# 行政院國家科學委員會補助專題研究計畫成果報告

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**周邊血液單核細胞內C型肝炎病毒之分子病毒學研究**( 2 / 2 )

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**計畫類別：**■**個別型計畫**　　□**整合型計畫**

**計畫編號：**NSC89－2315－B－002－009－

**執行期間：**88 **年** 8 **月** 1 **日至** 89 **年** 7 **月** 31 **日**

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**中　華　民　國** 89 **年** 10 **月** 12 **日**

# 中文摘要

自從 C 型肝炎抗體檢驗試劑問世以來，吾人已確知大多數之慢性非 A 非 B 型肝炎 (non-A, non-B hepatitis) 乃由 C 型肝炎病毒 (hepatitis C virus, HCV) 所引起。近年對 C 型肝炎病毒的分子生物學與感染後的臨床病理病程研究皆有長足之進步，但對 C 型肝炎病毒的持續感染機轉及病毒在慢性 C 型肝炎病患周邊血液單核細胞 (peripheral blood mononuclear cell, PBMC) 內之病毒學意義則尚待加強。為釐清上述極具重要性但懸而未決的問題，吾人在過去兩年內設計一系列的實驗由各個層面來探討 C 型肝炎病毒感染人類周邊血液單核細胞的機轉，諸如(1) 定量血清與周邊血液單核細胞之病毒濃度，藉此瞭解宿主細胞中病毒正股 RNA 和負股 RNA (複製中間體) 之比例並評估病毒複製速率之高低；(2) 探討血清與周邊血液單核細胞內病毒之物種類似 (quasispecies) 程度有無差異和 (3) 尋找病毒基因體外套高度變異區 (hypervariable region, HVR-1) 有無存在特定之胺基酸序列以決定病毒之細胞趨向性。本系列研究之結果顯示 (1) 血清中病毒之平均濃度為 $1.5 \times 10^4$ copies/mL，而周邊血液單核細胞中正股 RNA 和負股 RNA 之平均濃度分別為 $7.8 \times 10^2$ 和 $2.7 \times 10^2$ copies/μg RNA。其中正股 RNA 濃度約為負股 RNA 之 1~10 倍，顯示 HCV 在周邊血液單核細胞內之複製效率並不高。(2) HCV 在血清和周邊血液單核細胞內之平均 quasispecies complexity 分別為 $5.4 \pm 1.7$ 和 $7.0 \pm 2.5$，顯示不同生存環境對 HCV 有不同的選擇壓力 (selection pressure)。(3) HVR-1 之胺基酸 402 (L) 似乎和 HCV 感染周邊血液單核細胞有密切關係，但需做進一步確認。這些成果將有助於闡明 C 型肝炎病毒持續感染和其細胞趨向性的部分機轉，相信對未來相關分子病毒學與免疫學的研究會有相當程度的影響，同時也將裨益於有關慢性 C 型肝炎的治療與預防性疫苗的研發。

關鍵詞：C 型肝炎病毒，周邊血液單核細胞。

## Abstract

With the recent advent of cloning a novel hepatitis C virus (HCV) and subsequent development of several serological assays, at present HCV has been recognized as the major etiologic agent of parenteral non-A, non-B hepatitis worldwide [6, 14, 44, 45, 54]. In order to clarify the biological significance and clinical implications of HCV in PBMC from patients with chronic type C hepatitis, we have conducted a series of studies including (1) quantitation of HCV in serum as well as in PBMC and estimation of the frequency of HCV-infected PBMC; (2) whether diversity of virus quasispecies exists between serum and PBMC; (3) whether specific sequences for receptor recognition exist in the HVR-1 region of HCV genome to determine the PBMC tropism in the past two years. Our results showed that (1) the average serum HCV titer is $1.5x10^4$ copies/mL and the average titer of positive-strand and negative-strand HCV RNA in PBMC is $7.8x10^2$ and $2.7x10^2$ copies/ug RNA. In addition, the titer of positive-strand HCV RNA in PBMC is 1 to 10 times higher than that of negative-strand HCV RNA in PBMC, suggesting a low replication efficiency of HCV in such cells. (2) The quasispecies complexity of HCV genome in serum and PBMC is 5.4+1.7 and 7.0+2.5, suggesting different selection pressure in different tissue compartments. (3) The aa 402(L) of HVR-1 may be associated with the PBMC tropism of HCV; however, further studies are warranted to confirm this preliminary finding. Based on these results, we could understand, at least in part, the pathogenesis of viral persistence and cell tropism of HCV. In the meantime, these data will shed much light on the establishment of culture system for HCV infection and will significantly influence our future strategies of hepatitis C vaccine development and antiviral therapy.


**Key words:** Hepatitis C virus, peripheral blood mononuclear cell, HCV, PBMC

# Introduction

Since the application of sensitive serologic markers for the diagnosis of hepatitis A and hepatitis B infections, it becomes clear that there are other hepatitis viruses designating non-A, non-B hepatitis viruses [2]. Because of lacking reliable markers, the diagnosis of non-A, non-B hepatitis (NANBH) required the exclusion of ongoing HAV or HBV infections and hepatotoxin or drug exposure [19]. Clinically, more than 50% of acute NANBH cases develop chronic hepatitis and 20% of them will progress to liver cirrhosis [19, 80, 81]. Besides, a potentially causal association of NANB virus infection with hepatocellular carcinoma (HCC) has been reported [42, 68]. With the recent advent of cloning a novel hepatitis C virus (HCV) and subsequent development of several serological assays, at present HCV has been recognized as the major etiologic agent of parenteral non-A, non-B hepatitis worldwide [6, 14, 44, 45, 54]. The characteristics of HCV have been described [11, 15, 31, 40, 64, 67, 77]. It is an enveloped, 50-60 nm virus with a single positive-stranded RNA genome of 9,500 nucleotides. There is some homology between the HCV and the flavivirus or pestivirus group. The genome organization of HCV is similar to that of pestiviruses or flaviviruses and consists of 5' and 3' untranslated regions (5UT and 3UT) flanking the virus structural (core, envelope) and nonstructural region 1/envelope region 2 (E2/NS1) and nonstructural regions 2 to 5 (NS2 - NS5) [31]. Several HCV isolates cloned from different geographic areas show significant sequence divergence [11, 15, 40, 64, 67, 77]. Recent phylogenetic analysis of the NS5 region of HCV genome has classified the virus into 6 major groups and 11 subtypes at least 6 major genotypes of HCV have been proposed to classify all of the reported isolates [4, 76], but only genomes of 4 subtypes (1a, 1b, 2a and 2b) have been wholly sequenced [11, 15, 40, 64, 67, 77]. The 5UT region of HCV is the most conserved among different HCV isolates and has been shown useful in detecting the virus by polymerase chain reaction (PCR) assay [4, 11, 31, 38, 66]. By contrast, the envelope region (E1) and E2/NS1 are the moderately variable and hypervariable portions among different subtypes of HCV genomes, respectively [4, 11, 15, 27, 28, 31, 43, 58, 62, 63, 64, 67, 77, 82, 83]. In recent reports, the genotypes and the amino acid heterogeneity in the variable regions of E2/NS1 viral protein of HCV have been claimed to be closely associated with the response to interferon-$\alpha$ therapy [86]. Because of the sequence diversity, the envelope region can be used as an effective tool to elucidate unresolved issues about HCV infections such as tracing transmission routes [35, 38] as well as correlating the interrelationships between the acute exacerbations of chronic type C hepatitis and the evolution of hepatitis C viral genomes [34]. However, there had been no efficient primers suitable for amplification of the envelope region until recently [17, 23, 36, 66].

Although direct sequencing of the amplified hypervariable region has been used to identify the main HCV subtype [11, 15, 40, 64, 67, 77], it is sophisticated and time-consuming and thus cannot be applied to a large number of samples. Recently, several easier genotyping methods for

HCV have been introduced and the incidence of genotypes in different countries has been determined [76]. Moreover, the genotypes of HCV have been claimed to be associated with the severity of chronic hepatitis and the response to interferon-α therapy [86]. By using a PCR typing assay with specific primers, we have demonstrated that type 1b virus is the predominant genotype in chronic type C hepatitis in Taiwan [36, 39], and type 1b virus as well as mixed infection may trigger more severe liver disease [34, 37].

Chronic viral hepatitis, liver cirrhosis and hepatocellular carcinoma are common in Taiwan and most are HBV-related [8, 10, 85]. The possible etiology accounting for the remaining chronic liver disease in Taiwan has been less mentioned until Chen et al [6] reported that 65% of HBsAg negative patients with chronic hepatitis, 44% of HBsAg negative cirrhotics and 63% of HCC patients seronegative for HBsAg are positive for anti-HCV. These data indicate that HCV infection plays an important role in HBsAg negative patients with chronic liver disease in Taiwan.

The high frequency of acute HCV infection evolving into chronicity [19, 80, 81], like human immunodeficiency virus (HIV) infection, is rather unusual as compared to other virus-related diseases. The possible mechanisms of viral persistence are as follows [69]: (1) tolerance to viral antigens as the hepatitis B e antigen in the perinatal transmission of HBV; (2) immunosuppression and infection of lymphoid tissue as HIV and Epstein-Barr virus (EBV) [12, 32]; (3) antigenic variation of virus as HIV and HDV [29, 30, 51, 57, 70]; (4) inaccessibility of the immune system to the infected cells like the HIV-infected neuron cells. Among these possibilities, lack of adequate host immunity [13, 22, 34, 65, 71], frequent viral antigen mutation [21, 27, 62, 63] and infection of lymphocytes by organ-specific selection of viral variants [1, 3, 24, 61, 72, 79, 87] which enable HCV to escape from host immune pressures may be most likely involved in the pathogenesis of HCV persistence. Viral variants and their cell tropism have been documented to play major roles in the persistent infection of viruses. Recent studies of HIV indicated that antigenic variation in the hypervariable V3 loop sequence of envelope glycoprotein (gp120) not only can escape from host immune response, but also contributes to the determination of lymphocyte versus macrophage tropism [12, 32, 33]. Several recent studies revealed that replication intermediates of HCV can be demonstrated in the peripheral blood mononuclear cells (PBMC) of a proportion (70%-100%) of chronic HCV-infected patients, implying HCV may infect PBMC [24, 61, 72, 79, 87]. Whether these also contribute to the possible mechanisms of HCV persistence remains unknown. Moreover, a recent report indicated that determination of HCV RNA in PBMCs at the end of interferon (IFN) therapy may predict whether the disease will relapse after IFN discontinuation [25], suggesting the presence of HCV in PBMC may have its clinical implications.

Although several patterns of serum ALT activities in chronic hepatitis C have been identified [39], the viral mechanisms of fluctuating hepatitis activities are far from clear. Recent studies based on chimpanzee experiments and human observations have revealed partly pathogenesis

responsible for episodes of hepatitis flares in chronic HCV carriers [22, 34, 65, 71]. In chronic hepatitis B, superinfection with other hepatitis viruses and reactivation of the original hepatitis B virus are two major causes of clinical exacerbations [7, 10, 18, 49, 55, 73]. Whether similar mechanisms also contribute to the acute exacerbations of chronic hepatitis C remains to be explored. Several cross-challenge studies in chimpanzees have provided evidence that reinfection with either homotypic or heterotypic HCV along with chronic HCV carriage does occur [22, 23, 65, 71]. In human beings, Lai et al. [47] indicated that the multiple episodes of acute hepatitis in 2 of their 3 polytransfused thalassemic children are due to reinfection with a different HCV strain and in the third may be due to reactivation of primary infection. Our previous studies indicated that superinfection of heterotypic HCV indeed occurs in humans and mixed infections of heterotypic HCV may be important in causing hepatitis flares of chronic hepatitis C, and thus also reinforced the concept of multiple infection with HCV as well [34, 37].

The influence of virus amount of HCV on clinicopathological course of chronic type C hepatitis has been demonstrated [26, 46, 50]. In addition, viral genetic variation during chronic infection has been documented in hepatitis viruses [5, 16, 48, 52]. For chronic type B hepatitis, severe liver damage is related to the clustering missense mutation in the core gene [16]. Similarly, the evolution rates of HDV genomes appear to be correlated with the changes of clinical pictures of hepatitis [52]. Thus, the more drastic the change of symptoms of hepatitis, either related to HBV or HDV, the more nucleotide changes are detected. HCV, like many other RNA viruses, conforms extensive variability in genomic sequences [20, 29, 56], especially in the 5' end of E2/NS1 region (nt. 1156-1233, 78 nucleotides, 26 amino acids) so-called hypervariable region (HVR-1), appears to be one of the major targets of host immune response and the hypervariation is a result of the selection pressure like the V3 loop of HIV-1 [4, 11, 15, 21, 27, 28, 31, 43, 58, 62-64, 67, 77, 82-84]. Our recent data showed that quasispecies nature of HCV genomes exists in humans and patients with different clinicopathologic course of chronic type C hepatitis may have sequential variations in virus amounts as well as neutralization epitopes of the HVR-1 [35a].

The fact that no suitable animal model or in vitro cell culture system for experimental HCV infection except chimpanzee inoculation limits the biologic assay of HCV. Thus it is imperative to develop either a less expensive small animal model or an in vitro system for propagating HCV. Two recent reports have demonstrated that some human T cell leukemia lines can support the replication if HCV [74, 75], and this prompted us to establish our own in vitro cell culture system and small animal model for HCV propagation.

In order to clarify the biological significance and clinical implications of HCV in PBMC from patients with chronic type C hepatitis, in the following two years a series of studies including (1) quantitation of HCV in serum as well as in PBMC and estimation of the frequency of HCV-infected PBMC; (2) whether diversity of virus quasispecies exists between serum and PBMC; (3) whether specific sequences for receptor

recognition exist in the HVR-1 region of HCV genome to determine the PBMC tropism will be performed in our laboratory (Hepatitis Research Center, National Taiwan University Hospital).

# Patients and Methods

**I. Patients:**

A total of 30 patients with chronic type C hepatitis have been enrolled, and paired plasma and PBMC samples from these patients were stored at -70$^{o}$C until used.

**II. Methods:**

**1.** PBMC and plasma are separated using Ficoll-Hypaque gradient centrifugation.

**2.** Extraction of ribonucleic acids:

HCV RNA in serum and PBMC are extracted by single-step method with acid guanidinium thiocyanate-phenol-chloroform.

**3.** Reverse transcription and nested polymerase chain reaction (RT-PCR) genotyping with type-specific primers [38, 39, 78]. By distinct sizes of PCR products, 4 genotypes (1a, 1b, 2a and 2b) of HCV can be identified.

**4.** Amplification of the HVR-1 of HCV genome [34-36, 38, 53]. The primers that produce a PCR product encompassing E1 and HVR-1 regions are designed after comparing different genotypes of HCV (type 1a to 2b) and have been reported [11, 15, 64, 67].

**5.** To detect negative or antigenomic strand (replication intermediate) of HCV in PBMC, outer sense PCR primers instead of random primers are used in cDNA synthesis. After cDNA synthesis and heat inactivation of reverse transcriptase at 95$^{o}$C for 2 hours, RNase A (0.1 μg) is added subsequently (37$^{o}$C for 30 minutes) to ensure removal of residual positive strand or genomic HCV RNA [59].

**6.** The sequences of amplified HVR-1 PCR products are determined by using fluorescence labelled primers with 373A Sequencer (Applied Biosystems, Foster City, CA)

**7.** Comparative sequence analysis of the HVR-1 from serum and PBMC to detect conserved domain that can be specific for receptor recognition

**8.** Selected amplified HVR-1 PCR products from serum and PBMC are ligated to pCR-Script$^{TM}$ SK(+) vectors and then transformed to E. coli XL1-Blue MRF' competent cells (Stratagene, La Jolla, CA). Plasmid DNAs are extracted from white colonies by Winard$^{TM}$ minipress DNA purification system (Promega, Madison, WI).The sequences of inserted DNA are also determined by an automatic sequencer.

**9.** Quantitation of HCV RNA (positive and negative strands) in serum (copies/mL serum), PBMC (copies/μg RNA) and by a competitive PCR assay [53]. The estimated frequencies of HCV-infected PBMC are calculated accordingly.

## Results

**1.HCV RNA level in PBMC of patients with chronic type C hepatitis**

Table 1. Clinical and laboratory data of 10 patients with chronic type C hepatitis.

| Patient | Sex, age (years) | Serum ALT (U/L) | HCV genotype | | | |
|---|---|---|---|---|---|---|
| | | | Plus-strand | | Minus-strand | |
| | | | Serum | PBMC | Serum | PBMC |
| 1 | F, 52 | 51 | 1b | 1b | –* | 1b |
| 2 | F, 59 | 157 | 1b | 1b | – | 1b |
| 3 | M, 54 | 16 | 2a | 2a | – | 2a |
| 4 | F, 47 | 57 | 1b | 1b | – | 1b |
| 5 | M, 58 | 48 | 1b | 1b | – | 1b |
| 6 | F, 73 | 191 | 1b | 1b | – | 1b |
| 7 | F, 63 | 24 | 1b | 1b | – | 1b |
| 8 | M, 54 | 56 | 2a | 2a | – | 2a |
| 9 | M, 55 | 52 | 1b | 1b | – | 1b |
| 10 | M, 73 | 123 | 1b | 1b | – | 1b |

Note. ALT, alanine aminotransferase; M, male; F, female; PBMC, peripheral blood mononuclear cell.

- Negative

Table 2. HCV titers of serum , plus- and minus-strand in PBMC of 10 patients with chronic type C hepatitis.

| Patient | Genotype | Serum titer (copies/mL) | Titer in PBMC (copies/μg RNA) | |
|---|---|---|---|---|
| | | | Plus-strand | Minus-strand |
| 1 | 1b | $5x10^2$ | $10^2$ | $10^2$ |
| 2 | 1b | $5x10^3$ | $10^3$ | $10^2$ |
| 3 | 2a | $5x10^2$ | $10^2$ | $10^2$ |
| 4 | 1b | $2.5x10^4$ | $10^2$ | 10 |
| 5 | 1b | $2.5x10^3$ | $5x10^2$ | $10^2$ |
| 6 | 1b | $5x10^2$ | $10^3$ | $10^2$ |
| 7 | 1b | $5x10^3$ | $10^3$ | $10^3$ |
| 8 | 2a | 5x10 | $10^3$ | $10^2$ |
| 9 | 1b | $10^4$ | $2x10^3$ | $10^3$ |
| 10 | 1b | $10^5$ | $10^3$ | $10^2$ |
| Average | | $1.5x10^4$ | $7.8x10^2$ | $2.7x10^2$ |

Note. PBMC, peripheral blood mononuclear cell.

**2. Degree of quasispecies of HCV in PBMC of patients with chronic type C hepatitis**

Table 1. Clinical and laboratory characteristics of 5 patients with chronic hepatitis C included in the study

| Patient No. | Sex/ Age (yr) | Peak serum ALT (units/L) | HCV Genotype | HCV RNA* | |
|---|---|---|---|---|---|
| | | | | Plasma | PBMC |
| 1 | M/65 | 52 | 1b | +/– | +/+ |
| 2 | M/73 | 254 | 1b | +/– | +/+ |
| 3 | M/58 | 84 | 1b | +/– | +/+ |
| 4 | F/47 | 69 | 1b | +/– | +/+ |
| 5 | F/61 | 152 | 1b | +/– | +/+ |

NOTE. M. male; F, female; ALT, alanine aminotransferase; PBMC, peripheral blood mononuclear cells.

*Positive/negative strands of HCV RNA are indicated.

Table 2. Comparison of the quasispecies diversity of HCV genome between plasma and peripheral blood mononuclear cells

| Patient No. | No. of Clones Sequenced | | Quasispecies diversity* | |
|---|---|---|---|---|
| | Plasma | PBMC | Plasma | PBMC |
| 1 | 8 | 11 | 3 | 7 |
| 2 | 10 | 12 | 7 | 10 |
| 3 | 9 | 7 | 7 | 5 |
| 4 | 7 | 12 | 5 | 4 |
| 5 | 7 | 11 | 5 | 9 |
| Average (mean±SD) | | | 5.4 ± 1.7 | 7.0 ± 2.5† |

NOTE. PBMC, peripheral blood mononuclear cells.

* No. of clones with different amino acid sequences.

† Statistically non-significant by Student's t test.

3. **Specific sequences in the HVR-1 region of HCV genome and PBMC tropism**

Table 1 Demographic and clinical data of group A and group B chronic hepatitis C patients

| Group and patient no. | Sex/ age (years) | History of transfusion | Serum ALT (U/L) |
|---|---|---|---|
| A1 | F/63 | – | 24 |
| A2 | F/59 | – | 175 |
| A3 | F/58 | – | 61 |
| A4 | M/58 | – | 48 |
| A5 | M/73 | + | 254 |
| A6 | M/50 | – | 95 |
| A7 | F/61 | + | 152 |
| A8 | F/47 | + | 91 |
| A9 | M/63 | – | 45 |
| A10 | M/47 | + | 123 |
| A11 | F/47 | – | 69 |
| A12 | F/67 | + | 189 |
| A13 | F/65 | – | 140 |
| A14 | M/68 | – | 46 |
| A15 | M/31 | + | 156 |
| A16 | F/83 | + | 32 |
| A17 | F/64 | – | 243 |
| A19 | F/55 | – | 52 |
| A20 | M/67 | – | 69 |
| B1 | F/77 | + | 78 |
| B2 | F/60 | – | 74 |
| B3 | F/62 | + | 188 |
| B4 | M/33 | + | 136 |

Group A: chronic hepatitis C patients with HCV infection of peripheral blood mononuclear cells; Group B: chronic hepatitis C patients without HCV infection of peripheral blood mononuclear cells;

Table 2 Conservation of amino acids in the hypervariable region of hepatitis C virus genome between group A and group B chronic hepatitis C patients

| Amino acid | % of conservation | | P value |
|---|---|---|---|
| | group A | group B | |
| 385 (T) | 100% | 100% | NS |
| 389 (G) | 100% | 100% | NS |
| 390 (G) | 95% | 100% | NS |
| 402 (L) | 90% | 0% | 0.03 |
| 403 (F) | 90% | 100% | NS |
| 406 (G) | 100% | 100% | NS |
| 409 (Q) | 100% | 100% | NS |

Group A: chronic hepatitis C patients with HCV infection of peripheral blood mononuclear cells; Group B: chronic hepatitis C patients without HCV infection of peripheral blood mononuclear cells.

## Discussion

Our results showed that (1) the average serum HCV titer is $1.5x10^4$ copies/mL and the average titer of positive-strand and negative-strand HCV RNA in PBMC is $7.8x10^2$ and $2.7x10^2$ copies/ug RNA. In addition, the titer of positive-strand HCV RNA in PBMC is 1 to 10 times higher than that of negative-strand HCV RNA in PBMC, suggesting a low replication efficiency of HCV in such cells. (2) The quasispecies complexity of HCV genome in serum and PBMC is 5.4+1.7 and 7.0+2.5, suggesting different selection pressure in different tissue compartments. (3) The aa 402(L) of HVR-1 may be associated with the PBMC tropism of HCV; however, further studies are warranted to confirm this preliminary finding. Based on these results, we could understand, at least in part, the pathogenesis of viral persistence and cell tropism of HCV. In the meantime, these data will shed much light on the establishment of culture system for HCV infection and will significantly influence our future strategies of hepatitis C vaccine development and antiviral therapy.

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