determined.

3) Genomic copies of the RNA were calculated by conversion from weight in different concentrations.
3.4.5.5 Correlation of PCR cycles and genomic copies

Finally, the sensitivity of the RT-PCR system was measured by detection of serially diluted HCV-RNA as above. Briefly, after RT, there were 30 cycles in the first-PCR, followed by 10, 20, 30, 40, and 50 cycles in the nested-PCR amplification. The positive results from the lowest concentration in different cycles were correlated with the number of genomic copies in each serial dilution.

3.5 DEFINITION OF CLINICAL RESPONSE TO TREATMENT

The clinical responses to IFN treatment were classified according to the following definitions:

3.5.1 Complete Response

Complete response was defined as normalization of AST 1 month after receiving treatment and at the end of treatment.

3.5.2 Partial Response

Partial response was defined as an AST level < 1.5 times the upper limit of normal and < 50% of the pretreatment value at the end of treatment.

3.5.3 No Response

No response was defined as not meeting criteria above.

3.5.4 Relapse

Relapse was defined as AST elevation to at least 1.5 times the upper limit of normal at any testing interval after initial response or during the 6 months of follow-up period.
CHAPTER 4
RESULTS

4.1 RT-PCR DETECTION ASSAY

4.1.1 Sensitivity Analysis

4.1.1.1 Comparison of sensitivities between extraction and non-extraction methods

In order to select the most sensitive and practical technique, the HCV-RNA extraction method was compared with the non-extraction method on the same serially diluted serum samples followed by RT-PCR. In the final results, after guanidinium lysing followed by phenol/chloroform extraction HCV-RNA was detected at a serum dilution of $10^2$, compared to a dilution of $10^6$ with the non-extraction method (Figure 6). This indicates that the direct method is considerably more sensitive than the routine method, and different concentrations of HCV-RNA are detectable on serially diluted serum. Since the direct method is more sensitive and less time consuming, it was used for clinical serum HCV-RNA detection.

Figure 6. Electrophoresis gel from the comparative detection methods
4.1.1.2 Quantitative analysis by standard detection on synthesized HCV-RNA

The RT-PCR detection assay was quantitatively analyzed by the detection of synthesized HCV-RNA, which consisted of sequencing HCV-cDNA, synthesis of HCV-RNA, and quantitative detection of serially diluted HCV-RNA by RT-PCR.

4.1.1.2.1 Sequence of cloned HCV-cDNA

Figure 7. Sequence of pBluescript HCV-cDNA

5' T3 Promoter

GGGAACAAAAAGCTGGAGCTCCACCCGGCGGTGGCCGCCCTCTAGAACTAGTCCCCGC
GACACTCCACCATAGATCACTCCCTGAGGAACTACTGTCTTCACGCGAGAAAGC
GTCTAGCCATGGGGTTAGATGAGTGTC(T)GTGCACGCTCCAGGACCCCGGCTC
CCGGGAGAGCCATAGTGTTCGGAAGCCCGGTGAGTACACCGGAATTGCCAGG
ACGACCGGCTCCCTTTTCTTGGATA(C)AACCCGCTCAATGCCTGGAGATTTGGGC
GTGCCGCCCGCA(G)AGACTGCTAGCCGAGTAGATGTTGGTGGTGCAGAAGGCCCC
GTGGTACTGCTGATAGGGTGGTTGCGAGTGCCCCGGAGGTCTAGACCG
TGCATCATGACACAAATCTAAACCTCAAAGAAACCAAAACGTAAACCAAC
C#TTGCCAGGTTCGATATCAAGCTTTATCGATACCGTGC#GACTCGAGGGGGGGGCCC....

Hinc II 3' T7 Promoter

#HCV-cDNA: Length: 372bp.

HCV genome rt. 7-378.
RESULTS

The sequence of pBluescript HCV-cDNA is shown in Figure 7. This is a 372bp long DNA fragment corresponded to HCV genome nt. 7-378.

4.1.1.2.2 Synthesized HCV-RNA

Figure 8 shows the sequence of 460nt long synthesized RNA which includes the HCV genomic RNA nt.7-378.

Figure 8. Sequence of synthesized HCV-RNA from pBluescript HCV-cDNA

5’ T3 Promoter
GGGAACAAAGCUGGAGCUCACCGCUGGCGGCGGCUCUAAGAAACACGUAUCCCC#GC
GACACUCGAACCAUGACACUCCCCUGAGAAACUGUCUUCACGCAAGAAAG
CGUCUAUGCCAUUGGCGUUAGUAUAGUGUC(U)GUGCAACCUCCAGGACCCCCCC
UCCCCGGAGAGCCAUAGUGGUCUGCGGAACCCGUGAGAUACACCCGAAUUGCC
AGGACGACCGGUUUUCUUGGAUA(C)AACCCGCUCAUGCCUGGAAGUUG
GGCGUGCCCCGCA(G)AGACUCUGCUAGCCAGUAGAUGUUGGGUCGCAAGGG
CCUUGUGUACUGCUGCCUAGGGUGGUUGCGAGUGCCCGCCGGGAGGCCUCUGUA
GACCGUGCAUCAGAGCAAAAAUCUAACCCUUAAAGAAAAACCAACGUAA
ACCAACC#UUGCCAGGUUGCAGAUAAGCUUAGCUUAGCAUACCGUC

#HCV-RNA: Length: 372bp.
HCV genome nt. 7-378.
Synthesized RNA: Length: 460nt.
4.1.1.2.3 Quantitative analysis of serially diluted HCV-RNA

Based on optical density (O.D.) reading and identified sequence, the synthesized HCV-RNA was quantitatively analyzed. Table 6 shows the components, molecular weight, and conversion to genomic copies of the RNA. Table 7 shows the corresponding genomic copies of the template in serial dilutions used in reverse transcription.

**Table 6. Data of synthesized RNA**

<table>
<thead>
<tr>
<th>Component</th>
<th>Length (nt)</th>
<th>A: 109</th>
<th>C: 135</th>
<th>G: 126</th>
<th>U: 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.W.</td>
<td></td>
<td>37848.07</td>
<td>43632.0</td>
<td>45766.98</td>
<td>29177.1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>156424.15</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**O.D.:**

- 235nm: 0.066
- 260nm: 0.129
- 280nm: 0.075

Conversion of concentration to genomic copies:

1) Concentration of synthesized HCV-RNA:

\[
0.129 \times 40 \mu g/ml = 5.16 \times 10^{-3} g/L
\]

\[
5.16 \times 10^{-3} / 156424.15 = 3.29 \times 10^8 \text{ M}
\]
2) Genomic copies in solution:

\[ 3.29 \times 10^8 \times 6.023 \times 10^{22} = 1.9816 \times 10^{15} / \text{L} \]

**Table 7. Genomic copies in serial dilutions***

<table>
<thead>
<tr>
<th></th>
<th>1μl</th>
<th>5μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 10^9:</td>
<td>1.98 x 10^8</td>
<td>1 x 10^{10}</td>
</tr>
<tr>
<td>2. 10^2:</td>
<td>&quot; 10^7</td>
<td>&quot; 10^7</td>
</tr>
<tr>
<td>3. 10^4:</td>
<td>&quot; 10^6</td>
<td>&quot; 10^6</td>
</tr>
<tr>
<td>4. 10^6:</td>
<td>&quot; 10^4</td>
<td>&quot; 10^4</td>
</tr>
<tr>
<td>5. 10^8:</td>
<td>&quot; 10^2</td>
<td>&quot; 10^2</td>
</tr>
<tr>
<td>6. 10^{10}:</td>
<td>&quot; 10^1</td>
<td>&quot; 10^0</td>
</tr>
<tr>
<td>7. 10^{12}:</td>
<td>≈ 0</td>
<td>≈ 0</td>
</tr>
</tbody>
</table>

* serial dilutions of RNA converted to numbers of genomic copies
RESULTS

4.1.1.2.4 Correlation of PCR cycles and template copies

The sensitivity of the RT-PCR assay was evaluated by the detection of known amounts of synthesized HCV-RNA in 10 fold serial dilutions at different cycle amplifications. After 30 cycles in first-PCR amplification, nested-PCR was respectively performed in different cycles. Amplification signals were visible on the electrophoresis gel after first PCR at 10^6 molecules of HCV-RNA template. Typically, the sensitivities were consistently increased in proportion to the number of amplification cycles in nested PCR. Single copy of synthesized HCV-RNA became detectable as the amplification time was increased to 30 cycles (Figure 9, page 40). It appears that in order to obtain the best results by using the most reasonable hours, the optimum amplification cycle numbers were 30 for both first and nested PCR (Figure 10.). Based on the results, all of serum and liver tissue samples were detected by employing this condition.

Figure 10. RT-PCR results correlated with nested-PCR cycles and template copies

SENSITIVITY OF RT-PCR

Genomic copy 1x10^log

Cycles of nested-PCR
Figure 9. Electrophoresis gel of quantitative detections

First PCR

Nested PCR
10 cycles

20 cycles

30 cycles

50 cycles
RESULTS

To ensure that RT-PCR products resulted from amplification of RNA rather than amplification of residual DNA present in the RNA preparation, reactions in which the RT step was omitted were performed. As shown in lane 4-8 of Figure 11., no products were generated from as many as $10^6$ RNA copies in the absence of RT. However, products were seen at input RNA levels of $10^8$ or higher, which suggested: 1) the presence of residual DNA due to uncompleted digestion by DNaseI; or 2) possible low level of reverse transcriptase activity by DNA polymerase during DNA amplification.

Figure 11. Results of PCR detection without RT

Copies of HCV-RNA

(+) $10^4$ $10^6$ $10^8$ $10^6$ $10^8$ $10^9$ $10^6$ $10^8$ $10^6$ (-)
4.1.2 Specificity Analysis

4.1.2.1 Electrophoresis

The fragments of nested PCR products were located at the position of 145bp which was the expected size.

4.1.2.2 DNA Typing

Because the region amplified by PCR was located in the most conserved 5' end of the HCV genome, restriction endonuclease was chosen to digest nested PCR products to confirm the PCR results. According to genomic analysis, restriction endonuclease $RsaI$ was chosen to incubate with nested PCR products. As we expected, two pieces of digested DNA fragments were located at the position of 102 and 43bp. See Figure 12.

Figure 12. Results of Nested-PCR and $RsaI$ digested products
4.1.2.3 Southern Blot

Figure 13. shows the positive results of southern blot carried out on the nested-PCR products from three patients. Southern Blot was not done on every PCR product, since we have too many PCR products (total 58 benches, and 600 reactions), and it is not very necessary to do Southern Blot for each of them.

**Figure 13. Electrophoresis gel and results of Southern Blot**

4.1.3 Clinical Application

As soon as the RT-PCR method was established, it was used for clinical diagnosis. 32 serum samples, from the patients with chronic hepatitis, liver transplantation donors or recipients, and other unknown liver disease, have been tested. 25 of them (78%) were positive for HCV-RNA.
4.2 OBSERVATION OF SERUM HCV-RNA IN IFNα TREATED PATIENTS

Twelve patients received IFNα therapy for more than 6 months and none of them stopped treatment. All 12 were included in the study.

4.2.1 Changes of Serum HCV-RNA during Therapy

Quantitative RT-PCRs were performed to amplify the HCV-RNA from 5µl of original or diluted serum samples serially collected from the 12 patients over periods of 6 to 27 months of IFN treatment. HCV-RNA titer for each specimen was recorded as the lowest concentration of diluted serum as HCV-RNA was positive. HCV-RNA titers prior to, or at different times in the IFN treatment for all patients are given in Table 8. The general responses of HCV-RNA were interpreted as follows:

1) Prior to treatment, serum HCV-RNA was evident in the sera from all 12 patients. HCV-RNA titers ranges between 10⁹-10⁸, mean 10³.3.

2) Initial responses were shown as the HCV-RNA titers fell in 7 of 12 (58%) patients. The titers were down between 10¹-10⁸, (mean 10².6) after 3-6 months of treatment.

3) HCV-RNA titers climbed again in 4 of 7 (57%) initial responders still receiving 3 months later.

4) No patients were negative for HCV-RNA at 6 months into treatment. Eventually HCV-RNA became undetectable in 3 patients continuing to receive IFN treatment at 12, 21, and 24 months respectively). The patients with lower serum HCV-RNA titer before the treatment had more chance to become HCV-RNA negative. Clearance of HCV-RNA
RESULTS

occurred in 2/3 patients with initial titers ≤ 10^6 but in only 1/9 with initial titers > 10^6.

6) Because none of these 12 patients stopped treatment at the time this thesis was written, final results of HCV-RNA status at treatment cessation and post treatment are not yet available.

Table 8. HCV-RNA titers before and during treatment

<table>
<thead>
<tr>
<th>No.</th>
<th>0M</th>
<th>3M</th>
<th>6M</th>
<th>9M</th>
<th>12M</th>
<th>15M</th>
<th>18M</th>
<th>21M</th>
<th>24M</th>
<th>27M</th>
<th>30M</th>
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<tbody>
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<td>10^7</td>
<td>10^4</td>
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</tr>
<tr>
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<td>10^5</td>
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<td></td>
<td></td>
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</tr>
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</tr>
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<td>10^2</td>
<td></td>
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</tr>
<tr>
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<td>-</td>
<td>-</td>
<td></td>
<td></td>
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</tr>
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<td>6.</td>
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<td>7.</td>
<td>10^3</td>
<td>10^3</td>
<td>10^3</td>
<td>10^1</td>
<td>10^9</td>
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<tr>
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<td>10^0</td>
<td>-</td>
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<tr>
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<tr>
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<td>10^3</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>10^3</td>
<td>10^2</td>
<td>10^3</td>
<td></td>
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</tr>
</tbody>
</table>
4.2.2 Three Patterns of HCV-RNA Detected in the Patients

Although most of the patients were persistently positive for HCV-RNA during the treatment, three patterns of viraemia could be identified from the analysis of HCV-RNA titers: decreased, increased and unchanged HCV-RNA levels.

4.2.2.1 Decreased HCV-RNA titers

Eight (#1, 2, 4, 5, 6, 7, 8, and 9) of 12 patients showed constant trend toward decreased serum HCV-RNA titers. Three patients (#5, 8, and 9) became HCV-RNA negative in the final. Example for pattern I is shown in Figure 14 from patient #1. Titers of HCV-RNA were $10^5$ before the treatment, and $10^4$ 3 months after treatment presumably due to an initial response. Although the titer elevated to $10^7$ 3 months later, it fell down to $10^6$ at 15 months into treatment.

Figure 14. Pattern of decreased HCV-RNA titer

HCV-RNA DETECTION

Patient #1

Serial Dilution log.

Months in treatment

46
4.2.2.2 Increased HCV-RNA titers

Opposite to pattern 1, 2 of 12 patients (#3, 11) showed increased HCV-RNA titers during the treatment. An example for pattern II is shown in Figure 15 from patient #3. The titers of HCV-RNA was lower before the treatment, but rose to peak values 9-12 months in the treatment, though initial responses were found in both patients.

Figure 15. Pattern of increased HCV-RNA titer
4.2.2.3 Unchanged HCV-RNA titers

Two (#10, 12) patients showed static titers of HCV-RNA during the treatment. Example from pattern 3 is shown in Figure 16 from patient #10. No matter how high the titer was before the treatment, it remained unchanged during the treatment.

Figure 16. Pattern of unchanged HCV-RNA titer

HCV-RNA DETECTION
Patient #10

Serum dilution log.

Months in treatment
RESULTS

4.3 OBSERVATION OF LIVER HCV-RNA IN IFN-α TREATED PATIENTS

Among the 12 patients, 4 of them had liver biopsy before and after treatment. Both genomic and replicative forms of HCV-RNA were detected on the liver specimens. As the internal control, β-actin mRNA was also detected by RT-PCR among the extracted RNA samples, to exclude the possibility that a negative result was due to RNA loss and degradation during the process of preparation and extraction.

Because it is difficult to control biopsy liver tissue in same volume, quantitative analysis was not available for HCV-RNA detection. The results were recorded as "-" no band, ± "faint band", "+" definite band but not dense, and "++" dense band, based on the visually estimated density of band appearance of electrophoresed nested-PCR products.

The results are shown on the Table 9. Both genomic (positive strand) and replicative form (negative strand) were detectable in the liver specimens. However, we could not correlate liver HCV-RNA results with serum as well liver functions since we do not have enough specimens and the specimens were collected at different times from sera.
RESULTS

Table 9. HCV-RNA in the liver tissue

<table>
<thead>
<tr>
<th>HCV-RNA</th>
<th>Patient #1</th>
<th>Patient #2</th>
<th>Patient #4</th>
<th>Patient #10</th>
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<tr>
<td></td>
<td>-2M</td>
<td>12M</td>
<td>-1M</td>
<td>11M</td>
</tr>
<tr>
<td>Genomic Form</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>Replicative Form</td>
<td>±</td>
<td>++</td>
<td>±</td>
<td>++</td>
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</table>

4.4 OBSERVATION OF CLINICAL RESPONSE TO IFN-α TREATMENT

4.4.1 Liver Function

For the observation of clinical response, all patients were tested for liver function almost at the same time as serum samples were collected for the HCV-RNA detection. The results of liver function, measured by aspartate aminotransferase (AST), are listed in Table 10. The general responses to IFNα treatment were described as following:

1) Eight patients showed an initial response as determined by a decreased AST at the first time HCV-RNA was detected during treatment (3 patients at 3 months, 2 patients at 6 months, 3 patients at 12 months). 4 of them (#3, 4, 9, 12) achieved a partial response.

2) Seven of the 8 responders had recurrence of AST elevation 3 to 15 months later although they were continuing to receive IFN treatment.

3) Two patients were complete responders as demonstrated by normalization of AST
values while they were continuing their treatment 6 (#12) and 12 (#8) months.

4) The other 4 patients were classified as non-responders because they did not have improvement in their liver function at any time.

Table 10. AST levels before and during treatment

<table>
<thead>
<tr>
<th>No.</th>
<th>0M*</th>
<th>3M</th>
<th>6M</th>
<th>9M</th>
<th>12M</th>
<th>15M</th>
<th>18M</th>
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<tr>
<td>5.</td>
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<td>79</td>
<td>68</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>35</td>
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<td>73</td>
<td>34</td>
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<td></td>
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<td></td>
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<td>28</td>
<td>30</td>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>146</td>
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<td>75</td>
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<tr>
<td>11.</td>
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<td>43</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td></td>
<td></td>
<td>201</td>
<td>47</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* months of treatment,
4.4.2 Correlation between Clinical Response and HCV-RNA

The results of serum HCV-RNA and AST from the 12 patients are respectively shown in Figure 17. The relationship between clinical responses and serum HCV-RNA was demonstrated as follows:

1) 7 patients (#1, 4, 6, 7, 8, 10, 11) had roughly parallel patterns of both HCV-RNA titer and AST responses.

2) AST decreased in 5 of 7 initial responders measured by serum HCV-RNA. In 3 HCV-RNA negative converted patients, 1 (#8) had normal AST, 2 (#5, 9) had slightly abnormal AST value.

3) in 2 patients (#3, 11) with the pattern of increased HCV-RNA titers, AST responded parallel with HCV-RNA in the first 6 months, and decreased later.

4) in 2 patients with unchanged HCV-RNA titers. Patient #10 had no remarkable change for AST, and patient #12 had a normal AST value at 6 month in the treatment.

5) Among 4 patients with lower HCV-RNA titer (<10^3) prior to the initiation of treatment, 2 (#8, 12) of them had normal liver function, and 2 (#9, 11) had slightly abnormal AST levels. None of other 8 patients with HCV-RNA titer \( \geq 10^3 \) prior to treatment had normal liver function.

6) It is not possible to do statistical analysis on the results above due to the small sample size.
**Figure 17. Observation of serum HCV-RNA and liver function**

RESPONSE TO IFN TREATMENT

**Patient #1**
Serum dilution log - AST UI/liter

**Patient #2**
Serum dilution log - AST IU/liter

**Patient #3**
Serum dilution log - AST IU/liter

**Patient #4**
Serum dilution log - AST IU/liter

**Patient #5**
Serum dilution log - AST IU/liter

**Patient #6**
Serum dilution log - AST IU/liter

---

RESULTS
RESULTS
4.5 RESULTS IN SUMMARY

1. The specificity of the RT-PCR assay was verified by the Southern blot and DNA restriction digestion analysis performed on nested-PCR products.

2. The sensitivity of RT-PCR assay was analyzed by a piece of recombinant HCV-RNA which was in vitro transcribed from transcription vector HCV-cDNA. Amplification from single copy of HCV-RNA was detected reproducibly by gel electrophoresis after 30 cycles in both first and Nested-PCR amplification.

3. HCV-RNA was quantitatively assayed by the RT-PCR method on 53 serum specimens serially collected from 12 IFNα treated patients and 9 paraffin-embedded liver specimens from 4 of the patients above.

4. Interferon-α had a preliminary beneficial effect on 7 of 12 (58%) patients at 3-6 months into treatment as assessed by a fall in HCV-RNA titer. Four of 7 (57%) initial responders relapsed 3 months later. Eventually HCV-RNA became undetectable in 3 patients’ sera. Serum HCV-RNA titers showed parallel changes with liver function in 7 patients. Two patient had normal AST level at 6 and 12 months in treatment. The patients with lower serum HCV-RNA titers prior to initiation of treatment had a better therapeutic response.
Recently, interferon has been widely used and proved useful in the therapy of hepatitis C. However, most of the questions dealing with the efficacy of IFN treatment are:

How is the rate of response in patients with chronic hepatitis C infection?

Which parameter is the best one to monitor the treatment and predict the response?

What are the possible reasons for poor response?

What should the future studies focus on?

5.1 EVALUATION OF INTERFERON TREATMENT ON HEPATITIS C

In order to evaluate the IFN therapeutic function in a larger sample size, 11 reports published in 1993 were reviewed to measure the IFN treatment responses. In addition to the observation on the clinical features, biochemical responses of liver and viral markers were measured in most cases for the evaluation of IFN treatment effects.

5.1.2 IFN Treatment Effects on Liver Function

510 patients were included in the 13 trials, and the treatment period ranged between 6-12 month. At the time of 3-6 months in the treatment, 325 (63.7%) of them were normal in the ALT and were considered as initial response cases. However, 180 of them relapsed during the posttherapy follow up period. Eventually, 145 (28.4) patients were normal in ALT and considered as the complete responders (Table 11).
The results from our trial are similar to the results above although we do not have a big enough sample size. In total 12 patients, 8 (66.7%) had initial responses between 3 to 12 month in treatment. Seven (87.5%) had relapse as judged by rising AST. At last follow-up, 3 (25%) patients had maintained normal liver function.

### Table 11. Interferon treatment effects on ALT

<table>
<thead>
<tr>
<th>IFN</th>
<th>patient</th>
<th>Normal</th>
<th>Relapse</th>
<th>Normal</th>
<th>Reference</th>
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<td>&gt;6M No. (%)</td>
<td>final No. (%)</td>
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<td>1</td>
<td>a2a</td>
<td>234</td>
<td>143 (61.1)</td>
<td>84 (58.7)</td>
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<tr>
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<td>30</td>
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<td>12 (60.0)</td>
<td>8 (26.6)</td>
</tr>
<tr>
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<td>a Bs</td>
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<td>11 (50.0)</td>
<td>11 (36.7)</td>
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<td>4</td>
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<td>6 (100.0)</td>
<td>0 (0.0)</td>
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<td>11 (39.3)</td>
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<td>10</td>
<td>a2a</td>
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<td>5 (31.2)</td>
<td>3 (60.0)</td>
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<td>61</td>
<td>51 (83.6)</td>
<td>27 (52.9)</td>
<td>24 (39.3)</td>
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<tr>
<td>Total:</td>
<td>510</td>
<td>325 (63.7)</td>
<td>180 (55.4)</td>
<td>145 (28.4)</td>
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</table>
5.1.2 IFN Treatment Effects on Serum HCV-RNA

In a total population of 146 patients from 7 trials, 70 (47.9%) patients became serum HCV-RNA negative after 3-6 months, but 38 (54.3%) of them reverted to positive at 6-12 months after the end of treatment. Only 32 (21.9) were still HCV-RNA negative 12 months after treatment (Table 12). Our findings are consistent with these results. Seven (58%) patients had an initial response, and 4 (57%) initial responders relapsed 3 months later. It is interesting that serum HCV-RNA became undetectable in 3 patients after more than 12 months of treatment, which indicates that long term treatment may have better therapeutic efficacy than the more common 6 month course.

Table 12. Interferon treatment effects on serum HCV-RNA

<table>
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<tr>
<th>IFN</th>
<th>Patient No.</th>
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<th>&gt;12M</th>
<th>Reference</th>
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<td></td>
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<td>No. (%)</td>
<td>No. (%)</td>
<td></td>
</tr>
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<td>13 (43.3)</td>
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<td>1 (14.3)</td>
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<td>16</td>
<td>3 (18.8)</td>
<td>1 (6.3)</td>
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<tr>
<td>Total:</td>
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<td>70 (47.9)</td>
<td>32 (21.9)</td>
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</tbody>
</table>
5.2 MONITORING THE TREATMENT RESPONSE TO INF

The optimal duration of therapy is not currently known. Different patients may require different lengths of treatment. What is needed is a means of monitoring treatment that would correctly identify when HCV has been cleared and a sustained clinical response could be expected. Several clinical, serum biological, serological and histological features have been analyzed as possible means of monitoring therapy in chronic hepatitis C (Table 13).

Table 13. Potential means for monitoring therapy

| Serum aminotransferase levels |
| Liver histology               |
| Antibody to HCV               |
| IgM antibody to HCV           |
| HCV-RNA in serum              |
| HCV-RNA in liver              |
| HCV antigen in liver          |

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5.2.1 Detection of HCV-RNA by RT-PCR

To monitor the therapeutic response to IFN, the virological markers, serum or liver HCV-RNA detected by RT-PCR, is being used in more and more clinical trials at the present. The results of RT-PCR directly reveal the status of virus replication. However, more and more concerns appear with the method of RT-PCR itself.

5.2.1.1 Reliability of RT-PCR

Polymerase chain reaction multiplies minute quantities of genetic material to a detectable level. Therefore, PCR is used to detect HCV-RNA, and the results of this assay may have a bearing on management of patients. Therefore, the reliability of PCR itself is critical for the detection of hepatitis C virus.

With the aim of standardisation of HCV-RNA detection, a coded test panel of fresh frozen plasma samples were sent to 38 hepatitis C research laboratories in Europe, USA, and Japan (Zaaijer H.L., 1993). The final results showed that one-third of laboratories had errors determining the status of undiluted samples and half of the laboratories had one or more errors in the dilution series. Only 5 of 31 laboratories generated perfect results, and even these 5 laboratories reported a hundred-fold difference in sensitivity for the dilution series. This indicates that the results of HCV-RNA detection should be interpreted with caution, and standardisation of HCV-RNA detection is a first requisite for reliable HCV infection studies.

More advantage of the HCV-RNA detection assay was reported by using the recently developed RT-PCR method which combined step of RT with PCR by using a new Taq DNA polymerase with both DNA polymerase and reverse transcriptase functions.
DISCUSSION

Improved reliability of the assay may be beneficial for monitoring patients undergoing antiviral therapy to determine the treatment end point (Young KY, 1993).

5.2.1.2 Quality control in this project

In this study, great care was taken in each process to avoid both contamination and poor sensitivity during the assay. To avoid the degradation of HCV-RNA, all of the dH2O used in the experiment was treated with 0.1%DEPC, which is an inhibitor of RNase. The reliability of the result from each RT-PCR amplification was insured by the detection of both negative and positive as well as internal controls in the same time. All of the specimens in which results were considered as indeterminate were tested to confirm the results. In the RT-PCR assay, the specificity was verified by the Southern blot analyses and restriction endonuclease digestion. In particular, the sensitivity was enhanced by performing the direct non-extraction method and quantitative measurements on the synthesized HCV-RNA template by moderate cycles for amplification.

1) Direct RT-PCR method for HCV-RNA detection

Usually, the experimental steps are RNA extraction, reverse transcription and PCR. Guanidinum, SDS, or proteinase K are routinely used for serum lysing followed by phenol/chloroform, ethanol extraction. However, these methods are time consuming and RNA is lost during the different steps above. Since it does not need the steps for RNA extraction, the direct RT-PCR method is more sensitive than the conventional method. In addition, less manipulation minimizes the possibility of contamination, and reduces the hands-on work required.
2) Quantitative analysis by synthesized HCV-RNA

Determination of the efficiency of RT-PCR assays as applied to the detection of HCV-RNA has been hampered by the lack of a method for growing large quantities of virus. Chimpanzee infectious dose-titered virus stocks are not useful for this purpose because only infectious virus is measured. While measurement of infectious virus may be clinically relevant, it does not necessarily correlate with virus particle count, as demonstrated previously (Garson JA, 1990). To obtain a direct measure of the analytical sensitivity of our RT-PCR assay, we have used a transcription vector from which HCV-RNA is in vitro transcribed and the RNA concentration is determined photometrically by UV A_{260}. By using such well-characterized templates, the analytical sensitivity of the RT-PCR assay was found to be equal to a single copy of the RNA template when analyzed by gel electrophoresis.

5.2.1.3 Significance of quantitative RT-PCR

Most patients with chronic hepatitis C have HCV-RNA in serum in titers ranging from 10^9 to 10^5. Testing of serum from patients treated with IFNα has shown that levels of this viral marker decrease on treatment and HCV-RNA becomes undetectable in most patients with a beneficial response to treatment. Levels decreased only slightly or not at all in patients with no or only a partial response (Hoofnagle JH, 1993; Shindo M, 1991). In this study, 7 of 12 (58%) patients showed parallel change of both HCV-RNA titer and transaminase. Especially, in 7 initial responders measured by serum HCV-RNA, 5 of them showed improved transaminase. The patients with lower initial serum HCV-RNA titers (10^5) had more likelihood of becoming HCV-RNA seronegative and of developing normal
DISCUSSION

transaminase. Clearly, there are not enough patients for an adequate sample size in the study. Nevertheless, the preliminary results show that the quantitative analysis of HCV-RNA has the potential to be used to predict and measure the treatment response from IFN.

HCV-RNA reappears in patients with a relapse after therapy is stopped and loss of HCV-RNA, while correlating well with a response to IFNα treatment, does not predict a sustained response (Hoofnagle JH, 1993). Obviously, in addition to HCV-RNA detection, other better markers are needed to assess and monitor therapy, and to provide guidance in when to initiate and when to stop therapy. Among these may be factors that regulate the rate of viral replication.

5.2.2 Measurement of 2',5'-Oligoadenylate Synthetase Activity

Although the virological parameters in chronic viral hepatitis are seen to be very important in evaluation of the response to IFN therapy, there are other biological aspects that could be taken into account for monitoring treatment. As being reviewed in chapter 2, 2',5'-oligoadenylate (2-5A) synthetase is an intracellular enzyme induced by interferon. Measurement of 2-5A levels may serve as a means to monitor the treatment of IFN. Serum levels of this enzyme were evaluated in 25 patients affected by chronic hepatitis C and treated with recombinant IFN-α2b (Giannelli G. 1993). At the end of treatment, 14 patients were classified as responders and 11 as nonresponders. Before therapy initiation no significant difference in (2-5A) synthetase levels among the patients were detected, while during therapy responders showed higher mean levels of 2-5A synthetase than nonresponders. An increase in the enzyme activity was observed after 1 month of
therapy, and this trend was maintained in the following 2 months. The peak 2-5A synthetase activity was found at the end of therapy. 2-5A synthetase levels were negatively correlated with serum ALT.

Although no definitive explanation has been provided for the presence of this typically intracellular enzyme in the serum, it has been proposed that this may be due to degeneration or cellular lysis of lymphocytes (Sawai H, 1988). Because 2-5A synthetase is an enzyme profoundly involved in the induction of the antiviral state induced by IFN, it has been suggested that 2-5A synthetase levels during IFN therapy correlate with the efficacy of the IFN treatment (Shindo M, 1988). The results above indicate that such correlation does exist also in patients with chronic hepatitis C. This study suggests that 2-5A synthetase may be a useful marker to monitor IFN efficacy during IFN treatment and to predict the clinical response. As supplementary data, 2-5A synthetase may need to be detected in all of the serum specimens in this study to compare with the HCV-RNA detection results.

5.3 REASONS FOR POOR RESPONSE TO IFN TREATMENT.

Interferon is commonly used for treatment of type C hepatitis, but the effects are variable and many factors may be responsible.

5.3.1. Neutralizing antibodies against IFN

It is now well established that some patients undergoing treatment with interferon-α (IFN-α) produce antibodies that neutralize the activity of the IFN used (Trown 1983, Antonelli 1991). The clinical significance of these antibodies remains controversial. Some
trials have suggested that the antibodies may have a negative effect on clinical response (Lok 1990). Patients who develop high titers of neutralizing antibodies often relapse and fail to respond again if given a large dose of the same IFN-α2 (Von Wussow 1991). It was reported recently that anti-IFN antibodies developed in 32% of all IFN treated patients. Forty percent of nonresponders developed anti-IFN antibodies compared to only 14% of responders (Douglas 1993). Three hepatitis C patients out of 15 who received recombinant IFN-α2a therapy developed high levels of neutralizing antibody coincident with clinical relapse (Brand 1993). All of these data suggest that the antibodies may have an adverse effect on clinical response rate. It is necessary to detect the antibody on the sera of patients in our trial.

5.3.2. Sequence Variation of HCV during Chronic Infection

Recent reports demonstrate that there is some viral variation during the chronic HCV infection. Sequence analysis demonstrated that the RNA sequence varied in the large envelope glycoprotein (E2/NS1) of hepatitis C virus in the interferon treated patients with relapsing non-A, non-B hepatitis (Kumer U. 1993). These data provide evidence that viral persistence may be due to the emergence of "escape mutants" in the hypervariable region.

5.3.3. Effects on IFN Treatment by HCV Genotypes

The responses to interferon treatment have been found to vary in patients chronically infected with the different HCV genotypes (Carreno V, 1993, Shino M, 1991). HCV can be classified into 4 types (as reviewed in chapter 2). In patients with type II and type III virus infection, both liver function and serum HCV-RNA were observed at the end
DISCUSSION

of 8 weeks and 24 weeks in the interferon treatment (Takada N., 1993). The percentage of patients exhibiting a good response was significantly higher in the type III group than in the type II group at both observation periods. During the post-treatment periods, relapse following complete response was found to be higher in type II group than III. The final effects of interferon were significantly better in the type III than in the type II.

Whether or not the typing results will predict the clearance or non-clearance of HCV-RNA at the end of treatment remains to be proven in future trials, but the HCV genotype identification before initiation of treatment seems useful to predict a therapeutic response.

There are very few studies reported in Canada to introduce prevalence of different HCV genotype infection and possible variation in the process of IFN treatment. In this clinical trial, further studies should focus on the relationship between the HCV genotype and IFN treatment efficacy. Especially, we need to know whether there is any genotype variation in the process of IFN treatment, when the variation happened, and how the genotype variation is correlated with therapeutic responses. Based on sequence analysis, the method of DNA typing could be used to analyze PCR products from both most conserved and the hypervariable regions of HCV genome in the different periods of IFN treatment. The result should be very significant for the evaluation of IFN treatment, and may provide a new sight to explain the possible reason for a poor response to IFN.
5.4 THE FUTURE OF THERAPY OF HEPATITIS C

5.4.1 Other Approaches to the IFN Administration

In recent years, it has been demonstrated that recombinant interferon is efficacious in the treatment of chronic hepatitis C. Thus approximately 50-70% of patients treated with 3MU trice weekly over 6 months normalize ALT values and show improvement in their liver histology. However, the majority of patients suffer a reactivation of the liver disease at the end of treatment with an increase in ALT values. To improve the response rate, several following approaches may be tried in the administration of IFN:

1) Higher doses of IFN;
2) Prolongation of the treatment other period;
3) Retreatment;
4) Combination with other agents.

5.4.2 Other Potential Agents for the Therapy of Hepatitis C

An important task to be carried out in the future is to find new antiviral agents (perhaps with in vitro systems of HCV culture), and to eradicate HCV-RNA from all the compartments such as serum, peripheral blood mononuclear cells, and liver. Some of potential agents are listed in table 14.
Table 14. Potential agents for therapy of chronic hepatitis C

<table>
<thead>
<tr>
<th><strong>Antivirals</strong></th>
<th><strong>Biologic response modifiers</strong></th>
<th><strong>Immunomodulators</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>ribavirin</td>
<td>alpha-, beta-, and gamma-interferon</td>
<td>prednisone</td>
</tr>
<tr>
<td>acyclovir</td>
<td>interleukins 2, 4, and 6</td>
<td>thymosin</td>
</tr>
<tr>
<td>adenine arabinoside</td>
<td>colony-stimulating factors</td>
<td>levamisole</td>
</tr>
<tr>
<td>foscarnet</td>
<td>tumour necrosis factor</td>
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</tr>
<tr>
<td>azathymidine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dideoxynucleosides</td>
<td></td>
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</tr>
<tr>
<td>ganciclovir</td>
<td></td>
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<tr>
<td>suramin</td>
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In summary, a major goal on the treatment of hepatitis C is the prevention of the high relapse rate after cessation of therapy and the improvement of the initial response rate. Thus, future antiviral therapy in chronic hepatitis C will help to fill in the gap in knowledge about the correct use of alpha-interferon but will also focus on newer antiviral agents that alone or in combination with interferon promise to provide a relatively safe but highly effective therapy for all patients with liver disease due to hepatitis C.
REFERENCES


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**Esteban R. 1993.** Epidemiology of hepatitis C virus infection. *J Hepatol.* 17(S3): S35-41.


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