virological response (SVR) rates for those infected with the most resistant genotypes (HCV-1a and HCV-1b) still hover around 50% [3, 4].

To surmount this SVR rate with combination therapy, several trials have been undertaken, two of which are: (1) retreatment with combination therapy and (2) double-filtration plasmapheresis (DFPP). By the protocol-defined primary analysis of the former, the SVR rate has been 16% at most, even for a 72-week induction group [5].

The use of DFPP [approved in Japan in April 2008 for the retreatment of chronic hepatitis C (CHC) patients with genotype 1b and high viral loads] together with IFN administration has produced a substantial reduction in the viral load during the early stages of treatment and has effected a high SVR [6], suggesting that this treatment is a new modality for CHC patients in difficult-to-treat states. In this study, we used DFPP plus IFN to enhance the efficacy of the treatment of CHC patients whose HCV was not eradicated by earlier PEG-IFN/RBV combination therapy, and we assessed early viral dynamics associated with SVR.

Patients and Methods

Patients

Nine patients (aged 43–66 years) whose HCV had not been eradicated by earlier PEG-IFNα-2b plus RBV combination therapy carried out between 2008 and 2009 were enrolled in this study. The patients were divided into 2 groups: partial responders (PR; relapse after the end of therapy) and non-responders (NR; no disappearance of HCV RNA during therapy). All the patients were confirmed to be HCV RNA positive with high transaminase levels persisting for 6 months or longer, and with HCV RNA genotype 1b at levels exceeding 10^5 log IU/ml in blood (as determined before the start of therapy by real-time PCR). Also, the patients were negative for hepatitis B surface antigen. Patients with platelet counts of $\leq 10 \times 10^4/\mu l$, leukocyte counts of $\leq 3{,}000/\mu l$, or hemoglobin levels of ≤ 12 g/dl were excluded from the study.

Each patient gave written informed consent and agreed to receive concomitant DFPP, and the study was approved by the review board of the Kobe Asahi Hospital.

DFPP and Blood Collection

Blood collected from the peripheral vein for DFPP by a Plasmaflo™ OP-18W filter (Asahi Kasei Medical, Tokyo, Japan) was separated into plasma and cell components. The virus was then removed from the plasma by a second filter (Cascadeflo™ EC-50W; Asahi Kasei Medical) of an average pore size of 30 nm. For each session, the final volume of treated plasma was 50 ml/kg; the number of sessions was 5 over 2 weeks, and the time of DFPP, based on the reduced plasma fibrinogen levels during DFPP, was decided by the physicians and as required by the patients.

Types of IFN for 4 Weeks with DFPP

During DFPP, the patients were treated with different kinds of IFN: patient 1 with PEG-IFNα-2b plus RBV for 4 weeks; patients 2 and 3 with IFN-β 3 MU twice daily for 2 weeks and PEG-IFNα-2a plus RBV for 2 weeks; patients 4 and 9 with IFN-β 3 MU twice daily for 2 weeks and IFN-β 6 MU daily for 2 weeks; patient 5 with IFN-β 3 MU twice daily for 10 days and IFN-β 6 MU daily for 18 days, and patients 6, 7 and 8 with IFN-β 3 MU twice daily for 4 weeks. The dose of PEG-IFNα-2b was 1.5 μg/kg and 180 μg of α-2a per week. The RBV dose was 800 mg/day with α-2b and 600–800 mg/day with α-2a. After DFPP plus IFN treatment for 4 weeks, all patients were scheduled to receive PEG-IFN/RBV combination therapy (patient 1: PEG-IFNα-2b 1.5 μg/kg per week plus RBV 800 mg/day; patients 2–9: PEG-IFNα-2a 180 μg per week plus RBV 600–800 mg/day).

Amino Acid Substitutions in the Core Region (aa 30 and aa 91) and Number of IFN Sensitivity-Determining Region Mutations

We measured pre-treatment factors such as prediction of clinical outcome of therapy, amino acid sequence variation in the NS5A region (referred to as IFN sensitivity-determining regions) and in the core protein regions (aa 70 and aa 91) of HCV with a given genotype, and the viral load.

HCV RNA Measurement

The quantity of HCV RNA was measured by real-time PCR (detection limit 1.2 log IU/ml), by HCV core antigen (detection limit 20 fmol/l), and by RT-PCR (Amplicor HCV monitor v 2.0; Roche; detection limit 50 IU/ml).

Virus Removal at Second Filter Inlet and Outlet

Plasma was collected twice from the inlet and outlet of the second filter during 1 session of DFPP: once when the treated plasma volume reached half of the target quantity, and once when DFPP was completed. The change in the quantity of HCV RNA was evaluated through the plasma samples collected.

Viral Reduction and Viral Response Rate

The quantity of HCV RNA was converted to a log value at the beginning of the treatment (A) and at each of the virus measurement points (B). Δlog was then calculated: $\Delta\log = \log A - \log B = \log (A/B)$.

Evaluation of DFPP Safety

The subjective and objective adverse events of DFPP were observed, and five clinical factors were measured (platelet and lymphocyte counts, and hemoglobin, albumin and fibrinogen levels) before the first session of DFPP, before successive sessions on the second, third, fourth, fifth and sixth days, and 2 weeks after the last session.

Statistical Analysis

Statistical analysis consisted of analysis of variance for patient background factors, and the paired t test for quantities of HCV RNA at the second filter inlet during DFPP. The t test was used for viral load reductions and Fisher's exact test for viral response rates among the groups. The t test was 2-tailed, and differences of $p < 0.05$ were considered significant.

Table 1. Early viral dynamics with DFPP plus IFN treatment

Case	Age/ sex	Type of IFN for 4 weeks with DFPP	Viral dynamics after DFPP+IFN						Viral dynamics of previous treatment (PEG-IFN/RBV)				Viral mutation		
			before treatment	log drop				unit	before treatment	log drop	unit	outcome	aa 70	aa 91	ISDR
				24 h	1 wk	2 wks	4 wks			4 wks					
1	66/M	PEG-IFNα-2b/RBV 4 wks	6,510	0.5	0.6	0.6	1.1	fmol/l	452	0.7	KIU/ml	NR	wild	wild	0
2	65/F	IFN-β (3 MU 2/day) 2 wks → PEG-IFNα-2a/RBV 2 wks	7.5	0.4	1.3	2.6	1.0	log IU/ml	2,800	ND	KIU/ml	PR	wild	wild	0
3	52/F	IFN-β (3 MU 2/day) 2 wks → PEG-IFNα-2a/RBV 2 wks	5.8	0.4	1.0	1.6	+0.2	log IU/ml	6.3	0.2	log IU/ml	NR	wild	wild	1
4	47/F	IFN-β (3 MU 2/day) 2 wks → IFN-β (6 MU 1/day) 2 wks	6.8	0.6	0.3	0.4	0.4	log IU/ml	2,900	0.3	KIU/ml	NR	mutant	mutant	1
5	52/F	IFN-β (3 MU 2/day) 10 days → IFN-β (6 MU 1/day) 18 days	5.5	1.4	1.5	1.2	1.9	log IU/ml	782	0.6	fmol/l	NR	wild	mutant	1
6	61/F	IFN-β (3 MU 2/day) 4 wks	6.5	1.2	3.4	5.0	4.8	log IU/ml	8,450	2.6	fmol/l	NR	wild	wild	0
7	66/F	IFN-β (3 MU 2/day) 4 wks	5.3	0.0	0.8	1.2	1.3	log IU/ml	11,500	0.8	fmol/l	NR	mutant	wild	1
8	43/F	IFN-β (3 MU 2/day) 4 wks	3,460	0.5	0.2	1.3	2.2	fmol/l	745	0.1	fmol/l	NR	wild	mutant	1
9	43/M	IFN-β (3 MU 2/day) 2 wks → IFN-β (6 MU 1/day) 2 wks	7.2	0.6	1.4	2.5	2.9	log IU/ml	426	0.1	KIU/ml	NR	wild	wild	0

PEG-IFN/RBV: PEG-IFNα-2a (180 μg per week) plus RBV (600–800 mg/day) or PEG-IFNα-2b (1.5 μg/kg per week) plus RBV (800 mg/day). IFN-β: 3 MU twice daily or 6 MU daily. ND = Not done; aa = amino acid; ISDR = interferon sensitivity-determining region.

Results

Of the 9 patients, 1 was PR and 8 were NR. Virus mutation in the core region was as follows: wild type (7 patients) and mutant type (2 patients) at aa 70; wild type (6 patients) and mutant type (3 patients) at aa 91. IFN sensitivity-determining regions demonstrated mutation 1 (5 patients) and mutation 0 (4 patients), while mutation 2 was not seen in any patient. The overall viral dynamics of DFPP plus IFN treatment with or without RBV for 4 weeks showed a reduction in the viral load of ≥1 log in 22% (2 of 9 patients), 55.6% (5/9), 77.8% (7/9) and 77.8% (7/9) at 24 h, 1, 2 and 4 weeks after the start of treatment, respectively. The early viral dynamics after DFPP plus consecutive intravenous IFN-β treatment for 4 weeks showed a reduction in the viral load of ≥1 log in 33% (2 of 6 patients), 50% (3/6), 83.3% (5/6) and 83.3% (5/6) at 24 h, 1, 2 and 4 weeks after the start of treatment, respectively. The reduction of the viral load by ≥2 log was observed in 50% (3 of 6 patients) at 4 weeks after the start of treatment (table 1).

Discussion

New drugs to replace IFN as well as drugs that can be used in combination with IFN are being actively developed. Also, attempts are being made to find ways to physically remove HCV particles from the blood. Granulocyte apheresis, plasma exchange and hemofiltration have been applied to HCV-infected patients for the treatment of cryoglobulinemia and vasculitis, modalities which have been shown to reduce HCV RNA in the blood during treatment [6–11]. The mechanisms of the clinical results of plasmapheresis have been described, whereby HCV in the blood is related to the effects of IFN therapy that could be enhanced by removing the virus from blood [12–14]. Low-density lipoprotein-cholesterol apheresis and plasma exchange in hypercholesteremic patients with HCV infection reduces the quantity of HCV RNA in the blood of some patients [15]. Hemodialysis, hemofiltration and peritoneal dialysis in chronic dialysis patients infected with HCV significantly lower HCV RNA levels in the blood [16]. Combined granulocyte apheresis with IFN therapy for CHC [17–19] and the prerequisite for early reduction of the virus in the treatment of CHC [20, 21] are essential. Thus, the potential effectiveness of IFN therapy combined with early physical removal of the virus is of particular interest.

Asahina et al. [22] studied HCV dynamics in both serum and peripheral blood mononuclear cells in 44 patients, with HCV genotype 1b and high viral loads, randomly assigned to 4 treatment groups: (1) combination therapy with 6 MU daily of IFNα-2b plus 800 mg of RBV; (2) monotherapy with 6 MU daily of IFNα-2b; (3) monotherapy with twice-daily intravenous administration of 3 MU of IFN-β, and (4) monotherapy with daily intravenous administration of 6 MU of IFN-β. HCV RNA levels measured serially by highly sensitive real-time PCR and HCV dynamics in both serum and peripheral blood mononuclear cells have demonstrated a 'biphasic' pattern. The exponential decay slopes of the second phase have been significantly higher in the combination or the twice-daily dose regimen groups than in group 2 or 4 (0.10 ± 0.08 vs. 0.02 ± 0.09 or 0.16 ± 0.09 vs. 0.02 ± 0.04 day^{-1}; $p < 0.05$ and $p < 0.0005$, respectively) [22]. Kim et al. [23] observed that a daily dose of IFN-β 6 MU for 4 weeks effects a 2 log decrease in the HCV RNA load in 7 patients with genotype 1b and high viral loads.

In this study, early viral dynamics were assessed in the 9 patients non-SVR to the combination therapy. The overall viral dynamics of DFPP plus IFN treatment with or without RBV for 4 weeks reduced the viral load by ≥1 log in 22% (2 of 9 patients), 55.6% (5/9), 77.8% (7/9), and 77.8% (7/9) at 24 h, 1, 2 and 4 weeks after the start of treatment, respectively. DFPP plus consecutive intravenous IFN-β treatment for 4 weeks reduced the viral load by ≥1 log in 33% (2/6), 50% (3/6), 83.3% (5/6) and 83.3% (5/6) at 24 h, 1, 2 and 4 weeks after the start of treatment, respectively.

The prerequisite for early virological response (EVR; indicating negative HCV RNA at 12 weeks) has been emphasized in predicting SVR and non-SVR in CHC patients undergoing IFN treatment; those who do not reach EVR fail to respond to further therapy. Treatment discontinued in patients not reaching EVR would reduce drug costs by more than 20%; consequently, early confirmation of viral reduction after initiating antiviral therapy for CHC is highly desirable [24].

To be able to predict SVR with PEG-IFN/RBV treatment, reduction of the HCV RNA viral load by week 4 is considered essential. A 2 log reduction in the HCV RNA viral load by week 4 is a prerequisite to achieving SVR with PEG-IFN/RBV treatment [25]. In our study of DFPP plus consecutive intravenous IFN-β treatment for 4 weeks, a reduction in the viral load of ≥2 log was achieved in 50% (3 of 6 patients) at 4 weeks after the start of treatment.

From the above considerations, DFPP plus consecutive intravenous IFN-β treatment for 4 weeks is a promising regimen for non-SVR patients with genotype 1b and high viral loads, previously treated with PEG-IFN/RBV therapy. Further study is needed to elucidate the SVR rate in a larger number of patients given DFPP plus IFN treatment, especially with consecutive intravenous IFN-β.

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Disclosure Statement

No conflict of interest exists.

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Outcome and Early Viral Dynamics with Viral Mutation in PEG-IFN/RBV Therapy for Chronic Hepatitis in Patients with High Viral Loads of Serum HCV RNA Genotype 1b

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Key Words

Chronic hepatitis · Early viral dynamics · IFN/RBV resistance-determining region · HCV RNA genotype 1b · High viral load · PEG-IFN/RBV combination therapy · Virological response, prediction

Abstract

We investigated whether sustained virological response (SVR) and non-SVR by chronic hepatitis C patients to pegylated interferon plus ribavirin (PEG-IFN/RBV) combination therapy are distinguishable by viral factors such as the IFN/RBV resistance-determining region (IRRDR) and by on-treatment factors through new indices such as the rebound index (RI). The first RI (RI-1st; the viral load at week 1 divided by the viral load at 24 h) and the second RI (RI-2nd; the viral load at week 2 divided by the viral load at 24 h) were calculated. The subject patients were divided into 3 groups based on RI-1st and RI-2nd: an RI-A group (RI-1st ≤1.0), an RI-B group (RI-1st >1.0 and RI-2nd <0.7) and an RI-C group (RI-1st >1.0 and RI-2nd ≥0.7). The SVR rate was 71.4% (10/14) in the RI-A group, 46.2% (6/13) in the RI-B group and 20.0% (3/15) in the RI-C group (p = 0.005 between the RI-A group and the RI-C group). In IRRDR ≥6 and IRRDR ≤5 the SVR rate was 81.3% (13/16) and 23.1% (6/26) (p = 0.0002), respectively. By combining RI and IRRDR as a predicting factor, the SVR rate was 87.5% (7/8) in the RI-A group (≥6 mutations in the IRRDR) and 7.7% (1/13) in the RI-C group (≤5 IRRDR mutations) (p = 0.0003).

Introduction

Recently, global consensus has obtained that a combination of IFN or pegylated IFN plus ribavirin (PEG-IFN/RBV) is the treatment of choice for chronic hepatitis C (CHC). Notwithstanding this treatment regimen, sustained virological response (SVR) rates of those infected with the most resistant genotypes [hepatitis C virus (HCV)-1a and -1b] still hover at ~50% [1, 2]. It is therefore worthwhile to identify the predictive factors that allow the selection of patients who would achieve eradication

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of HCV RNA either before or during therapy, especially since IFN/RBV combination therapy is costly and has several side effects [3].

Predictors of the effectiveness of IFN-based therapy can be classified into pretreatment and on-treatment factors. Pretreatment factors comprise: (1) host factors such as age, gender, obesity, alcohol consumption, hepatic iron overload, fibrosis, immune responses and co-infection with other viruses, and (2) viral factors that mainly include viral genotypes and loads, particular amino acid sequence variations in the NS5A region [4, 5] and in the core protein region of HCV [6] within a given genotype. Moreover, the mean number of mutations in variable region 3 (V3) plus its upstream flanking region of NS5A [amino acid 2334–2379, referred to as IFN/RBV resistance-determining region (IRRDR)] is significantly higher in HCV isolates obtained from patients who later achieve SVR by PEG-IFN/RBV than in those from non-SVR patients. On-treatment factors are mainly related to viral kinetics within the first few weeks of treatment [7].

In the current study, with the aim of investigating whether SVR and non-SVR can be distinguished by viral factors such as IRRDR and by on-treatment factors through new indices such as the rebound index (RI), we calculated the first RI (RI-1st; the viral load at week 1 divided by the viral load at 24 h) and the second RI (RI-2nd; the viral load at week 2 divided by the viral load at 24 h), as proposed by Nomura et al. [8].

Patients and Methods

The 42 patients included in this study, who all demonstrated high viral loads (>100 KIU/ml) of serum HCV RNA of genotype 1b, had been diagnosed with CHC on the basis of abnormal serum alanine aminotransferase persisting for at least 6 months, and of positive HCV RNA assessed by RT-PCR. None of the patients was positive for hepatitis B surface antigen or other liver diseases (autoimmune hepatitis, alcoholic liver disease). All the patients received a regimen of PEG-IFNα-2b (peginterferon alpha-2b; PegIntron; Schering-Plough, Kenilworth, N.J., USA) (1.5 μg/kg/week, subcutaneously) in combination with RBV (ribavirin; Rebetol; Schering-Plough) 600–1,000 mg/day for 48 weeks. RBV was administered at a dose of 600 mg/day (3 capsules) to patients weighing <60 kg, 800 mg/day (4 capsules) to those weighing <80 kg and 1,000 mg/day (5 capsules) to those weighing ≥80 kg.

The efficacy of the combination therapy was evaluated by HCV RNA negativity determined by qualitative RT-PCR analysis at the end of therapy (end of therapy response) and 6 months after the completion of therapy (SVR). The amount of HCV RNA was also measured quantitatively by RT-PCR (Amplicor HCV monitor v. 2.0; Roche) before therapy. The lower detection limit of the assay was 5 KIU/ml. Samples collected during and after therapy were also determined by qualitative RT-PCR (Amplicor; Roche), which has a higher sensitivity than quantitative analysis, and the results were labeled as positive or negative. The lower limit of the assay was 50 IU/ml.

SVR was defined as undetectable serum HCV RNA at 24 weeks after the cessation of treatment, and non-SVR as detectable HCV RNA at 24 weeks after the discontinuation of treatment. Informed consent was obtained from all patients enrolled in the study after thoroughly explaining the aims, risks and benefits of the therapy.

The amount of HCV core antigen was assessed by the IRM assay (Ortho Clinical Diagnostics, Tokyo, Japan), which provides a good correlation between the amount of HCV core antigen and the amount of HCV RNA, as shown in our previous study [9]. The HCV core antigen was measured on days 0, 1 (24 h), 7 (1 week) and 14 (2 weeks) according to the detection limit of 20 fmol/l established by the manufacturer.

RI-1st was defined as the coefficient derived by dividing the viral load of HCV core antigen at week 1 by that at 24 h, and RI-2nd was defined as the coefficient derived by dividing the viral load at week 2 by that at 24 h [8].

The patients were divided into 3 groups based on RI-1st and RI-2nd: group A (RI-1st ≤1.0), group B (RI-1st >1.0 and RI-2nd <0.7) and group C (RI-1st >1.0 and RI-2nd ≥0.7).

NS5A sequence analysis (IRRDR) was performed as described [4]. Briefly, the sequences of the amplified fragments were determined by direct sequencing without subcloning with the use of a Big Dye Deoxy Terminator cycle sequencing kit and an ABI 337 DNA sequencer (Applied Biosystems, Japan). The aa sequences were deduced and aligned with Genetyx Win software v. 7.0 (Genetyx Corp., Tokyo, Japan). Numbering of aa throughout the manuscript is according to the polyprotein of HCV genotype 1b prototype HCV-J.

Statistical Analysis

Differences between the groups were assessed by the χ^2 test, Fisher's exact test or Student's t test, the Mann-Whitney test and the Kruskal-Wallis test. $p < 0.05$ was considered statistically significant.

Results

Of the 42 patients treated with combination therapy, 19 (45.2%) achieved SVR and 23 (54.8%) were still HCV RNA positive (non-SVR) 6 months after therapy. No significant differences were observed in patient characteristics between SVR and non-SVR, except in platelet counts and the degree of fibrosis (table 1), or among the RI-A, -B and -C groups (table 2).

The SVR rate was 71.4% (10/14), 46.2% (6/13) and 20.0% (3/15) in the RI-A, -B and -C groups, respectively, with a significant difference between the RI-A and -C groups ($p = 0.005$), but not significant between the RI-A and -B groups and the RI-B and -C groups (fig. 1). In the 14 patients of the RI-A group, HCV RNA turned negative

Table 1. Host-dependent, virus-related profile by response (SVR and non-SVR)

	SVR	Non-SVR	p value
Gender (M/F), n	11/8	13/10	NS
Age, years	56.7 ± 8.8	59.3 ± 10.5	NS
HCV RNA level, KIU/ml	1,685 ± 1,477	1,660 ± 1,363	NS
HCV core antigen, fmol/l	7,044 ± 6,763	9,343 ± 12,563	NS
Body weight, kg	59.9 ± 11.5	59.8 ± 13.6	NS
Treatment history (retreatment/naïve)	6/13	13/10	NS
Platelet count ($\times 10^4/mm^3$)	18.7 ± 4.4	14.8 ± 5.4	NS
F0, 1/F2, 3	12/2	5/10	0.004

Table 2. Host-dependent, virus-related profile by response (RI-A, -B and -C groups)

	RI-A	RI-B	RI-C	p value
Gender (M/F), n	7/7	9/4	8/7	NS
Age, years	60.0 ± 5.9	58.5 ± 9.4	56.1 ± 12.8	NS
HCV RNA level, KIU/ml	1,401 ± 1,014	2,053 ± 1,286	1,593 ± 1,772	NS
HCV core antigen, fmol/l	6,084 ± 5,106	7,674 ± 5,038	11,000 ± 15,837	NS
Body weight, kg	62.1 ± 16.6	59.5 ± 10.4	58.2 ± 10.1	NS
Treatment history (retreatment/naïve)	3/11	7/6	9/6	NS
Platelet count ($\times 10^4/mm^3$)	15.3 ± 3.5	18.3 ± 5.9	16.3 ± 6.0	NS
F0, 1/F2, 3	7/3	5/4	5/5	0.004

Table 3. SVR rate between IRRDR ≤5 and IRRDR ≤6 in RI-A, -B and -C groups

	RI-A		RI-B		RI-C	
	IRRDR ≤5	IRRDR ≥6	IRRDR ≤5	IRRDR ≥6	IRRDR ≤5	IRRDR ≥6
SVR	3	7	2	4	1	2
Non-SVR	3	1	5	2	12	0
SVR rate, %	50.0	87.5	28.6	66.7	7.7	100
p value	NS		NS		0.0024	
			0.0003			

by week 4 in 3 patients, week 8 in 5 patients, week 12 in 5 patients and was positive in 1 patient throughout the treatment. In the 13 patients of the RI-B group, HCV RNA was negative by week 4 in 1 patient, week 8 in 2 patients, week 12 in 4 patients, at and after week 16 in 5 patients and remained positive throughout the treatment in 1 patient. In the 15 patients of the RI-C group, HCV RNA was negative by week 12 in 1 patient, on and after week 16 in 6 patients and remained positive throughout the treatment in 8 patients (fig. 2).

The SVR rate was 81.3% (13/16) in the group with ≥6 mutations in IRRDR, and 23.1% (6/26) in those with ≤5 (fig. 3), with a significant difference between the 2 groups (p = 0.0002).

By combining RI and IRRDR, the SVR rate was 87.5% (7/8) in the RI-A group (IRRDR ≥6) and 7.7% (1/13) in the RI-C group (IRRDR ≤5) (table 3), with a significant difference between the 2 groups (p = 0.0003).

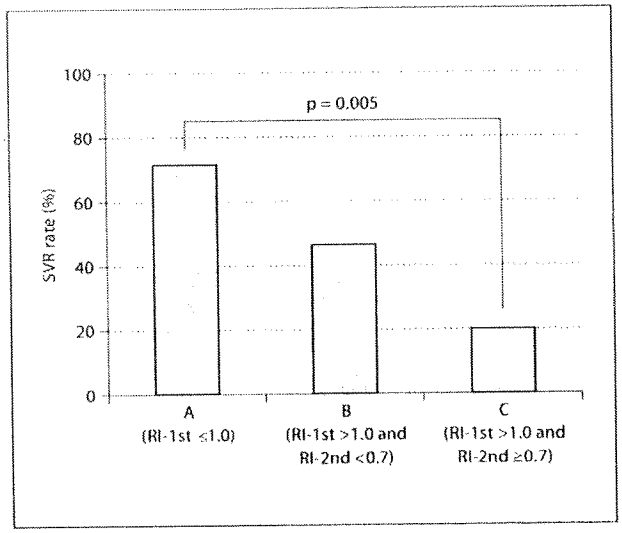

Fig. 1. SVR rate in RI-A, -B and -C groups. The overall SVR rate was 71.4, 46.2 and 20.0%, respectively. Significant difference in SVR rate is indicated.

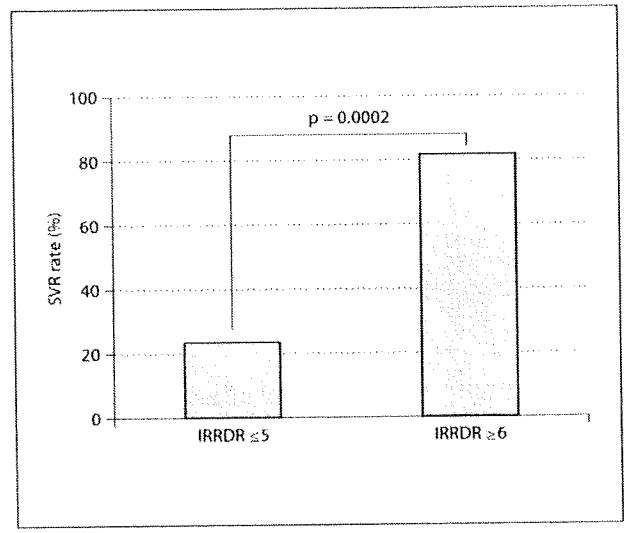

Fig. 3. SVR rate and IRRDR number. The SVR rate was 23.1% in IRRDR ≤5 and 81.3% in IRRDR ≥6, which was significantly different.

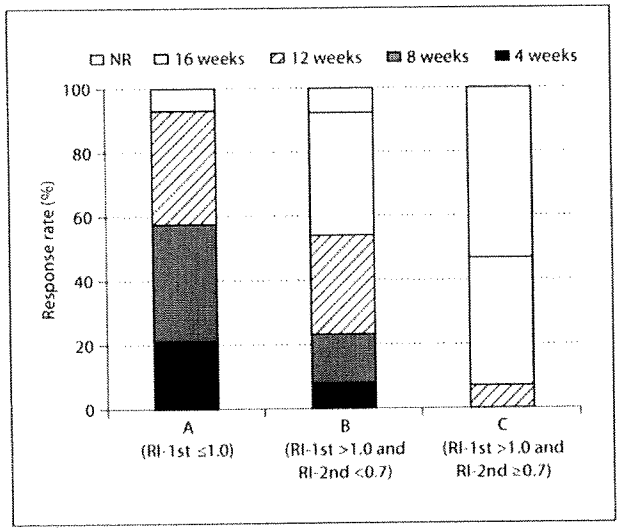

Fig. 2. Relation between response time and virus dynamics. In the 14 patients of the RI-A group, HCV RNA turned negative by week 4 in 3 patients, week 8 in 5 patients, week 12 in 5 patients and remained positive throughout the treatment in 1 patient. In the 13 patients of the RI-B group, HCV RNA was negative by week 4 in 1 patient, week 8 in 2 patients, week 12 in 4 patients, at and after week 16 in 5 patients and remained positive throughout the treatment in 1 patient. In the 15 patients of the RI-C group, HCV RNA was negative by week 12 in 1 patient, at and after week 16 in 6 patients and remained positive throughout the treatment in 8 patients.

Discussion

The importance of early virological response (EVR; signifying HCV RNA negative at 12 weeks) has been emphasized in predicting SVR and non-SVR in CHC patients undergoing IFN treatment; those not reaching EVR do not respond to further therapy. Discontinuation of treatment in patients not reaching EVR would reduce drug costs by more than 20%; consequently, early confirmation of viral reduction after initiating antiviral therapy for CHC is worth investigating [10].

Treatment with IFN induces a decline in HCV RNA levels that can be mathematically measured in 2 phases. The decline in the first phase, usually measured at 24 or 48 h, probably reflects direct inhibition of intracellular production and release of HCV [11], with IFN efficacy ranging from about 70% (approx. 0.7 log units) for standard IFN (given 3 times a week) to more than 90% (1 log unit) for high daily doses of standard IFN or PEG-IFN (given once a week) [12, 13]. The decline in the second phase, beginning after 24–48 h, is slower and more variable than that in the first phase, and is thought to reflect continued inhibition of replication and the gradual elimination of virus-infected cells [11]. The decay in the first phase has little correlation with the IFN dose, but is more rapid with PEG-IFN than with standard IFN preparations [10].

Lowering HCV RNA during the first phase is essential for efficient elimination of HCV during the second phase. Decreases in HCV RNA titers within the first 24–48 h after the start of IFN would, therefore, be a dependable estimate of antiviral efficacy [12, 13].

Early viral kinetics, determined up to week 2, are believed to express the therapeutic effect of PEG-IFN. The concentration of PEG-IFNα-2b in serum peaks after 24 h, then declines gradually [14, 15]. The viral load is thus reduced by 24 h but increases in week 1 [16, 17]; with a large dose of PEG-IFN at each administration, it decreases markedly at 24 h but then increases in week 1 regardless of the dose. In the responder group, however, the viral load continues to decline each week thereafter [17].

In this study, we used new indices proposed by Nomura et al. [8]: RI-1st and RI-2nd calculated from early viral kinetics. RI-1st was defined as the coefficient derived by dividing the viral load of HCV core antigen at week 1 by that at 24 h, and the RI-2nd was defined as the coefficient derived by dividing the viral load at week 2 by that at 24 h. In the SVR group, a number of patients demonstrated no increase in the viral load at week 1. Patients with a high RI-2nd were regarded as poor responders or non-responders to PEG-IFN. The RI-2nd of those other than non-responders was below 0.7; therefore, 0.7 was adopted as the reference value for RI-2nd, and the patients were divided into 3 groups based on RI-1st and RI-2nd: the RI-A group (RI-1st $\leq$1.0), the RI-B group (RI-1st >1.0 and RI-2nd <0.7) and the RI-C group (RI-1st >1.0 and RI-2nd $\geq$0.7). The SVR rate of the RI-A, RI-B and RI-C groups was 71.4% (10/14), 46.2% (6/13) and 20% (2/10), respectively ($p = 0.005$ between the RI-A group and the RI-C group). RIs are also associated with the early clearance of HCV RNA that is related to SVR.

In the RI-A group 21.4% (3/14), 35.7% (5/14) and 35.7% (5/14) became HCV RNA negative by weeks 4, 8 and 12, respectively. In the RI-B group 7.7% (1/13), 15.4% (2/13), 30.8% (4/13) and 38.5% (5/13) became HCV RNA negative by weeks 4, 8, 12, and at and after week 16, respectively. In the RI-C group 6.7% (1/15) and 40.0% (6/15) became HCV RNA negative by week 12, and at and after week 16, respectively. It is believed that the simplified RI-1st and RI-2nd are evidential indices for determining the therapeutic efficacy of PEG-IFN/RBV treatment.

We have previously reported that the high degree of sequence variation in IRRDR (IRRDR $\geq$6) significantly correlates with SVR, whereas the low degree of sequence variation in this region (IRRDR $\leq$5) correlates with non-SVR [4]. A significant correlation between the rapid reduction of HCV core antigen titers and the degree of sequence variation in IRRDR has been observed. This, in particular, suggests a possible influence of IRRDR $\geq$6 on HCV replication kinetics during IFN-based therapy, especially that the direct effect of IFN begins a few hours after the first dose.

In this study, the SVR rate was 81.2% (13/16) with IRRDR $\geq$6 and 23.1% (6/26) with IRRDR $\leq$5 ($p = 0.0002$), strongly suggesting that IRRDR $\geq$6 would be a useful marker for the prediction of SVR.

By combining RI and IRRDR as a predicting factor, the SVR rate was 87.5% (7/8) in the RI-A group (RI-1st $\leq$1.0) with IRRDR $\geq$6, signifying that about 90% of these patients turned SVR and were, thus, believed to be very good responders. An SVR rate of 7.7% (1/13) was obtained in the RI-C group with IRRDR $\leq$5 ($p = 0.0003$).

In conclusion, we propose that IRRDR combined with RIs is the most sensitive predictive factor for SVR and non-SVR. With the aid of RIs and IRRDR, a more effective PEG-IFN/RBV treatment could be within reach. A more detailed investigation with a larger number of subjects is needed to confirm the current results in patients given PEG-IFN/RBV combination therapy.

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Disclosure Statement

No conflict of interest exists.

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Analysis of Neutralizing Antibodies against Hepatitis C Virus in Patients Who Were Treated with Pegylated-Interferon *plus* Ribavirin

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RUNNING TITLE: ANTI-HCV NEUTRALIZING ANTIBODIES IN SERA OF HCV-INFECTED PATIENTS

Key words: hepatitis C virus, neutralizing antibody, pegylated-interferon *plus* ribavirin, treatment outcome

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ABSTRACT

The role of neutralizing antibodies (NAb) in determining responses to antiviral therapy has not been defined well. By using hepatitis C virus (HCV) cell culture system with the J6/JFH1 strain of HCV genotype 2a, we analyzed NAb responses in patients with chronic hepatitis C who received pegylated-interferon *plus* ribavirin (PEG-IFN/RBV) antiviral therapy. A total of 65 patients chronically infected with HCV genotype 1b were enrolled in this study. Of all the 65 patients, 34 (52%) patients achieved early virological response (EVR), with the remaining 31 patients (48%) being Non-EVR. Twenty-seven patients (42%) achieved sustained virological response (SVR), with the remaining 38 patients (58%) being Non-SVR. Thus, NAb titers were significantly higher in sera of patients who achieved EVR and SVR than those of Non-EVR and Non-SVR, respectively. Rather unexpectedly, NAb titers did not significantly decrease when measured even one year after disappearance of HCV RNA. On the other hand, when change ratios of NAb titers before and after disappearance of HCV RNA were compared between patients with different treatment outcomes, we noticed that the change ratio of NAb_{50} titers of patients who achieved an EVR was significantly lower than that of Non-SVR. In conclusion, our present results suggest that NAb titers were significantly associated with clinical responses to PEG-IFN/RBV therapy.

INTRODUCTION

Hepatitis C virus (HCV), an enveloped, positive-stranded RNA virus, is a member of the Genus *Hepacivirus*, the Family *Flaviviridae*. The life cycle of the virus, including viral attachment and entry to the cells, genome replication, protein synthesis and virion assembly, has recently been studied using an HCV cell culture system (12). An estimated 170 million individuals are infected with HCV worldwide. The current standard therapy is based on a combination of pegylated-interferon *plus* ribavirin (PEG-IFN/RBV) and, with this treatment regimen, viral eradication can be achieved in around 50% of the patients infected with HCV-1b.

Both viral and host factors play important roles in the control of viral infection. Whereas viral factors help to adjust the cellular environment to support viral replication, host factors generally function to combat the viral invasion either by actively blocking the virus replication through innate and/or acquired immune responses or by having the infected cells die out by themselves through apoptosis so that the virus can no longer replicate in the infected cells.

Acquired immune responses of the host involve cell-mediated immunity and humoral immunity. The importance of cellular immunity in combating HCV infection has been well documented (4, 14). On the other hand, humoral immune responses in protection against and/or recovery from HCV infection may be of less importance. Nevertheless, it has been reported that the neutralizing antibody (NAb) responses play an important role in the prevention of infection and in limiting viremia (10, 13, 16). Indeed, patients chronically infected with HCV were reported to possess relatively high titers of cross-reactive NAb (1). It is reported that patients with chronic hepatitis C infection also have high NAb titers to envelope protein of HCV-like particles (HCV-LPs) (2). Humoral and cellular immune responses are also important in determining response to antiviral therapy with IFN/RBV (7). We previously reported that the degree of antibody responses to the NS5A protein of HCV was correlated with early virological response after the initiation of PEG-IFN/RBV therapy (8). However, the role for NAb in determining responses to PEG-IFN/RBV antiviral therapy has not been well documented.

In the present study, we have established an experimental system to measure NAb titers using hepatitis C virus cell culture (HCVcc) model, and measured NAb titers in patients with chronic hepatitis C who were treated with PEG-IFN/RBV. Our data revealed that good treatment outcome was associated with higher NAb titers in patients chronically infected with HCV-1b.

MATERIALS AND METHODS

Cells

Huh-7.5 cells (3), a kind gift from Dr. C. M. Rice (Rockefeller University, New York, NY, USA), were propagated in Dulbecco's modified Eagle's medium (DMEM; Wako) supplemented with 10% fetal bovine serum (FBS; Biowest), 0.1 mM nonessential amino acids (Invitrogen), 100 IU penicillin per ml and 100 μg streptomycin per ml (Invitrogen). Cells were grown at 37°C in a CO_2 incubator.

Virus

The J6/JFH1 strain of HCV (11) was a kindly gift from Dr. C. M. Rice. Virus stocks were produced in Huh-7.5 cells, and the viral titers were determined by focus forming units (FFU) assay in Huh-7.5 cells, as described previously (5). The viral stocks were kept at -80°C until ready for use.

Patients and serum samples

Patients chronically infected with HCV-1b, who were treated with pegylated interferon α-2b (1.5 μg per kilogram body weight, once weekly, subcutaneously) and ribavirin (600-800 mg daily, per os), were described previously (8, 9). Sera were collected and stored at -80°C until ready for use. The sera were inactivated at 56°C for 30 min before being used for the virus neutralization test, as describe below.

HCV focus reduction neutralization assay

An HCV focus reduction neutralization assay was performed, as described elsewhere (Sasayama et al., in preparation). Briefly, one-tenth volume of serum obtained from uninfected healthy human, which had been inactivated at 56°C for 30 min, was mixed with HCV solutions at 37°C for 1 h to avoid nonspecific inhibition. Serial 3-fold dilutions of each serum sample were mixed with pre-treated HCV solution containing 10^4 FFU. After incubation at 37°C for 1 h, the mixtures were inoculated to naïve Huh-7.5 cells (2 x 10^5 cells per well in 24-well plates) and incubated in a 5% CO_2 incubator. After 3 h of virus adsorption, the inocula were removed and fresh complete DMEM were added to the cells. At 24 h postinfection, cells were washed with PBS, fixed with 100% methanol, blocked with 5% goat serum in PBS and subjected to immunofluorescence analysis using mouse monoclonal antibody against HCV core antigen (clone 2H9; a kind gift from Dr. T. Wakita, Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan) (15) and Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) (Invitrogen). The immunostained cells were washed with PBS, counterstained with Hoechst 33342 solution (Invitrogen) at room temperature for 5 min, mounted on glass slides, and observed under a fluorescence microscope (BZ-9000; Keyence). The number of HCV-infected foci in each well was counted by a software BZ-H1C (Keyence). The dilution that neutralized 50% of the initial virus infectivity was calculated by curvilinear regression analysis. Each neutralization titer was determined as the logarithmic value of the reciprocal antibody dilution that reduced 50% of viral foci to the total number. Titers were expressed as logarithmic values and means ± standard deviation (SD) were calculated.

Statistical analysis.

The statistical significance of comparisons between the two groups of patients was determined using Student's *t*-test. In all tests, a *p*-value lower than 0.05 was considered statistically significant.

RESULTS

Virological response of the patients underwent treatment with PEG-IFN/RBV

In this study, 65 patients infected with HCV-1b were enrolled. During and after PEG-IFN/RBV therapy, their clinical responses to the treatment were evaluated (Table 1.). Of all the 65 patients, 34 (52%) patients achieved early virological response (EVR) by week 12, with the remaining 31 patients (48%) being Non-EVR. Twenty-seven patients (42%) achieved sustained virological response (SVR), with the remaining 38 patients (58%) being Non-SVR, which is divided into 2 categories, complete non-response (CNR) and relapse as described previously (14). CNR was observed with 16 (25%) of the 65 patients and relapse was observed with 22 patients (34%).

Table. 1. Proportion of various virological responses of patients treated with PEG-IFN/RBV.

Virological response	Proportion
EVR	52% (34 / 65)*
Non-EVR	48% (31 / 65)
SVR	42% (27 / 65)
Non-SVR	58% (38 / 65)
CNR	25% (16 / 65)
Relapse	34% (22 / 65)

* Number of patients/Number of total.

Pre-treatment NAb titers in the serum and PEG-IFN/RBV treatment outcome

To study the possible impact of NAb on PEG-IFN/RBV treatment outcome of patients infected with HCV-1b, we measured NAb titers in patients sera using the J6/JFH1 strain of HCV. The mean dilution of antibodies required for 50% neutralization (NAb_{50} titer) of HCV J6/JFH1 in patients who achieved an EVR (2.28±0.36) was significantly higher than that of non-EVR (1.97±0.38) ($p<0.01$) (Fig. 1). Also, the NAb_{50} titers in patients who achieved an SVR (2.27±0.40) was significantly higher than that of non-SVR (2.04±0.37) ($p<0.05$) or CNR (1.91±0.43) ($p<0.05$) (Fig. 1). These data suggest that NAb_{50} titers in the pre-treatment sera are associated with treatment outcome.

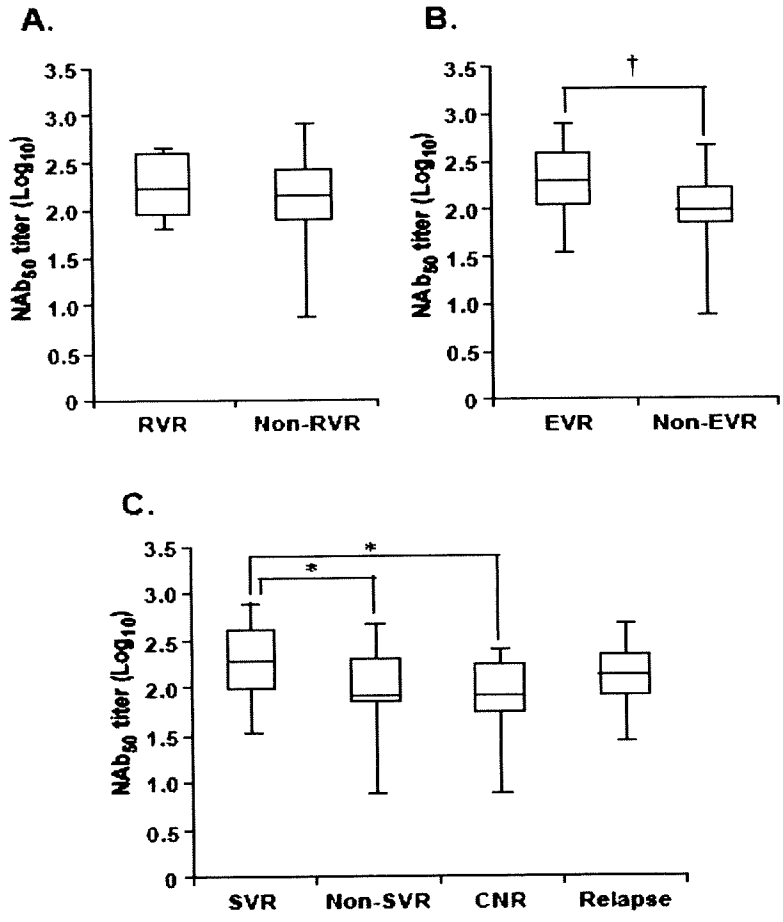

Fig. 1. NAb_{50} titers in the sera of HCV-infected patients. NAb_{50} titers in the pre-treatment sera of HCV-1b-infected patients were compared between RVR and Non-RVR (A), EVR and Non-EVR (B), SVR and Non-SVR (C), SVR and CNR (C), and SVR and Relapsers (C). *, $P<0.05$; †, 0.01.

Fate and change ratios of NAb titers before and after disappearance of HCV RNA in the serum of patients treated with PEG-IFN/RBV

In order to investigate as to whether or not the NAb titers decrease after disappearance of HCV RNA in the serum of patients treated with PEG-IFN/RBV, we measured NAb titers in patients' sera collected at both pre-treatment (before disappearance) and after disappearance of HCV RNA in the serum. Rather unexpectedly, NAb titers did not decrease when measured even one year after disappearance of HCV RNA. On the other hand, when change ratios of NAb titers before and after disappearance of HCV RNA were compared between patients with different treatment outcomes, we noticed that the change ratio of NAb_{50} titers of patients who achieved an EVR was significantly lower than that of Non-EVR (Fig. 2).

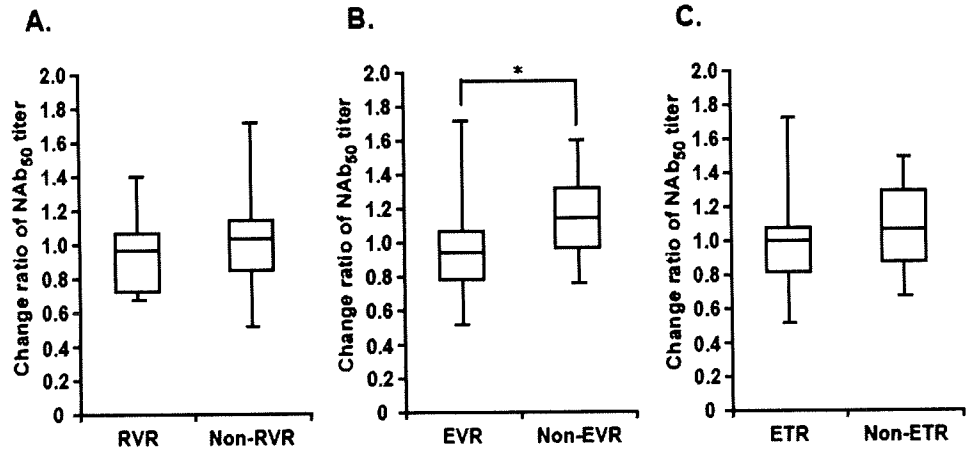

Fig. 2. Change of NAb ratios and before after

disappearance of HCV RNA in the serum. Change ratios (post-treatment/pre-treatment) in the NAb_{50} titers before and after disappearance of HCV RNA in the sera of patients who were treated with PEG-IFN/RBV were compared between RVR and Non-RVR (A), EVR and Non-EVR (B), and ETR and Non-ETR (C). *, $P<0.05$.

DISCUSSION

We previously reported that anti-NS5A antibodies were more frequently detected in sera of patients who achieved EVR compared to Non-EVR (8). In this study, we demonstrated that NAb_{50} titers in the pre-treatment patients' sera were associated with the good responses (EVR and SVR) to PEG-IFN/RBV combination therapy (Fig. 1). Consistent with our observations, it was reported that NAb titers to HCV-LPs were higher in patients who achieved an SVR with IFN/RBV therapy than in relapsers and non-responders (2). The better humoral responses, such as NAb and anti-NS5A antibodies, might be associated with better cell-mediated immune responses, which involve CD4+ and CD8+ T cells. It is well known that peripheral and intrahepatic CD8+ T cell responses, which play an important role in the control of and recovery from HCV infection, are also important in determining an SVR in response to PEG-IFN/RBV treatment (6). Thus our present results imply the possibility that individuals who can maintain harmonized good immune responses are able to achieve good responses to PEG-IFN/RBV therapy, such as EVR and SVR.

We also found that NAb_{50} titers did not decrease significantly when measured even one year after disappearance of HCV RNA in the serum (Fig. 2).

Another important finding in this study is that the NAb in patients infected with HCV-1b significantly cross-reacts to HCV-2a; we observed that average NAb titers of HCV-2a-infected patients were ca. 3 times higher than those of HCV-1b-infected patients when measured with the same experimental system using the J6/JFH1 strain of HCV-2a (439±2.72 vs. 139±2.48; $P<0.0001$). This information would be helpful when considering immunological prophylaxis against HCV infection, either active or passive immunizations using vaccines and NAb.

In conclusion, NAb_{50} titers were significantly higher in sera of patients who achieved EVR and SVR than those of Non-EVR and Non-SVR, respectively. Also, NAb_{50} titers declined only slightly during the course of one year after disappearance of HCV RNA in the sera.

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ORIGINAL ARTICLE

Characterization of anti-idiotypic antibodies mimicking antibody- and receptor-binding sites on hepatitis A virus

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Abstract Two anti-idiotypic monoclonal antibodies (mAb2s; named 94-2 and 94-7), were generated from a BALB/c mouse immunized with human monoclonal anti-hepatitis A virus (HAV) neutralizing antibody KF94. We characterized the properties of the mAb2s and determined interactions between mAb2s, KF94 and HAV using enzyme-linked immunosorbent assay, immunofluorescence assay and HAV infectivity assay. Inactivated HAV inhibited mAb2 binding to KF94, indicating that the mAb2s mimicked the HAV neutralization site that was complementary to the paratope of KF94. MAb2 94-7 competed with an anti-HAV cellular receptor antibody for binding to HAV-susceptible cells and partially blocked virus infection. We speculated that mAb2 94-7 mimicked a portion of the HAV receptor-binding site. The ability to generate mAb2 implies that HAV receptor-binding sites are exposed on the surface of HAV, permitting antibody access.

Introduction

Hepatitis A virus (HAV) is an epidemiologically important pathogen that causes acute hepatitis in humans [12]. It is a positive-strand RNA virus belonging to the family *Picornaviridae*, genus *Hepatovirus* [16].

Picornavirus particles contain 60 protomers arranged as 12 pentamers. Each protomer is composed of four capsid proteins, viral protein (VP) 1, VP2, VP3 and VP4. The HAV particle also contains 60 protomers, made up of the same capsid proteins, but the VP4 of HAV is truncated and smaller than the VP4 proteins found in other picornaviruses [5].

Unlike other picornaviruses [3], the antigenic structure of HAV has not been completely characterized. Its unique features, the difficulty of obtaining a high virus yield in tissue culture and the strict conformational dependence of its own neutralizing antigenic structure have hampered investigations of HAV morphology. Generating neutralizing antibodies in response to individual structural proteins, synthetic peptides or expressed polypeptides is reportedly difficult [7, 10, 15]. Fragmental structural proteins cannot maintain the function of the neutralizing site. Studies using escape mutants have generated information about the antigenic structure of HAV [17, 19] that supports the notion of an immunodominant neutralizing site involving residues of VP1 and VP3, and that a second, potentially independent site involves residue 221 of VP1.

We generated anti-idiotypic antibodies (anti-Id) by immunization with an HAV-specific human neutralizing monoclonal antibody to investigate the antigenic structure of HAV. Immunization with an antibody (Ab1) can induce anti-antibody antibodies (Ab2s), and those specific for idiotopes of Ab1 are considered anti-Ids. Idiotopes are confined to the variable region of immunoglobulin molecules, and they are crucial for antigen recognition [21]. Among the four known types of anti-Ids (Ab2α, Ab2β, Ab2γ and Ab2ε) [2], Ab2β binds directly to the

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idiotope within the paratope of Ab1. Ab2β has the potential to mimic the structure of the antigenic epitope complementary to the Ab1 paratope and it has an antigen-like function. Several reports have used anti-Ids to identify viral/bacterial antigenic structure or cellular receptors [1, 9].

Here, we describe the properties of our anti-Ids, designated mAb2 94-2 and 94-7, which mimic the HAV surface structure and allowed unique insight into the antigenic structure of HAV.

Materials and methods

Cell line and viruses

The GL37 line was derived from African green monkey kidney cells and established to support the optimal growth of HAV [20]. GL37 cells were cultured in Eagle's minimal essential medium (Nissui, Tokyo, Japan) supplemented with 50 μg/ml of gentamicin (Biological Industries, Kibbutz Beit Haemek, Israel) and 10% fetal bovine serum (FBS, GIBCO, Invitrogen Corporation, Auckland, NZ).

Human tissue-culture-adapted hepatitis A virus strains KRM003 (genotype IIIB), KRM031 (genotype IA) and TKM005 (genotype IB) were isolated from patients with hepatitis A [18] and propagated in GL37 cells. Each strain was purified from cell extracts by differential centrifugation, chloroform extraction, RNase, DNase and protein K digestion, extraction with a mixture of 2-ethoxyethanol and 2-buthoxyethanol, and gel filtration. After sucrose gradient centrifugation, the fraction containing intact particles was selected.

The HAV strain KRM003 was propagated and purified as described above. Purified virus was inactivated with 0.025% formalin at 37°C for 12 days and then diluted with 10 mM Tris–HCl, 2 mM EDTA, 100 mM NaCl, 0.02% Tween 80, 0.1% NP40, 0.2% BSA, 0.03% NaN_3 to 3 mg/ml and stored at 4°C.

Preparation of anti-HAV antibodies

Syngeneic anti-HAV human monoclonal antibodies designated KF6 and KF94 provided by Dr. Yasushi Kuwahara, Denka Seiken Co. Ltd. [4] corresponded to Ab1s derived from the blood of a convalescent patient with hepatitis A who was infected with strain IIIB. Here, the parental antibody was KF94, which cross-reacted with a mouse monoclonal antibody that recognized the HAV immunodominant site (data not shown).

Anti-HAV rabbit hyperimmune serum was prepared by immunization with the inactivated HAV.

Generation of mouse monoclonal anti-KF94 antibodies (mAb2s)

Hybridoma clones were selected from the fusion products of SP2/0 myeloma (Riken Cell Bank, Tsukuba, Japan) and spleen cells of BALB/c mice immunized with KF94.

The affinity of culture fluid from each hybridoma clone to KF94 was tested using an enzyme-linked immunosorbent assay (ELISA) (Table 1). Polystyrene microtiter plates (F96 CERT. MAXISORP, Nunc Laboratories, Roskilde, Denmark) were coated with 50 μl of appropriately diluted KF94, or with anti-HAV-negative human serum obtained from a healthy individual. Sera or specimens derived from human sources were collected after obtaining informed consent from the donors.

Nonspecific binding in the wells was blocked with bovine serum albumin. Culture fluid (50 μl) was applied to plates coated with either KF94 or anti-HAV-negative human serum. Bound antibody was detected by incubation with a horseradish-peroxidase-conjugated (HRPO) anti-mouse IgG (MBL, Nagoya, Japan) followed by *o*-phenylenediamine (Tokyo Chemical Industry Co, Ltd, Tokyo, Japan) substrate. Thirty minutes later, the reaction was stopped with 2 N H_2SO_4. Absorbance was measured at 492 nm in an ELISA plate reader (Corona, Hitachinaka, Japan). The procedures for color development and absorbance measurement subsequent to adding conjugated antibody were identical in every subsequent ELISA.

Antibodies that reacted with KF94 and not with anti-HAV-negative human serum were identified as KF94-specific mAb2s. The isotype of the mAb2 was determined using a mouse monoclonal antibody isotyping test kit (Serotec Ltd., Oxford, UK). The ascitic fluid of each mAb2 was obtained by intraperitoneal injection of hybridomas into pristane-primed BALB/c mice. Animals were handled and cared for in accordance with the "Guidelines for Animal Experimentation at NIID."

Cross-reactivity between the selected mAb2s was determined by competitive inhibition ELISA using biotin-labeled mAb2s and KF94-coated plates (Table 1). Briefly, non-labeled mAb2 was applied to KF94-coated plates, followed by biotin-labeled specific or non-specific mAb2. The mAb2 was labeled with biotin using EZ-Link NHS-Biotin (Pierce, Rockford, IL, USA) according to the manufacturer's instructions and detected using HRPO avidin (MBL, Nagoya, Japan).

Biotin-labeled mAb2s were also used in mAb2-HAV binding ELISA (Table 1). Serially diluted biotin-labeled mAb2s were applied to plates that were prepared as follows: polystyrene microtiter plates were coated with 50 μl of anti-HAV rabbit hyperimmune serum, and nonspecific binding was blocked with bovine serum albumin. Fifty microliters of formalin-inactivated HAV strain KRM003

Table 1 Summary of ELISA used in this study

Aim of ELISA	Plate coated with	First input	Second input	Third input
MAb2-KF94 binding	KF94	MAb2	HRPO anti-mouse IgG	–
MAb2 cross reactivity	KF94	MAb2	Biotin-labeled mAb2s	HRPO avidin
MAb2-HAV binding	Anti-HAV rabbit hyperimmune serum[a]	Inactivated HAV	Biotin-labeled mAb2s	HRPO avidin
Inhibition of binding of Ab1 to HAV by mAb2	Anti-HAV rabbit hyperimmune serum[a]	Inactivated HAV	The mixture of Ab1 and mAb2	HRPO anti-human IgG
Inhibition of binding of KF94 to mAb2 by HAV	KF94	Inactivated HAV	Biotin-labeled mAb2s	HRPO avidin
Competitive inhibition	GL37 cells	MAb2s	HRPO anti-HAV receptor antibody 190/4C	–

[a] We confirmed that anti-HAV rabbit hyperimmune serum did not bind to mAb2s

(20 ng/ml) was added, and the plates were incubated overnight at 4°C. Bound biotin-labeled mAb2s were detected as described above.

Inhibition assays

We determined the ability of the mAb2 to inhibit the binding of KF94 to HAV (Table 1). A mixture of diluted Ab1 and mAb2 was incubated at 4°C overnight and then applied to HAV-coated plates. Bound Ab1 was detected using a HRPO anti-human IgG (Dako, Glostrup, Denmark).

We examined the ability of inactivated HAV to inhibit the binding of KF94 to mAb2 (Table 1). Serially diluted inactivated HAV was incubated in KF94 plates overnight at 4°C, and then the wells were emptied. Biotin-labeled mAb2 (diluted 1:1,000) was applied to the wells, followed by HRPO avidin. Inhibition rates were measured using the formula:

Inhibition rate (%) = 100 × (absorbance without inhibitor − absorbance with inhibitor)/absorbance without inhibitor.

Anti-HAV-cellular-receptor mouse monoclonal antibodies

Anti-HAV-cellular-receptor mouse monoclonal antibodies (anti-HAV-receptor antibodies), designated 190/4, 235/4 and 263/6, were induced by immunizing GL37 cells and selected by their ability to block HAV propagation in GL37 cells [11]. We confirmed that the anti-HAV receptor antibodies recognized a common receptor. Anti-HAV receptor antibody190/4 was conventionally conjugated with horseradish peroxidase and named 190/4C.

Binding of mAb2 to HAV cellular receptor

The binding of each mAb2 to GL37 cells was examined using an immunofluorescence assay. The positive controls were the anti-HAV-receptor antibodies, 190/4, 235/4 and 263/6, and the negative control was a normal mouse serum (NMS) that was confirmed to be negative for anti-HAV antibody.

Confluent, unfixed GL37 cell monolayers on glass cover slips were gently washed with phosphate-buffered saline (PBS) and then incubated with mAb2 or control antibodies for 1 h at 37°C in a moist chamber. The cover slips were washed with PBS and then incubated with fluorescently labeled anti-mouse IgG (MBL, Nagoya, Japan) for 1 h at 37°C in a moist chamber. The labeled anti-mouse IgG was removed, and the cover slips were washed three times in PBS. The cell surface was immediately observed using a fluorescence microscope.

We confirmed using competitive inhibition ELISA that mAb2 94-7 and anti-HAV receptor antibody 190/4 shared the same HAV cellular receptors.

The anti-HAV receptor antibody 235/4 was the positive competitor, and an anti-human Fc mouse monoclonal antibody (anti-human Fc mAb) was the negative competitor in this ELISA. An anti-human Fc mAb bound non-specifically to KF94 despite being simultaneously generated with the mAb2s 94-2 and 94-7.

Confluent GL37 cell monolayers in 96-well cell culture plates (Corning, NY, USA) were incubated with serially diluted mAb2s and positive or negative competitors for 2 h at 37°C. After washing with PBS, 50 µl of HRPO anti-HAV-receptor antibody 190/4C was added to the corresponding wells and incubated for 2 h at 37°C. The wells were washed, and then bound HRPO anti-HAV receptor antibody was detected. Competition rates were measured using the formula:

Competition rate (%) = 100 (absorbance without competitor − absorbance with competitor)/absorbance without competitor.

Infectivity assays

We performed $TCID_{50}$-infectivity and immunofocus assays to investigate the mAb2-mediated protection of GL37 cells against HAV infection.

For $TCID_{50}$-infectivity assays, GL37 cell monolayers in 96-well cell culture plates were incubated with 50 µl of serially diluted mAb2 for 1 h at 37°C in a CO_2 incubator. Without washing, 25 µl of HAV, genotype IA, IB or IIIB, was added to the appropriate wells and incubated for 1 h at 37°C. Cells were infected with all HAVs at a multiplicity of infection of 100. Infected cells were maintained in Eagle's minimal essential medium (EMEM) supplemented with 2 mM L-glutamine, 50 µg/ml of gentamicin, 2% fetal bovine serum and 0.15% of sodium bicarbonate (2% FBS-EMEM) for 3 days. Infected cells were fixed with 80% methanol containing 0.03% H_2O_2, and air-dried, and then HAV propagated in infected cells was detected using anti-HAV hyperimmune rabbit serum. The plates were washed and incubated with HRPO anti-rabbit IgG (MBL, Nagoya, Japan), and then the rate of blocking of infection was measured using the formula:

Blocking rate (%) = 100 × (absorbance without inhibitor − absorbance with inhibitor)/absorbance without inhibitor.

The immunofocus assay was a modified radioimmunofocus assay [13] as follows: We mixed 50 µl of mAb2 (diluted 1:20 in PBS supplemented with 2% FBS) with an equal volume of HAV, genotype IA, IB or IIIB, containing 40–60 focus-forming units (FFU) in 2% FBS-EMEM. GL37 cell monolayers in six-well cell culture plates (Falcon, Franklin Lakes, NJ, USA) were inoculated with 100 µl of the mAb2-HAV mixture and incubated for 1 h at 37°C in a 5% CO_2 environment to allow mAb2 and virus adsorption to the cells. Without removing the inoculum, the GL37 monolayer was overlaid with 5 ml of Dulbecco's modified Eagle medium (Nissui, Tokyo, Japan) containing 0.6% agarose ME (Iwai Chemicals Company, Tokyo, Japan), 2% FBS and 0.22% sodium bicarbonate. After the agar had solidified, the cultures were placed upside down and incubated at 37°C for 10–12 days at 37°C in 5% CO_2. The agarose overlay was discarded, and the cells were fixed with 1.5 ml of 80% methanol containing 0.03% H_2O_2 for 1 h at 4°C. Anti-HAV rabbit hyperimmune serum (1 ml of 1:2,000 dilution) was added to each well and incubated overnight at 4°C. The wells were washed with PBS and then incubated with 1 ml of HRPO anti-rabbit IgG for 2 h at 37°C. The plates were washed once again, and then HAV foci were detected using 1.5 ml of DAB substrate [0.5 mg/ml diaminobenzidine, 0.03% $(NH_4)_2Ni(SO_4)_2$, 0.03% $CoCl_2$, 0.03% H_2O_2 in PBS]. Infection was considered blocked if the input FFU was reduced by ≥50%.

Results

Binding properties of mAb2s

Among the hybridoma clones secreting mAb2, two stable clones were selected by KF94-binding ELISA. The ascitic fluids of the selected clones, designated mAb2 94-2 and 94-7 were specific for KF94, and they did not react with either KF6 or HAV-negative human serum. The titers of mAb2 94-2 and 94-7, given as the reciprocal of the end-point dilution required to generate maximal absorbance in KF94-binding ELISA, were 102,400 and 25,600, respectively. Both were categorized as IgG1 subclass, κ chain. They did not cross-react or bind to HAV.

Interactions between mAb2s, KF94 and HAV

Figure 1a shows that both mAb2s inhibited KF94 binding to HAV. At a 1:1,000 dilution, mAb2 94-2 and 94-7

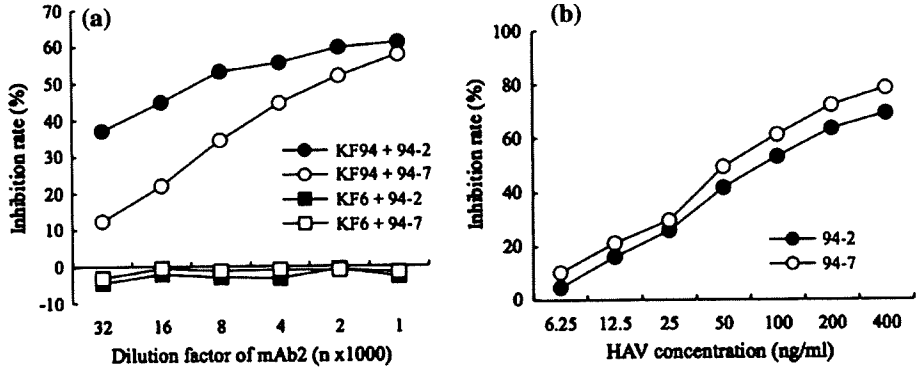

Fig. 1 Interactions between KF94, mAb2s and HAV. Anti-HAV antibodies KF6 and KF94 were incubated with serially diluted mAb2 94-2 or 94-7 (*filled circle*, KF94 and 94-2; *open circle*, KF94 and 94-7; *filled square*, KF6 and 94-2; *open square*, KF6 and 94-7; **(a)**, and then binding to inactivated HAV was determined by ELISA. The mAb2s (diluted 1:1,000) inhibited KF94 binding to HAV by 60%. The mAb2s did not interfere with KF6 binding to HAV, thus confirming their specificity. Anti-HAV antibody KF94 was incubated with serially diluted inactivated HAV, and binding of preincubated KF94 to mAb2 94-2 (*filled circle*) or 94-7 (*open circle*) was determined by ELISA. Inactivated HAV interfered with mAb2 binding to KF94 **(b)**