indicate that increases in dietary leucine intake substantially decrease diet-induced obesity and hyperglycemia [41]. In addition, elevated BCAA and/or loss of BCAA catabolism in peripheral tissues has been shown to play an important role in regulating energy expenditure [42]. In this regard, BCATm (-/-) mice were found to exhibit elevated plasma BCAA and a decreased body size along with increased energy expenditure and protection from diet-induced obesity [42].

Recent study demonstrated that BCAA supplementation did not regulate the energy expenditure and diet-induced obesity induced with a 60-KJ% fat diet [43]. Consistently, we found in our current experiments that BCAA did not ameliorate diet-induced obesity induced with a 60-KJ % fat diet. Given that BCAA does ameliorate diet-induced obesity associated with a 45-KJ % fat diet, the lipolytic effects of BCAA appear to be sensitive to fat levels in the diet.

Our study has some noteworthy limitations that must be highlighted. First, we are not able to explain the energy expenditure and weight loss in BCAA-treated mice by the changes of UCP2 and UCP3. The marker of energy expenditure may influence on the energy expenditure and weight loss in the present study. In the present study, we cannot determine the levels as physiological or pharmacological levels. In addition, it is possibility that high volume of nitrogen itself or nitrogen unbalance might be able to exert preferable effects.

In summary, BCAA treatment may prevent fat accumulation in muscle, liver, and WAT, accompanied by improved insulin resistance, in DIO mice. Associated changes in PPAR- alpha may also contribute to changes in adiposity in these tissues by affecting fatty acid oxidation and uptake. The results of our present study thus provide new insights into possible therapeutic approaches for obesity-related metabolic disorders.

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The incidence of hepatocellular carcinoma associated with hepatitis C infection decreased in Kyushu area

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B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
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Summary

Background: The incidence of hepatocellular carcinoma (HCC) in Japan has still been increasing. The aim of the present study was to analyze the epidemiological trend of HCC in the western area of Japan, Kyushu.

Material/Methods: A total of 10,010 patients with HCC diagnosed between 1996 and 2008 in the Liver Cancer study group of Kyushu (LCSK), were recruited for this study. Cohorts of patients with HCC were categorized into five year intervals. The etiology of HCC was categorized to four groups as follows; B: HBsAg positive, HCV-RNA negative, C: HCV-RNA positive, HBsAg negative, B+C: both of HBsAg and HCV-RNA positive, non-BC: both of HBsAg and HCV-RNA negative.

Results: B was 14.8% (1,485 of 10,010), whereas 68.1% (6,819 of 10,010) had C, and 1.4% (140 of 10,010) had HCC associated with both viruses. The remaining 1,566 patients (15.6%) did not associate with both viruses. Cohorts of patients with HCC were divided into six-year intervals (1996–2001 and 2002–2007). The ratio of C cases decreased from 73.1% in 1996–2001 to 64.9% in 2002-2007. On the other hand, B and -nonBC cases increased significantly from 13.9% and 11.3% in 1996-2001 to 16.2% and 17.6% in 2002-2007, respectively.

Conclusions: The incidence of hepatocellular carcinoma associated with hepatitis C infection decreased after 2001 in Kyushu area. This change was due to the increase in the number and proportion of the HCC not only nonBC patients but also B patients.

key words: hepatitis virus • hepatocellular carcinoma • Japan

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BACKGROUND

The three leading causes of death in Japan are malignancy neoplasms, cardiovascular diseases, and cerebrovascular diseases. Since 1981, malignant neoplasms have been the leading cause of death in Japan. For the last 30 years, liver cancer has been the third leading cause of death from malignant neoplasms in men. In women, liver cancer has ranked fifth during the past decade [1]. Hepatocellular carcinoma (HCC) accounts for 85% to 90% of primary liver cancers[2] and the age-adjusted HCC mortality rate has increased in recent decades in Japan [3]. Similarly, a trend of increasing rates of HCC has been reported from several developed countries in North America, Europe and Asia [4,5]. HCC often develops in patients with liver cirrhosis caused by hepatitis B virus (HBV), hepatitis C virus (HCV), excessive alcohol consumption, or nonalcoholic fatty liver disease. Of the hepatitis viruses which cause HCC, HCV is predominant in Japan [6–9].

Although the age-adjusted incidence of HCC has increased in Japan, sequential changes in etiology of HCC patients between 2001 and 2008 are not fully understood [10]. To clarify factors affecting epidemiological changes in Japanese HCC patients, especially the recent trend of HCC, we analyzed the epidemiological trend of HCC in the western area of Japan, Kyushu area.

MATERIAL AND METHODS

Patients

A total of 10,010 patients with HCC diagnosed between 1996 and 2008 in the Liver Cancer study group of Kyushu (LCSK), were recruited for this study. The diagnosis of HCC was based on AFP levels and imaging techniques including ultrasonography (USG), computerized tomography (CT), magnetic resonance imaging (MRI), hepatic angiography (HAG), and/or tumor biopsy. The diagnostic criteria for HCC were either a confirmative tumor biopsy or elevated AFP (>20 ng/mL) and neovascularization in HAG and/or CT.

Etiology of HCC

A diagnosis of chronic HCV infection was based on the presence of HCV-RNA detected by polymerase chain reaction (PCR), whereas diagnosis of chronic HBV infection was based on the presence of hepatitis B surface antigen (HBsAg). The etiology of HCC was categorized to four groups as follows; **B:** HBsAg positive, HCV-RNA negative, **C:** HCV-RNA positive, HBsAg negative, **B+C**: both of HBsAg and HCV-RNA positive, **nonBC:** both of HBsAg and HCV-RNA negative.

Statistical analysis

The data were analyzed by the Mann-Whitney test for the continuous ordinal data, the x2 test with Yates' correction and the Fisher exact test for the association between two qualitative variables. The standard deviation was calculated based on the binomial model for the response proportion. P<0.05 was considered statistically significant.

RESULTS

Clinical features of the studied patients

A total of 10,010 patients with HCC were diagnosed at our study group from 1996 to 2008. Table 1 show that the proportion of patients diagnosed with **B** was 14.8% (1,485 of 10,010), whereas 68.1% (6,819 of 10,010) had **C**, and an additional 1.4% (140 of 10,010) had HCC associated with both viruses. The remaining 1,566 patients (15.6%) did not associate with both viruses. In analysis of patients in HCC by category, the median age of patients at diagnosis of **B** was 57 years old significant younger than other types HCC(**C: 69, nonBC: 70,.B+C 65 years old)**.

As shown in Figures 1 and 2, the number and ratio of **B** cases remained unchanged from1996 to 2001 and thereafter increased and plateaued, whereas **C** rapidly increased from 1996 to 2000 and thereafter decreased and plateaued. In addition, the number and ratio of the **nonBC** cases has increased continued gradually and continued in this study period.

Change of etiology in patients with HCC during the period 1996–2007 with 6-years intervals

Cohorts of patients with HCC were divided into six-year intervals (1996–2001 and 2002–2007). Table 2 show that the incident rate of **C** decreased significantly from 73.1% in 1996-2001 to 64.9% in 2002-2007 (1996–2001 vs. 2002–2007, p=<0.001). On the other hand, the incident rate of **B** and **-nonBC** increased significantly from 13.9% and 11.3% in 1996-2001 to 16.2% and 17.6% in 2002-2007, respectively. Not only the incident rate but also number of **B** and **-nonBC** became larger in same 6 years periods.

Table 3 shows that male/female ratio of **C** and **-nonBC** decreased significantly from 2.2 and 4.0 in 1996-2001 to 1.8 and 2.7 in 2002-2007, respectively (p<0.001). The ratio became clearly smaller, indicates an increase in female patients with **C** and **nonBC.** On the other hand, the male/female ratio of **B** patients did not significantly change during the period. The median age at diagnosis of **B, C**, and **nonBC** in six-year intervals were significant increase from 56 to 58, from 67 to 71 and from 68 to 71 years of age during the period.

DISCUSSION

Our study was the twenty-three major liver center-based study designed to examine the sequential change in the background of HCC patients during the past 13 years, 1996–2008. More than 80% of our patients had chronic HBV or HCV infections. During this observation period, the number and proportion of HCC-C reached a peak in 2000 and thereafter decreased and became stabilized. Previous studies from Japan reported that the proportion of the HCC patients with HCV infection had been increased and reached a plateau in the period of 1981–2001 [1,3,10–12]. However, in our study, the number and proportion of the HCC patients with HCV infection cases decreased in 2001–2008. The reason may be explained as follows; interferon therapy for chronic hepatitis C may have been associated with a decreased incidence of HCC [13–17]. Oral supplementation with a oral branched-chain amino acids has been useful in the prevention HCC [18]. Finally, the chronically HCV-infected

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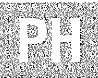

Table 1. The characteristic of HCC patients during the period of 1996–2008.

Age (y.o.)	B		C		nonB		B+C		Total
	Male	Female	Male	Female	Male	Female	Male	Female	
0–	1	0	0	1	0	0	0	0	**2**
10–	4	1	0	0	0	2	0	0	**7**
20–	6	2	1	0	1	1	0	0	**11**
30–	31	5	4	0	11	3	2	0	**56**
40–	204	22	130	12	32	15	12	0	**427**
50–	507	66	728	145	167	32	31	6	**1,682**
60–	287	118	1836	741	411	102	35	13	**3,543**
70–	140	64	1775	947	483	133	22	14	**3,578**
80–	9	18	271	214	97	65	1	4	**679**
90–	0	0	9	5	9	2	0	0	**58**
Total	**1,189**	**296**	**4,754**	**2,065**	**1,211**	**355**	**103**	**37**	**10,010**
	1,485 (4.8%)		6,819 (68.1%)		1,566 (15.6%)		140 (1.4%)		
Median	57	63	67	70	68	70	61	68	**67**
	57		69		70		65		
Mean	56	64	68	71	69	71	62	68	**67**
	58		68		68		63		
Range	1–87	14–89	27–94	0–93	28–96	17–90	36–82	55–82	**0–96**
	1–89		0–94		17–96		36–82		

Age: B vs. C $p \leq 0.001$; B vs. B+C $p \leq 0.001$; B vs. nonBC $p \leq 0.001$; C vs. BC $p \leq 0.001$; C vs. nonBC $p=0.043$; BC vs. nonB+C $p \leq 0.001$. IQR – interquartile range; SD – standard deviation.

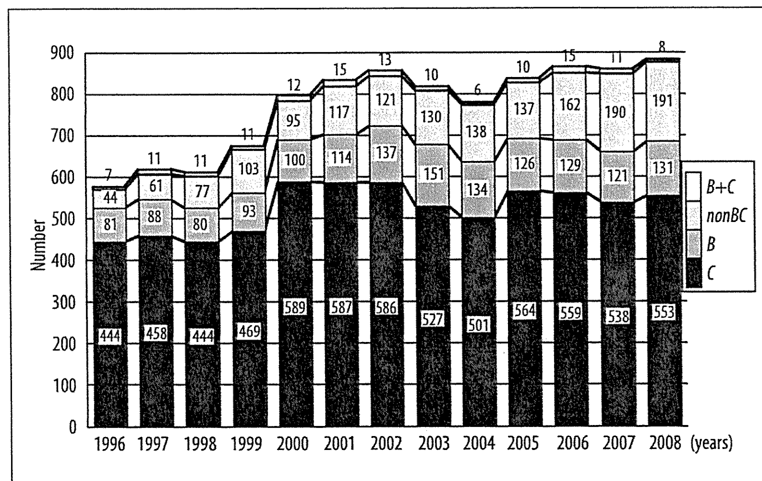

Figure 1. Sequential changes in the number of HCC patients categorized by etiology during the period 1996–2008.

population is aging in Japan. Yoshizawa et al. reported that age-specific prevalence for the presence of HCVAb among ~300,000 voluntary blood donors from Hiroshima in 1999 clearly increased with the age, reaching the highest proportion of 7% in individuals who were more than 70 years old [10,19]. In this study, the median age of the HCC patients with HCV infection steadily increased from 67 to 71 years of age during the studied period. In a word, HCV infected people become older with years in Japan and they were regarded as a high risk for HCC.

The prevalence rate of HBV in Kyushu area has been reported to be higher than other area in Japan [1]. In Kyushu area, 95% of patients with chronic HBV infection had HBV genotype C except for Okinawa [20]. HBV genotype C is thought to be associated with higher incidence of HCC

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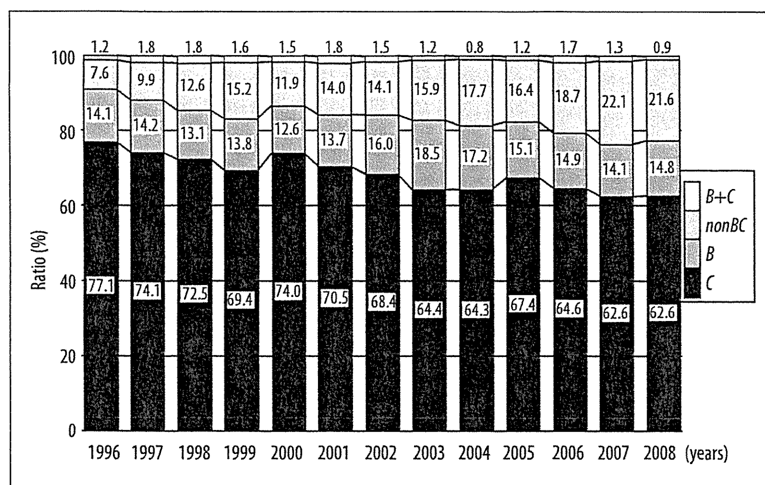

Figure 2. Sequential changes in the ratio of HCC patients categorized by etiology during the period 1996–2008.

Table 2. Change of etiology in patients with HCC during the period 1996–2007 with 6-years intervals.

Period		1996–2001	2002–2007	P value
Number		3,023	4,173	
Sex	Male	2,162	2,849	
	Female	861	1,324	
Ratio (male/female)		2.5	2.2	0.003
Age (y.o.) (IQR)		66 (14)	69 (12)	<0.001
Hepatitis virus (%)	B	13.9	16.2	
	C	73.1	64.9	
	B+C	1.7	1.3	
	nonBC	11.3	17.6	0.001

QR – interquartile range.

Table 3. The median age and male/female ratio of HCC patients during the period of 1996–2007.

Period		1996–2001	2002–2007	P value
B				
Age (y.o.) (IQR)		56 (14)	58 (15)	0.001
Sex	Male	331	519	
	Female	88	157	
Ratio (male/female)		3.8	3.3	0.391
C				
Age (y.o.) (IQR)		67 (9)	71 (11)	<0.001
Sex	Male	1,524	1,753	
	Female	687	955	
Ratio (male/female)		2.2	1.8	0.002
nonBC				
Age (y.o.) (IQR)		68 (12)	71 (13)	<0.001
Sex	Male	273	534	
	Female	69	201	
Ratio (male/female)		4.0	2.7	0.012

QR – interquartile range.

compared with other HBV genotypes [21]. In the present study, the incident rate of HCC patients with HBV infection became larger in this study period. To explain this change, we must consider from two viewpoints. The one is that the number of patients with HCC caused by HCV infection decreased, the other is that the proportion of chronic HBV infected patients who have reached the age of developing HCC is relatively high as described below.

Nationwide health survey for HBsAg in the over 40 years of age population had been done between 2002 and 2006 in Japan. This survey reports indicated that the average HBsAg prevalence was 1.2% in the total Japanese population patients with chronic HBV infection [10] and the age-specific prevalence of HBsAg was higher in the group aged between 50 (1.4%) and 55 years (1.5%). In the HCC patients with HBV genotype C, the mean age was 55 years in Japan [20]. This overlap between age-specific prevalence and hepatocellular carcinogenic age would be associated with the increase of HCC patients with HBV infection. Nucleoside analogue reverse transcriptase inhibitor (NARTI) therapy effectively reduces the incidence of HCC in chronic hepatitis B patients [22,23]. However, Interferon therapy for chronic hepatitis C started from 1992, whereas NARTI therapy for HBV started from 2000 in Japan [24,25]. Hence, HBV associated HCC will probably decrease in Japan during the next 10 to 20 years.

The survey of HCC patients associated with nonBC infection in Japan was conducted by Inuyama Hepatitis Research Group from 1995 to 2003. The ratio of HCC patients with nonBC accounted 9.3% [1]. In the present study, the ratio of HCC patients with nonBC was 14.1%. Furthermore, the number and the proportion of HCC patients with nonBC have been gradually increasing in the periods. The current two studies account for the increase in number and proportion of HCC patients with nonBC. First, Lai et al. reported

that type 2 diabetes increases the risk of developing HCC in those who are HCV negative or have a high level of total cholesterol [26]. Second, Nakano et al. reported that epidemiological studies on diabetes mellitus revealed that the number of patients with diabetes mellitus is gradually increasing in Japan along with development of car society and westernization of food intake. Since prevalence of diabetes mellitus increases with aging, proportion of individuals with diabetes mellitus aged over 60 has exceeded two-third of estimated total number of patients (7.40 million in 2002) in Japan where aging of society is rapidly progressing [27]. In a word, the number of type 2 diabetes people is increasing in Japan and they were regarded as a high risk for HCC. Then, the number and the proportion of HCC patients with nonBC have been increased recent twelve years in Japan.

It is known that 2 to 4 decades of chronic HCV infection are required to develop cirrhosis and subsequent HCC [28–31]. The number of HCC cases has increased in Japan, because individuals infected with HCV during the past have grown old and have reached the cancer-bearing age. The prevalence of HCV infection in young Japanese individuals is low and the incidence of HCVAb is very low because of preventative actions against HCV infection such as the screening of blood products for HCV and the use of sterile medical equipment [32]. Additionally, we showed that the number and proportion of patients with HCC-C cases decreased, whereas the number and ratio of HCC-nonBC steadily increased during the studied period. These findings may be expected that the incidence of HCC patients with nonBC in Japan may continue to increase even after the consequence of the HCV epidemic level off, a country that is far advanced with regard to HCC patients with HCV infection, in the near future.

Conclusions

In summary, HCC patients had increased from 1996 to 2000 and this increase was originated from HCC patients with HCV infection. The number and proportion of HCC patients with HCV infection reached a peak in 2000 and thereafter decreased and became stabilized. The incidence of hepatocellular carcinoma associated with hepatitis C infection decreased after 2001 in Kyushu area. This change was due to the increase in the number and proportion of the HCC not only nonBC patients but also B patients.

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Hepatology Research

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Original Article

Rapid reduction of hepatitis C virus-Core protein in the peripheral blood improve the immunological response in chronic hepatitis C patients

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Aim: The extracellular hepatitis C virus (HCV)-antigen, including HCV-Core protein, can suppress immune cells. Recently, the efficacy of double filtration plasmapheresis (DFPP) for chronic hepatitis C (CHC) was reported. However, the mechanism of efficacy of DFPP might not be only the reduction of HCV but also the effect of immune cells via direct and/or indirect mechanisms. The aim of this study is to analyze the virological and immunological parameters of difficult-to-treat HCV patients treated with DFPP combined with Peg-interferon and RBV (DFPP/Peg-IFN/RBV) therapy.

Methods: Twelve CHC patients were enrolled and treated with DFPP/Peg-IFN/RBV therapy. The immunological, virological and genetic parameters were studied.

Results: All patients (4/4) treated with the major IL28B allele (T/T) could achieve complete early virological response (EVR). The amounts of HCV-Core antigen in the peripheral blood of EVR patients treated with DFPP/Peg-IFN/RBV rapidly declined in comparison to those of late virological response (LVR) patients treated with DFPP/Peg-IFN/RBV and EVR patients treated with Peg-IFN and RBV (Peg-IFN/RBV). The amount of IFN-γ produced from peripheral blood gradually increased. On the other hand, the amount of IL10 gradually decreased in the EVR patients. The frequencies of HCV-Core binding on CD3+ T cells rapidly declined in EVR patients treated with DFPP/Peg-IFN/RBV therapy. Moreover, the distributions of activated $CD4^{+}$and $CD8^{+}$ T cells and CD16-CD56 high natural killer cells were significantly changed between before and after DFPP.

Conclusions: The rapid reduction of HCV-Core antigens and changes in the distribution of lymphoid cells could contribute to the favorable immunological response during DFPP/Peg-IFN/RBV therapy.

Key words: Core, double filtration plasmapheresis, hepatitis C virus, IL28B, natural killer cells, T cells

INTRODUCTION

HEPATITIS C VIRUS (HCV) is a non-cytopathic virus that causes chronic hepatitis and hepatocellular carcinoma (HCC).[1] Recently, the efficacy of double filtration plasmapheresis (DFPP) combined with Peg-interferon and RBV (DFPP/Peg-IFN/RBV) therapy for chronic hepatitis C (CHC), high viral loads and genotype 1b patients was reported.[2–4] DFPP has been applied in the first 2 weeks to mechanically eliminate HCV particle from blood in the induction phase.[2–4] In DFPP, the patients' blood is separated into plasma and blood cells by the first filter. Then, the second filter is used to eliminate target-sized molecules including HCV particles from the separated plasma.[2–4]

The mechanisms of the favorable effects for CHC patients might not be only the reduction of HCV particles but also the reduction of LDL-cholesterol and unknown effects of DFPP including that of immune systems as previously reported.[5–10] Plasmapheresis has been used in some T cell-dependent disorders, such as multiple sclerosis and ulcerative colitis.[11–16] One report indicated that DFPP could directly affect the distribution of lymphocyte subsets.[5]

The cellular immune response to HCV plays an important role in the pathogenesis of chronic hepatitis,

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cirrhosis, and HCC[17–19]. The hyporesponsiveness of HCV-specific T helper 1 cells and the excessive regulatory function of CD4+CD25+FoxP3+ regulatory T (Tregs) cells in the peripheral blood of patients with chronic hepatitis C has been demonstrated.[20–24] Recently, we reported that lymphotropic HCV might contribute to the suppression of the Th1 response.[25–27] In those studies, UV-irradiated HCV that theoretically could not replicate in T cells also suppressed primary human Th1 lymphocytes.[25] It has been reported that extracellular HCV-core protein could interact with the complement receptor gC1qR and upregulate the suppressor of cytokine signaling-1 (SOCS-1), accompanied by downregulation of the signal transducer and activator of transcription-1 (STAT-1) phosphorylation in T cells.[28] Moreover, the HCV-core proteins activate STAT-3 in human monocyte/macrophage/dendritic cells, which would play a critical role in the alteration of the inflammatory response, leading to impaired anti-viral T cell responses.[29,30]

Recently, the polymorphism of IL28B and the amino acid sequence of HCV-core 70 were reported to be strong indicators for the efficacy of Peg-IFN/RBV therapy. Major IL28B allele (T/T) and wild type amino acid of HCV-core 70 could easily achieve early virological response (EVR) and sustained virological response (SVR) in difficult-to-treat CHC patients.[31–35] Analysis of IL28B polymorphism and HCV-core 70 amino acid is necessary to understand the real effect of unique therapy like DFPP/Peg-IFN/RBV therapy.

Here we describe the immunological effects of the DFPP procedure and a novel mechanism of DFPP/Peg-IFN/RBV therapy for difficult-to-treat CHC patients.

METHODS

Patients

TWELVE DIFFICULT-TO-TREAT CHC patients with genotype1b, high viral HCV-RNA and advanced age were enrolled for DFPP/Peg-IFN/RBV therapy. (Table 1) Twelve HCV patients that received Peg-IFN/RBV therapy were enrolled as control subjects. None of the patients had liver disease due to other causes, such as alcohol, drugs, congestive heart failure, or autoimmune diseases. Permission for the study was obtained from the Ethics Committee at Tohoku University Graduate School of Medicine (permission no. 2009-166). Written informed consent was obtained from all participants enrolled in this study. Participants were monitored for at least 2 years before the start of therapy.

Treatment protocol

Double filtration plasmapheresis was performed on day 1 of the therapy to decrease the viral load, and administration of Peg-IFN and ribavirin was started at 1 h after the completion of DFPP. DFPP was performed five times, on day 1, 2, 4, 7 and 8, and blood samples were obtained before and after each DFPP. DFPP was performed using Plasmaflo OP (Asahi Kasei Kuraray Medical, Tokyo, Japan) as the first filter, and Cascadeflo EC-50W (Asahi Kasei Kuraray Medical) as the second filter. For each session, the final volume of treated plasma is shown in Table 2. The backgrounds of the patients were analyzed, including gender, age, weight, histology of liver biopsy (METAVIR SCORE), history of IFN based therapy, titer of HCV-RNA, genotype of HCV-RNA, polymorphism of IL28B (rs8099917) and ITPA (rs1127354). Biochemical laboratory testing was performed at each DFPP procedure and every 4 weeks during Peg-IFN/RBV therapy.

Isolation of peripheral blood mononuclear cells and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized blood by means of Ficoll-Hypaque density gradient centrifugation (Amersham Bioscience, Uppsala, Sweden). PBMCs were stained with CD3, CD4, CD8, CD16, CD25, CD56, HLA-DR and isotype control antibodies (BD Pharmingen, San Jose, CA, USA) for 15 min on ice to analyze the frequency of $CD3^+CD4^+HLA\text{-}DR^+$ cells, $CD3^+CD8^+HLA\text{-}DR^+$cells, $CD4^+CD25^+$ Tregs, $CD3^-CD16^-$CD56highNK cells, and $CD3^-CD16^+$CD56dim NK cells by FACS Canto-II (BD).

Staining of HCV-Core antigen binding on the $CD3^+$ lymphocyte

We used modified staining methods to analyze whether the mechanical stress of DFPP and dilution of HCV-Core antigen could affect the binding of HCV-Core antigen on $CD3^+$ T cells. Freshly isolated PBMCs were immediately stained with PE-conjugated anti-HCV-Core antibody (Abcam, Cambridge, MA, USA) and $CD3^+$ antibody for 15 min on ice. Then, antibody-bound PBMCs were diluted with plasma from the same patients. Finally, the frequency of HCV-Core binding $CD3^+$ T cells was analyzed by using FACS Canto-II without the washing step to investigate the effect of mechanical stress. Therefore, the positive rate of isotype control and healthy controls were a little bit higher than those with conventional staining methods.

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Table 1 Backgrounds of patients receiving with double filtration plasmapheresis combined with Peg-interferon and RBV (DFPP/Peg-IFN/RBV) therapy

	1	2	3	4	5	6	7	8	9	10	11	12
Age	61	61	73	54	59	45	66	55	43	66	59	55
Gender	Female	Male	Male	Female	Female	Male	Male	Male	Male	Male	Male	Male
Fibrosis	F3	F2	F2	F2	F1	F3	F2	F1	F2	F3	F3	F2
Activity	A2	A2	A2	A2	A2	A2	A2	A2	A2	A2	A2	A2
Previous Therapy	Peg-IFN/RBV Null-responder	none	Peg-IFN Null-responder	PEG-IFN/RBV Recurrence	IFN Recurrence	none	none	Peg-IFN/RBV Recurrence	none	Peg-IFN/RBV Recurrence	none	none
Kinds of Peg-IFN	Peg-IFN-α2b	Peg-IFN-α2b	Peg-IFN-α2b	Peg-IFN-α2b	Peg-IFN-α2b	Peg-IFN-α2b	Peg-IFN-α2b	Peg-IFN-α2a	Peg-IFN-α2b	Peg-IFN-α2b	Peg-IFN-α2b	Peg-IFN-α2b
Peg-IFN Dose (μg)	80	80	100	100	80	120	80	180	80	100	80	100
Ribavirin (mg)	600	600	200	800	600	800	600	800	600	800	800	800
HCV-RNA (log copies/mL)	7	5.6	6.3	5.5	6.4	6.8	5.8	7	6.8	6.2	6.6	6.5
T-bil (mg/dL)	1.6	0.7	0.7	1.4	0.8	0.9	0.8	1.2	0.7	1.1	1.4	1.2
D-bil (mg/dL)	0.2	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
ALP (IU/L)	627	116	242	326	285	155	172	234	216	299	146	111
γ-GTP (IU/L)	68	26	33	23	55	79	50	28	12	26	64	29
AST (IU/L)	33	35	28	42	43	42	46	21	22	46	39	31
ALT (IU/L)	39	39	33	63	64	86	63	21	20	46	37	19
LDH (IU/L)	207	200	203	225	252	162	193	135	169	252	155	194
TP (g/dL)	7.4	6.9	6.9	6.9	7.3	7.3	7.1	7.3	7	7.5	7	6
Alb (g/dL)	3.8	4.1	3.7	3.8	4.5	4.6	4.1	4.4	4.1	4	4.2	3.6
TG (mg/dL)	97	98	186	53	166	135	53	145	142	113	81	72
T-Cho (mg/dL)	116	118	149	148	216	133	123	190	164	182	144	133
HDL-Cho (mg/dL)	43	33	48	62	35	28	49	67	32	40	57	52
LDL-Cho (mg/dL)	55	68	65	76	148	79	62	97	108	116	71	59
HbA1c (%)	6.4	6.9	5.9	5.3	5.4	4.8	6.1	6.1	5.3	5.8	5.1	5.1
BMI	21.1	19.2	21.4	28.5	25.3	24.9	17.8	21.9	21.4	25.9	19.3	21.8
ANA (fold)	79	79	79	79	79	79	79	79	79	79	79	79
ASMA (fold)	19	19	19	19	19	19	19	19	19	19	19	19
AMA (fold)	19	19	19	19	19	19	19	19	19	19	19	19
IgG (mg/dL)	1492	1229	1523	1758	1389	1315	1349	1303	1330	1687	1475	1162
IgA (mg/dL)	323	72	279	200	117	124	344	284	208	307	170	113
IgM (mg/dL)	111	31	176	84	43	106	23	21	165	62	53	36
WBC ($\times10^3$/uL)	5800	4600	6700	4600	6500	7600	4100	4700	5100	4800	3900	4400
Hb (g/dL)	12.4	16.1	14.4	13.1	14.5	18	12.9	14.5	14.1	14	14.6	14.4
Plt ($\times10^3$/ul)	111	132	119	117	191	128	142	197	155	133	134	174
Neutrohil ($\times10^3$/uL)	3830	1470	3620	2580	3580	3650	1970	2350	3010	2590	2220	2420
lymph ($\times10^3$/uL)	1330	2530	2080	1610	2280	2740	1800	1790	1580	1250	1250	1500
Mono ($\times10^3$/uL)	580	370	800	320	520	910	250	470	410	530	270	480
IL28 SNP rs8099917	T/G	T/G	T/G	T/G	T/T	T/G	T/T	T/G	T/G	T/T	T/G	T/T
ITPA SNP rs1127354	C/A	C/C	C/C	C/C	C/C	C/C	C/C	C/A	C/C	C/C	C/A	C/A
HCV-Core 70	Q	R	R	Q	R	R	R	Q	R	R	R	R
HCV-Core 91	L	M	M	M	L	L	L	M	L	M	L	M

Biochemical, hematological, virological and genetical data of pre-treatment are shown.

Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AMA, anti-mitochondrial antibodies; ANA, anti-nuclear antibodies; ASMA, anti-smooth muscle antibody; AST, aspartate aminotransferase; BMI, body mass index; γ-GTP, gamma-glutamyltransferase; D-Bil, direct-bilirubin; HbA1c, hemoglobinA1c; HCV, hepatitis C virus; HDL, high-density lipoprotein; LDH, lactate dehydrogenase; LDL, low-density lipoprotein; Ig, immunoglobulin; ITPA, inosine triphosphatase; SNP, single nucleotide polymorphism; T-Cho, total cholesterol; TG, triglyceride; TP, total protein; T-Bil, total bilirubin; WBC, white blood cell count.

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Table 2 Detailed information of the procedure of DFPP for each case

	The amount of filtration (L)					Filter	Blood Access	Anti-coagulant	Side effect
	1st	2nd	3rd	4th	5th				
Patient-1	3.21	3.3	3.3	3.3	3.06	EC-50W	Right internal jugular vein	6′-amidino-2-naphthyl 4-guanidinobenzoate (30 mg/mL)	None
Patient-2	3.11	3.24	3.29	3.33	3.24	EC-50W	Right internal jugular vein	6′-amidino-2-naphthyl 4-guanidinobenzoate (30 mg/dL)	None
Patient-3	3.2	3.25	3.3	3.35	3.4	EC-50W	Right internal jugular vein	6′-amidino-2-naphthyl 4-guanidinobenzoate (30 mg/dL)	None
Patient-4	3.76	3.77	4.26	3.71	3.7	EC-50W	Right internal jugular vein	6′-amidino-2-naphthyl 4-guanidinobenzoate (40 mg/dL)	None
Patient-5	3.3	3.4	3.28	2.83	3	EC-50W	Right internal jugular vein	6′-amidino-2-naphthyl 4-guanidinobenzoate (40 mg/dL)	Nausea
Patient-6	4.26	4.07	4.06	4.27	4.23	EC-50W	Right Internal Jugular Vein	6′-amidino-2-naphthyl 4-guanidinobenzoate (40 mg/dL)	None
Patient-7	3.22	3.26	3.37	3.38	3.27	EC-50W	Right internal jugular vein	6′-amidino-2-naphthyl 4-guanidinobenzoate (40 mg/dL)	None
Patient-8	3.22	3.19	3.24	2.77	1.93	EC-50W	Median Antebrachial Vein	6′-amidino-2-naphthyl 4-guanidinobenzoate (40 mg/dL)	None
Patient-9	3.2	3.23	3.38	3.19	2.2	EC-50W	Median Antebrachial Vein	6′-amidino-2-naphthyl 4-guanidinobenzoate (40 mg/dL)	Nausea
Patient-10	3.17	3.28	1.21	3.22	3.23	EC-50W	Median antebrachial vein	6′-amidino-2-naphthyl 4-guanidinobenzoate (50 mg/dL)	None
Patient-11	3.23	3.21	3.25	3.24	3.2	EC-50W	Median antebrachial vein	6′-amidino-2-naphthyl 4-guanidinobenzoate (50 mg/dL)	none
Patient-12	3.19	3.18	3.22	3.22	3.2	EC-50W	Median antebrachial vein	6′-amidino-2-naphthyl 4-guanidinobenzoate (50 mg/dL)	None

Analysis of cytokines production from PBMCs

Isolated 10^6/mL PBMCs (2×10^5 cells/well) were incubated in round bottomed 96-well plates with serum-free complete medium established by our group[36] for 24 h to analyze the cytokines produced from PBMCs. Supernatants of the incubated PBMCs were analyzed by using IFN-γ and IL10 enzyme linked immunosorbent assay (ELISA) (eBioscience, San Diego, CA, USA). The concentrations of IFN-γ and IL10 in the plasma were also analyzed by using ELISA (eBioscience).

Real-time PCR analysis

CD4+ T Cells were collected sequentially at pre-treatment, 4 weeks, 12 weeks and 24 weeks after DFPP/Peg-IFN/RBV or Peg-IFN/RBV therapy. After the extraction of total RNA and the reverse transcription (RT) procedure, real-time polymerase chain reaction (PCR) using TaqMan Chemistry System was carried out. The ready-made set of primers and probe for the amplification of STAT-1 (ID Hs00234829) and T-bet (ID HS00203436) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Perkin-Elmer Applied Biosystems (Carlsbad, CA, USA). The relative amount of target mRNA was obtained by using the comparative threshold (CT) cycle method. The expression level of mRNAs of the non-stimulation sample of $CD4^+$ cells from Patient 1 at pre-treatment is represented as 1.0 and relative amount of target mRNA was calculated according to the manufacturer's protocol.

Detection of HCV RNA

RNAs were extracted from 250 μL of serum using TRIzol LS (Invitrogen, Tokyo, Japan). They were divided into two, and assayed by each RT-PCR with nested primers derived from the core region of the HCV genome. Nested PCR of the core region of the HCV genome was carried out with primers C008 (sense, 5′-AAC CTC AAA GAA AAA CCA AAC G-3′) and C011 (antisense, 5′-CAT GGG GTA CAT YCC GCT YG-3′) in the first round and C009 (sense, 5′-CCA CAG GAC GTY AAG TTC CC-3′) and C010 (antisense, 5′-AGG GTA TCG ATG ACC TTA CC-3′) in the second round.

Analysis of nucleotide and amino acid sequences

The PCR products were sequenced directly on both strands using the BigDye Terminator version 3.1 Cycle Sequencing Kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence

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analysis was performed using Genetyx-Mac ver. 12.2.6 (Genetyx Corp., Tokyo, Japan) and ODEN (version 1.1.1) from the DNA Data Bank of Japan (National Institute of Genetics, Mishima, Japan).

Detection of IL-28B and ITPA polymorphism

Genomic DNA was isolated from PBMC using an automated DNA isolation kit. Then, the polymorphism of IL28B (rs8099917) and ITPA were analyzed using realtime PCR (TaqMan SNP Genotyping Assay). The detection of these polymorphisms was approved by the Ethics Committee at Tohoku University Graduate School of Medicine (permission no. 2010-323)

Statistical analysis

Statistical analyses of the data in Figure 2b and d were made by Wilcoxon Test. The data in Figure 3a–f were analyzed by Dunnett Test. The data in Figures 3h, and i, and 5 were analyzed by independent Student's *t*-test. The data in Figure 4e were analyzed by analysis of variance (ANOVA) test. All statistical analyses were carried out using SPSS version 16.

RESULTS

Efficacy and tolerability of DFPP combined with Peg-IFN/RBV therapy

THE CHARACTERISTICS OF 12 patients treated with DFPP/Peg-IFN/RBV therapy are shown in Table 1. The subjects enrolled in this study were 43 to 73 years old. Six patients were previously treated with IFN-based therapy and failed to achieve SVR. Another six patients were treatment-naïve patients. The liver histology and stiffness of all patients were analyzed at the time of start of the therapy. All of the enrolled patients had over 5 log copies/mL HCV RNA and genotype 1b HCV-RNA. Eight patients (66%) had hetero/minor IL28B allele (T/G) (rs8099917) that was reported as a marker of difficult-to-treat with Peg-IFN/RBV therapy. Four patients (33%) had major homo IL28B allele (T/T) that was reported as favorable for achieving SVR. In Japanese, it was reported that approximately 80% of subjects had major IL28B allele.[32,33] These data indicated that most of the patients enrolled in this study were categorized as the most difficult-to-treat-patients among the difficult-to-treat-patients.

The amounts of filtration were about 3–4 L per one DFPP therapy (Table 2). The method of blood access for seven patients was the right internal jugular vein with UK catheter and that for the other five patients was the median antebrachial vein. Both methods were safe enough to complete the DFPP procedure. The anti-coagulant was 6′-amidino-2-naphthyl 4-guanidinobenzoate. The amount of anti-coagulant was 30 mg to 50 mg. None of the major side effects appeared during DFPP therapy.

Five patients achieved the complete EVR (cEVR) that means undetectable of HCV-RNA (minus and lower than 1.2 log copies/mL) within the first 12 weeks during therapy (Fig. 1). All of the patients with the major homo IL28B allele (4/4) achieved cEVR. A patient with hetero/minor IL28B allele (1/8) achieved cEVR. Four patients achieved late virological response (LVR) indicating undetectable HCV-RNA (minus and lower than 1.2 log copies/mL) within the first 24 weeks during therapy. Three patients showed no response to DFPP/Peg-IFN/RBV therapy at all (NVR: null virological responder).

Change of lymphocyte distribution due to DFPP procedure

The effects of DFPP on the distribution of lymphocytes were analyzed by flow cytometry. The frequencies of $CD3^+CD4^+HLA\text{-}DR^+$ activated $CD4^+$ T cells and $CD3^+CD8^+HLA\text{-}DR^+$ activated $CD8^+$ T cells were significantly increased after the 1st DFPP in the EVR patients (Fig. 2a,b). The frequencies of $CD4^+CD25$high Tregs and $CD4^+CD25^+IL7R^-$ Tregs were not changed after the 1st DFPP (data not shown). Then, we analyzed the NK cells that might contribute to the responsiveness of IFN-based therapy. The frequency of $CD3^-CD16^-CD56$ high NK cells that could have the ability of good cytokine production and low cytotoxic activity was significantly increased after the 1st DFPP (Fig. 2c,d). On the other hand, the frequency of $CD3^-CD16^+CD56$dim NK cells that could have poor cytokine production ability and high cytotoxic activity was not changed after the 1st DFPP. These data indicated that the distribution of activated T cells and $CD3^-CD16^-CD56$ high NK cells could be changed due to the DFPP procedure without the Peg-IFN effect.

Amount of IFN-γ and IL10 in the culture supernatant of PBMCs and in the plasma of peripheral blood during DFPP therapy

The amounts of IFN-γ and IL10 produced from isolated PBMCs were analyzed to detect the effect of DFPP on peripheral immune cells. At first, we analyzed the amounts of IFN-γ and IL10 in the plasma of peripheral blood before and after DFPP. Both cytokines could be detected in the plasma from several patients showing almost no change between before and after 1st DFPP

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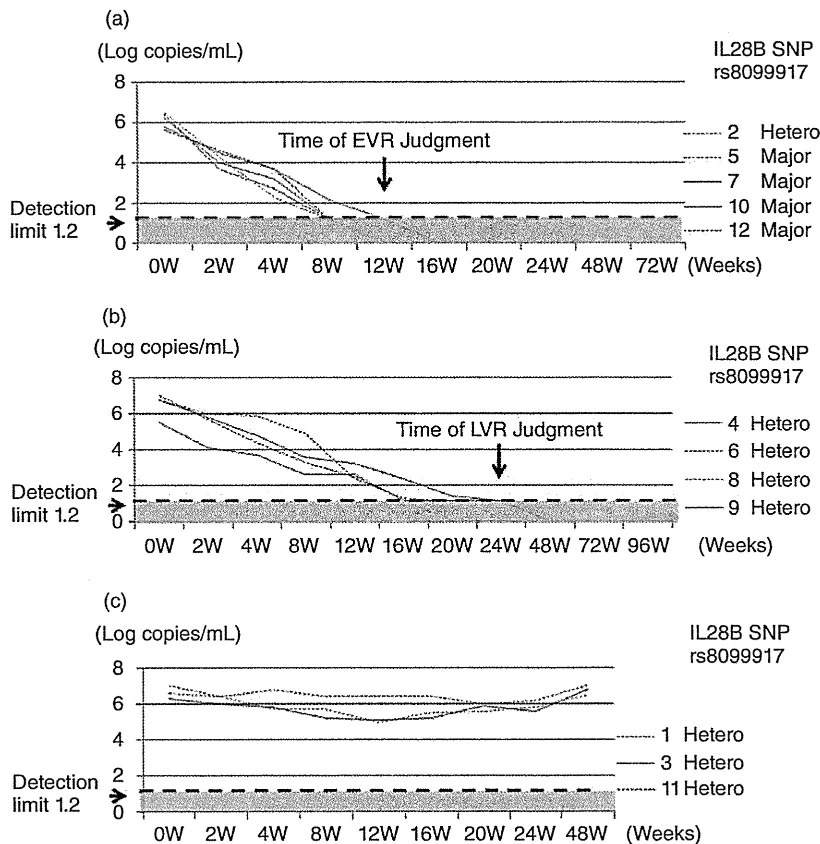

Figure 1 Viral dynamics of hepatitis C virus (HCV) during double filtration plasmapheresis combined with Peg-interferon and RBV (DFPP/Peg-IFN/RBV) therapy. Viral dynamics of early virological response (EVR) patients (a), late virological response (LVR) patients (b) and null virological response (NVR) patients (c) are shown in this figure. The *Y* axis indicates the viral titers. The *X* axis indicates the time course of therapy. The polymorphism of IL28B single nucleotide polymorphism (SNP) is shown on the left side of the graphs.

therapy (data not shown). However, the amounts of IFN-γ produced from PBMCs of EVR-patients, but not those of LVR and NVR patients, in the culture supernatant with serum-free medium for 24 h were increased after the 1st DFPP procedure. Moreover, the amount of IFN-γ gradually increased in EVR patients but not in LVR and NVR patients. On the other hand, the amounts of IL10 produced from PBMCs were significantly different between responders (EVR and LVR) and non-responders (NVR) at pre-treatment (Fig. 3d–f). The patterns of change in cytokine production after three DFPP procedures were clearly recognized in the three groups (EVR, LVR and NVR) (Fig. 3g).

Then, we compared the ability of cytokine production from PBMCs between DFPP/Peg-IFN/RBV therapy and Peg-IFN/RBV therapy. The amounts of IFN-γ produced from the PBMCs of EVR patients at 4 weeks post DFPP/Peg-IFN/RBV therapy were significantly higher than those after Peg-IFN/RBV therapy ($P < 0.05$)(Fig. 3h). On the other hand, the amounts of IL10 produced from the PBMCs of EVR patients at 4 weeks post DFPP/Peg-IFN/RBV therapy were significantly lower than those after Peg-IFN/RBV therapy ($P < 0.05$) (Fig. 3i)

Amounts of HCV-Core antigen in the plasma of peripheral blood and on the surface of CD3$^+$ T cells

The amount of HCV-Core antigen in the plasma of peripheral blood was quantified by using ELISA. This ELISA system can detect free HCV-Core antigen in addition to HCV-Core antigen in the HCV particle. The detection methods using realtime-PCR and ELISA could not detect the discrepancy between the titer of HCV-RNA and HCV-Core Ag before and after the first DFPP procedure since the sensitivity of realtime-PCR and ELISA might not be enough to detect an additional 10–15% reduction of amount of HCV-core Ag (data not shown). However, the amounts of HCV-Core antigen decreased very rapidly in the patients who could achieve EVR by receiving DFPP/Peg-IFN/RBV therapy in comparison to patients who could not achieve EVR by receiving DFPP/Peg-IFN/RBV

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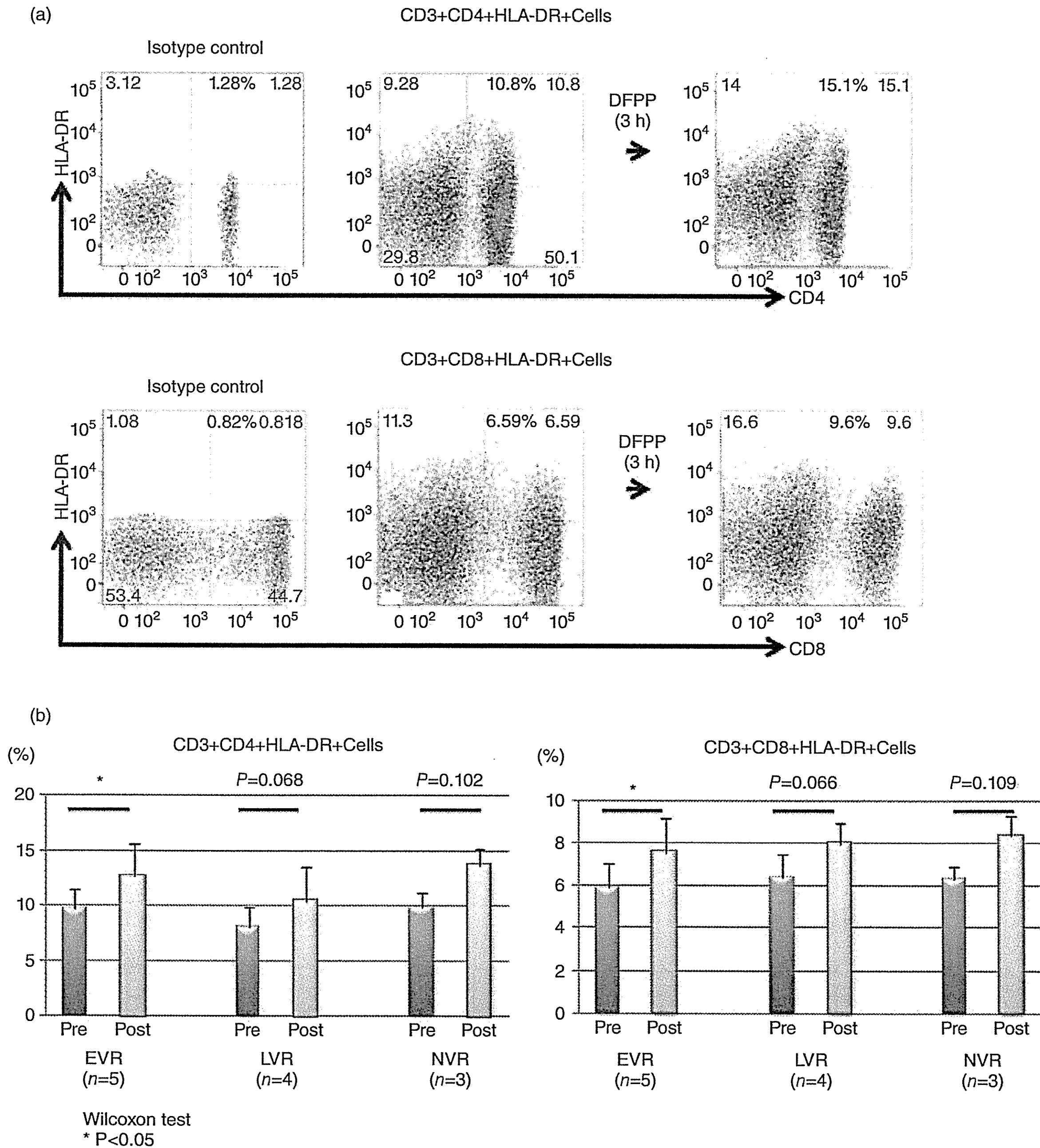

Figure 2 Change of lymphocyte distribution due to double filtration plasmapheresis (DFPP) procedure. Representative dot plots of CD3⁺CD4⁺HLA-DR⁺ and CD3⁺CD8⁺HLA-DR⁺ T cells are shown (a). The frequencies of HLA-DR⁺ activated CD4⁺ and CD8⁺ T cells are shown in the upper right side of the dot plots. The graphs indicating the frequencies of activated CD4⁺ and CD8⁺ T cells are shown in accordance with the responsiveness (b). Representative dot plots of natural killer (NK) cells and NK-T cells are shown (c). CD3 positive or negative cells were gated. Then, the gated cells were applied with CD16 and CD56 (c). The red box indicates the CD3⁻CD16⁻CD56high NK cells that could have the ability of good cytokine production and low cytotoxicity. The blue box indicates the CD16⁺CD56dim NK cells that could have the ability of poor cytokine production and high cytotoxicity. The bar graphs indicate the frequencies of CD3⁻CD16⁻CD56high NK cells and CD3⁻CD16⁺CD56dim NK cells before and after the 1st DFPP procedure (d). Statistical analyses of data were carried out by Wilcoxon test.

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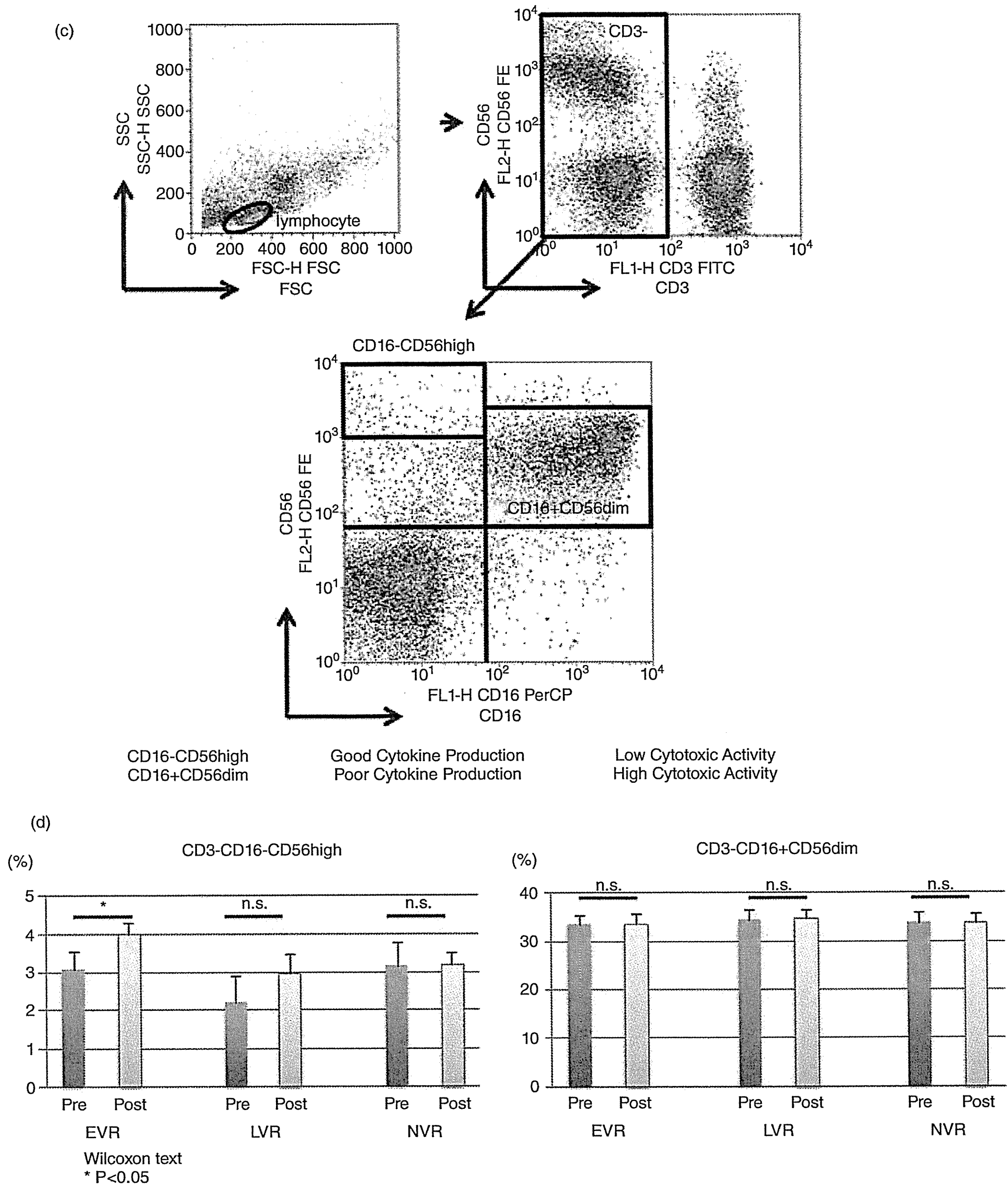

Figure 2 *Continued.*

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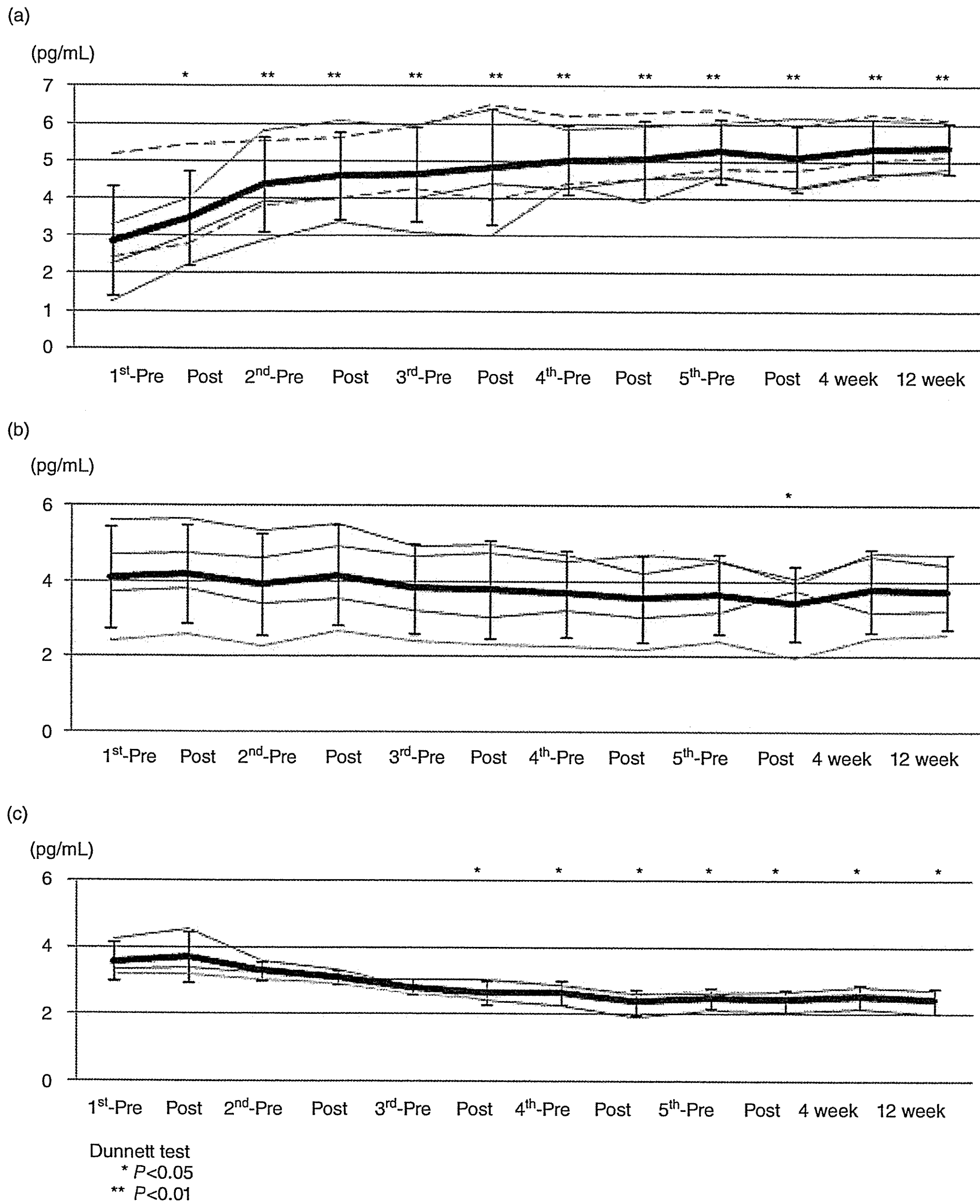

Figure 3 Change of cytokine production from peripheral blood mononuclear cells (PBMCs) during double filtration plasmapheresis combined with Peg-interferon and RBV (DFPP/Peg-IFN/RBV) therapy. The amount of IFN-γ produced from isolated PBMCs is shown in the graphs (a, b, c). The amount of IL10 produced from isolated PBMCs is shown in the graphs (e, f and g). The *X*-axis indicates the time course of DFPP/Peg-IFN/RBV therapy. The *Y*-axis indicates the amount of IFN-γ (a, b and c) or IL10 (e, f and g). Black broad lines indicate the mean amount of IFN-γ or IL10 from early virological response (EVR) (a and e), late virological response (LVR) (b and f) and null virological response (NVR) (c and g) patients' PBMCs. Error bars indicate the standard deviation. Statistical analyses of data were carried out by Dunnett test. The change-patterns of IFN-γ or IL10 productions after three DFPP procedures are shown (EVR, LVR and NVR) (g). The comparison of the changing amounts of cytokines between DFPP/Peg-IFN/RBV therapy and Peg-IFN/RBV therapy are shown (h and i). Statistical analyses of data were carried out by independent student t-test.

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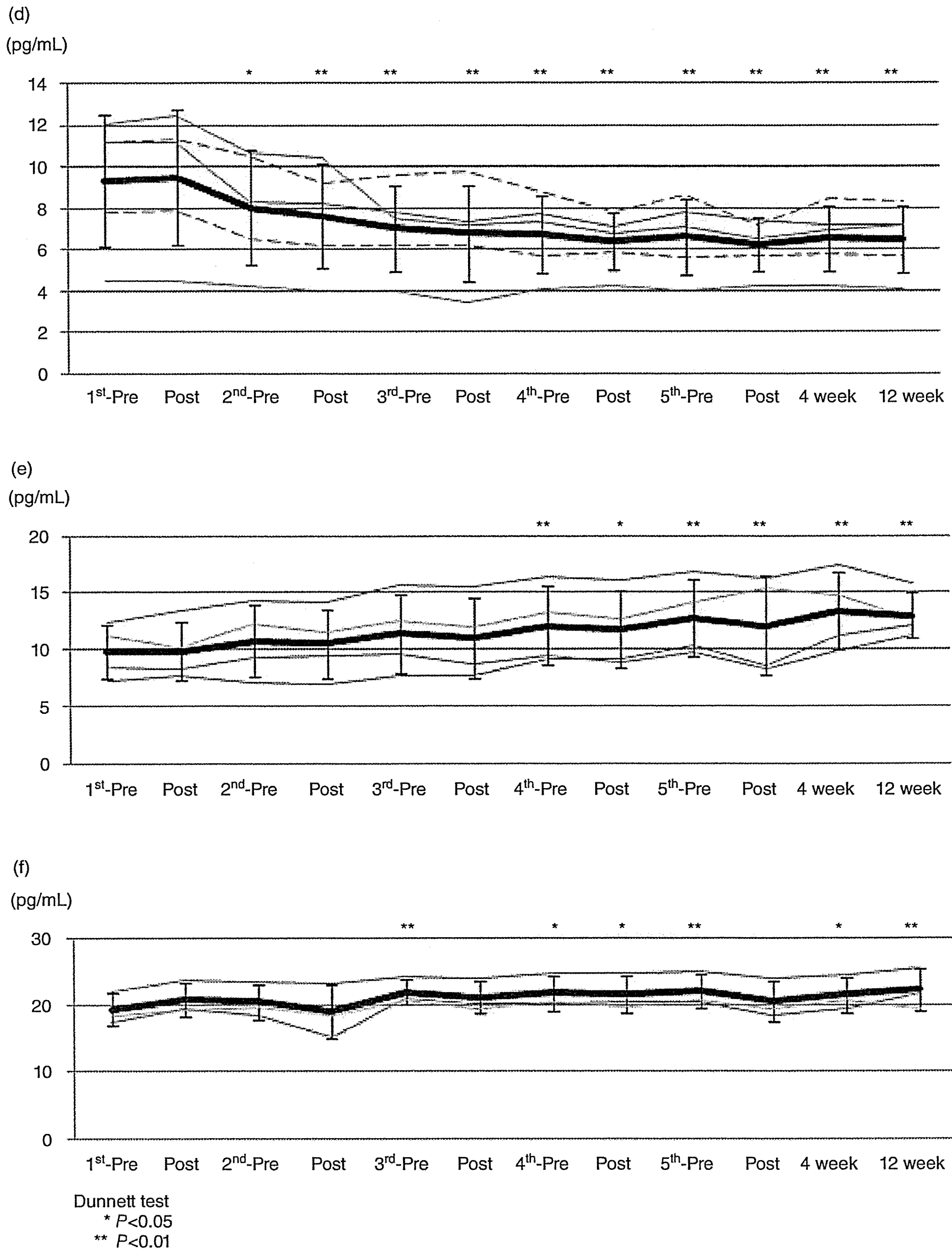

Figure 3 *Continued.*

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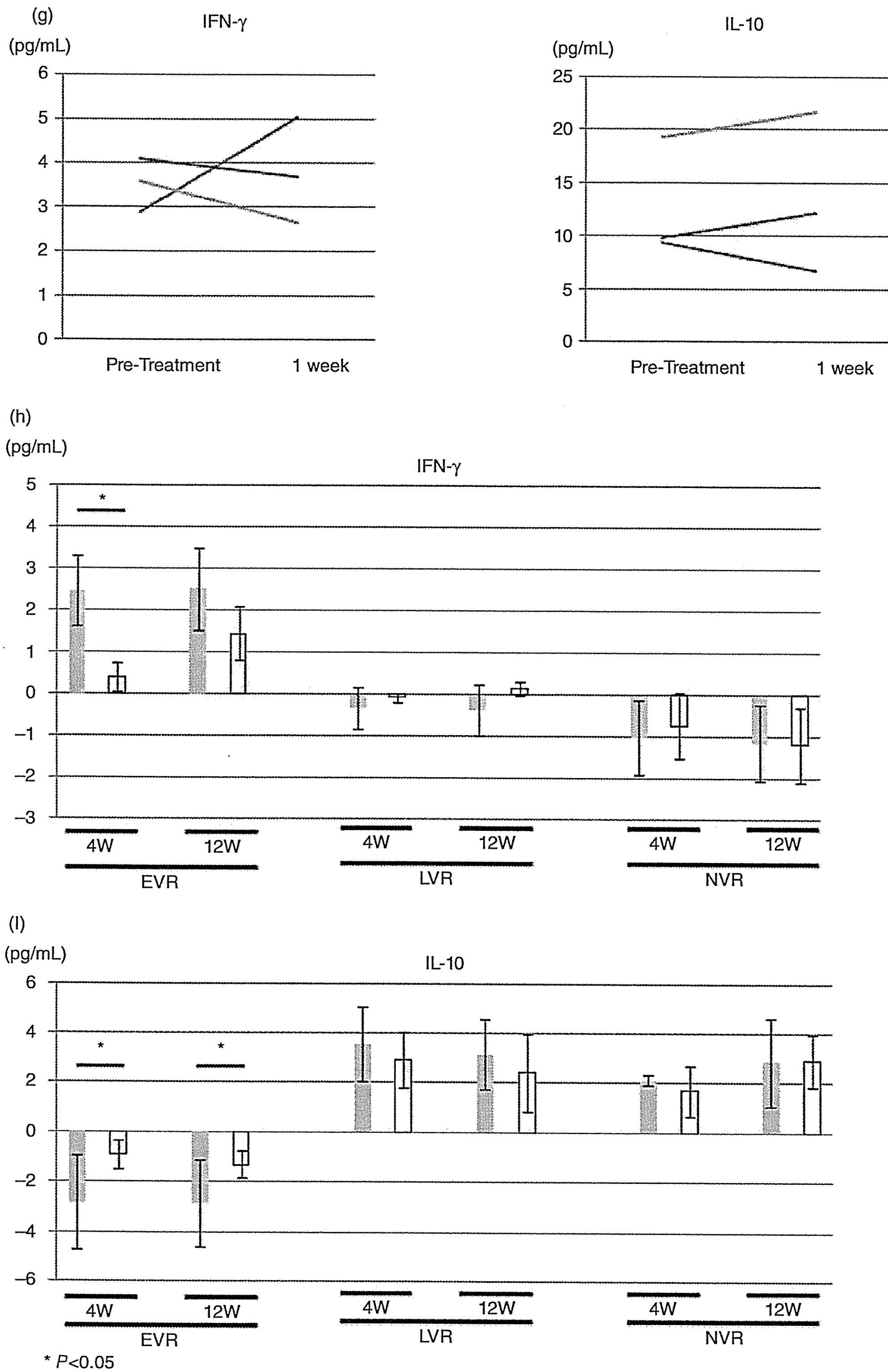

Figure 3 *Continued.*

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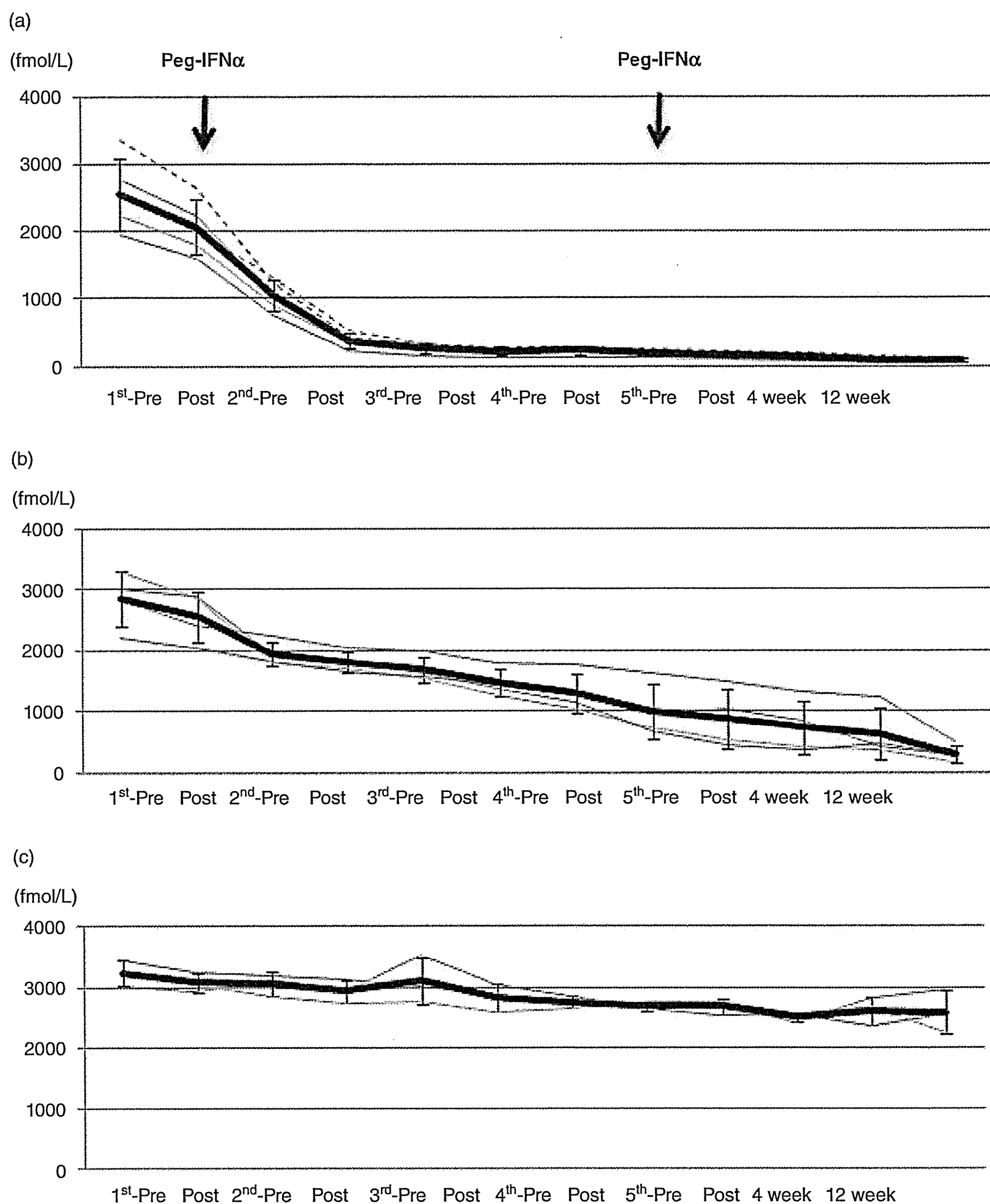

Figure 4 The amount of hepatitis C virus (HCV)-Core antigen in the plasma of peripheral blood and on the surface of $CD3^+$ T cells. The amounts of HCV-Core antigen in the plasma are shown in the line graphs (early virological response [EVR] patients; a, late virological response (LVR) patients; b, null virological response [NVR] patients; (c). The *Y*-axis indicates the amount of HCV-Core antigen (fmol/L). The *X*-axis indicates the time course of double filtration plasmapheresis combined with Peg-interferon and RBV (DFPP/Peg-IFN/RBV) therapy. The patients' numbers are shown on the right side of the line graphs (a, b and c). The representative histograms of HCV-Core staining of EVR patients are shown (d). The numbers in the histograms indicate the percentage of positive staining of HCV-Core antigen. The mean percentage of positive staining at pre-1st DFPP, post-1st DFPP and post 5th DFPP among the EVR, LVR and NVR patients are shown (e). The error bars indicate the standard deviation (SD).

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