IFN Receptor

IFN-α and IFN-β are classified as type I IFN whereas the mitogen-induced interferon gamma (IFN-γ) is a type II IFN. Type I IFNs are a family of homologous cytokines, which potently elicit an anti-viral and anti-proliferative state in cells. All human type I IFNs bind to a cell surface receptor consisting of two transmembrane protein, IFNAR1 [1] and IFNAR2 [2], which associate upon binding (Fig. 1).

IFNAR1, responding mainly to IFN-αβ, has been cloned from human cells by Uze et al. in 1990 [1]. However, the response of this receptor to other human IFN-α subtypes and to human IFN-β was very low. IFNAR1 displays low affinity for most human IFN-α isoforms and appears to be primarily a signal transducing subunit.

The ligand binding components of type I receptor was cloned by Novick et al. in 1994 [2]. This component (IFNAR2) responds equally well to IFN-β and to almost all IFN-α subtypes and is regarded as the major receptor to type I IFN. A soluble form of human IFNAR2 was first purified as an IFN-α binding protein from urine (IFNAR2a). Its identification led to the cloning of its own cDNA and those of two membrane-associated forms, human IFNAR2b and human IFNAR2c. Although all three forms bind type I IFN, only the long form (IFNAR2c) participates in signal transduction [3]. The functions of human IFNAR2a (extracellular soluble form) and human IFNAR2b, which possesses only a short intracellular domain, remain unclear. IFNAR2b is usually expressed at lower levels than IFNAR2c and may exert a dominant negative effect on IFN-α signaling [4].
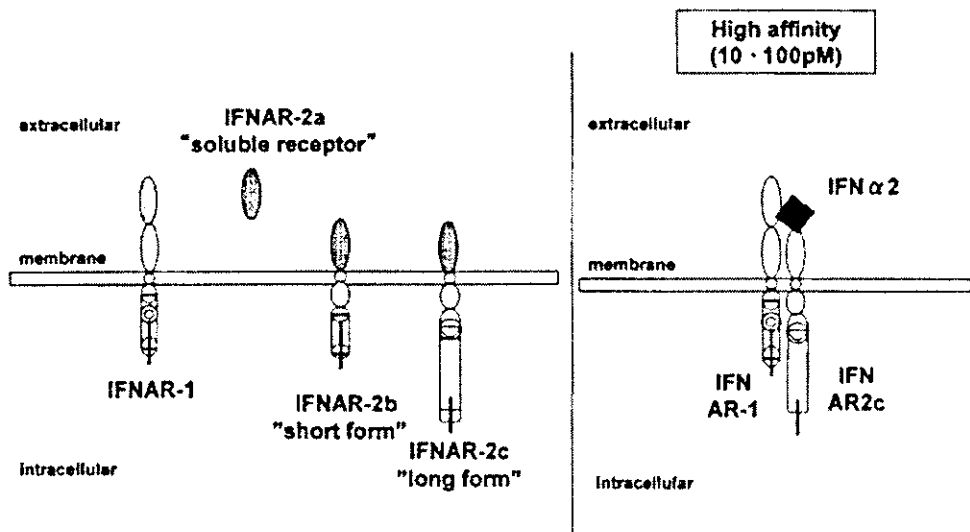

Fig. 1. Schematic representation of type I IFN receptors. IFNAR1 and three kinds of IFNAR2 (IFNAR2a: soluble form, IFNAR2b: short form, IFNAR2c: long form).

IFNAR2a (extracellular soluble form) is generally believed to be an antagonist for IFN signaling. Soluble forms of cytokine membrane receptors usually are produced either by proteolytic cleavage from a membrane form or by alternative splicing of mRNA precursor. Since these soluble forms often can bind ligand, they initially were proposed to be antagonists, blocking or, at the very least, reducing cytokine potency by competing with their membrane-anchored form for common ligands. As a result, recombinant extracellular portions of some receptors are being used to therapeutically reduce inflammatory, autoimmune, and other pathological events mediated by cytokine [5]. Another potential function for soluble receptor forms is to protect ligand from degradation or excretion. A soluble receptor subunit also may convert a ligand-resistant target into a sensitive one by substituting for an absent endogenous binding subunit. Recently, another new functional possibility was reported that the soluble extracellular domains of receptor subunits can mimic the activity of a cytokine even when the cytokine itself is absent [6].

Analysis of Hepatic IFNAR2 Expression by Immunohistochemical Techniques

We had developed quantitative analysis of hepatic IFNAR2 expression using immunohistochemical techniques [7]. We compared IFNAR2 expression in patients between viral and non-viral liver disease and found out the relationship between IFNAR2 expression and response to IFN. Liver biopsy samples were evaluated using an indirect immunoperoxidase staining technique. For this purpose, we used the specimens embedded in paraffin. The indirect immunoperoxidase method was employed with IgG class anti-IFNAR2 monoclonal antibody (mAb) developed by Muraguchi and Ohmoto (Cellular Technology Institute, Othsuka Pharmaceutical, Tokushima, Japan) and horseradish peroxidase-labeled anti-mouse rabbit IgG Fab fragments (MBL Co., Nagoya, Japan), as reported previously. Immunostaining was performed on streptavidin-biotin (Histofine SAB-PO (M) kit, Nichirei, Tokyo) stained sections. Computer-image quantitative analysis was performed in the following manner on specimens stained with SAB method. The staining intensity in each image (x400) was quantified by densitometry using an image analysis software. The expression intensity in one normal control sample was defined as 1 Unit (U).

Comparison of Hepatic IFNAR2 Levels in Viral and Non Viral Liver Disease

A diffuse staining of IFNAR2 was observed in hepatocytes, with a strong staining in the cytoplasm but weak staining in the cell membrane. A weak expression of IFNAR2 was detected in lymphocytes present in the portal area, compared with

that of hepatocytes. This is the first report of immunohistochemical analysis of hepatic IFNAR2 expression in patients with CHC. With regard to in situ distribution of IFNAR2 in hepatocytes, this staining pattern with a diffuse strong staining in the cytoplasma could be a subject to various interpretations. The main explanation is that the cytoplasmic pattern of hepatocellular reactivity might indicate receptor synthesis in hepatocytes or reflect internalization of this receptor. Similar results were reported by Navarro et al. [8] in the study of the expression of IFNAR1 and by Volpes et al. [9] in the study of IFN-γ.

The mean expression level of hepatic IFNAR2 in patients with acute hepatitis A (3.1±1.1 U, n=9) was significantly (P<0.01) higher than in chronic HCV infection (1.9±1.2 U, n=55) or control subjects (1.3±0.4 U, n=9). There was no significant difference in the mean expression level of hepatic IFNAR2 in patients with CHC infection and control subjects. These results demonstrated a significantly high expression of hepatic IFNAR2 in patients with acute hepatitis A compared with controls. Although there was no significant difference in hepatic IFNAR2 expression between patients with CHC and controls, the level of expression was higher in CHC than controls. Based on these results, we believe that hepatitis viral infection induces an increase in the expression of hepatic IFNAR2 and acute viral infection may result in a strong up-regulation of hepatic IFNAR2 compared with chronic viral infection. Although the precise stimulus responsible for enhanced hepatic IFNAR2 expression in hepatitis viral infection was not defined, such increase is compatible with the human protective system against viral infection. Our results are similar to those of previous studies showing a diffuse expression of IFN-γ receptor throughout the liver parenchyma and strong cytoplasmic as well as membranous and perinuclear reactivity in acute hepatitis [9].

Patients with chronic HCV infection were divided into four groups based on the degree of liver fibrosis (F 0-3). The mean expression level of hepatic IFNAR2 was 2.06±1.15 U in 14 patients with F 0, 2.00±1.16 U in 26 patients with F 1, 1.85±1.38 U, in 8 patients with F 2 and 0.89±0.38 U in 5 patients with F 3. Severe liver fibrosis was associated with a significant decrease in IFNAR2 levels (F0 vs F 3, P<0.01. F 1 vs F 3, P<0.05). Our results also demonstrated a close relationship between the expression of hepatic IFNAR2 and the extent of liver fibrosis in patients with CHC. These results are compatible with those of previous reports showing that the therapeutic effectiveness of IFN diminishes with the progression of histopathological abnormalities, and that a sustained response to IFN therapy in patients with CHC is influenced by the extent of hepatic fibrosis. In this regard, Ishimura et al. [10] also reported that the absence of IFN-α receptor mRNA was associated with severe fibrosis of the liver. Other investigators also identified IFNAR2 sites on hepatocytes in patients with chronic hepatitis C with and without cirrhosis [11]. In these studies, fewer IFNAR2 sites were identified on hepatocytes of patients with HCV-related liver cirrhosis than in chronic hepatitis C, using light microscopic autoradiography with radiolabeled HLBI. Combined together, these results suggest that a low expression of hepatic IFNAR2 might be partially responsible for the poor IFN response in liver cirrhosis.

Correlation Between Level of Hepatic IFNAR2 Levels and Response to IFN Therapy

We also examined the correlation between the outcome of IFN treatment and the level of hepatic IFNAR2 in 55 patients with chronic HCV infection [7]. Fifteen of 55 (27%) patients ,whose post-treatment level of serum HCV-RNA diminished to undetectable levels on RT-PCR, were judged as sustained viral responders (SVR). In contrast, serum HCV-RNA was still detectable after treatment in the remaining 40 (73%) non-sustained viral responders (Non-SVR) (Fig. 2).

The mean level of hepatic IFNAR2 in SVR (2.7±1.1U, n=15) was higher than that of Non-SVR (1.6±1.1 U, n=40) (P<0.001). Univariable and multivariable logistic regression analysis were also performed to evaluate the various factors influencing the efficacy of IFN therapy, such as the hepatic IFNAR2 level (IFN-R level), serum HCV-RNA level, HCV serotype, ALT level, age, gender, degree of fibrosis and inflamatory activity of the liver. Univariable logistic regression analysis showed that IFN-R level, HCV-RNA level, HCV serotype and degree of inflamatory activity of the liver achieved statistical significance and they were subjected to multiple logistic regression. Multivariate analysis was performed using these four factors. Among these factors, both IFN-R level (P=0.0056) and HCV-RNA level (P=0.0097) were important factors influencing the efficacy of IFN therapy in this study (Table 1).
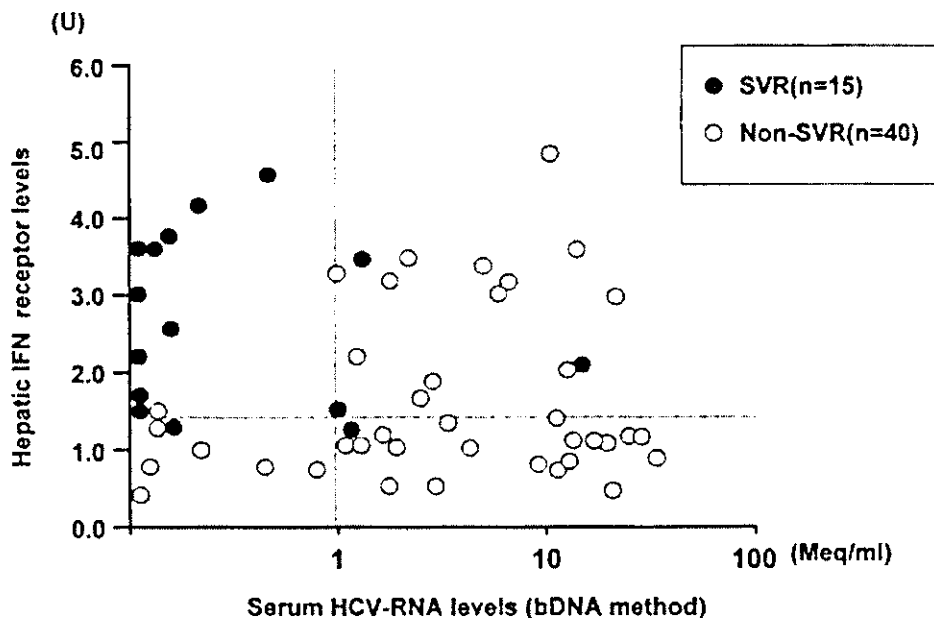

Fig. 2. Relationship between response to IFN therapy, level of expression of hepatic IFNAR2 and serum HCV-RNA levels in 55 patients with chronic hepatitis C. SVR: Sustained Responders (n=15), Non-SVR (n=40).

Table1. Predictive factors of the effectiveness of IFN monotherapy in patients (n=55) with chronic hepatitis C by multivariable logistic regression analysis

Predictor	Estimate	S.E.	P-value
IFN-AR2 level	− 1.4578	0.5266	0.0056
HCV-RNA level	0.3403	0.1316	0.0097
Activity (liver biopsy)	− 1.3815	0.7018	0.0490
HCV-genotype	− 1.2536	1.0601	0.2370

We observed a clear relationship between response to IFN therapy and the level of expression of hepatic IFNAR2. Notwithstanding the wide experience in the use of IFN treatment in CHC, little is known about the host mechanisms that influence the response, or the lack of response, to this agent in individual cases. Because the receptor is a key element in the IFN response, we examined the usefulness of a monoclonal antibody that recognizes IFNAR2 in monitoring the expression of type I IFN receptor in patients with liver disease. In our study, pretreatment levels of hepatic IFNAR2 expression were significantly higher in patients with SVR than Non-SVR, suggesting that a poor response to IFN might be due to a deficient expression of hepatic IFNAR2 in CHC.

In confirmation of the results of previous studies, SR in the current study had low pretreatment serum HCV RNA levels than NR, and the frequency of a sustained response in patients infected with HCV genotype Ib (serotype 1) was less than in those infected with other HCV genotypes (serotype 2). Both serum HCV RNA levels and HCV genotypes are considered as strong predictors of sustained response. Interestingly, our results showed that among patients with low level viremia or HCV serotype 2 who were expected to have SVR, hepatic IFNAR2 in these patients were significantly higher than in Non-SVR. To explain the reason of IFN resistance in patients with favorable virus-related factors on the response to IFN therapy, the level of hepatic IFN receptor should be considered as an important patient-related factor influencing the response to IFN therapy (Fig. 2). Logistic analysis in this study also demonstrated that the level of hepatic IFN receptor was an important predictor of IFN therapy. Furthermore, the monitoring of expression of type I IFN receptor in patients before IFN therapy may help to predict treatment outcome. Indeed, this approach would provide a better management of IFN therapy by deciding the time of IFN treatment.

In conclusion, we demonstrated that the expression level of hepatic IFNAR2 correlated with the response to IFN therapy in patients with CHC and that measurement of hepatic IFNAR2 levels might be useful in predicting the response to IFN therapy. Resistance to IFN treatment in patients with CHC might be due to low levels of hepatic IFNAR2. Further studies are necessary to understand the role of IFNAR2 and to allow the discovery of new therapies for patients resistance to treatment with IFN.

IFN Signals

As both the type I and type II IFN receptors lack intrinsic kinase domains, they require the association with a family of non-receptor tyrosine kinases known as Janus-Kinases (JAKs) to facilitate the tyrosine phosphorylation of STAT proteins. IFN exert their signals through receptor-associated Janus-Kinases (JAKs) and signal transducers by phosphorylation of STAT1 and STAT2. After binding to receptors, IFNs inititate gene activation through phosphorylation of intermediary proteins. The receptor-associated kinase Tyk2, JAK1 and JAK2, phosphorylate STAT1 and STAT2 (Fig. 3) [12].

Type I IFN (IFN-α and IFN-β) induces the formation of a heterotrimetric transcription factor complex, interferon-stimulated gene factor 3 (ISGF3), which consists of signal transducers and activators of transcription 1 and 2 (STAT1 and STAT2) and p48 (ISGF3γ), after binding to its receptor. ISGF3 translocates into the nucleus and binds to to a specific gene sequence termed the IFN-stimulated response element (ISRE), characterized by a conserved 15 base pair element. The ISRE has a consensus sequence GAAN(N)GAAA (N=any nucleotide) to a specific gene sequence termed the IFN-stimulated response element (ISRE), characterized by a conserved 15 base pair element. The ISRE has a consensus sequence GAAN(N)GAAA (N=any nucleotide). The ISRE is a necessary and sufficient component of the induction of many genes by IFN [13].

IFN-γ binds a different receptor and activates STAT1, which, as a homodimer, activates a related but distinct nucleotide sequence resulting in transcription of
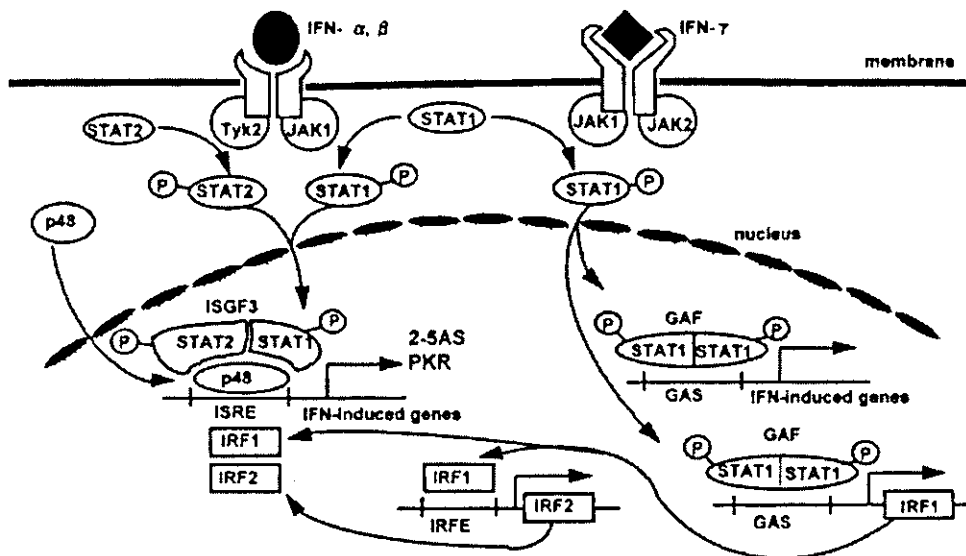

Fig. 3. The signal transduction pathway for IFNs

different genes and synthesis of a different set of proteins. An IFN-γ activation site (GAS) is composed of the consensus nucleotide sequence AANNNNNTT (N=any nucleotide) and confers responsiveness to IFN-γ after binding of a.STAT-1a homodimer [13].

Interferon regulatory factor-1 (IRF-1) is another important factor induced by type I and II IFNs. Since the sequence of IRF-1 binding site termed IRF-E overlaps with ISRE, IRF-1 also binds to ISRE sequence and activates the interferon-inducible gene transcription. p48 and IRF-1 show a homology within their amino-terminal regions and are members of the IRF family together with other IRFs [14].

IFN induces some interferon-inducible genes such as 2,5 oligo-adenylate synthetase (2-5AS) and double-stranded RNA-dependent protein kinase (PKR), inhibit the post-transcriptional stages of viral replication. The 2-5AS are a family of enzymes that convert ATP into unusual 2,5-oligo-adenylates (2-5A), which in turn activate a dominant ribonuclease, RnaseL, to degrade viral RNAs. The other antiviral enzyme, PKR, inhibits viral protein synthesis. It is activated by autophosphorylation of serine/threonine residues in the presence of dsRNA. Activated PKR phosphorylates eukaryotic translation initiation factor 2α (eIF-2α). Phosphorylated eIF-2α cannot participate in protein synthesis. Therefore, inhibition of PKR is essential for viral replication [15].

Recently it has been reported that JAB (JAK-binding protein), as a new molecule, strongly inhibits IFN-signaling by blocking JAK activity through directly binding to its kinase domain, and the loss of responsiveness to the antiviral effect of IFN-β in NIH-3T3 fibroblasts ectopically expressed JAB. It has been suggested that JAB might provide the influence on unresponsiveness and resistance to the IFN-therapy in some patients with viral disease [16].

Correlation Between Level of Hepatic IFN-Related Molecules and Response to IFN Therapy in Patients with Chronic Hepatitis C

Recently A.Katsume and M.Kohara, in the Department of Microbiol & Cell Biology in Tokyo Metro Institute Medical Science, developed quantitative method of IFN signals using real time detection polymerase chain reaction (RTD-PCR) based on Taq-Man chemistry. We examined IFN signals in liver tissue obtained from 22 patients with chronic hepatitis C later treated with IFN. According to viremia status after treatment, we divided into two groups: Sustained viral responders (SVR:n=9) and Non-sustained viral responders (Non-SVR:n=13). We examined mRNA level (expressed as Unit:U) of IFN receptor (IFN-R), double strand RNA-activated protein kinase (PKR), 2, 5 oligo-adenylate synthetase (2-5AS), interferon regulatory factor1(IRF1), IRF2, JAK-binding protein (JAB) and cytokine-inducible SH2 protein3 (CIS3) in liver tissue. It was shown that the mean levels of both IFN-R (1.195±0.311 U) and JAB (3.911±3.284U) in SVR were

significantly (P<0.01) higher than in NR (IFN-R:0.822±0.173U, JAB:1.471± 0.619). There was no difference of hepatic mRNA levels of PKR, 2-5AS, IRF1, IRF2 and CIS3 between SVR and Non-SVR (Table 2). This study also confirm that the levels of IFN-R are closely correlated with response to IFN therapy. But the expression levels of hepatic JAB mRNA, whose function was a strong inhibition of IFN-signaling by blocking JAK activity, were unexpectedly decreased in patients with Non-SVR group compared with SVR group. This paradoxical phenomenon may reflect the failure in IFN signal transduction in IFN resistant patients, because the expression of JAB is induced by IFN stimulation.

The action of IFN network such as IFN-R and JAB can contribute on the efficacy of IFN response. The resistance to IFN therapy might be due to low levels of hepatic IFN-R. A thorough understanding of intracellular IFN network that govern the IFN response will lay the foundation to identify the mechanisms that alter these effects in treatment of chronic hepatitis C.

Table 2. mRNA levels of several IFN related signals and IFN response in patients (n=22) with chronic hepatitis C

	SVR (n=9)	Non-SVR(n=13)	P-value
HCV-RNA	0.296±0.610	6.204±8.101	0.0424
IFN-R	1.195±0.311	0.822±0.173	0.0017
2-5AS	3.654±1.553	4.271±2.773	0.5539
PKR	1.507±0.637	1.272±0.621	0.3934
JAB	3.911±3.284	1.471±0.619	0.0157
CIS3	0.763±0.474	0.450±0.258	0.058
IRF1	4.648±1.343	3.595±1.530	0.1115
IRF2	3.633±0.953	3.062±0.674	0.1140

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Hepatitis B virus of genotype C persistence after recovery from acute hepatitis B virus infection in Japan

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Abstract

Background/aims: this study aimed to determine the viremia status after clinical, biochemical and serological recovery from acute hepatitis B viral (HBV) infection. *Methods:* we detected serum HBV-DNA in 19 patients with acute hepatitis B during followed-up 6–43 months after onset, and analyzed HBV genotypes. *Results:* 13 (72%) of 19 patients had detectable HBV DNA at the point of 6 months after onset, and four (33%) of 12 patients had persisted viremia for more than 1 year although they were recovery with normalization of alanine transaminase (ALT), disappearance of hepatitis B surface antigen (HBsAg) and appearance of antibody against HBsAg (anti-HBs). Eighteen (95%) of 19 patients were infected with HBV genotype C, one (5%) with genotype B. *Conclusions:* these results suggest genotype C of HBV is the predominant genotype of acute hepatitis B in Nagasaki region in Japan. HBV can persist in the serum for more than one year after complete clinical and serological recovery from acute viral hepatitis.
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Keywords: Hepatitis B virus; Acute hepatitis B; Viremia; Genotype; Precore mutation; Core promoter mutation; Direct sequencing

1. Introduction

Hepatitis B virus (HBV) DNA may persist in the serum and liver in patients with chronic hepatitis long after disappearance of hepatitis B surface antigen (HBsAg) and appearance of antibody against HBsAg (anti-HBs) in the natural course of the disease and after antiviral therapy [1–6]. Recent studies using polymerase chain reaction (PCR) have shown long-term persistence of HBV DNA in the serum and peripheral blood mononuclear cells (PBMCs) after serological recovery from acute hepatitis B [7–9].

It was confirmed in countries like Japan, where persistent HBV infection occurs almost exclusively from mother-to-newborn infection, seldom occurs

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after acute infection in adulthood, but in western countries, where HBV chronic carrier after acute infection in adulthood may be commonly observed. The different conditions of persisting HBV after acute HBV infection among those areas may be attributed to the HBV genomic variation. Recent advancement of genomic analysis has revealed at least seven genotypes (A–G) with distinct geographical distribution in the world [10–13]. Overall, genotype A is most prevalent in North Europe, North America and Africa; genotype B and C are mostly found in Asia; genotype D is predominant in the Middle East; genotype E is restricted to Africa; genotype F is found in American natives; genotype G is newly discovered in France and the US from patients chronically infected with HBV. Genotype A is a common genotype in Europe and about 10% of acute hepatitis B patients develop into chronic carrier state [14]. There were reports that adult Japanese patients became HBV carriers after acute hepatitis B; the causal HBV was genotype A[15,16]. These results suggest that genotype A has a tendency to cause persistent HBV infection after acute infection in adulthood.

The mutations in precore region and core promoter region have been studied in relation to HBeAg expression, liver disease severity as well as viral replication [17–24]. The mutations are often present in patients with fulminant hepatitis B, chronic hepatitis B and hepatocellular carcinoma, and less often in asymptomatic carriers and carriers without serological HBV markers. The mutations in preS1 region and preS2 region may contribute to viral persistence due to an evasion of the host immune surveillance [25].

It has been concerned that HBV DNA can persist in patients with resolved acute hepatitis B, but, the factors associated with becoming a HBV carrier after acute HBV infection in adulthood have not been well studied.

In this study, we detected serum HBV–DNA in patients with acute hepatitis B by enzyme-linked mini-sequence assay (ELMA) kit to determine whether HBV might be persistence in recovery stage, and analyzed HBV genotypes and mutations in precore region, core promoter region and preS region by direct sequencing to investigate the probable factors associated with persistent viremia.

2. Patients and methods

2.1. Patients

Nineteen Japanese patients who were admitted to the National Nagasaki Medical Center with acute HBV infection during the period 1990–1999 were studied. The diagnosis of acute hepatitis B was made based on the high titre of immunoglobulin (Ig) M antibody to hepatitis B core antigen (anti-HBc) in the absence of any evidence of acute infection with other hepatitis viruses. The diagnostic criteria for severe hepatitis was prothrombin time less than 40%. These patients were selected based on the availability of two or more serum samples over a follow-up period of at least 6 months. They included eight (42%) males and 11 (58%) females, aged 23–75 years (mean±S.D.: 39±18). The biochemical, serological data and serum samples were collected at various times (initiation, 6 months and more than 12 months after onset). Twelve patients were followed up for more than 12 months, seven patients for 6 months. Informed consent to the use of serum samples was obtained from each patient and study was approved by the Ethic Review Committee of Liver disease study in National Nagasaki Medical Center.

2.2. HBV DNA analysis

HBV DNA was extracted from 100 µl serum using a DNA/RNA extraction kit (Smitest EX-R&D, Sumitomo Metal Industries, Tokyo, Japan) according to the manufacturer's instructions. HBV DNA assay was performed with enzyme-linked mini-sequence assay (ELMA) kit (Nippon Roche, Tokyo, Japan), according to the manufacturer's instructions. The sensitivity of ELMA kit was to be reproducible until the HBV DNA concentration of 100 copies/ml [19].

2.3. HBV genotype and subtype assay

2.3.1. PCR amplification

The first PCR was performed on 5 μl extracted DNA in a 50-μl reaction mix containing 10 mmol/l Tris–HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 0.25 mmol/l of each of the dNTPs, 2.0 U Tag DNA polymerase (Takara Shuzo Co. Ltd. Japan), 0.25 μmol/l of each external primer (Table 1) and sterile water. Amplification was performed in a thermal cycler (GeneAmp PCR system 9600) under the following conditions: 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, followed by 38 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min and finally 94 °C for 1 min, 55 °C

for 2 min and 72 °C for 10 min. For the second PCR, 2 μl of the first PCR product was added to 48 μl of reaction mix with the same composition as the first PCR reaction, except the internal primers were used. Ten microliters of PCR products was analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide, and visualized with an ultraviolet transilluminator.

2.3.2. HBV DNA sequencing

Amplified PCR products were purified by Qiaquick Spin columns (Qiagen Inc.) according to the manufacturer's instructions. Purified HBV nucleotide was directly sequencing by an automated DNA sequencer ABI PRISM 310 (Perkin

Table 1
Overiew of the amplification and sequencing primers

Purpose	Target region	Primer	Primer sequence 5′–3′	Position
Genotype and subtype	PreS1, PreS2 and S	External		
		HBVgt–S1	TAC ACG CAG TGC CTC ATT CT	2792–2811
		HBVgt–AS1	TAC CCC AAC ATC CAA TTA CA	885–904
		Sequencing		
		HBVgt–SEQ1	TAC ACG CAG TGC CTC ATT CT	2792–2811
		HBVgt–SEQ2	AAT CGG CAG TCA GGA AGA CA	3139–3158
		HBVgt–SEQ3	TCG TGG TGG ACT TCT CTC AAT	254–274
		HBVgt–SEQ4	GTT TCT CCT GGC TCA GTT	660–677
Genotype	PreS1, PreS2	External		
		HBVgt–S2	TGG AAG GCT GGC ATT CTA TA	2760–2779
		HBVgt–AS2	TCC CCC TAG AAA ATT GAG AG	267–286
		Internal		
		HBVgt–S1	TAC ACG CAG TGC CTC ATT CT	2792–2811
		HBVgt–AS3	AGG AAT CCT GAT GTT GTG TT	161–180
		Sequencing		
		HBVgt–SEQ2	AAT CGG CAG TCA GGA AGA CA	3139–3158
Core promoter mutant and precore mutant	Core promoter region and precore region	External		
		HBV1601–S	ACG TCG CAT GGA GAC CAC CG	1601–1620
		HBV1974–AS	GGA AAG AAG TCA GAA GGC AAA	1954–1974
		Internal		
		HBV1653–S	CAT AAG AAG ACT CTT GGA CT	1653–1672
		HBV1959–AS	GGC AAA AAA GAG AGT AAC TC	1959–1978
		Sequencing		
		HBV1698–SEQ	GAG GCA TAC TTC AAA GAC TGT T	1698–1719

Elmer Corp., USA) on the basis of the fluorescence-labelled dideoxynucleotide chain terminating assay using the sequencing primers (Table 1).

2.3.3. Date analysis

The sequence of the PreS2 gene is used for genotyping [26]. Amino acid sequence of the PreS2 region is deduced from the nucleotide sequence, then according to the homologue of amino acid sequence, the genotype is decided by comparing with the reported sequences of the PreS2 region of the different HBV genotypes in the database bank. The subtype is determined by deduced amino acids at codon 122 and 160 in the S region [10].

2.4. Mutation assay of the precore and core promoter regions of HBV

2.4.1. PCR amplification and HBV DNA sequencing

The process of assay was same as genotype and subtype assay described above, except the changes of PCR primers and sequencing primer (Table 1).

2.4.2. Date analysis

The precore mutation is determined by the G to A mutation at nucleotide (nt) 1896 in the precore region. The core promoter mutation is determined by the double mutation, A to T mutation at nt 1762 and G to A mutation at nt 1764, in core promoter region. In addition, the nucleotide at position 1858 is analyzed as well.

2.5. Sequence analysis of preS region after HBsAg seroconversion

2.5.1. PCR amplification and HBV DNA sequencing

The serum samples of patients with HBV persistence more than one year after disappearance of HBsAg or appearance of anti-HBs were used to analyze the sequences of preS regions. The process of assay was same as genotype and subtype assay described above, using primers of the preS1 and preS2 region.

2.6. Statistical analysis

Differences in the proportion of cases infected with core promoter or precore HBV mutants between severe acute hepatitis B and self limited acute hepatitis B were evaluated by the χ^2-test with Yates' correction. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. HBV in serum

Table 2 lists the clinical, biochemical, serological data and HBV DNA detection in individuals convalescent from acute hepatitis B. At the beginning of the disease, all patients exhibited elevations of alanine transaminase (ALT) levels with serum HBV DNA positive and anti-HBs negative, and 17 (89%) of 19 patients were seropositive for HBsAg. At the point of 6 months after disease onset, all of them were recovery with normalization of ALT and disappearance of HBsAg, and 14 (78%) of 18 patients had developed anti-HBs, but 13 (72%) of 18 patients were positive for serum HBV DNA. Four (33%) of the 12 patients remained detectable HBV DNA in serum at the last observation, in which one patient (case 4) had persisted viremia for 43 months (Table 3).

3.2. HBV genotypes and subtypes

According to the homologue of amino acid sequence of the HBV preS2 region, 18 (95%) of 19 patients were infected with genotype C of HBV, 1(5%) with genotype B (Fig. 1). The result suggests that genotype C is the predominant genotype of acute HBV infection in Nagasaki region in Japan.

In addition, the subtypes were analyzed in eight patients who included seven patients with HBV genotype C and 1 with genotype B. Based on the presence of a lysine (K) at S region codon 122 and an arginine (R) at codon 160, all seven of genotype C were associated with subtype adr, based on K at both codon 122 and codon 160, genotype B with adw in our study.

Table 2
Clinical and laboratory data of patients with acute hepatitis B

Patient	Age	Sex	Clinical diagnosis	Time after onset (M)	AST	ALT	HBsAg	Anti-HBs	HBV DNA
1	26	F	SAH	0.1	1802	4026	+	−	+
				6	12	6	−	+	+
				12	10	6	−	+	+
2	25	F	SLAH	0.5	380	947	+	−	+
				6	19	14	−	+	+
				12	17	11	−	+	+
3	40	F	SLAH	0.2	292	784	+	−	+
				6	NT	NT	NT	NT	NT
				12	11	12	−	−	+
4	29	M	SLAH	0.2	965	2849	−	−	+
				6	11	18	−	+	−
				43	19	17	−	+	+
5	46	F	SLAH	0.2	750	1573	+	−	+
				6	16	13	−	+	+
				12	14	12	−	+	−
6	24	F	SAH	0.03	792	3124	+	−	+
				6	10	6	−	+	+
				12	11	6	−	+	−
7	64	M	SLAH	0	69	91	+	−	+
				6	13	7	−	+	+
				12	13	9	−	+	−
8	25	M	SAH	0.1	810	2507	+	−	+
				6	23	17	−	+	+
				12	24	15	−	+	−
9	75	F	SAH	0.07	438	1277	+	−	+
				5	31	29	−	+	+
				12	37	41	−	+	−
10	24	F	SLAH	0.07	666	1552	+	−	+
				6	20	13	−	+	+
				12	20	14	−	+	−
11	71	M	SLAH	3	101	181	−	−	+
				5	44	27	−	+	−
				12	15	11	−	+	−
12	30	M	SLAH	0.2	130	730	+	−	+
				6	9	12	−	−	−
				12	10	10	−	−	−
13	60	F	SLAH	0.1	123	428	+	−	+
				6	23	29	−	+	+
14	32	F	SLAH	0.1	768	1287	+	−	+
				6	13	8	−	+	+
15	41	M	SLAH	0.1	539	1082	+	−	+
				6	14	15	−	+	−
16	23	F	SAH	0.2	123	435	+	−	+
				6	9	7	−	−	+
17	30	M	SLAH	0.5	402	943	+	−	+
				6	17	38	−	−	+
18	37	M	SLAH	0.1	106	476	+	−	+
				6	26	46	−	+	−
19	33	F	SLAH	0.1	1404	2611	+	−	+
				6	16	11	−	+	+

SAH, severe acute hepatitis; SLAH, self-limited acute hepatitis; NT, not tested.

Table 3
Proportion of results of laboratory tests in patients with acute hepatitis B during follow-up

	Onset	6 M	≥ 12 M
ALT normal	0 (0/19)	100 (18/18)	100 (12/12)
HBsAg positive	89 (17/19)	0 (0/18)	0 (0/12)
HBsAb positive	0 (0/19)	78 (14/18)	83 (10/12)
HBV DNA positive	100 (19/19)	72 (13/18)	33 (4/12)

3.3. Precore mutation and core promoter mutation

All patients, irrespective of who were infected with either HBV genotype C or genotype B, had T1858 in this study (Table 4). Among the patients, two had G to A mutation at nt 1896, three had double A to T mutation at nt 1762 and G to A mutation at nt 1764, one had only G to A mutation at nt 1764. Of the five patients who had severe acute hepatitis B, and treated with corticosteroid, two (40%) had precore mutation, three (60%) had core promoter mutation. Of the other 14 patients who had self-limited acute hepatitis B, none had either precore mutation or core promoter mutation. Interestingly, case 19, who had only nt 1764 mutation, had low PT (46%), albeit disagreed with the diagnosis of severe

hepatitis. The results showed that the proportion of the core promoter mutation in severe acute hepatitis B (60%) was significantly higher than in self-limited acute hepatitis B (0%; $P < 0.01$). The proportion of the precore mutation in severe acute hepatitis B (40%) was higher than in self-limited acute hepatitis B (0%) although insignificantly. Among four patients with viremia, one (25%) had precore mutation and core promoter mutation, among eight patients without viremia, two (25%) had precore mutation and/or core promoter mutation. There are no differences of the proportions both in the precore mutation and in the core promoter mutation between group with viremia and group without viremia.

3.4. PreS sequences before and after HBs-seroconversion

Four patients with detectable HBV DNA more than one year were performed to compare in preS sequences before and after HBs-seroconversion. Sequence analysis revealed no differences in preS1 and preS2 regions before and after HBs-seroconversion in three patients with persistent HBV DNA, except one patient (case1) whose HBV preS region after seroconversion to anti-HBs was not able to be amplified by nested PCR.

Patient	Geno-types	Sub-types	Amino Acid Sequence of the PreS2 Region
1	C	adr	MQWNSTTFHQALLDPRVRGLYLPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
2	C	adr	MQWNSTTFHQALUDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
3	C		MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
4	C		MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
5	C	adr	MQWNSTTFHHALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
6	C	adr	MQWNSTTFRQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
7	B	adw	MQWNSTTFHQTLQDPRVRALYFPAGGSSSGTVSPAQNTVSAILSILSKTGDPVPN
8	C	adr	MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
9	C	adr	MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSITSRTGDPAPN
10	C		MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIRSRTGDPAPN
11	C	adr	MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIPSRTGDPAPN
12	C		MQWNSTTFHQTLLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
13	C		MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFLRTGDPAPN
14	C		MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
15	C		MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
16	C		MQWNSTTFHQALLDPRVRGLSFPAGGSSSGTVNPVPTTASPISTIFSRPGDPAPN
17	C		MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
18	C		MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
19	C		MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN

Fig. 1. Amino acid sequences of the preS2 region products of distinct genotypes and subtypes of the patients with acute hepatitis B. The determinant amino acids of genotypes were highlighted.

Table 4
HBV genotype and precore and core promoter mutation of patients with acute hepatitis B

Patient	Genotype	Precore nt 1858	Precore nt 1896	Core promoter nt 1762	Core promoter nt 1764	Max. ALT (U/dl)	Max. TB (mg/dl)	PT (%)	CS therapy	HBV DNA 6M	HBV DNA ≧12 M
1	C	T	A	T	A	4026	6.2	31	+	+	+
2	C	T	G	A	G	1139	2	77.8	−	+	+
3	C	T	G	A	G	797	7.4	98	−	NT	+
4	C	T	G	A	G	2990	1.8	67	−	−	+
5	C	T	G	A	G	2904	6.1	100	+	+	−
6	B	T	A	T	A	3124	15.7	22	−	+	−
7	C	T	G	A	G	1430	1.6	80	+	+	−
8	C	T	G	T	A	3265	7.8	24.5	+	+	−
9	C	T	G	A	G	1277	6	26	−	+	−
10	C	T	G	A	G	1552	1.7	94.8	−	−	−
11	C	T	G	A	G	209	0.9	96	−	−	−
12	C	T	G	A	G	730	4.5	88	−	+	−
13	C	T	G	A	G	558	0.4	70	−	+	NT
14	C	T	G	A	G	1287	0.9	68	−	−	NT
15	C	T	G	A	G	1134	20.9	76	+	+	NT
16	C	T	G	A	G	563	19.2	29	−	−	NT
17	C	T	G	A	G	1261	1.8	95	+	+	NT
18	C	T	G	A	G	476	3.6	92	−	−	NT
19	C	T	G	A	A	2611	3.3	46	−	+	NT

Max. ALT, maximal alanine transaminase during acute phase; Max. TB, maximal total bilirubin during acute phase; PT, prothrombin time; CS, corticosteroid; NT, not tested.

4. Discussion

Seroconversion to anti-HBs or the loss of HBsAg has been considered to be associated with complete elimination of the replicative HBV. However, this study showed that HBV DNA might persist in serum for more than one year after complete clinical and serological recovery from acute viral hepatitis, it was consistent with previous reports [7–9]. HBV must be replicating in the body, because HBV persistent in the blood for a long time. It has been reported that HBV was detected in PBMCs and liver decades after acute viral hepatitis [27], the reverse-transcribed HBV RNA and the covalently closed circular (ccc) HBV DNA, the template for the viral RNA transcription, were also detected in PBMCs or liver [28]. These suggest that, on occasion, HBV may not be in a latent state but undergoing low-level replication.

The rate of HBV DNA persistence by ELMA kit (sensitivity 100 copies/ml) in our study is 33%. Michalak et al. [7] reported that four (80%) of the five patients in Canada were detected HBV DNA in serum by nested PCR (approximate sensitivity 10 to ~100 copies/ml) after recovery from acute hepatitis Yotsuyanagi et al. [9] reported that ten (91%) of 11 patients were positive for HBV DNA in serum by PCR coupled with southern blotting (sensitivity 10 copies/ml). The discrepancy between our results and theirs might be due to the different sensitivity of HBV DNA assays. It is believed that more people in our study would have detectable HBV DNA in serum after serological recovery from acute hepatitis alone with improvement of sensitivity of HBV DNA assay.

In view of the difference in the rate of development of chronic hepatitis from acute hepatitis B in adults between Japanese and Western patients, the rate of HBV DNA persistence may be associated with the viral genetic variations. However, there are very little data on the genotypes associated with HBV DNA persistence after acute HBV infection. Four patients with persistent viremia reported by Michalak et al. [7] were infected with the adw subtype of HBV which is the most common subtype in North America. There are some correlations between subtypes and geno-types, although no specific subtype corresponds to each genotype. In general, genotype A and B were associated with subtype adw, genotype C with adr, and genotype D with ayw [26]. According to HBV genotype with distinct geographical distribution, genotype A is predominant in North America. Hence, we could infer that the four patients were infected with HBV genotype A. While in our study, the patients were mostly infected with HBV genotype C. The variation of HBV genotypes may influence the rate of HBV persistence after acute HBV infection. Lo et al. [29] reported that the prevalence of HBV DNA by nested PCR in liver tissues in HBsAg negative patients showed geographical variation. Two of 18 (11%) of Italian samples and 2/29 (6.9%) of Hong Kong samples were positive for HBV DNA while none of the 70 cases from the United Kingdom was positive. The different prevalence may be contributed to the viral genetic variations.

Our result confirmed that the core promoter mutation and precore mutation are associated with the severity of acute hepatitis. Analysis of HBV core promoter and precore mutation (especially core promoter mutation) may be useful to predict the clinical outcome of live disease in patients with acute HBV infection [19]. The mutation in precore region, a G to A change at nt 1896, which creates a premature stop codon, prevents the translation of the precore protein and abolishes the production of hepatitis B e antigen (HBeAg). The mutation in core promoter region, A to T change at nt 1762 and G to A change at nt 1764, decreases transcription of precore mRNA and secretion of HBeAg. These mutations result in a loss HBeAg and seroconversion to antibody against HBeAg (anti-HBe) with persistence of HBV replication. They may not be associated with HBV persistence after serological recovery from acute hepatitis B, because the proportions of the mutations were low in viral persistent patients in our study.

Mutations in the gene coding for HBsAg may result in infection or viral persistence despite the presence of anti-HBs ('vaccine escape' or 'immune escape') [25]. A recent investigation showed the all HBsAg-negative cases, who were hemodialysis patients and dialysis-unit staff members with

resolved acute hepatitis B, were infected by a mixture of the wild-type virus and a deletion (amino acids 58–118) mutant in the preS1 region [28]. The lack of detection of HBsAg in the presence of low viral levels of replication might be caused by the existence of viral genomes harboring deletions in the preS1 region that affect the S promoter, and produce a reduction of the HBsAg synthesis. However, our study before and after HBs-seroconversion revealed no mutation in preS1 and preS2 region in three patients with viral persistence more than 1 year.

The pathogenic significance of low-level viral persistence in the patients, who lost HBsAg or developed anti-HBs, is not clear. In Lo et al.'s study, one HBV DNA positive case had idiopathic chronic active hepatitis, but the other three HBV DNA positive cases did not suggest any aetiological connection between HBV DNA positivity and liver pathology [29]. A study was made to follow up patients with unresolved HBV infection, 25–27 years after the acute infection in Sweden. None of the 100 patients with acute HBV infection who were traced had become chronic carriers. Genotyping of the HBV strains showed that genotype D was the most prevalent [30]. However, some investigations indicated that the persistence of HBV DNA might play an important role in hepatocarcinogenesis in some cases even after serological recovery [31,32]. It is also important to determine whether HBV exists in a free state or immunoglobulin-bound form in the course of acute hepatitis B in the discussion of infectivity, such examination was not done in this study. But there were some documents which reported that immunoglobulin-bound HBV persists as immune complex in the recovery phase [7,9]. After seroconversion to anti-HBs, HBV remaining in circulation is believed to be unlikely to be infectious. The further investigations are necessary to clarify whether the low-level virus has the pathogenic significance.

In conclusion, we demonstrated that HBV DNA may persist in the serum for more than one year after complete clinical and serological recovery from acute hepatitis B. The rate of HBV persistence might be associated with the sensitivity of HBV DNA assay and the variation of HBV genotypes. HBV precore mutation and core promoter mutation are related to the severity of liver disease in patients with acute HBV infection, but no impact on HBV persistence in our study.

Acknowledgements

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6. C型慢性肝炎IFN単独治療および Ribavirin併用療法の治療成績

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はじめに

1992年にC型慢性肝炎に対するIFN治療が，また2001年12月からIFNにribavirinを併用する治療法が承認された．これらの治療法は臨床試験の審査を得て承認されたが，臨床試験での治療成績は，統計学的有意差を見いだすために設定された対象者数から得られた知見である．また臨床試験での対象者の年齢平均が40～50歳代にあることに比較して，わが国のC型肝炎患者集団は60～70歳代に大きなピークがあり，年々高齢化している．C型肝炎治療に関する臨床試験での患者年齢層と実際の医療現場での患者年齢層には大きな隔たりがある．

一方，全国22施設からなる国立病院・療養所肝疾患ネットワーク参加施設では，C型慢性肝炎に対する各種治療方法の問題点，治療困難例の実態を明らかにしたうえで，Evidence-based Medicine（EBM）に基づいたウイルス肝炎の治療法を確立，体系化することを目標とする治療共同研究を2000年から開始した（図1）．この研究班では，2000年1月以後，各施設でIFN治療を行った症例をprospectiveに登録し，順次ウイルス学的治療効果判定を行うことで，これらの治療成績の解析を行っている．本研究は現在も進行しており最終治療成績までには至っていないが，本稿では現時点での中間治療成績を紹介する．

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I. IFN単独治療成績

2000年1月1日～2002年12月31日までの3年間に，国立病院・療養所肝疾患ネットワーク参加施設22の施設において1,194例のC型慢性肝炎症例にIFN治療が導入された（表1）．うち初回治療症例は801例（67.1％），再治療例は393例（32.9％）であった．2000年の1年間の導入症例数は258例で，2001年は222例，2002年は714例で，2002年は，2000年，2001年と比較して3倍以上の症例においてIFNが導入されていた．2002年の治療導入症例数の増加は，新しい治療法であるribavirin併用療法とコンセンサスIFNが2001年12月以後使用可能になったことを反映していると思われる．またこの時期に再治療例の適応基準も緩和されたことを反映して，再治療例の比率も2002年には約20％から42％へと増加しており2002年には積極的に再治療が行われたと考えられる．

治療効果判定は，治療終了後6ヵ月目の時点でアンプコアHCV-RNA定性検査でHCV-RNA陰性をSVR：Sustained Viral Response（ウイルス学的著効）とし，それ以外をNon-SVRと判定した．IFN単独治療法の治療成績を明らかにする目的で，2000年1月1日～2001年12月31日までの期間に治療が行われたIFN単独治療（コンセンサスIFNを除く）症例中，HCV-RNA量，HCV genotypeともに判明している428例（効果判定不能，drop out 37例を含む）においてSVR率を算出した．