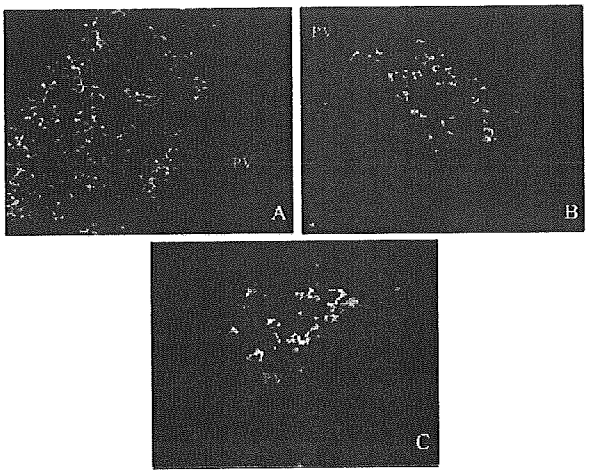


*Fig. 6.* Phenotype of infiltrated cells in the liver tissue in female C57BL/6 mice because of polyinosinic polycytidylic acid (poly I:C) administration for 16 weeks. The staining was done by an immunofluorescence method using monoclonal antibodies. (A) Cells stained green and red indicate B ($CD45R^+$) and T ($CD90.2^+$) lymphocytes, respectively. PV, portal vein. (B) Cells stained green and red represent $CD4^+$ and $CD8^+$ ($CD8\alpha^+$) T cells, respectively. (C) Cells stained green and red indicate $CD11b^+$ macrophages and $CD11c^+$ dendritic cells, respectively. Alexa Fluor 488(green) or Alexa Fluor 555(red)-conjugated secondary antibodies were used.

cannot be directly applied in patients because of ethical and safety concerns.

PBC is an intractable liver disease with no permanent remedy. The disease is usually detected in middle-aged females. In a considerable number of patients, the disease is progressive in nature and ultimately develops into liver cirrhosis and liver failure. Presently, ursodeoxycholic acid is used to provide symptomatic relief to PBC patients, but there is no curative therapy for this disease.

To study PBC pathogenesis and to test newly developed therapeutic regimens of PBC, several investigators have been trying to develop animal models mimicking PBC. It was observed that female C57BL/6 mice spontaneously develop cholangitis in the liver at 18–24 months (11, 12). However, it is difficult to study the cellular and molecular events underlying the pathogenesis of PBC because of late development of cholangitis in these mice. Indeed, the efficacy of newer drugs and therapeutic regimens could not be examined in this model because the life span of mice is usually around 24 months.

Here, we succeeded in developing a murine model of PBC at the age of 4 months by triggering autoimmunity in female C57BL/6 mice with the help of poly I:C. There are several studies about the effects of poly I:C in autoimmunity (22, 27–29). Most of these studies revealed that poly I:C had a role during the initiation and progression of these diseases in susceptible hosts. When 8-week-old female C57BL/6 mice were injected with poly I:C twice a week, there was increased production of IFN-α in the sera and all of these mice developed PBC-like lesions in the liver. The lesions were characterized by accumulation of mononuclear cells in the portal areas and were progressive in nature. Moreover, most of the mice developed some types of autoantibodies, including AMA. Extrahepatic inflammatory lesions in the salivary glands, pancreas, and kidneys mimicking PBC were also documented in considerable numbers of mice. When we made a comparison between poly I:C-induced and spontaneous development (11, 12) of PBC, two distinctive features were evident between these two animal models. First, the poly I:C-induced model was more rapid compared with the spontaneous model. Next and most importantly is the fact that all mice of poly I:C-induced model developed PBC-like lesions, on the other hands in the spontaneous model only some mice did.

Although we developed a mouse model of PBC within 8 weeks of injection commencement with poly I:C, several questions deserve further clarification, including the mechanism of development of cholangitis because of poly I:C injection. We have shown increased IFN-α levels because of poly I:C injection (Fig. 1). Moreover, other proinflammatory cytokines were increased in the sera at poly I:C injected female C57BL/6 mice. IFN-α can induce other proinflammatory cytokines (summarized in Stewart (30)). Moreover, poly I:C can also induce proinflammatory cytokines in addition to IFN-α. Taken together, IFN-α alone or in conjunction with other cytokines might have a role in the induction of PBC. Hanada et al. (31) revealed that tumor necrosis factor-α (TNF-α) or IFN-γ directly disrupt the barrier function of tight junction in cultured mouse cholangiocytes. This finding may provide a clue to investigating the mechanism of mononuclear cells' accumulation around the bile ducts in our model.

According to this protocol, the extent and ratio of infiltrating cells in the portal areas progressed in female C57BL/6 mice until 16 weeks after the commencement of poly I:C injection, but remained almost static thereafter (Fig. 3). At the same time, IFN-α levels in the sera decreased progressively (Fig. 1). Although the underlying mechanism is not completely clear, some studies have shown that repeated poly I:C administration might lead to reduced IFN-α levels (28). This might partially explain lower levels of IFN-α and the non-progressiveness of PBC-like lesions in our mice after 16 weeks.

The localization of macrophages and dendritic cells (Fig. 6) in the liver from poly I:C injected mice might be interesting. Both of these cells produce IFN-α because of poly I:C stimulation (32, 33). These cells are also activated by IFN-α (34, 35) and may in turn produce a variety of cytokines, chemokines, and immune modulators, which might have a role during the induction of autoimmunity. Type-1 IFNs also increase the secretion of antibodies (36), including autoantibodies; however, in our experimental protocol, although poly I:C-injected mice produced less IFN-α after 16 weeks (Fig. 1), AMA-positive mice increased after this time point (Fig. 5), indicating a differential regulation of AMA and IFN-α in this model.

In conclusion, we have shown that an animal model of PBC has been developed by poly I:C in genetically susceptible C57BL/6 female mice. We have detected higher levels of IFN-α and other proinflammatory cytokines in these mice because of poly I:C injection. This animal model of PBC would allow the analysis of the early cellular events of PBC. Moreover, as the PBC-like lesions developed in these mice within 4 months, this model would permit the investigation of the efficacy of newer drugs and therapeutic regimens for PBC.

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CLINICAL BIOCHEMISTRY

# The quantification of cytochrome P-450 (CYP 3A4) mRNA in the blood of patients with viral liver diseases

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**Abstract**

**Objective:** We quantified cytochrome P-450 (CYP) 3A4 mRNA in the blood and liver of patients with viral liver diseases to determine whether CYP 3A4 expression is related to disease progression.

**Design and methods:** Total RNA was extracted from 10 mL of blood from 12 normal volunteers, from 6 patients with acute hepatitis, 17 with chronic hepatitis, 12 with liver cirrhosis, and 16 with hepatocellular carcinoma. Total RNA from 1 mg of liver tissue was extracted simultaneously in 10 patients. CYP 3A4 mRNA was quantified by competitive reverse-transcription polymerase chain reaction and expressed as log copies/microliter.

**Results:** The CYP 3A4 mRNA titer in blood correlated with that of the liver ($r = 0.65$, $P < 0.05$). The CYP 3A4 mRNA titer was 1.6 ± 0.4 in normal controls, 1.0 ± 0.5 in acute hepatitis, 0.7 ± 0.2 in chronic hepatitis, 0.5 ± 0.2 in liver cirrhosis, 0.5 ± 0.2 in hepatocellular carcinoma, and decreased with progression of liver disease ($P < 0.05$).

**Conclusion:** These data suggest that the CYP 3A4 mRNA level in blood relates to progression of liver disease.


*Keywords:* Cytochrome P-450 3A4; Viral liver diseases; Hepatitis B virus; Hepatitis C virus

## Introduction

Cytochrome P-450 (CYP) is an important drug metabolic enzyme primary distributed in the liver [1]. The most highly expressed CYP is the CYP3A subfamily, which includes the isoforms 3A4, 3A5, 3A7, and 3A43. The most abundant CYP3A isoform expressed in the liver and gut is CYP 3A4 [2]. CYP 3A4 expression in liver and its enzymatic activity may vary between 20- and 50-fold among normal individuals [2,3].

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infection are worldwide health problems. Chronic hepatitis (CH) due to HBV and HCV may progress to liver cirrhosis (LC) or hepatocellular carcinoma (HCC) over the course of 20–30 years. In LC, liver function, including CYP 3A4 enzymatic activity, is decreased [4]. In addition, CYP 3A4 is also a procarcinogen metabolizer [1] and thus may play a role in hepatocarcinogenesis. However, the level of CYP 3A4 expression in blood and liver and its role in viral liver diseases is unclear. To clarify whether CYP 3A4 expression in blood and liver is related to hepatitic activity, progression of viral liver diseases, and occurrence of HCC, we quantified CYP 3A4 mRNA in the blood and liver of patients with viral liver diseases.

## Materials and methods

### *Patients*

The study population was comprised of 51 patients (40 males, 11 females, mean age ± SD: 52.4 ± 14.7 years, range:

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Table 1
CYP 3A4 mRNA titer in the blood in various liver diseases

| Diagnosis | No. | CYP 3A4 mRNA titer in blood (log copies/μL) |
|---|---|---|
| Normal controls | 12 | 1.6 ± 0.4 |
| Acute hepatitis | 6 | 1.0 ± 0.6[a] |
| Chronic hepatitis | 17 | 0.7 ± 0.2[a] |
| Liver cirrhosis | 12 | 0.5 ± 0.2[a,b] |
| Hepatocellular carcinoma | 16 | 0.5 ± 0.2[a,b] |

[a] $P < 0.01$ vs. normal controls.
[b] $P < 0.05$ vs. chronic hepatitis.

22–77 years) including 6 with acute hepatitis (AH), 17 with CH, 12 with LC, and 16 with HCC, admitted to our hospital between February 2001 and September 2002, consecutively. The number of HBs antigen positive and HCV antibody positive patients in CH was 14 and 3, respectively, and was 9 and 3 in LC, and 14 and 2 in HCC. In AH, 1 patient was HBs antigen positive, 3 were HCV antibody positive, and 2 were IgM anti-hepatitis A virus antibody positive. Twelve normal healthy people served as the controls (8 males, 4 females, age: 43.1 ± 15.6 years). Histological classification of CH was performed according to the criteria of the International Hepatitis Group [5]. There were 8, 5, and 4 patients with mild, moderate, and severe fibrosis, respectively. The grading and staging of HCC was performed according to The Liver Cancer Study Group of Japan [6]. There were 3, 5, and 8 patients with HCC stages III, IVA, and IVB, respectively. Informed consent was obtained from all patients. This study was approved by the Ehime University's ethical human research committee.

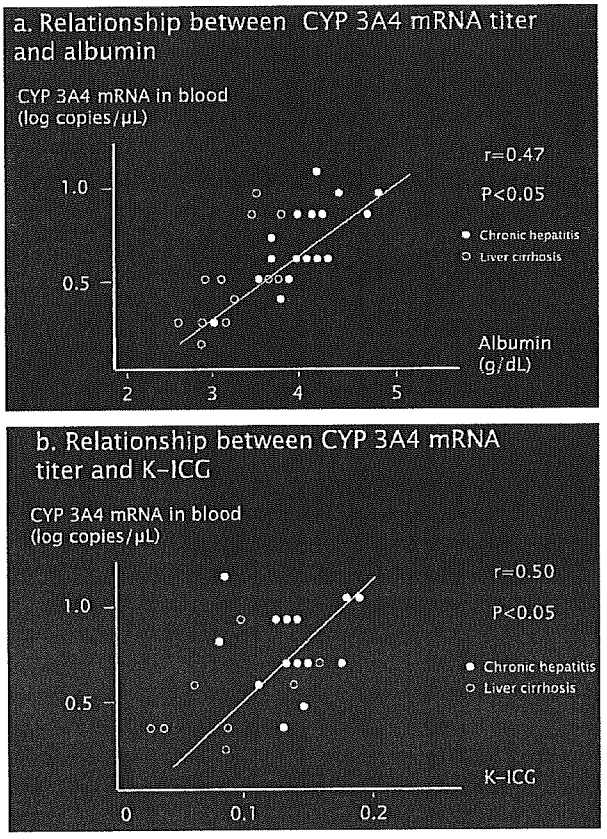


Fig. 1. Comparison of CYP 3A4 mRNA titer in the blood and a liver function test. The CYP 3A4 mRNA titer in blood was related to (a) serum levels of albumin and (b) K-ICG in patients with CH and LC ($P < 0.05$, $P < 0.05$). ○: CH; ●: LC.

*Estimation of HBV and HCV markers*

The presence of HBs antigen and anti-HCV antibody was determined using an enzyme immunoassay kit (AxSYM HbsAg; Dainabot, Tokyo, Japan, Imcheck-F-HCV; Kokusai-shiyaku, Kobe, Japan) according to the manufacturers' instructions.

*Liver function test*

Bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, γ-glutamyltranspeptidase, total protein, albumin, and K-indocyanine green (ICG) were examined for a liver function test. K-ICG was calculated by injecting ICG and evaluating its clearance by the liver at 5, 10, and 15 min. The value is calculated based on the levels of clearance and this indicates the functional capacity of liver. In all 29 patients with CH and LC, the liver function test was compared to the CYP3A4 mRNA titer. For K-ICG, 7 patients who could not be tested simultaneously with sampling of CYP3A4 mRNA were excluded.

*Assay of CYP450 3A4 mRNA in blood or liver*

Total RNA was extracted from 10 mL of whole blood from all subjects. To examine the relationship between CYP 3A4 mRNA in blood and liver, blood and liver samples (1 mg of frozen liver tissue) were collected simultaneously in 7 patients with CH and 3 patients with LC. RNA was extracted as previously reported [7]. CYP 3A4 mRNA was quantified using the competitive reverse transcription polymerase chain reaction (CRT-PCR) as reported [8]. The titer of CYP 3A4 mRNA was expressed as log copies/microliter. This was calculated from the expression of β-actin mRNA. Ten nanograms of β-actin mRNA corresponded to 1 microliter.

Table 2
CYP 3A4 mRNA titer in chronic hepatitis according to liver histology

| | No. | CYP 3A4 mRNA titer in blood (log copies/μL) |
|---|---|---|
| Activity | | |
| Mild | 8 | 0.7 ± 0.2 |
| Moderate, severe | 9 | 0.7 ± 0.2 |
| Fibrosis | | |
| Mild | 8 | 0.8 ± 0.3 |
| Moderate | 5 | 0.6 ± 0.2 |
| Severe | 4 | 0.6 ± 0.2 |

*Statistical analysis*

Statistical analysis was performed using the Student's *t* test, chi-squared test, Pearson's correlation test, and Fisher's Exact Test, with a *P* value of 0.05 or less regarded as statistically significant.

## Results

CYP 3A4 mRNA could not be detected in the peripheral mononuclear cells or serum isolated from the 10 mL of blood from the normal controls, but was detectable in 10 mL of whole blood (1.6 ± 0.4) from the same subjects. The CYP 3A4 mRNA titer varied about 20 fold in the 12 normal subjects.

In the 7 CH and 3 LC patients, the CYP 3A4 mRNA titer in the blood correlated with that of the liver ($r = 0.65$, $P < 0.05$). Table 1 shows the CYP 3A4 mRNA titer in the blood of the various liver disease patients. In the CH patients, the titer was significantly lower than that of the normal controls ($P < 0.01$). In addition, the titer was significantly lower in the LC and HCC patients than the CH patients ($P < 0.05$, $P < 0.05$, respectively). However, no significant difference was observed between the LC and HCC patients. Furthermore, no significant difference was observed between HCC stages III, IVA (0.6 ± 0.2), and IVB (0.4 ± 0.2).

Fig. 1 shows the relationship between the CYP 3A4 mRNA titer in blood and the liver function test with chronic liver diseases. The CYP 3A4 mRNA titer in blood was related to K-ICG and serum levels of albumin ($P < 0.05$, $P < 0.05$) but was not related to age, levels of total bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, γ-glutamyltranspeptidase, number of platelets, white blood cells, and lymphocytes.

Table 2 shows the CYP 3A4 mRNA titer in the blood of CH patients according to liver biopsy. No significant differences among the grading of histological activity and fibrosis were observed.

## Discussion

Examination of CYP 3A4 activity in liver disease is difficult. Sumida reported that CYP 3A4 activity is related to the CYP 3A4 mRNA titer in the liver [8]. To investigate CYP 3A4 expression and its role in viral liver diseases, we determined the CYP 3A4 mRNA titer in whole blood and liver tissue. A close relationship was detected between the CYP 3A4 mRNA titers in blood and liver, suggesting that the CYP 3A4 mRNA titer in blood is a surrogate marker for the CYP 3A4 mRNA titer and enzymatic activity in the liver. The reason why CYP 3A4 mRNA in blood and liver is correlated is unknown. CYP 3A4 induction is regulated by pregnane X receptor (PXR) and hepatocyte nuclear factor 4 in human hepatocytes [1,9]. Such common regulatory factors may exist and further investigation is needed. Interestingly, the CYP 2E1 mRNA titer in peripheral blood mononuclear cells has also been reported to have a close relationship with hepatic metabolic activity, i.e., CYP 2E1 titer in the liver [10]. On the other hand, there are some reports that CYP mRNA in blood has no correlation with that of the liver [11,12]. The reason for this discrepancy is unknown. We used fresh liver and blood samples similar to the report of Finnstrom et al. [11]. The differences of race and method (we performed a calculation by the expression of internal control) might have some influence in this regard.

In the present study, the CYP 3A4 mRNA titer in blood decreased with progression of various liver diseases and was related to serum levels of albumin and K-ICG, suggesting that assaying for CYP 3A4 mRNA in blood relates to progression of liver diseases and might reflect hepatic metabolic activity. On the other hand, no relationship was observed between the CYP 3A4 mRNA titer and histological hepatitic activity in the liver of CH patients. This is consistent with Finnstrom's report of no difference in the CYP 3A4 mRNA titer in liver between inflamed and steatotic/normal liver biopsy samples [13].

A decrease in CYP 3A4 activity and CYP 3A4 mRNA titer in the liver of patients with LC may be the result of a loss of liver volume. However, in the present study, the CYP 3A4 mRNA titer in blood standardized using an internal control (β-actin) was also lower in LC. Many drugs are ligands for PXR. Glucocorticoid and rifampicin enhance the expression of CYP [14]. On the other hand, IL-6 suppresses CYP expression [15] and is elevated in patients with LC [16], suggesting that IL-6 plays some role in the low CYP 3A4 mRNA titer in LC. The mechanism of the CYP 3A4 decrease in LC requires further analysis.

Both CYP 2C9 and 2C19 display polymorphisms that effect drug metabolism [17,18]. CYP 3A4 plays an important role in the metabolism of procarcinogens [1]. In this respect, it is interesting that CYP 3A4 polymorphism has also been reported in Caucasians and is related to occurrence of leukemia and prostatic tumor [19,20]. However, this polymorphism may not effect CYP3A4 expression [21], and for the present purposes, no CYP 3A4 polymorphism has been detected in the Japanese [22].

In the present study, no significant difference in the CYP3A4 mRNA titer in blood was observed between the HCC and LC patients. In addition, no significant differences were observed between HCC stages III, IVA and IVB, suggesting that CYP 3A4 has no relationship with carcinogenesis and staging of HCC. On the other hand, CYP 3A4 expression has been reported to be higher in the non-tumorous portion and some carcinoma tissue from patients with HCC [23]. Further study of CYP 3A4 expression in situ in patients with HCC is required.

In conclusion, assessment of CYP 3A4 mRNA in blood relates to the progression of viral liver disease and might reflect liver metabolic activity.

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MAJOR ARTICLE

# Detection of Hepatitis C Virus (HCV) in Serum and Peripheral-Blood Mononuclear Cells from HCV-Monoinfected and HIV/HCV–Coinfected Persons

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**It has been speculated that hepatitis C virus (HCV) replicates in peripheral-blood mononuclear cells (PBMCs), which, therefore, may be a site for interaction with human immunodeficiency virus (HIV). We used strand-specific real-time polymerase chain reaction to detect HCV RNA in 28 HCV-monoinfected and 20 HIV/HCV–coinfected women. At the first visit, positive-strand HCV RNA was detected in serum samples from 89% of the women, whereas positive-strand HCV RNA was detected in PBMC samples from 32% and 55% of the HCV-monoinfected and HIV/HCV–coinfected women, respectively. After initiation of antiretroviral therapy, the HIV/HCV–coinfected women were significantly more likely to have detectable positive- and negative-strand HCV RNA in the PBMC compartment than were the HCV-monoinfected women. HIV and HCV RNA levels were not correlated. Serum HCV RNA levels were correlated over time; HCV RNA levels in the serum and PBMC compartments were not. These data suggest differential regulation of HCV RNA in the serum and PBMC compartments and may partially explain the limited HCV antiviral response rates observed in coinfected persons.**

Hepatitis C virus (HCV) is a positive-strand RNA virus that infects >170 million people worldwide. Because of the inability to infect small animals with HCV and the lack of efficient cell-culture models, much of the current understanding of the HCV life cycle has been inferred from studies that use samples from infected humans. Although hepatocytes are the major site of infection, there is a broad clinical spectrum of disease and extrahepatic complications, including cryoglobulinemia, non-Hodgkin lymphoma, and porphyria cutanea tarda [1].

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Some studies have reported evidence for extrahepatic replication of HCV in peripheral-blood mononuclear cells (PBMCs); however, these studies have typically involved a small number of patients and have often yielded contradictory results [2–7]. Other studies have reported evidence for HCV replication in granulocytes, monocytes/macrophages, dendritic cells, and B lymphocytes, as well as in extrahepatic tissues [8–16]. Because certain amplification methods lack strand specificity, which may influence the reliable detection of replication intermediates (i.e., negative-strand HCV RNA), it has been challenging to definitively demonstrate extrahepatic HCV replication. Recently, modification of the real-time polymerase chain reaction (rtPCR) assay to include the *Tth* enzyme, which has high strand specificity and independent reverse-transcriptase and DNA-dependent polymerase activity, has been used to detect negative-strand HCV RNA in the liver and/or PBMC compartment [7, 9, 17–19].

In the United States, 150,000–300,000 people are coinfected with HCV and HIV [20]. Multiple studies have demonstrated the adverse effects of HIV coinfec-

tion on liver fibrosis, HCV RNA levels, HCV disease progression [21], and treatment response rates [22–24]. The mechanisms by which these 2 viruses interact remain unclear, because no direct virus-virus interactions have been demonstrated to date. However, recent in vitro data suggest that HCV and HIV proteins cooperatively induce hepatic apoptotic pathways [25, 26] and secretion of proinflammatory cytokines [27] without requiring cell infection and viral replication. Thus, it is reasonable to speculate that similar signaling cascades in PBMCs may also permit indirect interactions between HCV and HIV.

We have previously investigated serum HCV diversity in HIV/HCV–coinfected persons initiating antiretroviral therapy (ART) for HIV infection [28]. We found significant evolution of the hypervariable region 1, but not the adjacent envelope 1 region, after ART initiation. However, few studies have addressed the effects of ART on HCV in HIV/HCV–coinfected persons in compartments other than serum. The demonstration of extrahepatic HCV replication in the PBMC compartment would have important implications for transmission of the virus and efficient treatment of HCV infection. Nonetheless, previous studies have not assessed this phenomenon in HIV/HCV–coinfected persons in a longitudinal manner, nor have they addressed it in coinfected persons initiating ART [15, 29, 30]. Therefore, we sought to investigate whether HCV replication could be detected in the serum and PBMC compartments of persons coinfected with HIV and HCV and to assess the effect of ART on extrahepatic HCV replication.

## PARTICIPANTS, MATERIALS, AND METHODS

***Study population.*** From April 1993 to February 1995, the HIV Epidemiology Research (HER) Study, a prospective natural-history study of HIV infection, enrolled 871 HIV-infected women and 439 demographically matched HIV-uninfected women [31]. The women participated in clinic visits at 6-month intervals through 1999. By study design, one-half of the women reported injection drug use (IDU), and the other half reported only sexual risk behavior.

As described elsewhere, HCV serostatus was determined by either Abbott HCV EIA (version 2.0) or Ortho HCV ELISA (version 3.0) [32]. Overall, the seroprevalence of HCV was 56.5%, with rates of 48.0% and 60.8% in HIV-uninfected and HIV-infected women, respectively. Of the women who acknowledged prior IDU, 88.3% were HCV seropositive; of these women, 76.9% had detectable HCV RNA [33]. Because the HER Study cohort was formed before the widespread use of combination therapy, only 30% were receiving ART at the beginning of the study. By 1999, 31.3% were still not receiving any ART [34].

HER Study participants were included in the present study if they (1) were HCV seropositive, regardless of their HIV status; (2) had serum and PBMC samples available from at least 2 consecutive study visits conducted at the Providence, RI, site; and (3) were not receiving ART at the beginning of the study (for the HIV-infected women). For the HIV-infected women, study visits corresponded to the visit immediately before ART initiation (denoted "visit A") and the visit immediately after ART initiation (denoted "visit B"). The median intervals between visits were 5.8 months and 6.6 months for the HCV-monoinfected and HIV/HCV–coinfected women, respectively. The drug regimens initiated by the HIV-infected women were as follows: $\geq$2 nucleoside reverse-transcriptase inhibitors (NRTIs) ($n = 4$); $\geq$1 NRTI plus $\geq$1 protease inhibitor (PI) ($n = 11$); $\geq$2 NRTIs plus 1 nonnucleoside reverse-transcriptase inhibitor (NNRTI) ($n = 4$); and 2 NRTIs plus 1 NNRTI plus 2 PIs ($n = 1$). One HIV/HCV–coinfected woman was missing serum samples at both visits, and 3 HIV/HCV–coinfected women were missing PBMC samples at visit B.

***Cellular RNA extraction and strand-specific* Tth *rtPCR.*** RNA was extracted from serum samples by use of the QIAamp Viral RNA Kit (Qiagen). For PBMC samples, the number of cells available was limited. Because the number of cells varied per sample (range, 1.4–7.6 $\times$ $10^6$ cells/mL), we normalized all quantitative HCV RNA data on the PBMC compartment to the copy number of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Five hundred microliters of a PBMC suspension was washed with diethopyrocarbonate (DEPC)–treated $dH_2O$, and cellular RNA was extracted by use of TRIzol (Invitrogen). The resultant RNA was resuspended in 40 $\mu$L of DEPC-treated $dH_2O$ and treated 2 times with DNase I (Ambion). Positive- and negative-strand HCV cDNAs were quantified by a validated strand-specific rtPCR assay using SYBR green dye I, as described elsewhere [17, 19]. Extracted RNA was heated at 95°C for 1 min and then incubated at 70°C. A mixture containing 10 pmol/$\mu$L HCV-1 antisense primer (5′-TGGATGCACGGTCTACGAGACCTC-3′; nt 342–320, according to the numbering of H77 [35]; GenBank accession number AF009606) for HCV positive-strand synthesis or HCV-2 sense primer (5′-CACTCCCCTGTGAGGAACT-3′; nt 38–56) for HCV negative-strand synthesis, 1$\times$ reverse-transcriptase buffer, 1 mmol/L $MnCl_2$, 200 mmol/L each deoxynucleoside triphosphate, and 5 U of *Tth* enzyme (Applied Biosystems) was added. The cDNA reaction consisted of an annealing step for 2 min at 60°C, followed by an extension step for 20 min at 70°C. To inactivate the reverse-transcriptase activity of the *Tth* enzyme, chelating buffer was added after cDNA synthesis. cDNA was purified by use of the High Pure PCR Template Preparation Kit (Roche Diagnostics).

Positive- and negative-strand HCV PCR amplification was performed with 2 $\mu$L of purified cDNA in a mixture containing LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics), 4 mmol/L $MgCl_2$, and 5 pmol/L each antisense primer KY78 (5′-CTCGCAAGCACCCTATCAGGCAGT-3′; nt 311–288) and sense primer KY80 (5′-GCAGAAAGCGTCTAGCCA-

**Table 1. Characteristics of the study cohort.**

| Characteristic, parameter | HCV monoinfected | HIV/HCV coinfected | *P* |
|---|---|---|---|
| Risk factor | | | NS[a] |
| Injection drug use | 24 (86) | 18 (90) | |
| Heterosexual contact | 4 (14) | 2 (10) | |
| Age at enrollment, mean ± SD, years | 34.4 ± 6.0 | 35.2 ± 3.9 | NS[b] |
| Race | | | .008[a] |
| Black | 4 (14) | 11 (55) | |
| White | 21 (75) | 8 (40) | |
| Hispanic | 3 (11) | 1 (5) | |
| HCV genotype | | | NS[a] |
| 1 | 7 (25) | 11 (55) | |
| 2 | 3 (11) | 0 (0) | |
| 3 | 3 (11) | 1 (5) | |
| 4 | 2 (7) | 3 (15) | |
| Unknown | 13 (47) | 5 (25) | |
| Visit A | | | |
| CD4 cell count, mean ± SD, cells/μL | 1139 ± 345 | 285 ± 144 | <.0001[c] |
| Plasma HIV RNA level, median (IQR), $\log_{10}$ copies/mL | ... | 4.1 (2.9–4.6) | ... |
| Receipt of ART | ... | 0 (0) | ... |
| Visit B | | | |
| CD4 cell count, mean ± SD, cells/μL | 1101 ± 264 | 376 ± 191 | <.0001[c] |
| Plasma HIV RNA level, median (IQR), $\log_{10}$ copies/mL | ... | 2.1 (<1.7–3.4) | ... |
| Receipt of ART | ... | 18 (90) | ... |

**NOTE.** Data are no. (%) of women, unless otherwise noted. ART, antiretroviral therapy; HCV, hepatitis C virus; IQR, interquartile range; NS, not significant ($P > .05$).

[a] Fisher's exact test.
[b] Student's *t* test for normal data.
[c] Wilcoxon rank sum test.

TGGCGT-3′; nt 68–91). The PCR consisted of an initial denaturation step for 10 min at 95°C, then 40 cycles under the following conditions: 15 s at 95°C, 5 s at 70°C, and 15 s at 72°C. For generation of GAPDH mRNA, cDNA synthesis was performed with an oligo d(T) primer under standard conditions. For PCR amplification, we used a commercial GAPDH primer set (Roche Search LC), with the conditions recommended by the manufacturer.

For each PBMC sample, we determined the positive- and negative-strand HCV RNA copy numbers and normalized them to the GAPDH copy number, to provide standardized values (i.e., positive-strand HCV RNA copies and negative-strand HCV RNA copies per molecule of GAPDH). Serum HCV quantities were expressed as HCV RNA copies per microliter (extracted from 140 μL of serum). Previous studies have reported very low rates of negative-strand HCV RNA detection in serum [11, 14, 15]; thus, we did not systematically measure negative-strand HCV RNA in this compartment. To avoid potential cross-contamination, samples for each time point and each compartment from an individual were handled separately. Additionally, all rtPCR amplifications included a negative control that contained no template.

***Statistical analyses.*** Demographic and clinical data were compared by Fisher's exact test for categorical variables and either Student's *t* test or the Wilcoxon rank sum test for continuous variables. The Wilcoxon rank sum test was used to compare HCV RNA levels between the HCV-monoinfected and HIV/HCV–coinfected women; values for undetectable levels were set at 0 for serum samples ($\log_{10}$ transformed) and at 0.01 for PBMC samples (untransformed). Spearman's correlation test was used to investigate the linear relationships between CD4 cell count, plasma HIV RNA level, and serum and PBMC HCV RNA levels. All *P* values reported are 2-sided; $P < .05$ was considered to be statistically significant. No adjustments were made for multiple comparisons. All analyses were performed by use of SAS software (version 9; SAS Institute).

## RESULTS

***Study cohort characteristics.*** Twenty-eight HCV-monoinfected and 20 HIV/HCV–coinfected women from the HER Study cohort were selected for the present study. These 2 groups of women did not differ with respect to the reporting of IDU as the main risk factor for HCV acquisition, age at enrollment, or HCV genotype; however, HIV/HCV–coinfected women were more likely to be black (table 1). None of the women reported receiving HCV treatment during the visits included in the present study. Mean CD4 cell counts were lower in the HIV/HCV–

**Table 2. Strand-specific hepatitis C virus (HCV) RNA detection rates.**

| Visit, clinical variable | Total | HCV monoinfected | HIV/HCV coinfected | *P* |
|---|---|---|---|---|
| Visit A | | | | |
| Serum positive-strand HCV RNA | 89 (42/47) | 82 (23/28) | 100 (19/19) | NS |
| PBMC positive-strand HCV RNA | 42 (20/48) | 32 (9/28) | 55 (11/20) | NS |
| PBMC negative-strand HCV RNA | 35 (17/48) | 32 (9/28) | 40 (8/20) | NS |
| Visit B | | | | |
| Serum positive-strand HCV RNA | 91 (43/47) | 93 (26/28) | 89 (17/19) | NS |
| PBMC positive-strand HCV RNA | 44 (20/45) | 29 (8/28) | 71 (12/17) | .01 |
| PBMC negative-strand HCV RNA | 38 (17/45) | 25 (7/28) | 59 (10/17) | .03 |

**NOTE.** Data are percentage (no. positive/no. tested) of women, unless otherwise noted. $P > .05$); PBMC, peripheral-blood mononuclear cell.

coinfected women than in the HCV-monoinfected women at both time points (visit A, 285 vs. 1139 cells/$\mu$L [$P < .0001$]; visit B, 376 vs. 1101 cells/$\mu$L [$P < .0001$]). After ART initiation (between visits A and B), median plasma HIV RNA levels decreased, from 4.1 to 2.1 $\log_{10}$ copies/mL, in the HIV/HCV–coinfected women ($P = .0002$), whereas mean CD4 cell counts increased, from 285 to 376 cells/$\mu$L ($P = .4$), in these women.

***Strand-specific HCV RNA detection rates.*** At visit A, positive-strand HCV RNA was detected, by a strand-specific rtPCR assay, in serum from 42 (89%) of 47 women, including 23 (82%) of 28 HCV-monoinfected women and 19 (100%) of 19 HIV/HCV–coinfected women (table 2). At visit B, 43 (91%) of 47 women had detectable levels of positive-strand HCV RNA in serum. Rates of detection in the serum compartment were not significantly different between the HCV-monoinfected and the HIV/HCV–coinfected women at either visit. We did not systematically measure levels of negative-strand HCV RNA in the serum compartment. However, of 47 women tested, 34 (72%) had undetectable or negligible levels (<1000 copies/$\mu$L) of negative-strand HCV RNA (data not shown). Furthermore, among those women in whom both strands were detected, the ratio of negative-strand:positive-strand HCV RNA in the serum compartment was <1% in both groups, suggesting that there is a vast excess of positive-strand HCV RNA in the serum compartment (data not shown). In contrast, the proportion of negative-strand HCV RNA relative to positive-strand HCV RNA in the PBMC compartment was significantly higher (particularly in the HIV/HCV–coinfected women), a finding that is consistent with higher rates of HCV replication in the PBMC compartment.

Our findings regarding detection of positive- and negative-strand HCV RNA in the PBMC compartment by the strand-specific rtPCR assay were strongly suggestive of extrahepatic HCV replication. At visit A, positive-strand HCV RNA was detected in the PBMC compartments of 20 (42%) of 48 women, including 9 (32%) of 28 HCV-monoinfected women and 11 (55%) of 20 HIV/HCV–coinfected women. Negative-strand HCV RNA was detected in 17 (35%) of 48 women, including 9 (32%) of 28 HCV-monoinfected women and 8 (40%) of 20 HIV/HCV–coinfected women. At visit B, after the HIV-infected women had initiated ART, positive-strand HCV RNA was still more readily detected in the HIV/HCV–coinfected women than in the HCV-monoinfected women (8/28 [29%] vs. 12/17 [71%]; $P = .01$). The negative-strand HCV RNA detection rate was also significantly different in the HCV-monoinfected and the

**Table 3. Median strand-specific hepatitis C virus (HCV) RNA levels.**

| Visit, clinical variable | HCV monoinfected | HIV/HCV coinfected | *P* |
|---|---|---|---|
| Visit A, median (75th percentile) | | | |
| Serum positive-strand HCV RNA level | 3.6 (5.0) | 5.2 (5.6) | .002 |
| PBMC positive-strand HCV RNA level | 0 (31.2) | 2.5 (30.7) | NS |
| PBMC negative-strand HCV RNA level | 0 (2.3) | 0 (20.0) | NS |
| Visit B, median (75th percentile) | | | |
| Serum positive-strand HCV RNA level | 4.4 (5.0) | 5.5 (5.9) | .003 |
| PBMC positive-strand HCV RNA level | 0 (48.0) | 5.4 (38.2) | NS |
| PBMC negative-strand HCV RNA level | 0 (2.3) | 0.6 (19.3) | NS |

**NOTE.** Data for serum HCV RNA levels, which are $\log_{10}$ transformed, are no. of HCV RNA copies per microliter (extracted from 140 $\mu$L of serum); data for PBMC HCV RNA levels, which are untransformed, are no. of HCV RNA copies per molecule of glyceraldehyde-3-phosphate dehydrogenase. The medians for several of the clinical variables are 0 because of low rates of detection. NS, not significant ($P > .05$); PBMC, peripheral-blood mononuclear cell.

HIV/HCV–coinfected women at visit B (7/28 [25%] vs. 10/17 [59%]; $P = .03$). Negative-strand HCV RNA was detected in the PBMC compartment only when positive-strand HCV RNA was also detected.

***Strand-specific HCV RNA levels.*** Strand-specific HCV RNA levels were determined in the serum and PBMC compartments and, in the latter case, were normalized to the GAPDH copy number (table 3). Using dilutions of serum samples for which HCV RNA levels had previously been determined (by use of the Roche Amplicor Monitor Kit), we determined that the lower level of detection for the strand-specific rtPCR assay was ~260 copies/$\mu$L. The HIV/HCV–coinfected women had higher positive-strand HCV RNA levels in serum than did the HCV-monoinfected women, both before and after ART initiation (visit A, 3.6 vs. 5.2 $\log_{10}$ copies/$\mu$L [$P = .002$]; visit B, 4.4 vs. 5.5 $\log_{10}$ copies/$\mu$L [$P = .003$]). Because of the low rates of detection of HCV RNA in the PBMC compartment, medians could not be defined in several instances; therefore, 75th percentiles are presented in table 3. At visit A, there was no significant difference in either positive- or negative-strand HCV RNA levels in the PBMC compartment between the 2 groups. At visit B, after ART initiation, both positive- and negative-strand HCV RNA levels in the PBMC compartment were higher in the HIV/HCV–coinfected women than in the HCV-monoinfected women, but these differences did not reach statistical significance.

***Correlation analyses.*** We also analyzed potential correlations between specific immunologic parameters and strand-specific HCV RNA levels (table 4). Age and plasma HIV RNA levels were not correlated with either positive- or negative-strand HCV RNA levels in either compartment. CD4 cell count was inversely correlated with positive-strand HCV RNA levels in the serum ($P \leq .0001$), but not in the PBMC, compartment. Positive-strand HCV RNA levels in the serum compartment were consistent over time ($P < .0001$), as were both positive- and negative-strand HCV RNA levels in the PBMC compartment ($P < .0001$). However, in the absence of ART, positive-strand HCV RNA levels in the serum compartment were not correlated with either positive- or negative-strand HCV RNA levels in the PBMC compartment. Between visits, neither positive- nor negative-strand HCV RNA levels in the PBMC compartment were correlated.

**Table 4. Correlation between clinical variables and strand-specific hepatitis C virus (HCV) RNA levels.**

| Comparison | Coefficient[a] | *P* |
|---|---|---|
| Serum positive-strand HCV RNA level (A) vs. | | |
| Serum positive-strand HCV RNA level (B) | 0.75 | <.0001 |
| PBMC positive-strand HCV RNA level (A) | 0.02 | NS |
| PBMC negative-strand HCV RNA level (A) | 0.01 | NS |
| PBMC positive-strand HCV RNA level (A) vs. | | |
| PBMC negative-strand HCV RNA level (A) | 0.89 | <.0001 |
| PBMC positive-strand HCV RNA level (B) | 0.08 | NS |
| PBMC positive-strand HCV RNA level (B) vs. PBMC negative-strand HCV RNA level (B) | 0.90 | <.0001 |
| PBMC negative-strand HCV RNA level (A) vs. PBMC negative-strand HCV RNA level (B) | 0.06 | NS |
| CD4 cell count vs. | | |
| Serum positive-strand HCV RNA level (A) | −0.54 | <.0001 |
| PBMC positive-strand HCV RNA level (A) | −0.10 | NS |
| PBMC negative-strand HCV RNA level (A) | 0.02 | NS |
| Plasma HIV RNA level vs. | | |
| Serum positive-strand HCV RNA level (A) | 0.11 | NS |
| PBMC positive-strand HCV RNA level (A) | 0.31 | NS |
| PBMC negative-strand HCV RNA level (A) | 0.18 | NS |

**NOTE.** A and B refer to the visit. NS, not significant ($P > .05$); PBMC, peripheral-blood mononuclear cell.

[a] Spearman correlation coefficient for all women.

## DISCUSSION

Researchers have long sought to establish whether HCV replicates outside the liver, because detection of HCV RNA in extrahepatic reservoirs has important implications for transmission, disease progression, and effective treatment. Nonetheless, achieving a definitive demonstration of extrahepatic HCV replication has been limited by several biological and technical considerations. Foremost, the lack of a robust cell-culture system has made it exceedingly difficult to compare HCV replication in different cell populations. To date, the dynamics of HCV replication have typically been examined by intensive study of serum-specific or liver-specific HCV RNA; however, viral replication in such extrahepatic reservoirs as PBMCs may not reflect replication in these other compartments. Furthermore, although detection of positive-strand HCV RNA cannot distinguish between nucleic acids participating in replication and those already incorporated into viral particles, detection of replication intermediates, such as negative-strand HCV RNA, is a more biologically relevant measure of active virus replication. Negative-strand HCV RNA is generally present at levels 10–100-fold lower than those of positive-strand HCV RNA [36, 37]; thus, highly sensitive and specific detection assays must be used. Although distinguishing between positive- and negative-strand HCV RNA is critical, not all strand-specific detection methods have high specificity for detection of negative-strand HCV RNA. Here, we have used a validated strand-specific rtPCR assay that includes the *Tth* enzyme. Because this enzyme contains separate reverse transcriptase and DNA-dependent polymerase functions, it is highly specific and is ideal for discriminating between positive- and negative-strand RNA [17, 19].

Although several studies have measured HCV replication in the PBMC compartment, only a subset have used a bona fide *Tth*-based amplification assay to distinguish between positive- and negative-strand HCV RNA [4, 9, 17, 29, 38–41]. Our rate of detection of negative-strand HCV RNA in the PBMC compartment was somewhat elevated, compared with the results of these previous studies. Such differences could reflect minor dis-

crepancies in the amplification assay, study populations, HCV antiviral receipt, and/or sample preparation. However, it has previously been demonstrated that both HIV coinfection and testing of multiple extrahepatic samples are associated with an increased likelihood of detection of negative-strand HCV RNA [29, 38, 41]. For example, Laskus et al. demonstrated the presence of negative-strand HCV RNA in 5 of 14 PBMC samples from HIV/HCV–coinfected patients [29]. The authors also suggested that factors governing HCV replication at hepatic and extrahepatic sites may differ. Thus, one might anticipate increased detection of negative-strand HCV RNA in a population such as ours, because no participant received HCV antiviral therapy, a high prevalence of HIV coinfection existed, and we tested multiple samples for each participant. It is also theoretically possible that our use of an all-female cohort is responsible for increased detection of negative-strand HCV RNA, although sex-specific detection rates have not been reported to date.

Our study design has several distinct advantages over those of previously published studies. First, to date, most studies of extrahepatic replication have been restricted to a small population analyzed in a cross-sectional, rather than a longitudinal, manner. Second, paired serum and PBMC samples have not usually been analyzed, making intercompartment comparisons difficult. Third, despite clinical data suggesting that HIV adversely affects HCV replication, disease progression, and treatment response rates, HCV-monoinfected and HIV/HCV–coinfected persons have not typically been analyzed as distinct groups. Fourth, not all previously published studies used a strand-specific rtPCR assay that had high strand specificity.

The present study design does have several limitations. First, very low levels of negative-strand HCV RNA were detected in the serum compartments of a subset of women. Because "naked" negative-strand HCV RNAs are not known to circulate outside of cells, we suggest that these very low levels of negative-strand RNA likely represent a small amount of contaminating RNA from residual PBMCs that were not completely removed during the initial processing of whole blood. Second, given the limited number of PBMCs available, we were not able to more precisely define the cell population(s) within PBMCs that are responsible for HCV replication. Nonetheless, there is growing evidence that HCV may infect several peripheral-blood cell types, including B lymphocytes, granulocytes, monocytes/macrophages, and dendritic cells [8, 12, 40]; it is, however, important to note that each of these previous studies either excluded persons coinfected with HIV or did not report HIV status.

Results from our pilot study should be interpreted with caution, given its limited sample size. Nonetheless, we here report several novel findings regarding extrahepatic HCV replication. First, rates of detection of HCV RNA in the PBMC compartment were higher for HIV/HCV–coinfected women than for HCV-monoinfected women. Previous studies have suggested that serum HCV RNA levels are higher in HIV/HCV–coinfected persons [21]; however, this phenomenon has not been investigated in the PBMC compartment until now. Importantly, negative-strand HCV RNA, indicative of active viral replication, was detected at higher rates in the PBMC compartments of HIV/HCV–coinfected women, highlighting an important interaction between these 2 viruses in this compartment. Second, there was no correlation between plasma HIV RNA levels and positive- or negative-strand HCV RNA levels in either the serum or PBMC compartment. Moreover, ART initiation appeared to have a minimal effect on HCV detection rates and HCV RNA levels, although, because of the limited number of HIV/HCV–coinfected persons included in the present study, we cannot rule out a possible association. The finding of elevated HCV RNA levels in the serum and PBMC compartments even after ART initiation may suggest that immune reconstitution after suppression of HIV is not sufficient to control HCV replication. Third, there was an inverse correlation between CD4 cell counts and positive-strand HCV RNA levels in the serum, but not the PBMC, compartment (i.e., as the CD4 cell count increased, the serum, but not the PBMC, HCV RNA level decreased). Given that several components of PBMCs may support HCV replication [8, 12, 40], it is provocative to speculate that the PBMC compartment may be a site in which HCV is partially protected from adaptive and/or innate immune responses. Fourth, there was a positive correlation between positive- and negative-strand HCV RNA levels in the PBMC compartment. However, serum and PBMC HCV RNA levels did not correlate with each other. Thus, HCV RNA may be regulated differently in these compartments.

The precise mechanisms by which HIV influences extrahepatic HCV replication have yet to be determined. It is possible that HIV-induced immunosuppression results in less immunologic control of HCV replication, although reproducible correlations between HCV RNA levels in the serum compartment and CD4 cell counts have not been confirmed [30]. Moreover, the presence of replicative viral forms in extrahepatic sites does not correlate with CD4 cell count [29]. Interestingly, in the present study, HCV RNA levels in the PBMC compartment did not correlate with CD4 cell counts, although positive-strand HCV RNA levels in the serum compartment and CD4 cell counts were inversely correlated. These data imply that immunosuppression alone is not the sole driving force behind increased detection of HCV RNA in the PBMC compartment. It is also possible that HIV, through the induction of interferon antagonists, blunts host innate antiviral responses that would otherwise inhibit HCV replication. HIV may also render specific types of PBMCs more susceptible to HCV infection and replication [41].

In summary, low-level HCV replication in the PBMC compartment, as indicated by detection of negative-strand HCV RNA, may adversely influence the effectiveness of HCV anti-

viral therapies [38, 42], particularly in HIV/HCV–coinfected persons. Furthermore, the PBMC compartment may be a privileged site for HCV that is capable of reinitiating viral replication after termination of HCV treatment, when conditions once again become more favorable. Thus, even if clearance of HCV from hepatocytes is achieved by treatment, reinfection from such extrahepatic sites as the PBMC compartment may occur [43]. Future studies of HCV quasispecies diversification in serum and PBMCs may provide additional evidence that HCV replication—and evolution—is distinct in these compartments and may require targeted therapeutic approaches.

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# Administration of dendritic cells in cancer nodules in hepatocellular carcinoma

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**Abstract.** Dendritic cells (DCs), the most potent antigen-presenting cells *in vivo*, are now used for cancer immunotherapy during which they are usually administered to the blood of patients with cancer. However, the route of administration of DCs affects the magnitude of immune responses. This study was conducted to assess the safety of the direct administration of DCs into cancer nodules. DCs were generated by culturing peripheral blood mononuclear cells with granulocyte-macrophage colony-stimulating factor and interleukin-4 for 7 days. After confirming the phenotype and function, one hundred thousand DCs were injected directly into the cancer nodules of 4 patients with hepatocellular carcinoma (HCC) under ultrasonography guidance 48 h after the administration of 100% ethanol. All patients were monitored for any alteration in generalized condition, signs of inflammation, and liver and kidney function for the next 14 days. In addition, the final assessment of the safety of the administration of DCs into cancer nodules was performed 6 months after therapy commencement. The injection of 100% ethanol disrupted the HCC nodules in all 4 patients. DCs were distributed uniformly in the cancer nodules as assessed by ultrasonography. The administration of DCs into cancer nodules was well tolerated by all patients and there were no immediate or delayed side effects. The tumor marker decreased in one patient after the direct administration of DCs. Direct administration of DCs into the cancer nodules of patients with HCC was safe.

## Introduction

About 500 million people globally are chronically infected with the hepatitis B or hepatitis C virus, and a considerable number of them will eventually develop severe complications such as liver cirrhosis and hepatocellular carcinoma (HCC). In Japan at present, there are an estimated 40,000 patients with HCC, and in most Asian and African countries, the number of patients with HCC is increasing (1). In addition to surgery, various local interventional therapies, such as percutaneous ethanol injection therapy (PEIT), and radio frequency ablation (RFA) are widely used for treating these patients. Although local interventional therapies destroy HCC nodules, new HCC nodules develop either at the original site or in different parts of the liver (2). These features indicate that the recurrence of HCC may be blocked if adequate and powerful immune responses against HCC can be initiated in these patients.

Antigen-presenting dendritic cells (DCs) are able to induce both innate and adaptive immunity, and play a cardinal role in the pathogenesis of various forms of cancer, including HCC (3,4). In fact we, along with others, have demonstrated that the functions of DCs are impaired in patients with HCC (5-7). Moreover, mature and activated DCs are almost absent in most cancer tissue, including HCC nodules (7,8).

Accordingly, it has been speculated for a decade that mature DCs may have an antitumor effect in cancer patients. In fact, DC-based therapy is now used for various forms of cancer, including HCC (9,10). However, the outcome of DC-based therapy is not yet promising. Although several factors are responsible for this, the route of administration, the number and the level of maturation of DCs are prominent factors in this regard (11). In most previous clinical trials, DCs have been injected into patients with cancer to achieve an anticancer effect with the speculation that DCs will internalize tumor associated antigens (TAAs), process them, and induce a TAA-specific immune response (9,10,12). However, it is unlikely that DCs administered through circulation will move to cancer nodules or lymphoid tissue to induce a TAA-specific immune response. One of the maneuvers to overcome this limitation is to administer DCs directly into the tumor nodules. However, the administration of immature DCs into a tumor nodule may cause a tolerance to TAAs (13). Moreover, TAA should be available to DCs along with proinflammatory signals for antitumor effects. However, the administration of DCs into cancer nodules in which inflammatory signals are present may constitute a new therapeutic approach.

In order to validate this new concept of DC-based therapy, we performed an animal study in an experimentally-

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*Key words:* hepatocellular carcinoma, dendritic cells, immunotherapy, safety

Table I. Clinical profiles of the patients.

| | Patient-1 | Patient-2 | Patient-3 | Patient-4 |
|---|---|---|---|---|
| Age (years) | 73 | 67 | 67 | 65 |
| Sex | Male | Male | Male | Male |
| W.B.C. (/mm$^3$) | 5900 | 3900 | 2800 | 6500 |
| Platelet (/mm$^3$) | 134000 | 117000 | 41000 | 119000 |
| Total bilirubin (mg/dl) | 0.9 | 0.9 | 1.7 | 1.1 |
| Serum albumin (g/dl) | 3.2 | 2.7 | 3.1 | 3.2 |
| Alanine aminotransferase (U/l) | 43 | 88 | 80 | 82 |
| Serum creatinine (mg/dl) | 0.9 | 0.6 | 0.9 | 0.9 |
| C-reactive protein (mg/dl) | 0.04 | 0.13 | 0.08 | 0.63 |
| Child Pugh score | B | B | B | A |
| HCC nodule (mm) | 40 | 12 | 25 | 18 |
| Alpha feto protein (ng/ml) | 106 | 31 | <10 | 70 |
| PIVKA-2 (mAU/ml) | 341 | 92 | 1460 | 18 |

W.B.C, white blood corpuscles; PIVKA-II, protein induced vitamin K absence or antagonist II. Normal range: WBC, 3900-9800/mm$^3$; Platelet, 131000-369000/mm$^3$; Total bilirubin, 0.1-1.1 mg/dl; Serum albumin, 3.9-4.9 mg/dl; Alanine aminotransferase, 3-49 U/l; Serum creatinine, 0.5-1.2 mg/dl; C-reactive protein, <0.20 mg/dl; Alpha feto protein <10 mg/dl; PIVKA-II, <28 mAU/ml.

implanted murine colon cancer model. The cancer nodules were destroyed by ethanol injection. Forty-eight hours after ethanol administration, DCs were administered to the cancer nodules directly. This therapeutic approach showed an increased survival rate in mice with implanted colon cancer and a decrease in the size of cancer nodules (14).

Inspired by the outcome of this animal experiment, here we report on our pilot study during which human DCs were administered directly into the cancer nodules of patients with HCC after destroying them using 100% ethanol.

## Materials and methods

*Clinical history of patients.* Four patients with HCC were enrolled in the study. The clinical profiles of these patients just before starting this study are given in Table I. All patients were male aged ≤65 years. The white blood corpuscle count was within the normal range in 3 patients and only 2800/mm$^3$ in one patient. A slight elevation of total bilirubin was seen in one patient. The levels of C-reactive protein were either normal or slightly elevated. The levels of tumor markers such as alpha-feto protein and protein-induced vitamin K absence or antagonist II (PIVKA-II) were mostly elevated. All patients were suffering from liver cirrhosis. The presence of multiple HCC nodules was confirmed by ultrasonography and abdominal computed tomography. Liver specimens were also taken from all patients. According to clinical criteria based on the Liver Cancer Study Group of Japan (15), the TNM classification for HCC was worse than stage 3 in all cases. Although all patients had multiple HCC nodules, the nodules at segment 5, 7, 4 and 6 were used for DC-based therapy in patient 1, 2, 3, and 4, respectively (Table I). The diameters of the HCC nodules into which the DCs were administered are shown in Table I. All patients were infected with the hepatitis C virus and one, patient 3, was infected with both the hepatitis C and hepatitis B virus.

The clinical trial was approved after a hearing at the Ehime University School of Medicine in which the technical side, safety concerns, and the scope and limitations of the study were discussed. Written consent was obtained from each patient and the study protocol conforms to the ethical guidelines of the 'Declaration of Helsinki' as reflected in a priori approval by the Institution's Human Research Committee.

*DC preparation.* We have previously reported on the isolation of DCs for human usage (16). In brief, DCs were isolated in a special culture room. It was prohibited to culture the DCs of more than one person at a time. All reagents used in this study were free from endotoxin and toxoplasma. DCs were enriched from an adherent population of peripheral blood mononuclear cells (PBMCs), according to our previous report. In short, PBMCs were cultured in RPMI-1640 (Nipro, Osaka, Japan) plus 10% autologous sera or plasma and human grade granulocyte-macrophages colony stimulating factor (800 U/ml) and interleukin-4 (400 U/ml) (Pepro Tech EC Ltd, London, UK) for 7 days in plastic dishes. The cells were then retrieved from the dishes and washed thrice with phosphate-buffered saline. DCs were then counted and used for phenotypic and functional analyses.

The expression of HLA DR and CD86 on DCs was assessed by direct flow cytometry using fluorescein isothiocyanate-conjugated monoclonal antibody to human

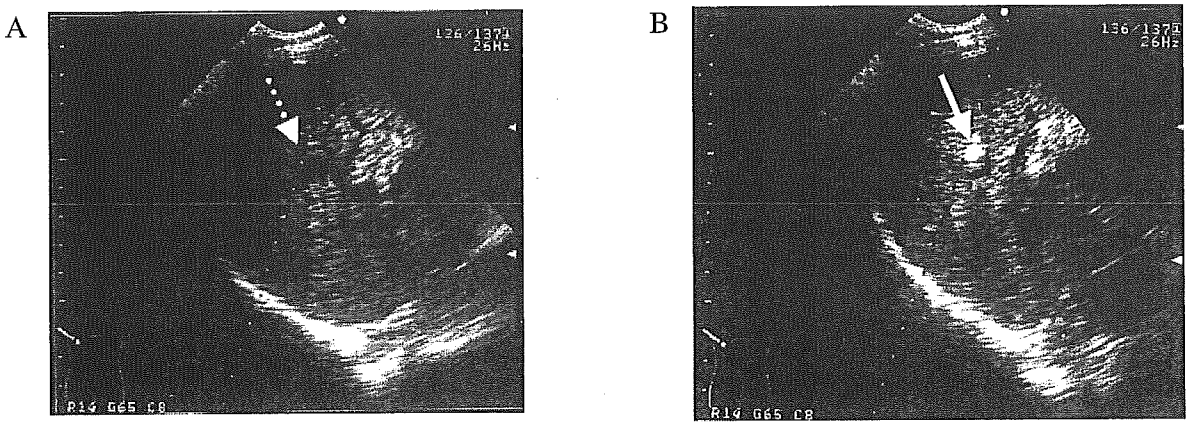


Figure 1. Hypoechoic tumor with a diameter of 12 mm, is visualized at segment 5 of the liver using ultrasonography in patient 2 (A, dotted arrow). After an injection of ethanol into the tumor, injected area changes to hyperechoic (B, solid arrow).

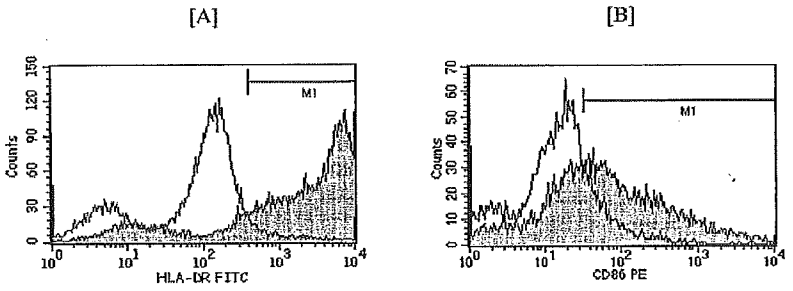


Figure 2. Expression of HLA DR (A) and CD86 (B) in DCs enriched by culturing peripheral blood mononuclear cells with granulocyte-macrophages colony stimulating factor and interleukin-4.

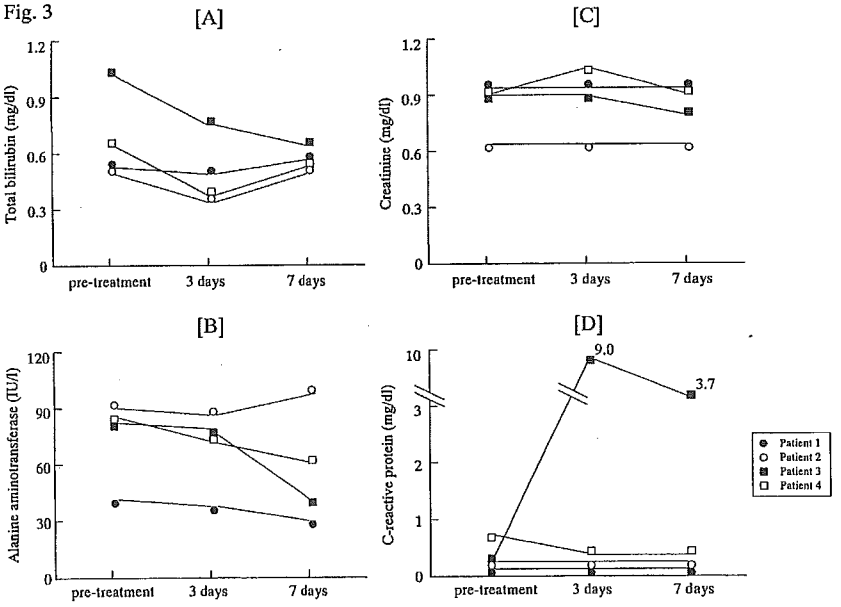


Figure 3. Administration of DCs was safe for patients with HCC. The level of total bilirubin, alanine transaminoferase (ALT), creatinine and C-reactive protein (CRP) in the sera was checked before, 3, and 7 days after the administration of DCs to assess the safety of the treatment. The normal levels of parameters are as follows: total bilirubin, 0.1-1.1 mg/dl; ALT, 3-49 IU/l; creatinine, 0.5-1.2 mg/dl; CRP, <0.2 mg/dl.

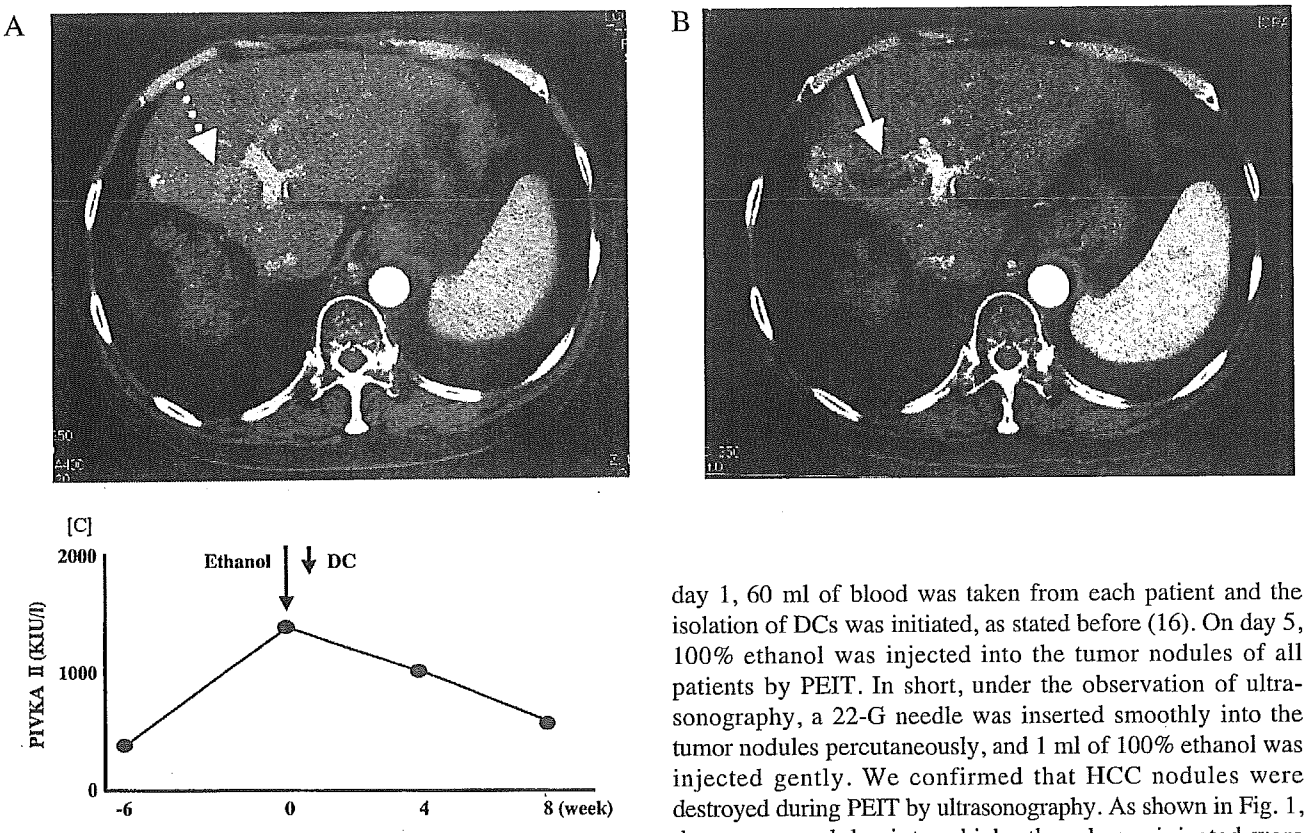


Figure 4. Enhancement in segment 4 of the liver on computed tomography of arterial phase (A, dotted arrow) altered to low-density area 2 weeks after the administration of DCs followed by the injection of only 1 ml of 100% ethanol in patient 3 (B, solid arrow). After the therapy, the level of PIVKA-II progressively decreased in this patient for the next 8 weeks (C).

HLA DR (Clone L243) and phycoerythrin-conjugated monoclonal antibody to human CD86 (clone 2331 [FUN-1]) (all from BD Pharmingen, San Jose, CA, USA). Data acquisition and analysis were performed on fluorescein-activated cell sorter (Becton Dickinson Biosciences, San Jose, CA, USA).

*Study parameters.* All patients were admitted to our hospital for this clinical trial and were admitted at least 7 days prior to the administration of DCs. The general conditions of the patients such as body weight, appetite, bowel movement, and body temperature, pulse rate and blood pressure were regularly monitored on an 8-hourly to daily basis. Parameters of generalized inflammation such as white blood corpuscles, red blood corpuscles, platelet count and C-reactive protein were also checked. In addition, all patients underwent the parameters of liver function test (alanine aminotransferase, aspirate aminotransferase, gamma-glutamyl transpeptidase, total bilirubin, total albumin and prothrombin time) and the kidney function test (routine test of urine, serum creatinine levels, blood urea nitrogen levels). The marker of autoimmunity, such as anti-nuclear antibody, was also checked in all patients.

*Study protocol.* After admission, the patients were given a rest of 7 days to acclimatize to the conditions of the hospital. On day 1, 60 ml of blood was taken from each patient and the isolation of DCs was initiated, as stated before (16). On day 5, 100% ethanol was injected into the tumor nodules of all patients by PEIT. In short, under the observation of ultrasonography, a 22-G needle was inserted smoothly into the tumor nodules percutaneously, and 1 ml of 100% ethanol was injected gently. We confirmed that HCC nodules were destroyed during PEIT by ultrasonography. As shown in Fig. 1, the cancer nodules into which ethanol was injected were mostly disrupted. On day 7 (48 h after performing PEIT), DCs were retrieved from culture and one hundred thousand DCs were injected into the cancer nodules of each patient using a 22-G needle adjacent to site of injection of ethanol.

## Results

*Nature and function of DCs.* First, we confirmed whether DCs isolated from patients with HCC expressed DC-specific surface antigens, and if they possessed an antigen-presenting cell function. As shown in Fig. 2, DCs expressed moderate levels of HLA DR and CD86. The functions of these cells were assessed by allogenic mixed leukocyte reaction, as described previously (16). DCs from patients with HCC stimulated allogenic T cells from a normal volunteer in a dose-dependent manner (data not shown).

*Administration of DCs was safe for all patients.* After performing PEIT, we checked the different parameters of inflammation and the liver and kidney function tests. There were no significant abnormalities in these parameters. After the administration of DCs at the site of the disrupted cancer nodules, different parameters of generalized inflammation were exclusively checked. There were no major alterations in heart rate, body temperature, and blood pressure in any patient following the administration of DCs. Only one patient (patient 3) showed an increased body temperature and slight elevation of the C-reactive protein for 14 days, which returned to the normal level after 21 days. The parameters of liver function test and kidney function tests were also checked serially. There was no major alteration of these parameters in

the next 14 days. The levels of creatinine, C-reactive protein, total bilirubin and alanine aminotransferase in 4 patients with HCC at different times after the administration of DCs are shown in Fig. 3.

Six months after the administration of DCs in HCC nodules, all patients were alive and none had developed any signs of autoimmunity or showed anti-nuclear antibody in the sera.

Although this study was designed to assess the safety of the administration of DCs into cancer nodules, we also checked whether this approach would bring anti-tumor effects. All patients enrolled in this study had multiple HCC nodules. Accordingly, it is very difficult to assess the anti-tumor effects of this therapy in these patients. However, one patient, patient 3, exhibited a decreased level of PIVKA-II within 4 weeks of DC administration and the findings on computed tomography changed (Fig. 4A and B). The level of PIVKA-II progressively decreased in this patient for the next 2 months (Fig. 4C).

## Discussion

DCs are professional antigen-presenting cells that induce both innate and acquired immune responses against various agents including tumor cells. Accordingly, the presence of cancer nodules in cancer-bearing hosts indicates that DC-induced immune surveillance was impaired in those patients. In fact, impaired function of DCs has been documented in all types of cancer patients, including those with HCC (3,4,6,8). Further studies have revealed that mature and activated DCs, those that induce and maintain tumor-specific immune responses, are almost absent in cancer nodules (7,8).

Accordingly, several investigators tried to develop DC-based therapeutic approaches for cancer during the last decade. In general, *in vitro*-cultured DCs or DCs pulsed with tumor products are administered to cancer patients with the postulation that these DCs would induce anticancer immunity *in situ* (9,10). However, it is unclear how injected DCs will induce anticancer immune responses by this approach, especially in the context of solid cancer when TAA may be available mainly in the cancer nodules.

Based on these realities, we planned a pilot study in which cancer nodules were destroyed through the administration of 100% ethanol in patients with HCC. This therapeutic approach is routinely used for destroying HCC nodules in clinics (17). After confirming that the cancer nodules had been destroyed by 100% ethanol, we administered DCs 48 h after ethanol injection. A time lag of 48 h was given to minimize the effect of ethanol on DCs *in situ*. Our study revealed that the administration of DCs into cancer nodules in HCC patients was safe. Firstly, no patient exhibited any sign or symptoms of acute inflammatory reactions. Secondly, no patient developed features of autoimmunity. This study was originally planned to assess the safety of this newly devised technique.

We are not sure whether anti-tumor immunity was induced due to the administration of DCs in HCC nodules by this approach. It is true that we administered 100% ethanol by PEIT to cancer nodules, 48 h before DC injection. This may cause necrosis of the cancer tissue and may provide a maturational signal for DCs. Maturation of DCs may have induced antitumor immunity. In general, to destroy a cancer nodule of 2.5 cm, at least 8.2 ml of 100% ethanol is required. However, after the administration of only 1 ml of 100% ethanol, patient 3 showed a progressive decrease of tumor marker PIVKA-II. This circumstantial evidence suggests that our therapeutic approach might have induced an anti-tumor effect, although this needs to be verified in more cases.

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