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なし

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Matrix effects in clinical immunoassays and the effect of preheating and cooling analytical samples

Hiroshi Yoshida*, Yuji Imafuku and Toshihiko Nagai

Department of Clinical Laboratory Medicine, Fukushima Medical University School of Medicine, Fukushima, Japan

Abstract

Immunological reactions are influenced by various factors including antigens, antibodies and other variables. We focused on two items: i) matrix effects, especially of detergents and ii) temperature effects: preheating sera, especially effects on rheumatoid factor (RF) measurement and false-positive reactions in ELISAs, and cold storage of sera, especially effects on complement. Among various additives, detergents affected the agglutination reaction for fecal hemoglobin and hepatitis B surface (HBs) antigen. Some of the detergents examined abolished these antigenicities, however, polyethylenglycols enhanced the reactions. Heat-inactivation of sera at 56°C for 30 min was employed in serological testing. However, in RF measurement, 10 min of preheating was sufficient to abolish C1q (subcomponent of C1), which could participate in the agglutination reaction. In ELISA for antibodies, false-positive reactions were caused by preheating sera. By the analyses of assays for antibodies to hepatitis C virus (HCV) and cardiolipin, it was found that they were induced by immunoglobulin G (IgG) modified by preheating. Cold storage induced activation of complement (cold activation) in anti-HCV antibody positive sera. CH50 titers in the sera were lowered by one cycle of freezing at −20°C and thawing, and the decrease was affected by the containers.

Keywords: detergent; false-positive reaction; immunological testing; matrix effects; pre-analytical issues; temperature effect.

Introduction

Immunological reactions are sometimes affected by various factors or components other than analytes; these are called matrix effects (1).

Various factors influencing antigen-antibody reactions are separated into two groups: the first group contains antigens and antibodies that include ratio, cross-reaction of antibodies, heterophile antibodies and rheumatoid factor (RF), and the second group, in addition to antigens and antibodies, contains numerous factors including temperature, pH, ionic strength, endogenous components such as enzymes, immunoglobulins, bile and salts, and exogenous substances such as drugs, polymers and detergents (2).

Concerning temperature, heat-pretreatment of sera in serological testing was begun as early as 1906 by Wasserman, and the term heat inactivation (Inaktivierung) was introduced by Sachs in 1927. Since then, sera have been routinely heat-pretreated for qualitative and semi-quantitative serological tests, especially in complement fixation and agglutination reactions. Heating serum at 56°C for 30 min affects the complement of the classical pathway: complete inactivation of C1 and C2 and to a lesser degree of C4 (3). However, antibodies have been recognized to be generally unaffected by the same conditions (2).

Reactions of antibodies and complement are usually more efficient at 37°C than at a lower temperature of around 4°C. Antibodies are more stable at −70°C than at −20°C. Sera stored for a long period at −20°C developed anti-complementary properties in vitro. This phenomenon was noticed in 1900. However, details of the mechanism have not been thoroughly investigated (3). When comparing incubation conditions between 4°C and room temperature, 4°C is preferable for preventing decreases of most antibodies and complement. However, the serum hemolytic complement activity (CH50) was decreased by cold storage, and cold activation of the classical pathway of complement was proposed by Kitamura et al. in 1977 (4). The phenomenon was first observed by Jordan in 1953 (5).

We describe here the effects of matrices, especially detergents, on immunological reactions, preheating sera at 56°C on RF measurement and on autoantibodies measured by ELISA and cold storage of cold activation-positive sera on CH50.

Materials and methods

Serum

Sera collected from healthy staff and patients in our hospital and stored at −70°C were made anonymous or pooled for examinations. They were thawed in a water bath at 37°C before use.

Preheating and cold storage

Sera were preheated in a water bath at 56°C for 30 min or under different conditions, cooled with water and used. The

*Corresponding author: Hiroshi Yoshida, Department of Clinical Laboratory Medicine, Fukushima Medical University School of Medicine, 1 Hikarigaoka, Fukushima 960-1247, Japan
Phone: 0081-245-47-1348, Fax: 0081-245-48-6016,
E-mail: hyoshida@fmu.ac.jp

Table 1 Effects of detergents on HBs antigen.

HBs antigen-positive serum	HBs antigen (%)*
+ Saline	100%
+ Detergent**	
CA	6.7
CHAPS	77.6
CHASO	79.2
MEGA-8	108.9
TX-100	39.1
PEG 200	127.1
PEG 1000	117.2

*Values (%), compared with that of saline control (100%, S/N ratio: 19.2), **Final concentration of detergent was 0.1%, except TX-100 (0.02%).

temperature in the water bath was estimated using a standard thermometer. To study the effects of cold storage, especially on CH50, sera were stored at 4°C for various periods and used for the measurement. Freezing and thawing were performed to determine the effects on antibodies and complement. Freezing at −70°C or −20°C and thawing at 37°C or room temperature (22°C~24°C) were repeated up to 5 times.

Antigen measurements

Hepatitis B surface (HBs) antigen and prostate-specific antigen (PSA) were measured by ELISA (AxSYM, Dainabot Co, Tokyo, Japan) and carcinoembryonic antigen (CEA), α-fetoprotein (AFP) and carbohydrate antigen (CA) 19-9 were measured by chemiluminescent immunoassay (Fujirebio Co, Tokyo, Japan). Immunoglobulins (Ig) (G, A and M) and complement components (C3 and C4) were measured by nephelometric immunoassay (Behring nephelometer II, Dade Behring, Marburg, Germany). C1q, one of the subcomponents of C1, was measured by single radial immunodiffusion prepared in our laboratory.

Antibody measurements

RF was measured by latex photometric immunoassay (LPIA; Dia-iatron, Tokyo, Japan) and its subclasses (IgG, and IgA and IgM) were measured by ELISA (Toyobo Co, Shiga, Japan). Anti-nuclear antibodies were measured by ELISA, which included anti-dsDNA antibody (Dia-iatron), anti-U1-RNP antibody (Medical Biological Laboratory (MBL), Nagoya, Japan), anti-scleroderma-70 (Scl-70) antibody (MBL) and anti-Jo-1 antibody (MBL). Anti-cardiolipin (CL) antibody (MBL) and anti-HCV antibody were also included.

Hemolytic complement activity (CH50)

CH50 was measured using a Cobas Mira Plus (Nippon Roche Co., Tokyo, Japan) with a reagent purchased from Denka Seiken Co. (Niigata, Japan). Serum CH50 was measured immediately after thawing and incubation at 4°C for 16 hours. Sera of more than 30% of the decrease in CH50 titer after cold incubation compared to that before were regarded as cold activation-positive. The effects of containers on CH50 of a cold activation-positive serum were examined. Four plastic tubes (Sumitomo Bakelite Co. (Tokyo, Japan), Terumo Co. (Tokyo, Japan), Nipro Co. (Tokyo, Japan) and Sekisui Medical Co. (Osaka, Japan)) and a glass tube (Nipro Co.) were employed. Cold activation-positive serum, which was divided into these tubes, was stored at 4°C for 24 hours and measured for CH50.

Detergents

To determine the effects of detergents on immunoassays, we examined the effects on immunological and chemical occult blood tests (6, 7). The effects of detergents on HBs antigen were examined. These included cholic acid (Sigma Chemical Co., St. Louis, USA), dodecyltrimethylammonium bromide (DTAB; Sigma), 3-[(3-cholamindopropyl)dimethylammonio]-1-propane sulfonate (CHAPS; Boehringer Mannheim, Germany), 3-[(3-cholamindopropyl) dimethylammonio]-2-hydroxy-1-propane sulfonate (CHAPSO; Boehringer), octanoyl-N-methylglucamide (MEGA8; Boehringer), TritonX-100 (TX100; Boehringer), polyethylene glycol (PEG) 200 and 1000 (Sigma). The effects of the detergents on HBs antigen were examined by mixing a positive serum with equal volumes of detergent solutions, and after incubation at room temperature for 1 hour the antigen was measured.

Others

Human monoclonal IgG and polyclonal IgG were purified from sera by protein G affinity chromatography (Mab Trap G II, Pharmacia Biotec, Uppsala, Sweden).

Results

Effects of detergent on HBs antigen

The effects of some detergents on the HBs antigen assay are shown in Table 1. Cholic acid and TX-100 reduced the antigenicity of HBs antigen, however, PEGs enhanced the reactions.

Preheating effect on RF

Pooled sera from five rheumatoid arthritis (RA) patients were preheated at 56°C for 2~30 min and measured for RF and its subclasses and complement components, C1q, C3, and C4. As shown in Figure 1, RF and IgM class RF were shown to be affected similarly by preheating. Reductions of RF and IgM class RF titers after 30 min of preheating were 24% and 40%, respectively. C1q concentration was markedly decreased and was not detectable after 10 min of preheating at 56°C. A marked reduction of C1q antigenicity and a slight to moderate reduction of IgM class RF activity could be due to the direct effects of heating on these molecules. Based on this experiment, it is recommended that preheating for 10 min is sufficient to inactivate C1q for RF measurement.

False-positive reaction in ELISA

We measured several antigens and antibodies in native or non-treated and preheated sera comparatively by ELISA. As shown in Table 2, among the antigens examined, the concentration of PSA was

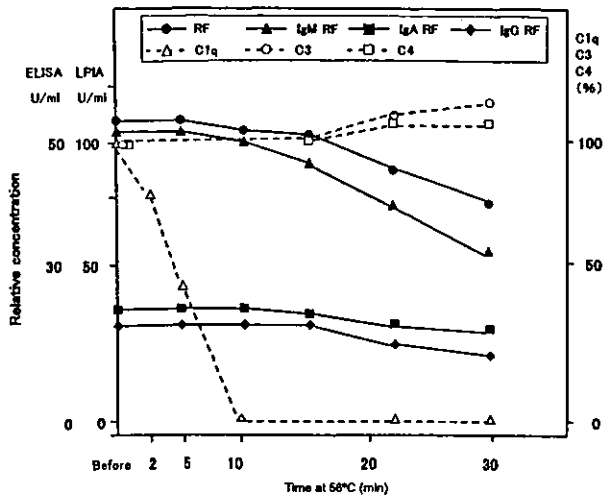

Figure 1 Effects of preheating sera at 56°C on rheumatoid factor (RF) and complement components. Pooled rheumatoid arthritis sera were incubated at 56°C for 2 min to 30 min. RF was measured by LPIA and its subclasses by ELISA. The concentrations of C1q were measured by single radial immunodiffusion and C3 and C4 by nephelometric immunoassay. The concentrations in native sera were regarded as 100% and those heat-incubated were compared.

markedly reduced and that of AFP was slightly reduced, due to the direct heating effect. Other items were not affected. Conversely, antibody titers were elevated slightly to markedly in all items examined. Antibodies to HCV, dsDNA, Scl-70 and CL were markedly increased by preheating the sera. We compared anti-CL antibody in sera before and after pretreatment by heating at 56°C for 30 min. Anti-CL antibody-negative sera with hyper-γ-globulin and monoclonal IgG developed greater increases in the ratio betweeen titer of 56°C-heated serum and that of native serum, however, sera with hypo-γ-globulin and monoclonal IgA and IgM showed smaller increases (Table 3). The titers of purified monoclonal IgG preparations were also increased by the preheating. These results could suggest that IgG participates in the false-positive reactions.

Cold and container effects on CH50

The effect of the container tube on cold activation was examined. As shown in Table 4, after incubation at 4°C for 16 hours, the residual CH50 of a cold activation-positive serum in one plastic tube was 53% higher than those (32%~37%) in the glass tube and the three plastic tubes. This reproducible difference could be induced under cold conditions by some matrix effect related to the tube wall.

Discussion

Matrix effects are important issues and have been discussed especially in the context of control surveys. According to the NCCLS guideline (1), matrix effects are defined as follows: i) the influence of a sample property, other than analyte, on the measurement, and thereby on the measured values, and ii) the physicochemical effect(s) of the matrix on the analytical method's ability to accurately measure an analyte. Through a conference on the matrix effect and accuracy assessment in clinical chemistry organized by the College of American Pathologists in 1992 (8), laboratory staff realized the importance of the matrix effects not only in clinical chemistry but also in other fields, including serology and clinical immunology.

Various factors are recognized to influence the antigen-antibody reaction. In blood group serological testing, various factors were disclosed to affect agglutination reactions; these include pH, ionic strength and temperature, which affect the equilibrium constant of antigen-antibody reactions, non-specific attachment of IgG, enzyme treatment, polymers and macromolecules (2). Natural and synthetic polymers have been used to enhance the hemagglutination reaction with anti-D antibody. Such polymers are serum albumin, gelatin, methyl cellulose, polyvinyl alcohol (9), casein gum acasia (10), gum tragacanth, dextran (11), polyvinyl-pyrrolidone (12) and glycerol (13). In a physicochemical investigation, such parameters as cell shape, cell distance, extracellular col-

Table 2 Effects of preheating serum at 56°C for 30 min on antigens and antibodies in pooled serum of healthy subjects measured by ELISA.

Item	Manufacturer of reagent	Ratio*
(Antigen)		
HBs	Dainabot (Tokyo, Japan)	1.05
CEA	Fuji rebio (Tokyo, Japan)	1.04
AFP	Fuji rebio (Tokyo, Japan)	0.94
CA19-9	Fuji rebio (Tokyo, Japan)	0.99
PSA	Dainabot	0.30
(Antibody)		
HCV	Dainabot	24.5
ds DNA	Medical Biological Laboratory (Nagoya, Japan)	76.8
U1 RNP	Medical Biological Laboratory	1.6
Sm	Medical Biological Laboratory (Nagoya, Japan)	2.6
SS-A	Medical Biological Laboratory	1.4
Scl-70	Medical Biological Laboratory	2.4
Jo-1	Medical Biological Laboratory	1.9
CL	Medical Biological Laboratory	19.3

*Pooled serum of healthy subjects: preheated at 56°C for 30 min/non-treated.

Table 3 Effects of preheating sera on anti-cardiolipin antibody.

Serum*	Ratio**
Healthy, pooled	19.3
Hyper-γ-globulin	29.1
Hypo-γ-globulin	7.8
Monoclonal IgG	150.0
Monoclonal IgA	9.2
Monoclonal IgM	21.6

*Negative for anti-cardiolipin antibody. **Anti-cardiolipin antibody ratio: serum preheated 56°C for 30 min/native serum. Mean values of three sera examined are shown.

loid osmotic pressure, cell surface hydration and cell zeta-potentials were changed by additions of polymers, actions of enzymes, salt ion changes and interaction with antibodies (14).

Various detergents have been used for stabilizing the components of reagents, reducing non-specific reactions and solubilizing cell membranes. Tween 20 has been used in the diluents of ELISA since 1971 (15) and was shown to enhance antiphosholipid antibody activity (16). However, details of detergents, especially in reagents, have not been provided by manufacturers. We examined the effects of representative detergents on the agglutination reaction for fecal hemoglobulin measured by means of nephelometry. Anionic, cationic, zwitterionic and some nonionic detergents reduced agglutination titers markedly, however, six kinds of PEG enhanced the agglutination reaction (7). Toilet sanitizers composed of various detergents showed potent destructive effects on the antigenicity of hemoglobin (6). We show the results of the effects of some detergents on HBs antigen. Similar to our previous observations, some detergents abolished the antigenicity of HBs and, conversely, PEG enhanced the reaction.

Heat-pretreatment of sera has been performed in serological tests, especially in complement fixation and agglutination reactions. When measuring RF, preheating sera at 56°C has been believed to be essential to abolish complement, especially C1q, which reacts with aggregated IgG (17, 18) to increase RF titer. By preheating sera at 56°C for 30 min, RF titers did not seem to be reduced significantly in a qualitative (RA test) or semiquantitative assays (Waaler Rose test and others). However, our quantitative measurement showed that titers of RF, as well as IgM class RF, were reduced by preheating sera at 56°C for 30 min, and the antigenicity of C1q was abolished completely by 10 min of preheating at 56°C (19). Based on this observation, we recommend that heating at 56°C for 10 min is sufficient to prevent the participation of complement. In recent automated or semi-automated quantitative measurement systems, native serum samples have been used, since the participation of C1q is slight in diluted samples due to the preheating influence to decrease RF titers.

Table 4 Effects of container tubes on CH50 of serum with positive cold activation.

Incubation*	CH50 (%)**
Before	100
At 4°C,16 hours in:	
Glass tube	32
Plastic tube	
A	53
B	37
C	34
D	32

*The serum was divided and incorporated into each tube, incubated at 4°C for 16 hours and measured for CH50. **CH50 after cold incubation was designated as %, compared with that (100%, 33 400 U/l) before the incubation.

False-positive reactions were observed in ELISA. In the anti-HCV-antibody assay, sera of patients with autoimmune chronic active hepatitis (20), paraproteinemia (21) and RA (22), and long-stored sera (23) showed higher positive incidences, which were suspected to be due to false-positive reactions induced by some components of the sera. We showed that one of the agents inducing a false-positive reaction was heat-aggregated IgG, which bound to HCV antigen(s) detected by recombinant immunoblot assay (24).

In an anti-CL antibody assay, Cheng et al. (25) reported that sera preheated at 56°C showed increased binding of the antibody in ELISA. A heat-labile inhibitor of anti-CL antibody was speculated to be one of the causers. However, as the sera depleted of IgG by protein A did not elevate the anti-CL antibody titer by the preheating, they suggested the participation of IgG in the false-positive reaction. We showed that anti-CL antibody-negative sera with hyper-γ-globulin and monoclonal IgG developed greater increases of antibody titers with preheating, however, sera with hypo-γ-globulin and monoclonal IgA and IgM showed smaller increases. The titers in purified monoclonal IgG preparations examined were also increased by the preheating. In an anti-endothelial cell (EC) antibody assay, D'Cruz et al. (26) reported that the anti-EC antibody binding index increased after preheating sera at 56°C for 30 min, and removing immunoglobulins with protein A abolished the increased binding seen after heating. These results could suggest that IgG participates in the false-positive reactions. However, it could be speculated from differences in the increases of titers among sera that some heat-modified serum constituents other than IgG might react non-specifically with antigens or some additives, which differ among ELISA systems.

In serological tests, especially complement fixation and agglutinations, sera has been preheated to inhibit the participation of complement, especially C1q. However, pretreatment has not been performed in recent automated as well as manual measurements. From the prevention of viral infections such as hemorrhagic fevers through handling sera and plasmas, the effects of heat-preatment on routine biochemical analytes was reported (27). In ELISA, some manufacturers suggest prohibiting the use of preheated samples in their illustrations, however, others do not. Based on our results, we recommend using native sera for the measurements by ELISA.

Antibodies and complement components are generally believed to be more stable at lower temperatures. However, two exceptions have been recognized: one comprises cold autoantibodies such as cold agglutinin and Donath-Landsteiner antibody and the other is cold activation of complement. Cold autoantibodies react only at low temperatures. However, the reason why they react at low temperatures is still unknown. Cold activation is frequently observed in sera with positive anti-HCV antibody (28). Details of the mechanism have not been clarified. However, several examples of evidence have been reported. Cold activation was inhibited by the addition of anticoagulants, and classical pathway components were reduced (4), it was observed in cryoglobulin-positive, but not all, sera (29), however, it proceeded without the participation of C1q (30).

When handling cold activation-positive sera, one cycle of freezing at −20°C and thawing at room temperature decreased hemolytic complement activity (CH50) significantly, and three repetitions decreased the activity to about 50%. However, freezing at −70°C and thawing at 37°C 3 times did not decrease the activity (28).

The effect of the container tube on cold activation was examined in this experiment and moderate inhibition of the decrease of CH50 titer in one plastic tube was observed. It could be speculated that some matrix effect related to the tube wall developed under cold conditions to affect the complement system, although the details are still unclear.

Matrix effects are important issues to be investigated and have received attention in quantitative measurements, especially in surveys and clinical chemistry (1, 8). To reduce or abolish matrix effects in immunological assays, it is necessary to pay more attention to the effects and have more communication between users and manufacturers.

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Prediction of treatment outcome with daily high-dose IFN α-2b plus ribavirin in patients with chronic hepatitis C with genotype 1b and high HCV RNA levels: relationship of baseline viral levels and viral dynamics during and after therapy

Shiro Iino[a,*], Eiichi Tomita[b], Hiromitsu Kumada[c], Hiroshi Suzuki[d], Joji Toyota[e], Kendo Kiyosawa[f], Kyuichi Tanikawa[g], Michio Sata[h], Norio Hayashi[i], Shinichi Kakumu[j], Takashi Matsushima[k], Tomoyoshi Ohno[l]

[a] *Kiyokawa Hospital, 2-31-12, Asagaya Minami, Suginami-ku, Tokyo 166-0004, Japan*
[b] *Gifu Municipal Hospital, Gifu, Japan*
[c] *Toranomon Hospital, Tokyo, Japan*
[d] *Yamanashi University, Nakakoma, Japan*
[e] *Sapporo Kosei General Hospital, Sapporo, Japan*
[f] *Shinsyu University School of Medicine, Matsumoto, Japan*
[g] *International Institute for Liver Research, Japan*
[h] *Kurume University School of Medicine, Kurume, Japan*
[i] *Osaka University School of Medicine, Suita, Japan*
[j] *Aichi Medical University, Nagakute, Japan*
[k] *Municipal Hakodate Hospital, Hakodate, Japan*
[l] *Nagoya City University School of Medicine, Nagoya, Japan*

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Abstract

Data on 334 patients with HCV genotype 1b and high viral levels were extracted from two multicenter double-blind studies conducted in Japan comparing IFN α-2b plus ribavirin ($n = 209$) with IFN α-2b alone ($n = 125$) for 24 weeks. HCV RNA assay was conducted before and 4, 12, and 24 weeks after the start and 4, 12, and 24 weeks after the end of treatment. Both sustained viral response (SVR) rate and relapse rate after the end of treatment were analyzed in relation to baseline viral levels and the time of first disappearance of virus. In the combination treatment group, the percentage of patients who were HCV RNA-negative within 4 weeks decreased with increase in baseline viral levels (i.e. 42%, 15%, and 11% were HCV RNA-negative in the groups exhibiting <500, 500 to <850, and ≥850 kcopies/mL, respectively). In the IFN monotherapy group, the response rates were lower at 13%, 15%, and 1%, respectively. Disappearance of virus within 12 weeks after the start of combination treatment was indicative of higher probability of SVR. The risk of relapse was more highly correlated with the timing of initial viral disappearance than with baseline HCV levels; it was 4.8 and 10.3 times higher in patients who became HCV-negative at 4–12 and 13–24 weeks compared with in those who were HCV-negative within 4 weeks.

Keywords: Chronic hepatitis C; Interferon α-2b; Ribavirin; Multicenter randomized double-blind study

1. Introduction

Global consensus obtains that PEG-interferon (PEG-IFN) plus ribavirin combination therapy is the treatment of choice

* Corresponding author. Tel.: +81 3 3312 0151; fax: +81 3 3312 2222.

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for chronic hepatitis C (CHC). That the duration of treatment should be 12 months for hepatitis C virus (HCV) genotype 1 and 6 months for other genotypes is also nearing consensus [1,2]. High-dose daily IFN monotherapy in patients with CHC was originally reported from Japan [3], and since then several reports of better efficacy using high-dose daily IFN therapy similar to that used in Japan plus ribavirin have appeared in both the USA and Europe [4–12].

Recently, much attention has been focused on the relationship between the timing of HCV RNA negativity and antiviral efficacy of IFN therapy. Many reports have investigated this relationship in patients receiving IFN or PEG-IFN and ribavirin combination therapy [13–18]. In Japan, the age of patients with CHC is increasing, and both nonresponders to previous IFN therapy and IFN-treatment-naïve patients usually are given high doses of IFN. In two Japanese studies of high-dose IFN therapy, SVR including in patients with HCV with genotype other than 1 was observed in 27.5% (316/1148) [19] and 30.6% (313/1022) [20], respectively, whereas in the USA and Europe where IFN 3 MIU is normally administered three times/week, SVR was observed in 6–19% even in patients undergoing treatment for 1 year [21–23]. For this reason, Japanese nonresponders to prior IFN therapy cannot be considered the same as non-Japanese patients, and hence direct application of the results of trials conducted outside Japan to Japanese patients is of limited use.

It has also been reported that reducing HCV relapse after the end of treatment enhances the efficacy of combination therapy [24]. Longer-term combination treatment has been confirmed to reduce the rate of HCV relapse after the end of drug administration [22,23], but the mechanism of this effect is not clear. The present study was performed to examine the relationship between the timing of disappearance of HCV RNA and HCV eradication in Japanese patients receiving IFN plus ribavirin combination therapy. Moreover, we attempted to clarify factors related to relapse after the end of treatment by analyzing the relationship between the time of HCV eradication and baseline HCV levels.

2. Materials and methods

2.1. Patient selection

Two randomized comparative studies of IFN α-2b plus ribavirin were conducted using IFN (-2b monotherapy as control; 1 in patients with HCV genotype 1b CHC with high viral levels (the most difficult CHC patients to treat) [25] and 1 in nonresponders and relapsers to previous IFN therapy [26] who are thus in urgent medical need. No bias was observed in patient distribution between groups in these studies (data not shown). Both studies were conducted after approval by the institutional review boards of each medical institution and informed consent was obtained in writing from each patient. IFN α-2b (Intron A, Schering Plough, Kenilworth, NJ) was administered six times/week for 2 weeks at a dose of 6 or 10 MIU and then three times/week for 22 weeks at a dose of 6 MIU. Ribavirin (Rebetol, Schering Plough, Kenilworth, NJ) was administered for 24 weeks at a dose of 600 mg/day (three capsules) in patients weighing <60 kg and 800 mg/day (four capsules) in those whose weight was ≥60 kg. The control group received IFN (-2b together with ribavirin placebo capsules. From these two clinical studies, we extracted data on patients with HCV genotype 1 and high viral levels and retrospectively analyzed the time of initial HCV RNA negativity, the percentage of patients with sustained viral negativity after the end of treatment, and the percentage of patients who relapsed after the end of treatment. The database subjected to retrospective analysis included data on sex, age, body weight, extent and activity of liver tissue lesion, history of IFN therapy, HCV RNA level, aspartate aminotransferase (AST), alanine aminotransferase (ALT), hemoglobin, white blood cells (WBC), red blood cells (RBC), platelet count, and serum creatinine. Virological response was defined as qualitative negative by qualitative Amplicor assay (Mitsubishi Kagaku BCL, Tokyo, Japan). In addition, HCV quantitative analysis was conducted by Amplicor HCV monitor method (Mitsubishi Kagaku BCL, Tokyo, Japan) with a detection limit of 100 copies/mL.

Qualitative and quantitative analyses of HCV RNA were performed immediately before and 4, 12, and 24 weeks after the start and 4, 12, and 24 weeks after the end of treatment. Viral levels ≥100 kcopies/mL were considered high. Genotype was determined immediately before the start of treatment by RT-PCR (Mitsubishi Kagaku BCL, Tokyo, Japan). All liver tissue was evaluated by the same examiner.

2.2. Enrollment and exclusion criteria

Enrollment criteria for the two studies were: (1) abnormal ALT and HCV RNA-positive in tests conducted within 12 weeks before the start of treatment; (2) HCV genotype 1, and HCV genotype 2 nonresponders and relapsers to prior IFN therapy; (3) age 20–64 years; (4) hemoglobin ≥12 g/dL and platelet count ≥100,000 mm^{-3} within 12 weeks before the start of treatment; (5) availability to stay in hospital for 4 weeks after the start of treatment; and (6) agreement to take contraceptive measures during and for 6 months after the end of treatment. Patients with the following characteristics were excluded: (1) pregnant or possibly pregnant and lactating women; (2) depression tendency; (3) severe complications; (4) hepatitis C complicated by other types of hepatitis; (5) liver cirrhosis or cancer as diagnosed in tests conducted within 12 weeks before the start of treatment; (6) history of hepatic encephalopathy, rupture of esophageal varices, or ascites; (7) HIV coinfection; (8) taken antiviral therapy or immunotherapy within 12 weeks before the start of treatment; (9) previous ribavirin therapy; and (10) history of allergy to IFN or nucleoside analogues.

2.3. Subgroup analysis

Baseline HCV RNA levels were categorized into three groups: 100 to <500; 500 to <850; and ≥850 kcopies/mL as determined by Amplicor HCV monitor assay. Disappearance of virus and relapse were judged by qualitative Amplicor assay. The time of initial HCV RNA negativity was recorded as the measurement time point (4, 12, and 24 weeks after the start of treatment) at which negativity was first observed; the time of initial relapse was the three measurement time point (4, 12, and 24 weeks after the end of treatment) at which HCV RNA was first detected in patients who achieved HCV RNA negativity during the treatment period. Patients who remained HCV RNA-negative for 6 months after the end of treatment were considered to have achieved SVR.

2.4. Statistical analysis

HCV RNA negativity rate, SVR rate, and relapse rate were compared by baseline viral load between the combination treatment and IFN monotherapy groups by Mantel–Haenzel test using modified RIDIT scores after the lack of interactions in efficacy was confirmed by the Breslow–Day test. Logistic regression analysis was used to identify factors contributing to initial HCV RNA negativity and SVR. The degree of risk of relapse was analyzed using the proportional hazards and grouped exponential models. Intergroup differences in patient profiles were tested by Fisher's exact test, Wilcoxon–Mann Whitney test, and Mantel–Haenzel test. $P < 0.05$ was regarded as statistically significant (two-sided). All calculations were performed by SAS program version 6.12 (SAS Institute, Cary, NC).

3. Results

3.1. Patient characteristics

Table 1 shows the main characteristics of the 209 patients in the combination treatment and 125 patients in the IFN monotherapy groups. In the study in nonresponders and relapsers to previous IFN therapy [26] 41 and 40 patients were allocated to the combination treatment and IFN monotherapy groups, respectively; in the study in patients with genotype 1b and high viral titers, the numbers were 168 and 85, respectively (i.e. 2:1 randomization) [25]. A total of 107 patients (51%) in the combination treatment and 68 (54%) in the IFN monotherapy group had HCV RNA levels ≥850 kcopies/mL. About half of patients in both treatment groups were relapsers after previous IFN therapy. Forty-nine patients (23%) in the combination treatment and 24 (19%) in the IFN monotherapy group had not received prior IFN therapy. No imbalance was observed in background variables between the two groups.

Table 1
Baseline patient characteristics

	IFN + ribavirin	IFN	*P* value
No. of patients	209	125	–
Sex (male/female)	164/45	94/31	0.503[a]
Mean age (years)	48	49	0.539[b]
Viral load (kcopies/mL)			0.792[c]
Low (<500)	23.0% (48)	24.8% (31)	
Moderate (500 to <850)	25.8% (54)	20.8% (26)	
High (≥850)	51.2% (107)	54.4% (68)	
Previous IFN therapy			0.295[ad]
Treatment-naive	23.4% (49)	19.2% (24)	
Relapsers	50.7% (106)	50.4% (63)	
Nonresponders	22.5% (47)	30.4% (38)	
Unknown	3.3% (7)	0	

[a] Fisher test.
[b] *U*-test.
[c] Mantel–Haenzel test.
[d] Excluding unknown.

3.2. Response to therapy

The SVR rate was 18% (38/209) with IFN and ribavirin combination therapy and 2% (2/125) with IFN monotherapy. The results of subgroup analysis by baseline viral levels are shown in Table 2. Patients receiving IFN and ribavirin combination therapy had a significantly higher chance for SVR than those receiving IFN monotherapy at any baseline viral level.

3.3. Initial viral negativity

In patients with viral titers of 500 to <850 kcopies/mL, initial viral negativity occurred in 20% by the first 4 weeks, in 46% by 12 weeks, and in 17% by 24 weeks of treatment in the combination therapy group (Fig. 1a). In the IFN monotherapy group the figures were 15%, 31%, and 12%, respectively (Fig. 1b). In patients with viral titers of ≥850 kcopies/mL, the HCV negativity rates at the same time points were 11%, 50%, and 20%, respectively, in the combination therapy group (Fig. 1a) and 1%, 28%, and 15%, respectively, in the monotherapy group (Fig. 1b). The time to initial viral negativity was slightly earlier in patients with viral titers of <500 kcopies/mL (42%, 25%, and 6% at the same time points, respectively) than in those with ≥500 kcopies/mL in the combination therapy group (Fig. 1a). Logistic regression analysis indicated that low HCV RNA levels and high ALT and creatinine levels before treatment are factors related to achieving HCV RNA negativity by week 4 of combination therapy. High baseline creatinine level was associated

Table 2
SVR rate by baseline viral load

Viral load (kcopies/mL)	IFN + ribavirin (n = 209)	IFN (n = 125)	*P* value
Low (<500)	29% (14/48)	0 (0/31)	0.001
Moderate (500 to <850)	17% (9/54)	8% (2/26)	0.001
High (≥850)	14% (15/107)	0 (0/68)	0.001

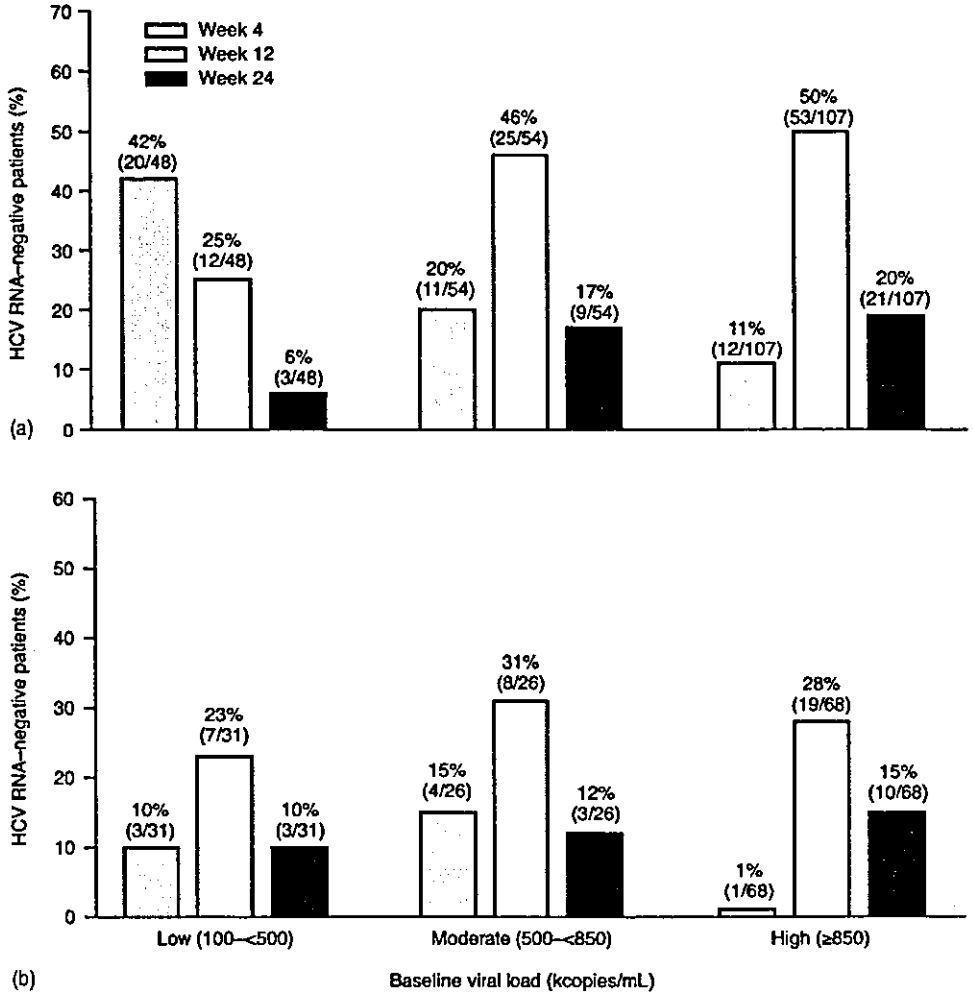

Fig. 1. Percentage of patients testing HCV RNA negative receiving combination therapy (a) and monotherapy (b). Patient numbers are in parentheses.

with achieving HCV RNA negativity by week 12 of treatment

3.4. SVR by baseline viral level and timing of initial viral negativity

Fig. 2 shows the SVR rate with respect to the timing of initial viral disappearance for each baseline HCV level. In patients with <500 kcopies/mL, SVR was observed only in those HCV RNA-negative within 4 weeks after the start of combination treatment, with a high SVR rate of 70% (14/20). However, in patients HCV RNA-negative by week 12 or 24, SVR was not observed in either treatment group. In the IFN monotherapy group, three patients were HCV RNA-negative within 4 weeks and the SVR rate was 0% (0/3). Among patients with 500 to <850 kcopies/mL, SVR was observed in 55% (6/11) and 12% (3/25) of patients HCV RNA-negative within 4 and 12 weeks of the start of combination treatment, respectively, and in none of the nine patients HCV RNA-negative within 24 weeks. Among patients treated with IFN alone, SVR was observed only in 50% (2/4) of patients HCV RNA-negative within 4 weeks. In patients with ≥850 kcopies/mL at baseline, SVR was observed in the combination treatment group in 42% (5/12) and 19% (10/53) of patients HCV RNA-negative within 4 weeks and 12 weeks, respectively. However, SVR was not seen in any of the 21 patients HCV RNA-negative within 24 weeks. SVR was not observed in patients with viral levels ≥850 kcopies/mL treated with IFN alone.

In patients HCV RNA-negative within 4 weeks, low baseline viral levels and high body weight were factors contributing to SVR; in those who were HCV RNA-negative within 12 weeks, low baseline viral levels and high baseline platelet count were contributing factors.

3.5. Relapse rate after end of treatment by baseline viral levels

In the combination treatment group, the relapse rate was 60% (21/35), 80% (36/45), and 83% (71/86) and in the IFN (-2b alone group 100% (13/13), 86% (13/15), and 100% (30/30) in patients with baseline viral levels <500, 500 to

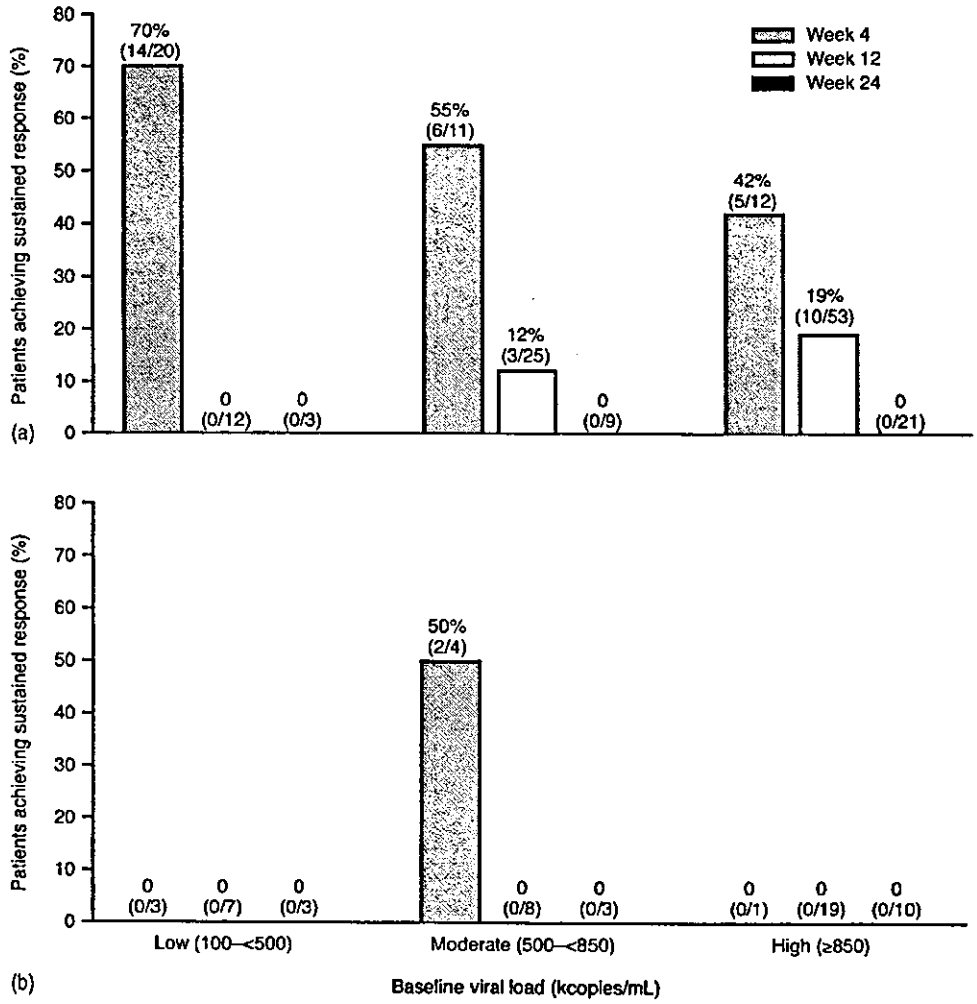

Fig. 2. Sustained response rate in patients receiving combination therapy (a) and monotherapy (b). Patient numbers are in parentheses.

<850, and ≥850 kcopies/mL, respectively (Table 3). Patients in the combination treatment group were 3.7 times (95% CI 1.9–7.3; $P < 0.001$) less likely to relapse than those in the IFN monotherapy group. If the probability of relapse in patients with <500 kcopies/mL is set at 1, then probability was 1.5 (95% CI 0.8–2.9) in patients with 500 to <850 kcopies/mL and 2.0 (95% CI 1.1–3.5) in patients with ≥850 kcopies/mL. However, baseline viral level was not a significant factor ($P = 0.3441$) with regards to risk of relapse. On the other hand, patients HCV RNA-negative within 12 and 24 weeks after the start of treatment were 4.8 (95% CI 2.8–8.1) and 10.3 (95% CI 5.4–19.7) times, respectively, more likely to relapse after the end of treatment than patients HCV RNA-negative within the first 4 weeks ($P < 0.001$).

Table 3
Rate of relapse at 6 months by baseline viral load

Viral load (kcopies/mL)	IFN + ribavirin ($n = 209$)	IFN ($n = 125$)	P value
Low (<500)	60% (21/35)	100% (13/13)	0.001
Moderate (500 to <850)	80% (36/45)	87% (13/15)	0.001
High (≥850)	83% (71/86)	100% (30/30)	0.001

The relative risk of relapse after the end of treatment by initial viral level and time of first viral negativity is shown in Table 4. The risk of relapse in the combination group versus in the IFN monotherapy group was significantly lower by a factor of 0.3 (95% CI 0.1–0.9) and 0.5 (95% CI 0.3–0.8) in patients HCV RNA-negative within 4 weeks ($P = 0.032$) and 12 weeks ($P = 0.011$), respectively. Regarding baseline HCV levels, in patients HCV RNA-negative by 12 weeks, the

Table 4
Relative risk of relapse at 6 months (95% CI)

	First HCV RNA-negative test result		
	4 weeks	12 weeks	24 weeks
Treatment group			
IFN + ribavirin	0.3 (0.1–0.9)*	0.5 (0.4–0.9)*	1.2 (0.6–2.2)
IFN	1	1	1
Baseline viral load (kcopies/mL)			
Low (<500)	1	1	1
Moderate (500 to <850)	0.9 (0.4–2.7)	0.6 (0.4–1.2)	0.8 (0.3–2.3)
High (≥850)	2.1 (0.8–5.6)	0.5 (0.3–0.9)†	0.9 (0.4–2.2)

* $P < 0.05$ vs. monotherapy.
† $P < 0.05$ vs. low baseline viral load.

hazard for relapse was significantly higher (odds ratio: 0.5; $P = 0.021$) in patients with ≥850 kcopies/mL than in those with <500 kcopies/mL. In patients HCV RNA-negative by 24 weeks, no effect on hazard for relapse was observed by treatment group or viral levels.

The relationship between baseline HCV levels and the time of relapse by time of initial HCV negativity is shown in Table 5. The relapse rate in patients HCV RNA-negative within 4 weeks with combination treatment was ≤18% at 4, 12, and 24 weeks after the end of treatment regardless of baseline viral level. In the IFN(-2b alone group, almost all patients relapsed soon after the end of treatment even when HCV RNA-negativity occurred within the first 4 weeks after the start of treatment. The circumstance of relapse in patients HCV RNA-negative within 12 weeks of the start of treatment differed from that in patients HCV RNA-negative within 4 weeks. In those receiving combination treatment, relapse was seen within 4 weeks in 11 (92%), 13 (52%), and 30 (57%) patients whose baseline viral levels were <500, 500 to <850, and ≥850 kcopies/mL, respectively. Relapse within 12 weeks was seen in 1 (8%), 5 (20%), and 13 (26%) patients, respectively. However, even with combination treatment, most patients who first became HCV RNA-negative after 12 weeks from the start of treatment relapsed within 4 weeks after the end of treatment (data not shown)

4. Discussion

In Japan, various IFN regimens for the treatment of CHC have been tried. Under the Japanese health insurance system, the duration of treatment was restricted to 6 months at the time that the present study was conducted. Standard treatment comprises high doses of IFN (6–10 MIU) administered daily in the initial stage of treatment followed by further doses at three times/week for ≤6 months with the aim of eradicating the virus [3]. In 1998, remarkable improvement in efficacy was reported when ribavirin is added to IFN α-2b [21–23], and clinical studies of IFN α-2b plus ribavirin combination therapy were initiated in Japan. Outside Japan, the standard treatment regimen with IFN α-2b was 3 MIU administered three times/week; for combination therapy, ribavirin was added to this standard regimen. The clinical studies in Japan were likewise conducted with ribavirin (600 or 800 mg/day depending on body weight) added to the standard Japanese regimen. When SVR rates by baseline viral levels were compared, combination therapy was superior to monotherapy at all viral levels. A number of reports have been published concerning the timing of first HCV RNA disappearance and its effect on the SVR rate [13–18,27]. To date, however, no study of the SVR rate analyzed in relation to HCV RNA levels has been published. The present study suggests that the timing of first disappearance of HCV RNA is significantly affected by baseline HCV RNA levels. In patients with low baseline viral levels (100 to <500 kcopies/mL), 42% became HCV RNA-negative in comparison with only 11% with high viral levels (≥850 kcopies/mL) following 4 weeks' combination therapy. The study suggests that >4 weeks' treatment is required to achieve HCV RNA negativity in patients with viral levels >500 kcopies/mL. Moreover, with IFN alone the proportion of patients achieving HCV RNA negativity within 4 weeks was especially low among those with HCV genotype 1b and high viral levels; >4 weeks' treatment is required to achieve HCV RNA negativity in this group. Vrolijk et al. [5] reported that when ribavirin was administered in combination with IFNα-2b, HCV RNA negativity was observed by week 4 in nearly half of patients and all patients achieved SVR when treatment was continued for 1.5 years. Tassopoulos et al. [8] reported that when ribavirin was administered in combination with 10 MIU IFNα-2b for 8 weeks, almost half of patients achieved HCV RNA negativity. Treatment was continued thereafter for 48 weeks, and the final SVR rate was roughly 25%. The differences between these studies conducted outside Japan and our results may be explained by the high viral levels in our patients. We also noted that low HCV RNA, high ALT, and high creatinine levels before the start of dosing were factors associated with early disappearance of HCV RNA after treatment was initiated. High serum creatinine levels are related to high serum ribavirin concentrations [28], and this may explain early HCV RNA disappearance.

Kasahara et al. [29] compared the results of 6-month and 1-year treatment and reported that judging from the degree of improvement in ALT, longer duration of treatment with IFN monotherapy may inhibit relapse after the end of treatment. However, no significant difference of SVR rate in CHC patients with genotype 1b and high viral levels between 52 weeks and 78 weeks treatment with IFN monotherapy was reported [30]. Poynard et al. [22] reported that the relapse rate with combination therapy after 48 weeks of treatment in patients not previously treated with IFN and including patients with HCV genotypes other than genotype 1 was significantly lower than after 24 weeks of treatment. McHutchison et al. [23] also reported a similar trend. Portal et al. [6] compared the relapse rate in HCV genotype 1 patients with high viral levels treated with IFN plus ribavirin for 1 year or IFN plus ribavirin for 6 months followed by IFN monotherapy for 6 months, and observed a significantly higher relapse rate in the latter group, indicating the importance of duration of combination treatment in reducing the relapse rate. Although our study of 6-month combination treatment in genotype 1 patients was not adequate to analyze the effects of duration of treatment, our analysis of HCV RNA levels in relation to relapse revealed that relapse is much more likely in patients with high rather than low baseline viral levels. Furthermore, compared with in patients who were HCV RNA negative within 4 weeks, the relative risk for relapse is significantly higher in patients HCV RNA-negative at both 4–12 weeks and 13–24 weeks after the start of treatment. Relative risk of relapse is also reduced by about 0.5 with combination therapy compared with monotherapy. Moderate antiviral effects of ribavirin remaining in the body for long periods after end of

Table 5
Relapse rate by baseline HCV level in patients HCV RNA-negative within 4 weeks and within 12 weeks

	Baseline viral load in patients receiving combination therapy (kcopies/mL)			Baseline viral load in patients receiving monotherapy (kcopies/mL)		
	Low (<500)	Moderate (500 to <850)	High (≥850)	Low (<500)	Moderate (500 to <850)	High (≥850)
(a) HCV RNA-negative within 4 weeks						
Relapse						
4 weeks	15% (3/20)	18% (2/11)	17% (2/12)	67% (2/3)	50% (2/4)	100% (1/1)
12 weeks	5% (1/20)	9% (1/11)	17% (2/12)	0 (0/3)	0 (0/4)	0 (0/1)
24 weeks	10% (2/20)	18% (2/11)	17% (2/12)	0 (0/3)	0 (0/4)	0 (0/1)
Unknown	–	–	8% (1/12)	33% (1/3)	–	–
Total	30% (6/20)	45% (5/11)	55% (7/12)	100% (3/3)	50% (2/4)	100% (0/1)
(b) HCV RNA-negative within 12 weeks						
Relapse						
4 weeks	92% (11/12)	52% (13/25)	57% (30/52)	100% (7/7)	100% (8/8)	84% (16/19)
12 weeks	8% (1/12)	20% (5/25)	26% (13/53)	0 (0/7)	0 (0/8)	5% (1/19)
24 weeks	0 (0/12)	4% (1/25)	0 (0/53)	0 (0/7)	0 (0/8)	5% (1/19)
Unknown	–	12% (3/25)	–	–	–	5% (1/19)
Total	100% (12/12)	88% (22/25)	81% (43/53)	100% (7/7)	100% (8/8)	100% (19/19)

Intergroup within 4 weeks: $P = 0.0319$; time $P = 0.0026$, intergroup within 12 weeks: $P = 0.0109$; time $P = 0.0001$.

treatment might explain the better end-of-treatment response with combination therapy [31]. Although HCV eradication may not be expected in patients HCV positive at 12 weeks, combination therapy should be continued so as to suppress liver inflammation and progression of liver cirrhosis. Moreover, the duration of treatment should be 12 months in patients with genotype 1 and high viral levels.

Many attempts have been made to improve the efficacy of combination therapy. Extending the dosing period from 6 months to 1 year does not affect the HCV RNA negativity rate at the end of treatment [22,23]; improved efficacy with the longer course is attributed to decreases in relapse rate after the end of treatment [32]. Thus it is necessary to conduct a prospective study to determine the optimal duration of combination treatment after HCV RNA becomes negative to improve the efficacy of IFN plus ribavirin combination therapy.

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A significant reduction in serum alanine aminotransferase levels after 3-month iron reduction therapy for chronic hepatitis C: a multicenter, prospective, randomized, controlled trial in Japan

Motoyoshi Yano[1], Hisao Hayashi[9], Kentaro Yoshioka[1], Yutaka Kohgo[2], Hiroyuki Saito[2], Yoshiro Niitsu[3], Junji Kato[3], Shiro Iino[4], Hiroshi Yotsuyanagi[5], Yoshimasa Kobayashi[6], Kinya Kawamura[6], Shinichi Kakumu[7], Masahiko Kaito[8], Jiro Ikoma[8], Shinya Wakusawa[9], Takeshi Okanoue[10], Yoshio Sumida[10], Fumiaki Kimura[11], Eiji Kajiwara[12], Michio Sata[13], and Kei Ogata[13]

[1]Third Department of Internal Medicine, Nagoya University School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Japan
[2]Third Department of Internal Medicine, Asahikawa Medical College, Asahikawa, Japan
[3]Fourth Department of Internal Medicine, Sapporo Medical University School of Medicine, Sapporo, Japan
[4]Department of Medicine, Seizankai Kiyokawa Hospital, Tokyo, Japan
[5]Department of Gastroenterology-Hepatology, St. Marianna Medical College, Kawasaki, Japan
[6]Second Department of Internal Medicine, Hamamatsu University School of Medicine, Hamamatsu, Japan
[7]Division of Gastroenterology, Department of Medicine, Aichi Medical University School of Medicine, Aichi, Japan
[8]Third Department of Internal Medicine, Mie University School of Medicine, Tsu, Japan
[9]Department of Medicine, Faculty of Pharmaceutical Sciences, Hokuriku University, Kanazawa, Japan
[10]Department of Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Graduate School of Medicine, Kyoto, Japan
[11]Department of Internal Medicine, Tamano Municipal Hospital, Tamano, Japan
[12]Department of Gastroenterology, Nippon Steel Yawata Memorial Hospital, Kitakyushu, Japan
[13]Second Department of Internal Medicine, Kurume University School of Medicine, Kurume, Japan

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Background. Increasing evidence indicates that iron cytotoxicity plays an important role in the pathogenesis of chronic hepatitis C (CHC). However, the biochemical effects of iron reduction therapy on CHC remain to be confirmed in a controlled study. This study aimed to test whether iron removal by repeated phlebotomy improves serum alanine aminotransferase (ALT) levels in patients with CHC. ***Methods.*** Patients were randomly assigned to an iron reduction therapy or control group. The patients in the treatment group received 3-month iron reduction therapy by biweekly phlebotomy, while the patients in the control group were followed up for 3 months with regular blood tests alone. ***Results.*** Thirty-three patients completed the 3-month treatment, while 29 patients received the complete follow-up. The serum ALT levels were reduced from 118 ± 79 to 73 ± 39 IU/L in the treatment group, but did not change in the control group (106 ± 45 versus 107 ± 48 IU/L). Post-treatment enzyme activity was decreased significantly from the baseline. Furthermore, it was significantly lower than the 3-month control level. Although 5 patients withdrew from the study, none was affected by any side effects of repeated phlebotomy that required them to discontinue the treatment. ***Conclusions.*** This short-term controlled trial demonstrated the biochemical efficacy and safety of iron reduction therapy for patients with CHC.

Key words: oxidative stress, free radicals, phlebotomy

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Reprint requests to: M. Yano

Introduction

Without treatment, chronic hepatitis C (CHC) may result in hepatic cirrhosis or may be complicated by hepatocellular carcinoma. At present, interferon (IFN) is the only antiviral agent known to clear hepatitis C virus (HCV) RNA. A number of factors contribute to predicting the response to IFN, including the gender and age of the patient, the disease stage, viral load, and HCV genotype.[1] Ribavirin, an antiviral nucleotide analogue, reduces the viral load of patients, resulting in an enhanced response with IFN when combined.[2] Recently, it was reported that the use of pegylated IFN improved the response rate of CHC.[3–6] When pegylated IFN is combined with ribavirin, the elimination rate might be increased to 50%.[7,8] However, IFN monotherapy or combination therapy is not completely effective and the majority of patients with CHC do not receive any benefit from these antiviral agents. Therefore, alternative therapies are required.

Iron reduction therapy for CHC was first introduced in practice based on the histochemical detection of iron deposits in the liver,[9] and then on the detection of lyso-