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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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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 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

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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 CO₂ 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 (2×10^5 cells per well in 24-well plates) and incubated in a 5% CO₂ 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 $\pm$ 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 I.). 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. I. 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.

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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 (NAb₅₀ 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 NAb₅₀ 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 NAb₅₀ titers in the pre-treatment sera are associated with treatment outcome.

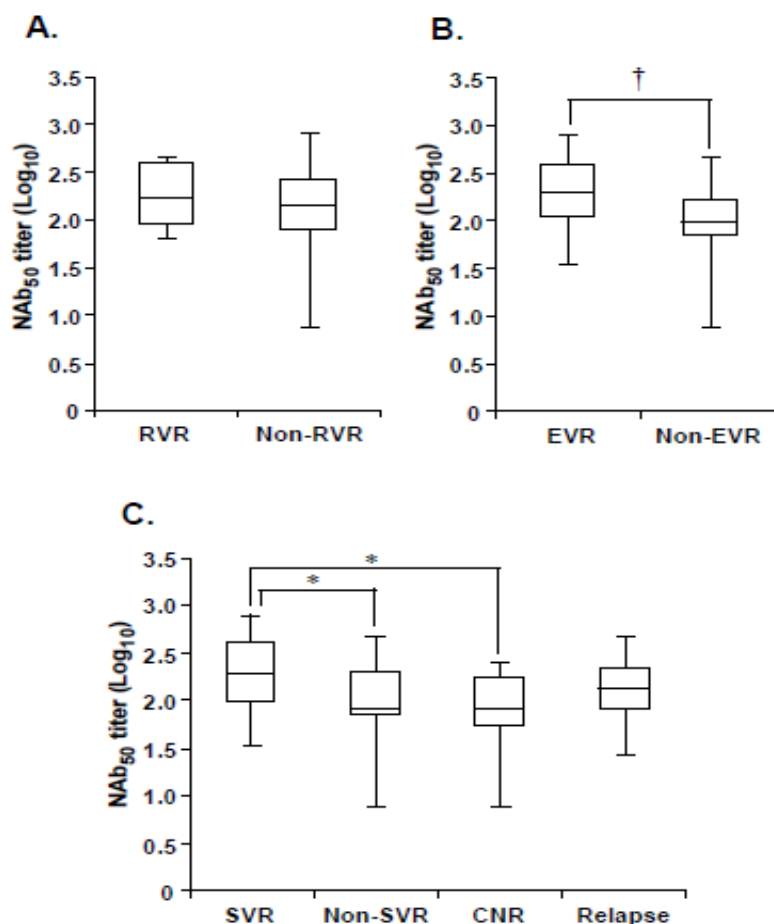


Fig. 1. NAb₅₀ titers in the sera of HCV-infected patients. NAb₅₀ titers in the pre-treatment sera of HCV-1b-infected patients were compared between RVR and Non-RVR (A), EVR and Non-EVR (B), SVR and Non-SVR (C), SVR and CNR (C), and SVR and Relapsers (C). *, $p<0.05$; †, $p<0.01$.

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 NAb₅₀ titers of patients who achieved an EVR was significantly lower than that of Non-EVR (Fig. 2).

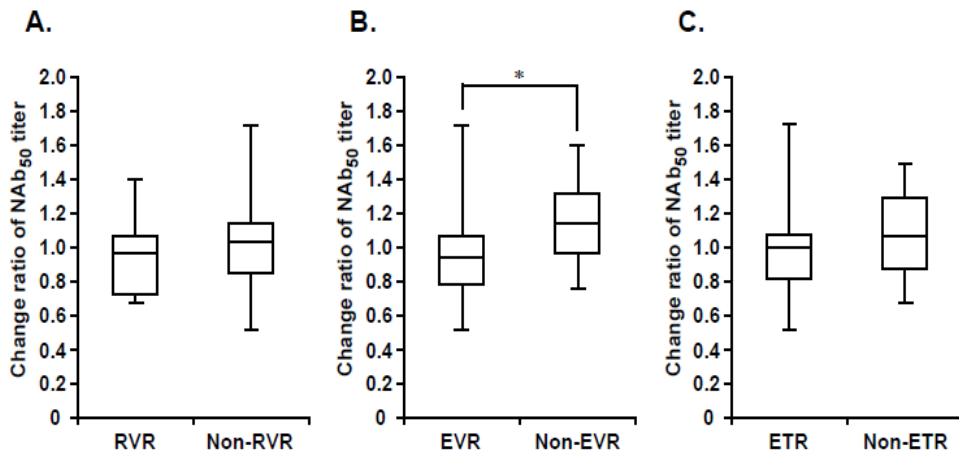


Fig. 2. Change ratios of NAb before and after disappearance of HCV RNA in the serum. Change ratios (post-treatment/pre-treatment) in the NAb₅₀ titers before and after disappearance of HCV RNA in the sera of patients who were treated with PEG-IFN/RBV were compared between RVR and Non-RVR (A), EVR and 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 NAb₅₀ 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 NAb₅₀ titers did not decrease significantly when measured even one year after disappearance of HCV RNA in the serum (Fig. 2).

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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, NAb₅₀ titers were significantly higher in sera of patients who achieved EVR and SVR than those of Non-EVR and Non-SVR, respectively. Also, NAb₅₀ titers declined only slightly during the course of one year after disappearance of HCV RNA in the sera.

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