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Table 1. Laboratory Findings on Admission*

Parameter	Value	Parameter	Value
Hematology		K	4.2 mEq/l
White blood cells	5000/μl	Cl	107mEq/l
Hemoglobin	15.1g/dl	CRP	1.0mg/d
Platelets	25.6×10⁴/μl	Coagulation test	
Blood chemistry		Prothrombin test	89.2%
Total protein	7.2g/dl	Viral markers	
Albumin	3.9 g/dl	IgM anti-HAV(EIA)	0.2(-)
Total bilirubin	0.5mg/dl	IgM anti-HBV(CLIA)	21.9(+)
AST	150IU/l	HBsAg (RPHA)	2048(+)
ALT	434IU/l	HBeAg (CLIA)	1460(+)
LDH	221IU/l	Anti-HBe(CLIA)	0%(-)
ALP	293	HBV DNA(TMA)	8.2 LEG/ml
γ-GTP	127 IU/l	HBV genotype	H
Creatinine	0.8 mg/dl	Anti-HCV(CLEIA)	0.3(-)
Na	143mEq/l	Anti-HIV(CLEIA)	(-)

* AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, gamma glutamyl transpeptidase; CRP, C-reactive protein; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; anti-HBe, antibody to HBeAg.

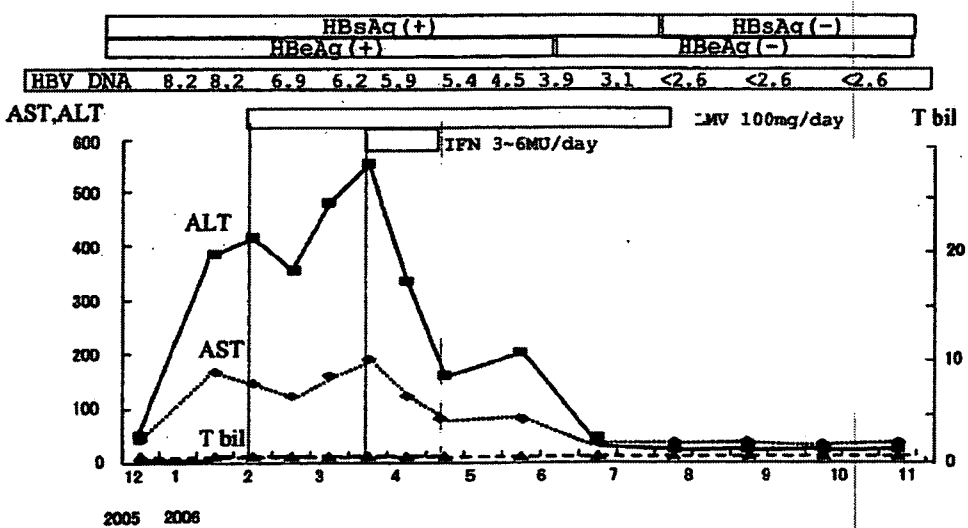

Figure 1. Clinical course of the patient with acute hepatitis B and genotype H.

month later, he just complained of slight fatigue and showed elevated AST and ALT.

He was admitted to our hospital for suspected acute hepatitis B in January, 2006. On admission he showed no jaundiced and was relatively healthy. He was positive for hepatitis B e antigen (HBeAg) and 8.2 LGE/ml of serum HBV-DNA as measured by transcription-mediated amplification and hybridization protect assay [Chugai Daiagnostics Science Co., Tokyo, Japan (5)]. Serum levels of AST and ALT were relatively low. Serological markers for HBsAg, HBeAg were strongly positive and serum level of HBV-DNA was high. IgM antibody to hepatitis B core antigen was high (21.9 S/CO) by the CLIA method (Abbott Japan Co., Ltd., Tokyo, Japan) as shown in Table 1. Therefore, he was diagnosed as having acute hepatitis B. No personal or family history of liver disease was recorded. Serological markers for antibodies to hepatitis C virus and antibodies to HIV type 1 and 2 were negative. However, he was a homosexual habit and went to a 'meeting' two to three times each month near his residence. In the meeting he had sexual contacts with unknown persons.

Lamivudine (LMV), a nucleoside analogue, was prescribed for him to reduce activity in the liver and HBV-DNA serum levels. He was given 100 mg of LMV daily. One month later from the initiation of lamivudine, his transaminase level began to increase, and natural interferon (IFN) beta (Toray Industiries, Inc., Tokyo, Japan) was started by intravenous injection from one more week later. Interferon was started at 6 MU daily. But neutropenia was seen in one week. The dose was then decreased to 3 MU. Unfortunately, three more weeks later, he had complained of depression which was suspected to be an interferon related side effect and IFN therapy was discontinued within one month. Over that time, HBV-DNA had gradually decreased (Fig. 1). Mu-

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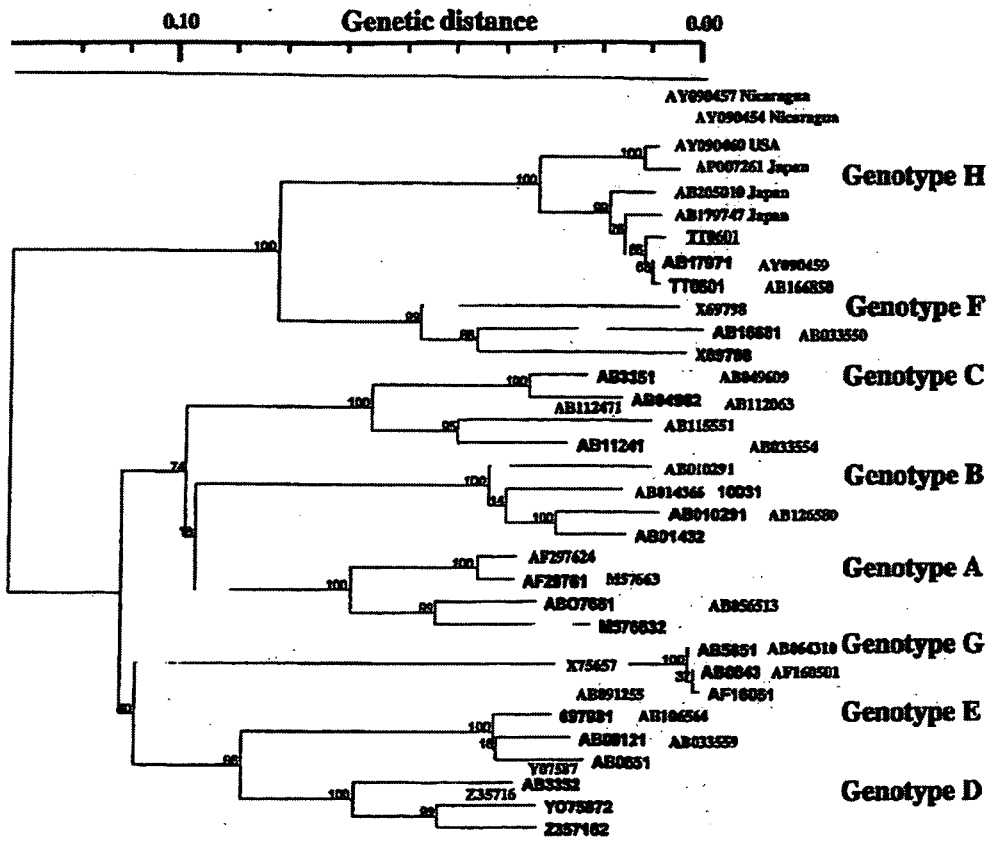

Figure 2. Phylogram generated by neighbor-joining analysis of genetic distance in the full-length sequence of HBV. Thirty strains (without TT0601; indicated by underline) were retrieved from the GenBank/EMBL/DDBJ database.

tation of the HBV DNA polymerase gene (rtM204I/V, L180M) was determined using polymerase chain reaction and restriction fragment length polymorphism as described previously (6). This patient did not show mutations at rt180 or 204 in the HBV DNA polymerase gene at the initiation of IFN therapy.

Full genome sequence analysis by PCR direct sequencing technique before treatment revealed that the patient was infected with genotype H virus (Fig. 2). The sequence was named HBV-TT0601. When compared with previously reported HBV isolates with full genome sequences, ST0404 showed high overall identity (99.2%) with a prototype of the Los Angeles strain (AY090460) and 97.5% identity with a Nicaragua strain (AY090457) of the genotype H group at the nucleotide level. Moreover, ST0404 showed higher overall identity (99.8%, 99.4% and 98.8%) with Japanese strains (AB179747, AB205010 and AP007261 respectively) (7-9).

Five months after the onset, needle liver biopsy under laparoscopy was performed. Portal Tracts had edematous enlargement with lymphocytic infilitration and increased collagen fiber. Moreover, the lobular area showed necroinflammatory activity. Inflammatory changes remained within the liver five months after the onset of acute hepatits B (Fig. 3). With the continuous treatment by LMV, eight months after onset from acute hepatitis, serum HBsAg converted to negative.

Discussion

Here, we report a 60-year-old man infected with genotype H HBV, who had a prolonged clinical course after onset of acute hepatitis B. The present case was suspected for infection from homosexual contact. The genotype H of this patient was reported three times in Japan previously (7-9).

This patient had several features. First, he showed a low level of serum aminotransferase and total bilirubin in spite of a high titer of serum HBV DNA level. In our previous report, we described that patients with a low serum level of aminotransferase and total bilirubin in acute hepatitis B have a high possibility of persistence (10). Low maximum ALT levels (<500 IU/l) and high baseline HBV-DNA levels (>8.7 LGE/ml) were going to persistent in patients with genotype A. Thus, we selected the intensive care for the present case of acute hepatitis B in order to prevent disease progression from acute to the chronic phase.

Second, acute hepatitis B with genotype H has the possibility of being prolonged or persistent in spite of intensive treatment. Generally, acute hepatitis B with HBV genotype A tends to be persistent (11). On the other hand, most patients with acute hepatitis B due to genotype B and C are

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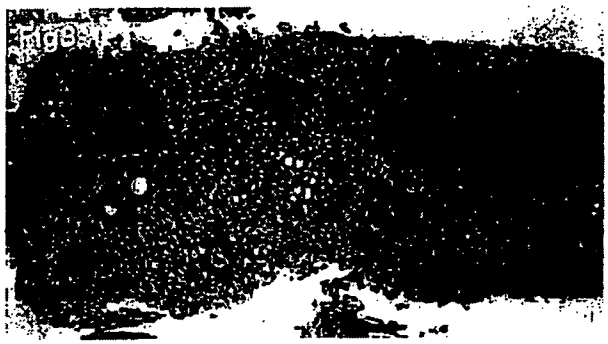

Figure 3-1. Picture of liver biopsy. Panacinar necrosis of the portal tracts and parenchymal remnants leads to disruption of the lobular architecture. Portal tracts exhibited increased fibrosis.

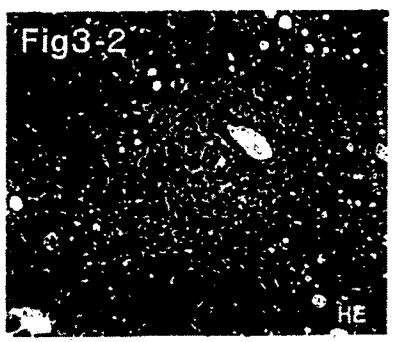

Figure 3-2. Picture of liver biopsy. Edematous enlargement with light lymphocytic infiltration of the portal tracts and parenchymal remnants was clear.

Figure 3-3. Picture of liver biopsy. Kupffer cells underwent hypertrophy and hyperplasia and were laden with lipofuscin pigment: red spot in D-PAS stain, indicating inflammatory persistence.

usually cured without antiviral drugs. The present patient showed a prolonged course after the onset of acute hepatitis by histological examination. HBV replicates by reverse transcription of an RNA intermediate, pregenomic RNA (pgRNA). For pgRNA to be encapsulated, its 5′ end is folded into a stem-loop structure, known as the encapsidation signal. PgRNA is transcribed from the distal Precore region and proximal C gene and consists of 60 nucleosides (positions 1847-1906, numbering from the EcoR1 site) (12-14). In general, the patients with HBV genotype A show adenosine at position 1858 in sequence. On the other hand, the patients with HBV genotype B or C show uracil at position 1858 in sequence. The present patient with genotype H had adenosine at position 1858 in sequence. We suggest that stability of pgRNA in HBV genotype A and H is associated with the clinical course after the onset of acute hepatitis B.

Thirdly, the present patient did not show a good response after lamivudine therapy. In most cases, acute hepatitis is cured with rest and observe. Therefore, antiviral treatment is rarely used for such cases. When antiviral drugs, such as lamivudine, are given the patients with acute hepatitis B in one or two months after onset, most patients show a decrease in the serum levels of ALT and HBV DNA level decrease (9). However, the present patient responded poorly to LMV treatment and had prolonged hepatitis. The serum level of ALT decreases slowly after the initiation of IFN therapy. IFN therapy may aid in decreasing aminotransferase.

Eight genotypes (A-H) of HBV have now been described. In brief, genotypes B and C are prevalent in Asia and the Far East, while genotype A is prevalent in northwestern Europe, North America and Africa. Genotype D is predominant in the Mediterranean area and India (15), while genotype E circulates in sub-Saharan Africa (16). Genotype F is found in Central and South America (17). Genotype G has been reported from France and North America (18). Genotype H has been described only recently, and the first report was from Central America (4). The strain in the present case showed high homology with those reported in Japan (7-9) and Los Angeles (4). However in the future, acute hepatitis B due to genotype H could be spread. Moreover, based on the difference of HBV-genotype, persistence rate is different (2, 10). Limitation of this case was other immunosuppressive factors. The patient was a homosexual. Homosexual men can be associated with poor responsibility for treatment of hepatitis (19).

In conclusion, the acute hepatitis B patients in Japan have shown various genotypes recently. We encountered a rare case of acute hepatitis B with genotype H which led to a prolonged state of acute hepatitis. LMV and IFN were effective for changing HBsAg to negative.

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CLINICAL RESEARCH STUDY

Viral Elimination Reduces Incidence of Malignant Lymphoma in Patients with Hepatitis C

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ABSTRACT

PURPOSE: A high prevalence of malignant lymphoma among patients with hepatitis C virus (HCV) infection has been reported. The aim of this retrospective study was to determine the incidence of malignant lymphoma and the relationship between malignant lymphoma and viral elimination in patients with HCV.

METHOD: We studied 501 consecutive HCV-infected patients who had never received interferon therapy and 2708 consecutive HCV-infected patients who received interferon therapy.

RESULTS: In the non-interferon group, the cumulative rates of malignant lymphoma development were 0.6% at the 5th year, 2.3% at the 10th year, and 2.6% at the 15th year. The cumulative rates of malignant lymphoma development in interferon-treated patients with sustained virologic response were 0% at the 5th year, 0% at the 10th year, and 0% at the 15th year. The cumulative rates of malignant lymphoma development with persistent infection were 0.4% at the 5th year, 1.5% at the 10th year, and 2.6% at the 15th year. The malignant lymphoma development rate was higher in patients with persistent infection than in patients with sustained virologic response ($P = .0159$). The hazard ratio of lymphomagenesis in 1048 patients with sustained virologic response was significantly lower than in patients with persistent infection (hazard ratio: 0.13; $P = .049$).

CONCLUSION: Our retrospective study is the first to determine the annual incidence of malignant lymphoma among patients with HCV at 0.23%. Our results indicate that sustained virologic response induced by interferon therapy protects against the development of malignant lymphoma in patients with chronic HCV.

KEYWORDS: Cohort study; Hepatitis C virus; Hepatocellular carcinoma; Interferon; Malignant lymphoma; Sustained virologic response; Viral elimination

Hepatitis C virus (HCV) is a major risk for hepatocellular carcinoma.[1-10] The incidence of hepatocellular carcinoma in patients with HCV-related cirrhosis is estimated at 5% to 10% per year, and it is one of the major causes of death, especially in Asian countries.[10] On the other hand, HCV has been detected not only within infected hepatocytes but also in blood cells, such as lymphocytes,[11] and has been implicated as a putative agent of cryoglobulinemia.[12] The virus sustains clonal expansion of B lymphocytes in HCV-infected patients.[13] Moreover, the prevalence of HCV infection in B-cell non-Hodgkin's lymphoma also is high,[14] and anti-HCV seropositivity is a risk factor of malignant lymphoma.[15] Zuckerman et al[16] suggested that HCV might induce clonal proliferation of B-cell and t(14;18) translocation. However, the mechanism of lymphomagenesis is not well known in patients with HCV.

There are many reports on the prevalence of HCV infection in malignant lymphoma,[14] but there is little or no information on the cumulative incidence and influence of

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interferon therapy on the development rate of malignant lymphoma. In our hospital, we evaluate a large number of patients with HCV-related hepatitis and often find hepatocellular carcinoma among our cases. We also find a proportion of patients with HCV-related hepatitis in whom malignant lymphoma develops. In the present retrospective study, we examined the incidence of malignant lymphoma among HCV-infected patients and determined the relationship between malignant lymphoma and interferon therapy in such patients.

CLINICAL SIGNIFICANCE

- The annual incidence of malignant lymphoma in patients with HCV infection who have never received interferon therapy is 0.23% per year.
- The risk of malignant lymphoma in patients with persistent HCV infection is approximately 7 times that in patients with sustained virologic response induced by interferon therapy.
- The risk of malignant lymphoma is low in patients with chronic HCV who show a sustained virologic response to interferon therapy.

PATIENTS AND METHODS

Study Population

In the retrospective cohort study, we analyzed all patients in our database of chronic HCV between 1969 and 2006 in the Department of Hepatology, Toranomon Hospital, Tokyo, Japan: 511 consecutive patients who did not receive interferon therapy (non-interferon group) and 2960 consecutive patients who received interferon therapy (interferon group). The patients were positive for anti-HCV antibody and HCV-RNA, and negative for hepatitis B surface antigen. Among them, 10 patients of the non-interferon group and 252 patients of the interferon group were excluded for the following reasons: possible association with hepatocellular carcinoma; possible association with malignant lymphoma and other hematologic malignancy; association with hemochromatosis, autoimmune liver disease, primary biliary cirrhosis, α-1-antitrypsin deficiency, or Wilson disease; or a short follow-up period of 6 months or less. Consequently, 501 patients of the non-interferon group and 2708 patients of the interferon group were retrospectively evaluated for the malignant lymphoma development rate and the efficacy of interferon therapy. All patients who did not show a sustained virologic response and persistently high alanine aminotransferase level (normal range of alanine aminotransferase: 6-50 IU/L) received liver protection therapy, consisting mainly of glycyrrhizin and ursodeoxycholic acid (300-600 mg/d), during this research.

In these groups, the observation starting point was the time of the first medical examination at our hospital.

Background and Laboratory Data

Table 1 summarizes the profiles and laboratory data of the 2708 patients who received interferon therapy and the 501 patients who did not receive interferon therapy. Patients of the interferon group were younger than those of the non-interferon group. The observation period was significantly shorter in the interferon group than in the non-interferon group (median 4.5 vs 14 years; $P < .0001$). Although all patients were HCV-RNA positive during the clinical course, the serum concentration of HCV-RNA using initial sera was analyzed in 2976 patients (92.7%). HCV subtype was analyzed in every patient. Serologic grouping of HCV showed that the percentage of HCV-2 in the interferon group was significantly higher than in the non-interferon group. The initial serum concentration of HCV-RNA was assessed in 2878 patients (89.7%). There was no significant difference between the 2 groups with

Table 1 Patient Profiles and Laboratory Data at the Time of the First Medical Examination at Our Hospital

	Non-IFN Group	IFN Group	*P* Value
No. of patients	501	2708	
Sex (M/F)	300/201 (1.49:1)	1735/973 (1.78:1)	.077
Age (y)	53 (21-79)	51 (10-83)	<.0001
Observation period (y)	14 (0.7-35.8)	4.5 (0.5-17.9)	<.0001
AST (IU/L)	66 (12.8-704)	59 (9-1266)	<.0001
ALT (IU/L)	96 (12-832)	92 (1-1620)	.927
HCV serologic group			
1	256 (84%)	1749 (66%)	<.001
2	50 (16%)	921 (34%)	
Viral load*			
Low	72 (28%)	807 (31%)	.566
High	183 (72%)	1816 (69%)	
Chronic hepatitis/liver cirrhosis	449/52	2533/175	.003

IFN = interferon; AST = aspartate aminotransferase; ALT = alanine aminotransferase; HCV = hepatitis C virus.
*Viral load: low; Amplicor <100 KIU/mL or Probe < 1 MEq/mL, high; Amplicor ≥ 100 KIU/mL or Probe ≥ 1 MEq/mL.

regard to the initial viral load (low viral load; Amplicor < 100 KIU/mL [Cobas Amplicor HCV Monitor Test, v2.0, Roche Molecular Systems, Inc, Belleville, NJ] or Probe < 1 MEq/mL, high viral load [branched DNA probe assay; version 2.0; Chiron, Dai-ichi Kagaku, Tokyo, Japan]; Amplicor ≥ 100 KIU/mL or probe ≥ 1 MEq/mL). In this study, the percentage of patients with chronic hepatitis was significantly higher in the interferon group than in the non-interferon group.

Type of Interferon and Judgment of Interferon Effect

Among 2708 patients with interferon therapy, 1675 patients received interferon-alpha, 415 patients received interferon-beta, 33 patients received both interferon-alpha and interferon-beta, and the remaining 585 patients received a combination therapy of interferon and ribavirin. The response to interferon therapy was based on a sustained virologic response (elimination of HCV-RNA at 6 months after the end of treatment). Among patients treated with interferon, 1048 patients (38.7%) acquired a sustained virologic response. Among 1660 patients in the nonsustained virologic response group, there were 1012 patients with a relapse of HCV RNA after temporal viral clearance, and the remaining 648 patients had nonviral clearance during treatment.

Viral Markers of Hepatitis B and C Viruses

Diagnosis of HCV infection was based on the detection of serum HCV antibody and positive RNA. Anti-HCV was detected using a second-generation enzyme-linked immunosorbent assay (Abbott Laboratories, North Chicago, Ill). HCV-RNA was determined by the Amplicor method (Cobas Amplicor HCV Monitor Test, v2.0, Roche Molecular Systems, Inc, Belleville, NJ) or the branched DNA probe assay (branched DNA probe assay; version 2.0; Chiron, Dai-ichi Kagaku, Tokyo, Japan). Hepatitis B surface antigen was tested by radioimmunoassay (Austria, Abbott Laboratories, Detroit, Mich). The used serum samples were stored −80°C at the first consultation.

Histopathologic Examination of Liver

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim-Silverman needle with an internal diameter of 2 mm. All specimens for examination contained at least 6 portal areas. Chronic hepatitis was diagnosed on the basis of histopathologic assessment according to the scoring system of Desmet et al.[17] Patients who did not undergo liver biopsy were diagnosed with chronic hepatitis on the basis of the presence of irregular liver surface, portal-hypertension, and/or ascites by ultrasonography, computed tomography (CT), and/or endoscopy.

Follow-up, Diagnosis, and Classification of Malignant Lymphoma

Patients were followed up monthly to trimonthly after the first medical examination at our hospital. Physical examination and biochemical tests were conducted at each examination together with a regular checkup with CT or ultrasonography imaging in each patient. When a patient had any symptoms in relation to malignant lymphoma (unexplained weight loss, fever, and lymphadenopathy), we further explored possible malignant lymphoma. Malignant lymphoma was diagnosed by histopathologic examination. Classification was based on the Revised European-American Classification of lymphoid neoplasms/new World Health Organization classification[18] revised by Harris.[19] Staging and extranodal involvement were determined according to the Ann Arbor classification by physical examination, total body CT scan, and bone marrow biopsy. The number of cases lost to follow-up included 78 patients (15.6%) in the non-interferon group and 184 patients (6.8%) in the interferon group.

Statistical Analysis

Nonparametric procedures were used for the analysis of background features of the patients, including the Mann-Whitney *U* test and chi-square method. The cumulative appearance rate of malignant lymphoma was calculated from the period between the first medical examination at our hospital to the appearance of malignant lymphoma, using the Kaplan-Meier method. Differences in lymphomagenesis curves were tested using the log-rank test. Independent factors associated with the incidence rate of malignant lymphoma were analyzed by a time-dependent Cox proportional hazard model, using the term of interferon therapy with "waiting time" as a time-dependent variable. The following 9 variables were analyzed for potential covariates for incidence of malignant lymphoma at the time of first medical examination at our hospital: age, sex, state of liver disease (chronic hepatitis or liver cirrhosis), viral serotype, viral load, history of interferon therapy, efficacy of viral clearance by interferon therapy, serum concentrations of aspartate aminotransferase, and alanine aminotransferase. A *P* value of less than .05 in a 2-tailed test was considered significant. Data analysis was performed using the computer program SPSS version 11.0 (SPSS Inc, Chicago, Ill). The physicians in charge explained the purpose and method of this clinical trial to each patient, who gave their informed consent for participation. This study was approved by the institutional review board of our hospital.

RESULTS

Incidence of Malignant Lymphoma in Patients Without Interferon Therapy

In the interferon group, malignant lymphoma developed in 12 patients (2.4%) during a median observation period of 14 years. The cumulative rate of newly diagnosed malignant lymphoma was 0.62% at the end of the 5th year, 2.26% at the 10th year, and 2.62% at the 15th year (Figure 1). Table 2 summarizes the characteristics of patients who developed malignant lymphoma. The period between the first medical

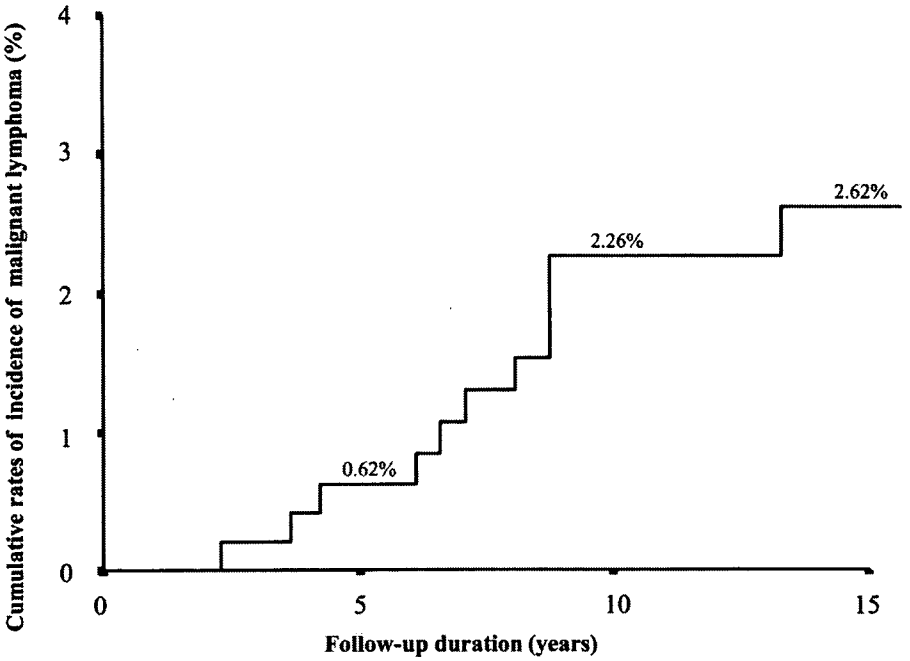

Figure 1 Cumulative rate of the incidence of malignant lymphoma from the first medical examination at our hospital in patients with chronic HCV who did not receive interferon therapy.

examination at our hospital and development of malignant lymphoma ranged from 2.2 to 26.1 years (median of 7.5 years). The patients who develop malignant lymphoma included 4 men and 8 women, aged 45 to 79 years (median, 70 years). With regard to the histologic type of malignant lymphoma, diffuse large cell lymphoma was found in 7 patients, follicular lymphoma was found in 3 patients, Hodgkin disease (nodular lymphocyte predominant) was found in 1 patient, and unclassified lymphoma was found in 1 patient. With regard to the background liver tissue, 6 patients had chronic hepatitis and 6 patients had cirrhosis at the time of malignant lymphoma development.

In our cohort, hepatocellular carcinoma developed in 102 patients (20.4%). The hepatocarcinogenesis rate in this cohort was 4.7% at the end of the 5th year, 11.9% at the 10th year, and 21.0% at the 15th year.

Incidence of Malignant Lymphoma in Patients with Interferon Therapy

In the interferon group, 14 patients (0.49%) developed malignant lymphoma during a median observation of 3.9 years. The cumulative rates of newly diagnosed malignant lymphoma were 0.16% at the end of the 5th year, 0.61% at the 10th year, and 1.81% at the 15th year. There was no significant difference in the incidence rate of malignant lymphoma between the non-interferon and interferon groups (Figure 2). Table 3 summarizes the characteristics of pa-

Table 2 Characteristics of Patients Not Treated with Interferon

Case	Sex	Age (y)	Histology	Stage	Extranodal	Serologic Group	Viral Load*	Liver Disease
1	F	45	Follicular	IV	BM	1	High	CH
2	F	50	Diffuse large cell	III	None	2	High	CH
3	F	59	Follicular	II	None	1	High	CH
4	F	67	Diffuse large cell	IV	BM	1	High	LC
5	F	69	Follicular	II	None	1	High	LC
6	F	69	ND	IV	Lung	ND	Low	LC
7	F	73	Hodgkin disease (nodular LP)	I	None	2	High	LC
8	F	74	Diffuse large cell	II	None	1	High	CH
9	M	71	Diffuse large cell	IV	BM	1	High	CH
10	M	71	Diffuse large cell	IV	Liver	ND	ND	CH
11	M	76	Diffuse large cell	IIE	Stomach	1	High	LC
12	M	79	Diffuse large cell	II	None	2	High	LC

IFN = interferon; BM = bone marrow; CH = chronic hepatitis; LC = liver cirrhosis; ND = not determined.
*Viral load: low; Amplicor < 100 KIU/mL or Probe < 1 MEq/mL, high; Amplicor ≥ 100 KIU/mL or Probe ≥ 1 MEq/mL.

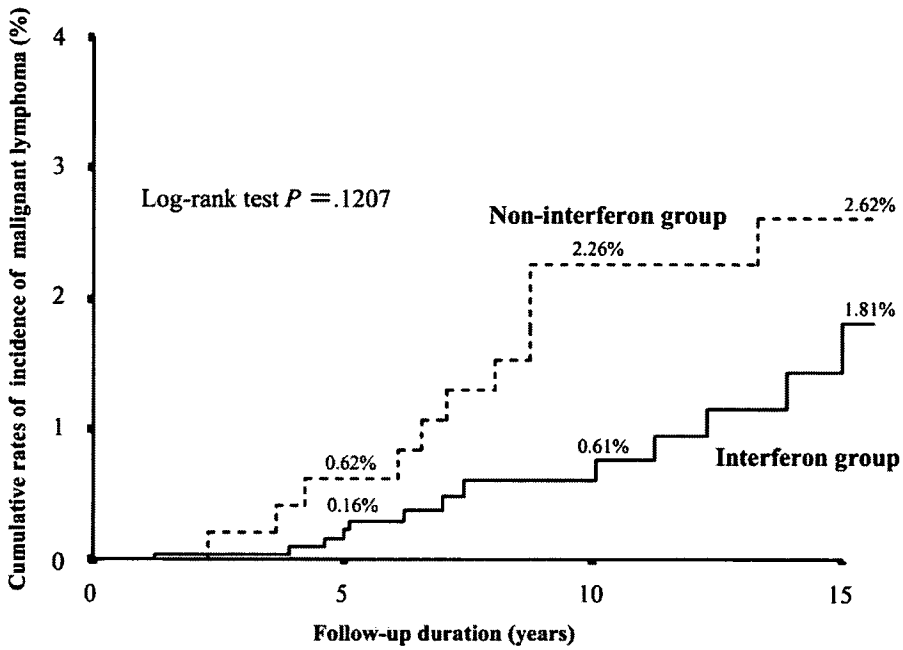

Figure 2 Cumulative rate of the incidence of malignant lymphoma from the first medical examination at our hospital in patients with chronic HCV who were treated or not treated with interferon.

tients who developed malignant lymphoma. Their median age was 61 years, and the period between the start of first interferon therapy and development of malignant lymphoma ranged from 0.7 to 14.5 years, with a median of 6.1 years. They included 7 men and 7 women aged 45 to 76 years (median, 67.5 years). Histologically, diffuse large cell lymphoma was found in 8 patients, follicular lymphoma was found in 3 patients, extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue was found in 1 patient, extranodal natural killer/T-cell lymphoma was found in 1 patient, and angioimmunoblastic T-cell lymphoma was found in 1 patient. With regard to the background liver disease, 10 patients had chronic hepatitis and 4 patients had cirrhosis at the time of malignant lymphoma development.

In our cohort, hepatocellular carcinoma developed in 154 patients (5.7%), and the rate of hepatocarcinogenesis was 2.5% at the end of the 5th year, 7.3% at the 10th year, and 15.3% at the 15th year.

Impact of Viral Elimination on the Incidence of Malignant Lymphoma

Among all 3209 patients, during the observation period, 1 patient developed malignant lymphoma among those with a

Table 3 Characteristics of Patients Treated with Interferon

Case	Sex	Age (y)	Histology	Stage	Extranodal	Serologic Group	Viral Load*	Liver Disease	Effect of IFN Treatment
1	F	46	Follicular	IV	BM	1	High	CH	Non-SVR
2	F	52	Diffuse large cell	III	None	2	High	CH	Non-SVR
3	F	59	MALT type	IE	Trachea	2	High	CH	Non-SVR
4	F	68	Diffuse large	II	None	1	High	LC	Non-SVR
5	F	70	Diffuse large	IV	Liver	1	High	LC	Non-SVR
6	F	74	Extranodal NK/T cell	IE	Nose	1	Low	LC	Non-SVR
7	F	76	Follicular	II	None	2	High	LC	Non-SVR
8	M	45	Diffuse large cell	IIS	Spleen	1	High	CH	Non-SVR
9	M	61	Diffuse large cell	II	None	2	High	CH	Non-SVR
10	M	64	Diffuse large cell	IVE	Omentum	1	ND	CH	Non-SVR
11	M	67	Diffuse large cell	IV	BM	1	High	CH	Non-SVR
12	M	68	Follicular	IIIE	Left pleural effusion	1	High	LC	Non-SVR
13	M	70	Diffuse large cell	IV	Lung	ND	High	LC	Non-SVR
14	M	73	Angioimmunoblastic T-cell lymphoma	III	None	2	Low	CH	SVR

IFN = interferon; BM = bone marrow; CH = chronic hepatitis; LC = liver cirrhosis; SVR = sustained virologic response; MALT = mucosa-associated lymphoid tissue; NK = natural killer; ND = not determined.

*Viral load: low; Amplicor < 100 KIU/mL or Probe < 1 MEq/mL, high; Amplicor ≥ 100 KIU/mL or Probe ≥ 1 MEq/mL.

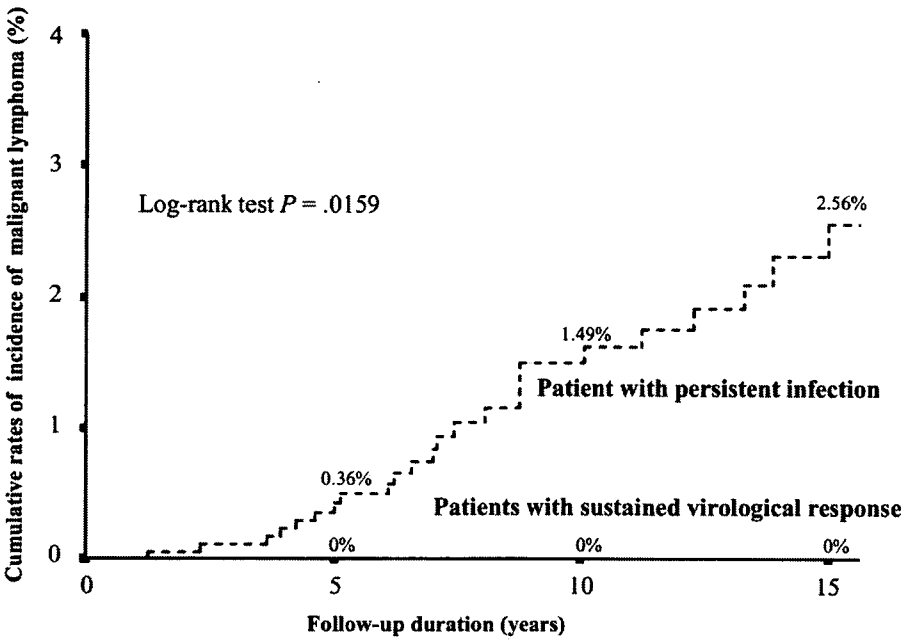

Figure 3 Cumulative rate of the incidence of malignant lymphoma from the first medical examination at our hospital in patients with sustained virologic response and those with persistent chronic HCV infection.

sustained virologic response, and 25 patients developed malignant lymphoma among those with persistent infection. The malignant lymphoma development rates were 0% at the end of the 5th year, 0% at the 10th year, and 0% at the 15th year among patients with a sustained virologic response, and 0.36% at the 5th year, 1.49% at the 10th year, and 2.56% at the 15th year among patients with persistent infection (Figure 3). Among patients with a sustained virologic response, 1 patient developed malignant lymphoma after 19.8 years from the first medical examination and after 466 days from the end of interferon therapy. In patients with persistent infection, the development rate of malignant lymphoma was significantly high ($P = .0159$).

Determinants of Malignant Lymphoma Incidence

We then investigated the factors associated with the incidence of malignant lymphoma in all 3209 patients. Univariate analysis identified the following 6 factors that influenced incidence of malignant lymphoma: age (<60/≥60) ($P < .0001$), alanine aminotransferase (<100/≥100) ($P = .0006$), viral elimination (yes/no) ($P = .016$), sex (male/female) ($P = .025$), state of liver disease (chronic hepatitis/liver cirrhosis) ($P = .045$), and viral load (low/high) ($P = .060$).

These 6 parameters were entered into multivariate Cox proportional hazard analysis (time-dependent model). The incidence rate of malignant lymphoma was significantly higher for patients with persistent infection (hazard ratio: 7.49; $P = .049$), aged 60 years or more (hazard ratio: 3.25; $P = .005$), and with serum alanine aminotransferase less than 100 IU/L (hazard ration: 3.02; $P = .030$) (Table 4).

Mortality and Causes of Death

During the observation period, 102 patients (3.18%) died: 65 of the non-interferon group and 37 of the interferon group. The estimated 5-year survivals of the non-interferon and interferon groups were 98.3% and 99.8%, 10-year survivals were 96.0% and 98.5%, and 15-year survivals were 88.4% and 90.4%, respectively. There was no significant difference in the overall survival between the non-interferon and interferon groups (log-rank test, $P = .60$). When examined according to the curative effect, the estimated 5-year survivals for patients with sustained virologic response and patients with persistent infection were 99.8% and 99.3%, 10-year rates were 99.8% and 97.1%, and 15-year rates were 98.7% and 88.9%, respectively. The survival of patients with sustained virologic response was significantly higher than that of patients with persistent infection (log-rank test, $P = .0005$). There were 2 and 3 malignant lym-

Table 4 Factors Associated with Malignant Lymphoma in Patients with Hepatitis C-related Hepatitis (Multivariate Cox Proportional Hazard Analysis: Time-Dependent Model)

Factors	Category	Hazard Ratio (95% CI)	P Value
Viral elimination*	1: Yes	1	.049
	2: No	7.488 (1.01-55.8)	
Age	1: <60 y	1	.005
	2: ≤60 y	3.247 (1.42-7.42)	
ALT	1: ≤100 IU/L	1	.030
	2: >100 IU/L	3.02 (1.11-8.20)	

ALT = alanine aminotransferase; CI = confidence interval.
*Viral elimination means sustained virologic response.

phoma-related deaths in the non-interferon group and the interferon group, respectively.

DISCUSSION

The reported significant incidence of HCV infection in B-cell non-Hodgkin's lymphoma in several areas of the world indicates a link between viral infection and this subset of lymphoproliferative disorder. The controversial results of the different research groups may be explained by the low probability of HCV carriers who develop lymphoma; thus, an accurate assessment of the exact risk could only come from a large cohort study.[20] Recent reports attesting to the efficacy of interferon therapy for HCV-related, low-grade B-cell non-Hodgkin's lymphoma[21] support the hypothesis of a link between HCV infection and B-cell lymphoma.

Little is known about the relationship between the incidence of malignant lymphoma and interferon therapy. The aim of this research was to clarify the relationship in patients with HCV. Our retrospective cohort study showed that 12 of 501 cases without interferon therapy (non-interferon group) developed malignant lymphoma and 14 of 2708 cases with interferon treatment (interferon group) developed malignant lymphoma. This epidemiologic study demonstrates the malignant lymphoma occurrence rate in HCV-positive patients: The annual appearance rate was 0.23% in the non-interferon group. The annual appearance rate was higher than that in the general Japanese population (~0.008%). Furthermore, our results clearly indicate that the hazard ratio for malignant lymphoma development in patients with HCV elimination is 0.133 compared with that of patients with persistent infection (Table 4).

Multivariate analysis identified age, HCV elimination, and alanine aminotransferase level as significant determinants of malignant lymphoma development. Interpretation of this finding requires further examination and analysis (Table 4). Zuckerman et al[16] reported that chromosome translocation of B-cell improved after interferon therapy in patients with malignant lymphoma complicating HCV infection. Our results are in agreement with those of a previous study that showed interferon-induced improvement of HCV-related lymphoma.[21] In our study, the incidence rate of malignant lymphoma was significantly lower among patients with a sustained virologic response than in patients with persistent infection for both the non-interferon and interferon groups (Figure 3). Thus, our results indicate that interferon therapy coupled with sustained virologic response reduces the likelihood of development of malignant lymphoma. We again emphasize the high prevalence of malignant lymphoma in HCV-positive patients without interferon therapy and the significance of viral elimination by interferon in regard to the suppression of lymphomagenesis.

Although it is safe to conclude that malignant lymphoma is not a risk in patients who achieve a sustained virologic response by interferon therapy, malignant lymphoma developed in 1 patient after achieving an interferon-induced sustained virologic response. However, the cause of malignant lymphoma is not clear, that is, whether it is de novo, mutation of genome by infection of HCV, or other factors. Studies are under way in our laboratories to investigate this issue.

We also analyzed the incidence of malignant lymphoma according to the subtype of malignant lymphoma. The results showed a significant prevalence of diffuse large-cell lymphoma ($P = .029$) and follicular lymphoma ($P = .005$) in our cohort compared with the distribution of the same subtypes of malignant lymphoma in Japan.[22] Malignant lymphoma cases with HCV-related hepatitis may develop a specific subtype of non-Hodgkin's lymphoma. However, our cohort included only a small number of malignant lymphoma cases, and this finding should be confirmed in another study with a large number of malignant lymphoma cases with HCV-related hepatitis.

Although the design of our study was retrospective in nature, multicenter prospective studies are needed to confirm the results described in this report.

CONCLUSION

Our retrospective cohort study reported for the first time the cumulative incidence rate of malignant lymphoma in HCV-infected patients and indicated that interferon therapy reduces the incidence of malignant lymphoma in patients with HCV-related hepatitis.

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Hepatitis C virus non-structural proteins responsible for suppression of the RIG-I/Cardif-induced interferon response

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Viral infections activate cellular expression of type I interferons (IFNs). These responses are partly triggered by RIG-I and mediated by Cardif, TBK1, IKKε and IRF-3. This study analysed the mechanisms of dsRNA-induced IFN responses in various cell lines that supported subgenomic hepatitis C virus (HCV) replication. Transfection of dsRNA into Huh7, HeLa and HEK293 cells induced an IFN expression response as shown by IRF-3 dimerization, whilst these responses were abolished in corresponding cell lines that expressed HCV replicons. Similarly, RIG-I-dependent activation of the IFN-stimulated response element (ISRE) was significantly suppressed by cells expressing the HCV replicon and restored in replicon-eliminated cells. Overexpression analyses of individual HCV non-structural proteins revealed that NS4B, as well as NS34A, significantly inhibited RIG-I-triggered ISRE activation. Taken together, HCV replication and protein expression substantially blocked the dsRNA-triggered, RIG-I-mediated IFN expression response and this blockade was partly mediated by HCV NS4B, as well as NS34A. These mechanisms may contribute to the clinical persistence of HCV infection and could constitute a novel antiviral therapeutic target.

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INTRODUCTION

Type I interferon (IFN) plays a central role in eliminating virus, not only following clinical therapeutic application but also as a cellular immune response (Samuel, 2001; Taniguchi & Takaoka, 2002). Hepatitis C virus (HCV) infection is characterized by persistence and replication of the virus in the liver, despite an intact host immune system (Alter, 1997). Indeed, even after administration of the currently most potent IFN reagents, as many as half of the patients are refractory to the treatment and fail to eradicate the virus (Fried *et al.*, 2002). These features have led to speculation that HCV escapes from or attenuates the host antiviral response (Katze *et al.*, 2002).

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Cellular antiviral responses are primarily mediated by IFN and IFN-stimulated genes (ISGs), including 2,5-oligoadenylate synthetase, dsRNA-dependent protein kinase R (PKR) and MxA proteins, as well as by as yet uncharacterized genes (Itsui *et al.*, 2006; Stark *et al.*, 1998). A study of experimental chimpanzee HCV infection has shown that various cytokines and chemokines are induced in the liver during the course of acute HCV infection and its clearance, and that a considerable proportion of the genes is induced by type I IFN (Bigger *et al.*, 2001).

Control of expression of ISGs is mediated by binding of type I IFNs to their receptors. Following receptor binding, STAT1 and STAT2 are phosphorylated to form ISGF-3, which translocates to the nucleus and binds the IFN-stimulated response element (ISRE), located in the promoter/enhancer region of ISGs, and activates transcription of ISGs (Samuel,

2001; Taniguchi *et al.*, 2001; Taniguchi & Takaoka, 2002). ISRE-dependent gene expression is also mediated by binding of the ISRE by molecules such as IRF-1, IRF-3 and IRF-7 (Kanazawa *et al.*, 2004). IRF-3 is a transducer of virus-mediated signalling and plays a critical role in the induction of cellular antiviral responses (Lin *et al.*, 1998; Sato *et al.*, 2000; Taniguchi *et al.*, 2001; Yoneyama *et al.*, 1998). Transcriptional activation and suppression of IRF-3 are inversely correlated with the level of HCV replication *in vitro* (Yamashiro *et al.*, 2006). Following virus infection, IRF-3 is phosphorylated by two cytoplasmic kinases, TBK1 and IKKε (Fitzgerald *et al.*, 2003; Sharma *et al.*, 2003). The phosphorylated IRF-3 forms a homodimer, translocates to the nucleus and predominantly activates expression of the IFN-β gene and certain ISGs (Doyle *et al.*, 2002; Nakaya *et al.*, 2001; Taniguchi & Takaoka, 2002).

RIG-I is a recently identified cytoplasmic DExD/H box RNA helicase that participates in recognition of virus-related dsRNA as a pathogen-related molecular pattern (Yoneyama *et al.*, 2005). RIG-I contains two caspase-recruitment domains (CARDs) in the N terminus and a DExD/H box RNA helicase in the C terminus. MDA5 has been identified as another CARD-containing DExD/H box RNA helicase (Andrejeva *et al.*, 2004). More recently, an adaptor molecule of RIG-I and MDA5, Cardif (also known as IPS-I, MAVS and VISA), has been identified by four independent groups (Kawai *et al.*, 2005; Meylan *et al.*, 2005; Seth *et al.*, 2005; Xu *et al.*, 2005). On association with dsRNA, RIG-I or MDA5 causes conformational changes and homo-oligomerization, and binds the CARD of Cardif (Saito *et al.*, 2007). Cardif subsequently recruits the kinases TBK1 and IKKε, which catalyse phosphorylation and activation of IRF-3 (Yoneyama *et al.*, 1998).

The IRF-3-mediated IFN-β induction pathway could be a target for viruses to counteract antiviral responses and promote their replication in host cells. Ebola virus, bovine viral diarrhea virus (BVDV) and influenza A virus interfere with the activation of IRF-3 through interactions of their virus-encoded proteins (Basler *et al.*, 2003; Schweizer & Peterhans, 2001; Talon *et al.*, 2000). There are several reports that HCV proteins interact with IFN-mediated antiviral systems. The NS5A and E2 proteins have been reported to interfere with the action of IFN by inhibiting the activity of PKR (He & Katze, 2002). It was reported recently that the HCV NS34A protease blocks virus-induced activation of IRF-3, possibly by proteolytic cleavage of Cardif (Foy *et al.*, 2003; Meylan *et al.*, 2005).

The HCV subgenomic replicon is an *in vitro* model that simulates autonomous cellular replication of HCV genomic RNA (Lohmann *et al.*, 1999). Expression of the HCV replicon can be abolished by treatment with small amounts of type I and type II IFNs (Blight *et al.*, 2000; Frese *et al.*, 2002; Guo *et al.*, 2001), suggesting intact IFN receptor-mediated cellular responses. In contrast, viral expression persists in the absence of the exogenous IFN. Baseline expression levels of ISG were substantially decreased in cells expressing the HCV replicon compared with parental Huh7 cells (Kanazawa *et al.*, 2004). These findings led us to speculate that intracellular virus-induced antiviral responses are attenuated or caused to malfunction by the expression of viral proteins.

In this study, we investigated cell lines that support subgenomic HCV replication and HCV cell culture for the dsRNA-induced cellular IFN expression pathway. Here, we report that RIG-I- and Cardif-mediated IFN gene activation is uniformly attenuated in several replicon-expressing cell lines of different lineages and, more importantly, that the HCV NS4B protein is involved in the suppression of antiviral IFN responses.

METHODS

Plasmids. Plasmids pEF-flagRIG-I and ΔRIG-I expressed full-length and C-terminally truncated RIG-I protein, respectively (Yoneyama *et al.*, 2004). The plasmid pER-flagRIG-IKA (RIG-IKA) has a point mutation in the putative ATP-binding site of the RIG-I helicase domain and was used as a negative control for ΔRIG-I and RIG-I full transfection assays. Expression plasmids for full-length Cardif (Cardif), Cardif CARD (CARD) and CARD-truncated Cardif (ΔCARD) were provided by Dr J. Tschopp (University of Lausanne, Switzerland) (Meylan *et al.*, 2005). Expression plasmids for toll-like receptor 3 (TLR3) and TIR domain-containing adaptor inducing IFN-β (TRIF), the transmembrane receptor of dsRNA and the adaptor molecule of TLR3, respectively, were provided by Dr S. Akira (Osaka University, Japan). Plasmids expressing HCV NS345, NS3, NS34A, NS4A, NS4B, NS5A and NS5B were amplified from HCV pCV-J4-L4S (Yanagi *et al.*, 1997) by PCR and subcloned. The DNA fragments were inserted into the vector pcDNA4/TO/*myc*-His (Invitrogen). Nucleotide sequences were confirmed by sequencing. Plasmids TOPO-NS34A (HCV N), TOPO-NS4B (HCV N) and pcDNA-NS4B (HCV JFH1) expressed Myc-tagged NS34A and NS4B proteins derived from the HCV N (Beard *et al.*, 1999) and HCV JFH1 (Wakita *et al.*, 2005) strains, as indicated. Plasmid pISRE-TA-Luc (Invitrogen) contained five copies of consensus ISRE motifs upstream of the firefly luciferase gene. Plasmid pIFNβ-Fluc was constructed by cloning the human IFN-β promoter region, spanning nt −110 to −36, upstream of the firefly luciferase gene of pGL3 Basic (Promega). Plasmid pcDNA3.1 (Invitrogen) was used as an empty vector for mock transfection. pRL-CMV (Promega), which expressed the *Renilla* luciferase protein, was used for correction of transfection efficiency.

Cell culture. HCV strain JFH1-infected Huh7.5.1, Huh7, Huh7.5.1 (kindly provided by Dr F. Chisari, The Scripps Institute, CA, USA; Zhong *et al.*, 2005), HeLa and HEK293 cells were maintained in Dulbecco's modified minimal essential medium (Sigma) supplemented with 2 mM L-glutamine and 10 % fetal calf serum at 37 °C with 5 % CO_2. Cells expressing the HCV replicon were cultured in medium containing 100 μg G418 (Wako) ml^{-1}.

HCV replicon constructs and transfected cell lines. An HCV subgenomic replicon plasmid, pHCVIbneo-delS (designated pRep-N), was derived from an HCV clone of strain N, genotype 1b, and pSGR-JFH1 was derived from HCV JFH1, genotype 2a (Guo *et al.*, 2001; Wakita *et al.*, 2005). These replicons were reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising *Renilla* luciferase and neomycin phosphotransferase to construct pRep-Reo-1b and pRep-Reo-2a, respectively (Tanabe *et al.*, 2004; Yokota *et al.*, 2003). RNA was synthesized from the replicons using T7 polymerase (Promega) and transfected into Huh7,

HeLa and HEK293 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established (Huh7/1bReo, Huh7/2aReo, HeLa/2aReo and 293/2aReo). We have previously reported that firefly luciferase activities of Feo-replicon-expressing cells correlate well with HCV NS3, NS4A and NS5A protein expression levels and with the levels of replicon RNA (Yokota *et al.*, 2003).

Transient transfection. Transient DNA transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. ISRE reporter assays were carried out as previously described (Nakagawa et al., 2004). To analyse IFN expression in HCV JFH1 cell cultures, a total of 1×10^5 Huh7.5.1, JFH-1 infected Huh7.5.1 and IFN-treated Huh7.5.1 cells were seeded into 24-well plates the day before transfection. Plasmids pISRE-TA-Luc and ΔRIG-I (200 ng each) were transfected using 1 μl Lipofectamine 2000. RIG-IKA was used as a control. Luciferase assays were performed on day 3 post-transfection.

For further study, 400 ng of each non-structural protein was added to 1×10^4 Huh7 or HEK293 cells that had been seeded into 96-well plates the day before transfection. pISRE-TA-Luc and ΔRIG-I (40 ng each) were transfected using 0.5 μl Lipofectamine 2000. RIG-IKA was used as a control.

Western blotting. Preparation of the cytoplasmic and nuclear fractions of cell lysates was carried out as described previously (Tanabe *et al.*, 2004). Protein (20 μg) was separated using NuPAGE 4–12 % Bis/Tris gels (Invitrogen) and blotted onto an Immobilon PVDF membrane (Roche). The membrane was immunoblotted with anti-IRF-3 (Santa Cruz) and detected by chemiluminescence (BM Chemiluminescence Blotting Substrate; Roche).

RT-PCR. Interleukin (IL)-8 mRNA was detected by RT-PCR as described previously (Itsui *et al.*, 2006). The primers used were IL8-S (5′-GCACAAACTTTCAGAGACAGCAGAGCACAC-3′) and IL8-AS (5′-CAGAGCTGCAGAAATCAGGAAGGCTGCCAA-3′).

Indirect immunofluorescence assay. Cells seeded onto tissue culture chamber slides were fixed with cold acetone. The cells were incubated with anti-protein disulphide isomerase (PDI) or anti-Myc antibodies and subsequently with Alexa 488- or Alexa 568-labelled secondary antibodies. Cells were mounted with VECTA SHIELD Mounting Medium and DAPI (Vector Laboratories) and visualized by fluorescence microscopy (BZ-8000; Keyence).

Luciferase reporter assays. Luciferase activity was measured using a 1420 Multilabel Counter (ARVO MX; PerkinElmer) using a Bright-Glo Luciferase Assay System (Promega) or a Dual Luciferase Assay System (Promega). Assays were carried out in triplicate and the results expressed as means ± SD.

MTS assay. To evaluate cell viabilities, dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assays were performed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega) according to manufacturer's instructions.

Statistical analyses. Statistical analyses were performed using an unpaired, two-tailed Student's *t*-test. *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

IRF-3 dimer formation is attenuated in cells expressing the HCV replicon

In the HCV replicon-expressing cell lines Huh7/Rep-Reo-2a, Hela/Rep-Reo-2a and 293/Rep-Reo-2a, replicon expression levels corresponded well to internal *Renilla* luciferase activities. Expression of the HCV replicon was suppressed by IFN in a dose-dependent manner (data not shown).

Activation of RIG-I or MDA5 induces phosphorylation and homodimerization of IRF-3. Following transfection of poly(I : C) into Huh7, HeLa or HEK293 cells, IRF-3 dimers were detected (Fig. 1). However, in cells supporting HCV replicons, IRF-3 dimer formation was almost completely abolished. These findings showed that expression of HCV proteins blocked activation of dsRNA-mediated IFN expression and that these effects were consistently found in several cell lines of different origin.

The HCV replicon suppresses RIG-I/Cardif-induced IFN responses

ISRE reporter activities did not increase in naïve Huh7, HeLa or HEK293 cells following transfection of poly(I : C), whilst overexpression of full-length RIG-I increased poly(I : C)-mediated ISRE reporter activity in Huh7 and HEK293 cells (data not shown). In RIG-I-overexpressing Huh7 cells, transduction with an HCV replicon abolished the poly(I : C)-induced ISRE activation, and elimination of the replicon by IFN treatment restored these ISRE responses (Fig. 2a). Consistent results were obtained by overexpression of ΔRIG-I, a constitutively active form. Transfection of ΔRIG-I in Huh7 and HEK293 cells induced ISRE activation, whilst these responses were abolished or significantly suppressed in cell lines expressing HCV replicons and were recovered by eliminating the replicon by IFN treatment (data not shown). Similarly, ISRE activation by overexpression of Cardif, an adaptor molecule of RIG-I, was almost completely blocked in replicon-expressing cells and was recovered by eliminating the replicon from the cells (data not shown). The RIG-I-mediated IFN response was

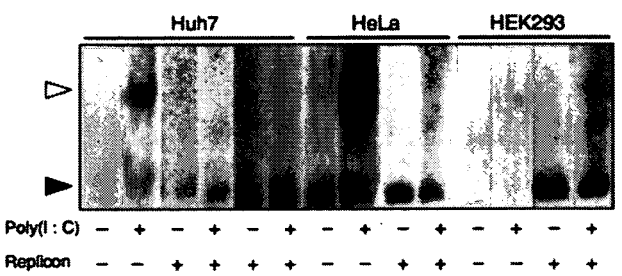

Fig. 1. Double-stranded RNA-induced IRF-3 dimer formation in cell lines that support HCV subgenomic replication. Poly(I : C) was transfected into naïve Huh7, HeLa and HEK293 cells, and into corresponding cell lines expressing the HCV replicon. Six hours after transfection, cell lysates were prepared, separated in polyacrylamide gels and blotted onto PVDF membrane. The membrane was immunoblotted with anti-IRF-3 and visualized by chemiluminescence (see Methods). The positions of the IRF-3 dimer (open arrowhead) and monomer (closed arrowhead) are indicated.

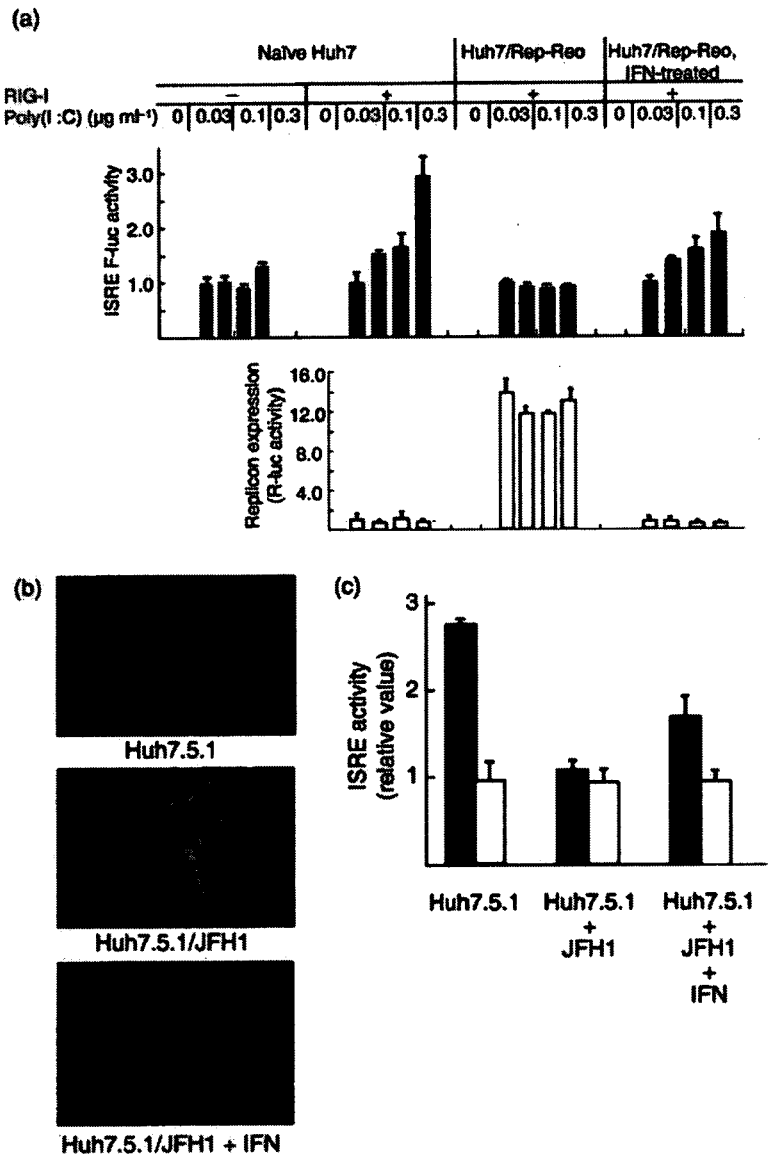

Fig. 2. Suppression of dsRNA-induced, RIG-I-mediated ISRE activation by HCV replication. (a) The HCV replicon suppresses transcriptional activation after poly(I : C) stimulation. The RIG-I expression plasmid and pISRE-TA-Luc were transiently transfected into the cell lines indicated. The following day, the amounts of poly(I : C) indicated were transfected into the corresponding cell lines and dual luciferase assays were carried out 8 h after transfection. Filled bars indicate ISRE-regulated firefly luciferase (F-luc) activities and open bars indicate *Renilla* luciferase (R-luc) activities representing replicon expression levels. In both graphs, scales for the *y*-axis are shown as relative values. Assays were carried out in triplicate and results are shown as means ± SD. (b) Immunofluorescence microscopy results. Huh7.5.1 cells infected with HCV JFH1 (Huh7.5.1/JFH1) and JFH1-infected cells from which the virus had been eliminated by IFN treatment (Huh7.5.1/JFH1 + IFN) were incubated with anti-core primary antibodies followed by Alexa Fluor-conjugated secondary antibody (green). Nuclei were stained with DAPI (blue). (c) ISRE activation by ΔRIG-I overexpression. The plasmid pISRE-TA-Luc was co-transfected with ΔRIG-I (filled bars) or RIG-IKA (empty bars) into naïve Huh7.5.1, Huh7.5.1/JFH1 or Huh7.5.1/JFH1 + IFN cells. Luciferase assays were carried out 8 h after transfection. The *y*-axis indicates ISRE-regulated luciferase activity shown as relative values. Assays were carried out in triplicate and results are shown as means ± SD.

also suppressed in HCV JFH1 virus cell culture. In JFH1-infected Huh7.5.1 cells, ΔRIG-I-induced ISRE reporter activation was significantly suppressed, but was recovered in IFN-treated, virus-eliminated cells (Fig. 2b and c). These results demonstrated that RIG-I- and Cardif-mediated antiviral responses were substantially suppressed by both subgenomic and genomic viral replication in both hepatocyte- and non-hepatocyte-derived host cells.

NS34A and NS4B are responsible for suppressing RIG-I-mediated IFN responses

We next sought to define which HCV proteins were responsible for inhibition of the RIG-I- and IRF-3-mediated IFN induction pathway. We constructed expression plasmids that expressed the non-structural proteins NS345, NS3, NS34A, NS4A, NS4B, NS5A and NS5B (Fig. 3a). We transfected each expression plasmid with simultaneous activation of the RIG-I pathway by overexpression of ΔRIG-I, Cardif, TBK1 and IKKε (Fig. 3b–e). Expression of full-length non-structural (NS345) and NS34A proteins inhibited ISRE activation mediated by expression of RIG-I and Cardif but not that mediated by TBK1 and IKKε. Interestingly, it was found that NS4B also inhibited ISRE activation mediated by expression of RIG-I and Cardif, but not by TBK1 and IKKε. Consistent with Fig. 3(b), overexpression of NS4B significantly suppressed ΔRIG-I-induced activation of the authentic IFN-β promoter (Fig. 3f).

Another group has studied IFN antagonism of flavivirus non-structural proteins and has reported that HCV NS4B did not affect IFN responses (Muñoz-Jordán *et al.*, 2005).

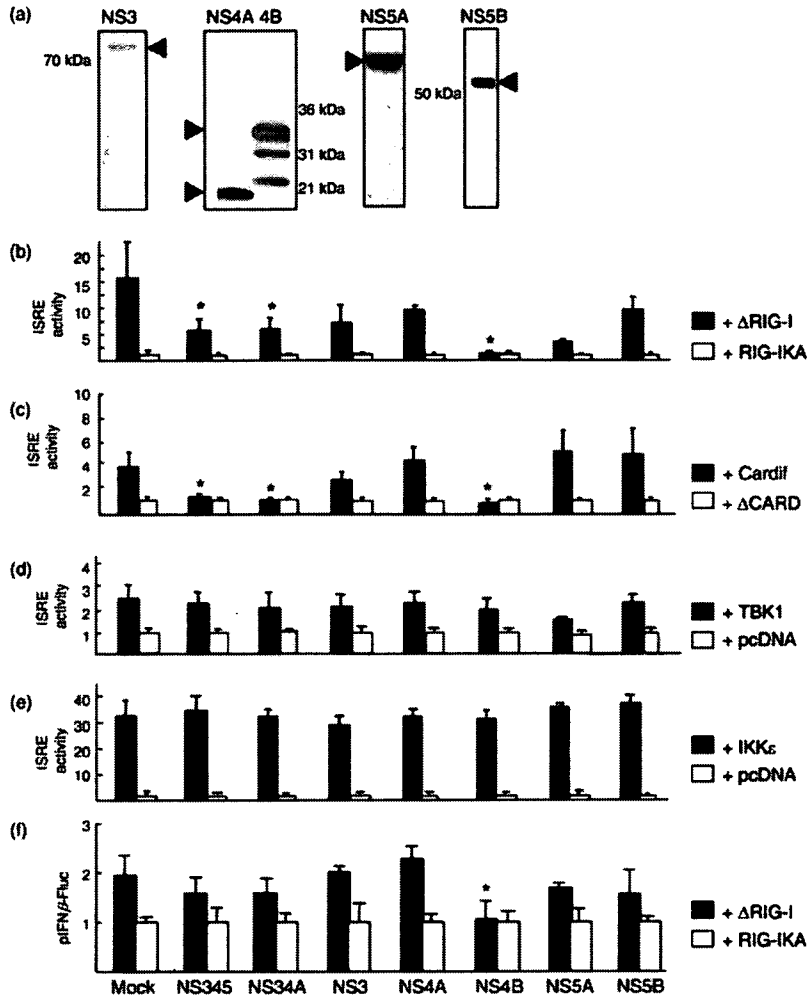

Fig. 3. Co-transfection analyses using plasmids that express individual HCV non-structural proteins. (a) Western blotting. Plasmids expressing the indicated Myc-tagged HCV proteins were transfected into Huh7 cells. Western blotting was carried out using anti-Myc antibody. (b–e) The following plasmids were co-transfected into Huh7 cells: pISRE-TA-Luc, pRL-CMV, the indicated plasmids expressing ΔRIG-I (b), Cardif (c), TBK1 (d) and IKKε (e), and the indicated plasmids expressing individual HCV non-structural proteins. Plasmids RIG-IKA, ΔCARD or pcDNA were used as negative controls as indicated. Twenty-four hours after transfection, luciferase activities were measured. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. *, $P<0.05$. (f) pIFN-β and pRL-CMV were co-transfected into Huh7 cells, with plasmids expressing individual HCV non-structural proteins and plasmid expressing ΔRIG-I. Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. *, $P<0.05$. Plasmid RIG-IKA was used as a negative control.

To investigate strain-specific differences in the characteristics of NS4B proteins, we performed co-transfection assays using NS4B expression constructs from HCV N (Beard *et al.*, 1999) and JFH1 (Wakita *et al.*, 2005) strains, as well as HCV strain J4 (Fig. 4a and b). All NS4B constructs suppressed ΔRIG-I- or Cardif-mediated ISRE activation. These results suggested that the above-described effects of NS4B were independent of HCV strain.

NS4B has been reported to induce an unfolded protein response or endoplasmic reticulum (ER) stress through ATF6 or IRE1-X box protein (XBP1) pathways (Zheng *et al.*, 2005). The ER stress induces production of IL-8, which has been reported to interfere with the IFN system (Polyak *et al.*, 2001). Therefore, we detected expression of IL-8 using RT-PCR in cells with and without overexpression of NS4B. As shown in Fig. 4(c), no significant difference was observed in IL-8 mRNA levels among mock-, NS34A- and NS4B-transfected cells. These results showed that NS4B overexpression in the present study did not induce expression of IL-8 and that the IFN-antagonizing effects of NS4B were independent of IL-8.

It has been reported that NS34A suppresses the TLR3-mediated IFN response (Breiman *et al.*, 2005; Ferreon *et al.*, 2005). However, overexpression of HCV non-structural proteins did not suppress ISRE activation that was induced by overexpression of TLR-3 or TRIF (Fig. 5a and b), nor did NS34A from two different HCV strains, J4 and N, show significant suppression of TRIF-mediated ISRE activation (Fig. 5c). Although strain-specific differences might be involved, these data suggest that neither NS34A nor NS4B affect the TLR3-triggered, TRIF-mediated IFN expression signalling pathway.

The NS4B N terminus is involved in inhibition of the RIG-I-mediated pathway

Given the result that NS4B suppressed the RIG-I-mediated IFN expression pathway, we next investigated which domain of NS4B was responsible. We constructed plasmids that expressed truncated NS4B in which the protein-coding frame was truncated at four positions corresponding to the five transmembrane domains (Lundin *et al.*, 2003) (Fig. 6a).

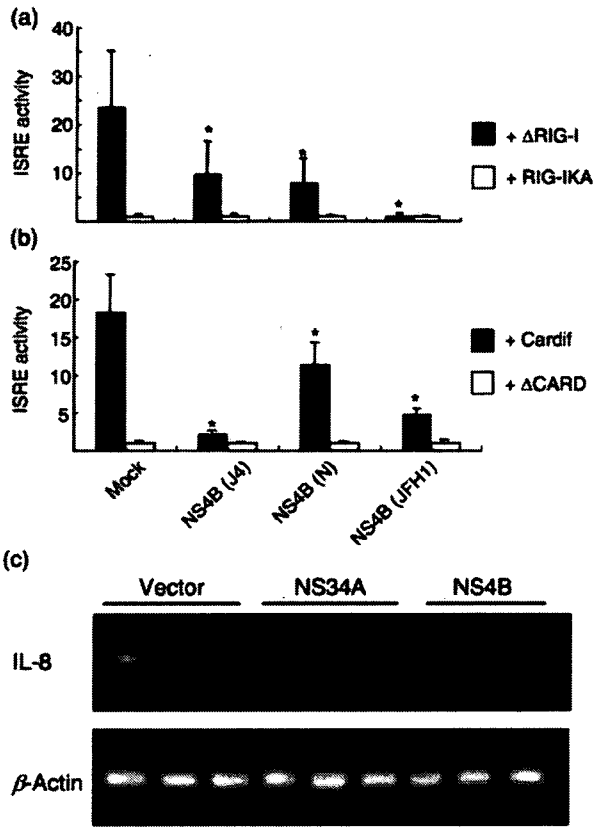

Fig. 4. Co-transfection analyses of HCV NS4B proteins of different origins. NS4B (J4), NS4B (N) and NS4B (JFH1) denote HCV NS4B proteins that were cloned from HCV strains J4, N and JFH1, respectively. The NS4B plasmids indicated were co-transfected with plasmids expressing ΔRIG-I (a) or Cardif (b). Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. *, $P<0.05$. (c) Semi-quantitative detection of IL-8 mRNA by RT-PCR. cDNA was prepared from Huh7 cells transfected with empty vector or with NS34A or NS4B expression plasmid.

Expression and subcellular localization of NS4B truncated proteins were visualized by indirect immunofluorescence assays (Fig. 7). Each of the NS4B truncated proteins was localized predominantly to the perinuclear rim as dense spots. Some of the spots were similar to the staining of the ER-resident host protein PDI, consistent with previous reports (Lindström *et al.*, 2006; Lundin *et al.*, 2006). These truncated expression plasmids were co-transfected with Cardif expression plasmids into Huh7 cells. As shown in Fig. 6(b), Cardif-mediated ISRE activation was significantly suppressed by co-transfection of NS4Bt1–156 and NS4Bt1–186, as well as full-length NS4B, whilst transfection of NS4Bt90–260 and NS4Bt110–260 did not significantly suppress Cardif-mediated ISRE activation. The shortest construct, NS4Bt131–260, partially retained the ability to reduce ISRE activity. These results suggested that the N-terminal domain of NS4B, which includes aa 1–110, might function directly to suppress RIG-I-mediated IFN expression responses.

DISCUSSION

The recent discovery of cytoplasmic dsRNA sensor molecules has resulted in rapid expansion of knowledge about the IFN-mediated virus defence pathway (Yoneyama *et al.*, 2004). Several reports suggest that viruses target the IFN system to establish replication in the host cells (Kato *et al.*, 2006). We have confirmed that the dsRNA-triggered, IRF-3-mediated IFN activation pathway was blocked in several replicon-supporting cell lines (Fig. 1). Similarly, the dsRNA responses were substantially suppressed in HCV JFH-1 cell culture compared with parental Huh7 cells (Fig. 2b and c). Overexpression analyses showed that RIG-I- and Cardif-mediated ISRE activation was significantly suppressed in HCV replicon-expressing cells, which recovered after elimination of the replicon by IFN treatment (Fig. 2a). In contrast, TBK1- or IKKε-mediated ISRE activation was not suppressed in replicon-expressing cells. Overexpression of individual HCV non-structural proteins revealed that not only NS34A but also NS4B inhibited the ISRE activation signal (Figs 3, 4 and 5). These results suggested that HCV non-structural proteins suppress the IFN induction pathway and that the target host molecule could be Cardif or an unknown adaptor molecule acting between Cardif and TBK1/IKKε.

NS4B protein is a 27 kDa hydrophobic integral membrane protein that is localized in the ER with other non-structural proteins. Studies on other flaviviruses such as Kunjin virus and BVDV support the notion that NS4B may indeed be an essential part of the replication mechanism (Grassmann *et al.*, 2001; Khromykh *et al.*, 2000; Li & McNally, 2001; Qu *et al.*, 2001). These systems have demonstrated that intact NS4B is necessary in a *cis* configuration in the polyprotein for maintaining viral replication (Grassmann *et al.*, 2001; Khromykh *et al.*, 2000). Furthermore, single mutations in NS4B alter the cytopathic effects of BVDV and even mediate changes in the cellular tropism of Dengue virus (Hanley *et al.*, 2003; Qu *et al.*, 2001). In HCV, the search for cell-culture-adaptive mutations in HCV subgenomic replicons has led to the generation of mutations in the NS4B region that confer higher replication levels and resistance to IFNs, as well as broadening the tropism for different cell lines (Lohmann *et al.*, 2003; Sumpter *et al.*, 2004; Zhu *et al.*, 2003). These pieces of evidence may imply that NS4B is not only part of the replication machinery but may also have other functions that enable establishment of viral replication.

NS4B truncation assays showed that RIG-I/Cardif-mediated ISRE activation was significantly suppressed by expression of N terminus-containing constructs (Fig. 6). These results imply that the N-terminal domain of NS4B, which is located between positions 1 and 110, may be essential for suppressing IFN expression responses in host

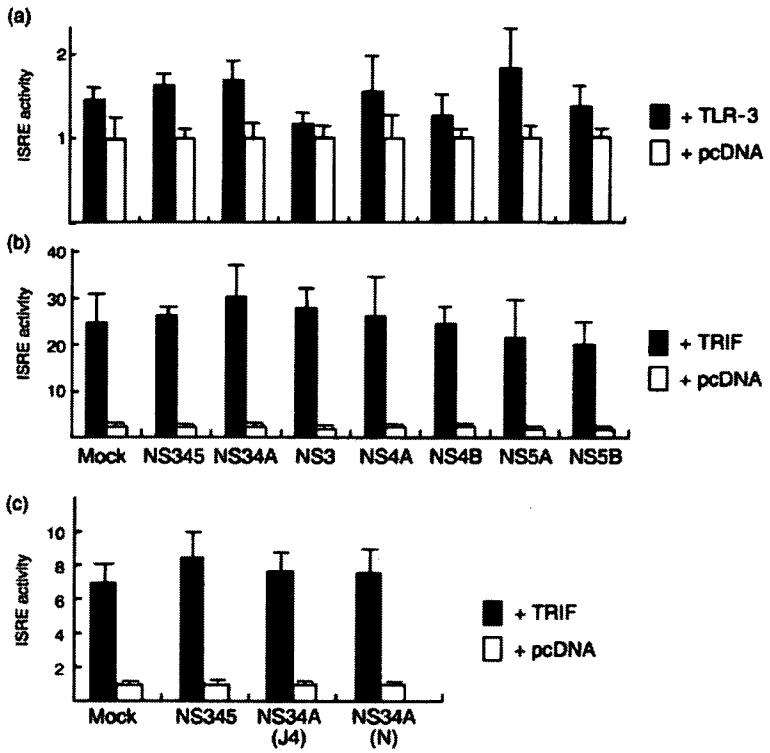

Fig. 5. Co-transfection analyses of HCV non-structural proteins and plasmid expressing TLR-3 or TRIF. (a, b). The following plasmids were co-transfected into Huh7 cells: pISRE-TA-Luc, pRL-CMV, plasmids expressing TLR-3 or TRIF and plasmids expressing individual HCV non-structural proteins, as indicated. Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. (c) NS34A (J4) and NS34A (N) denote plasmids expressing HCV NS34A derived from HCV strains J4 and N, respectively. The NS4B plasmids indicated were co-transfected with pISRE-TA-Luc, pRL-CMV and plasmids expressing TRIF or pcDNA. Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD.

cells. Lindström *et al.*, (2006) investigated single point mutations in NS4B that negatively affected expression efficiency of the HCV replicon and reported that most of the active mutations were located around the N-terminal domain. A distinctive feature of NS4B is that it requires membrane rearrangement to form its native structure

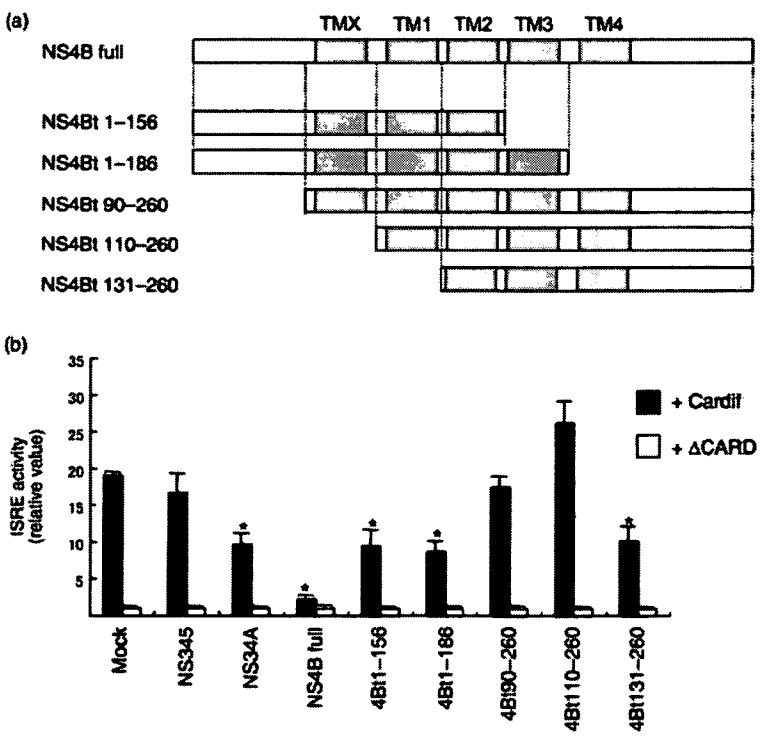

Fig. 6. Co-transfection analyses using truncated NS4B expression constructs. (a) Truncated constructs of NS4B. The protein-coding frame of NS4B was truncated in five constructs corresponding to the five transmembrane domains, as reported by Lundin *et al.* (2003). (b) The truncated NS4B plasmids, pISRE-TA-Luc and the Cardif- or ΔCARD-expressing plasmids indicated were co-transfected into Huh7 cells. Luciferase activities were measured 24 h after transfection. Results are given as means ± SD.

(Lindström *et al.*, 2006; Lundin *et al.*, 2006). The HCV polyprotein is translated from a single reading frame and subjected to proteolytic cleavage by the host signal peptidase and two viral proteases (Grakoui *et al.*, 1993). The mature form of NS4B is localized in the ER and constitutes a subcellular structure called the membrane-associated focus (MAF) (Gretton *et al.*, 2005). Once the NS4B is cleaved, the N-terminal peptide of NS4B is translocated from the cytoplasmic to the luminal side, giving it a fifth transmembrane region (Lundin *et al.*, 2006). The N-terminal amphipathic helix (AH) 1 of NS4B is necessary for this translocation and for MAF formation; NS4B molecules that were truncated at the AH1 lacked the ability to create the MAF, to translocate and to replicate (Elazar *et al.*, 2004; Lindström *et al.*, 2006).

In our assay, NS4Bt131–260 regained the ability to reduce ISRE activity. As we confirmed that all mutants co-localized with the ER, there may be some effect of the N-terminal localization of this mutant. The precise mechanism of NS4B suppression is still not clear and further experiments are needed.

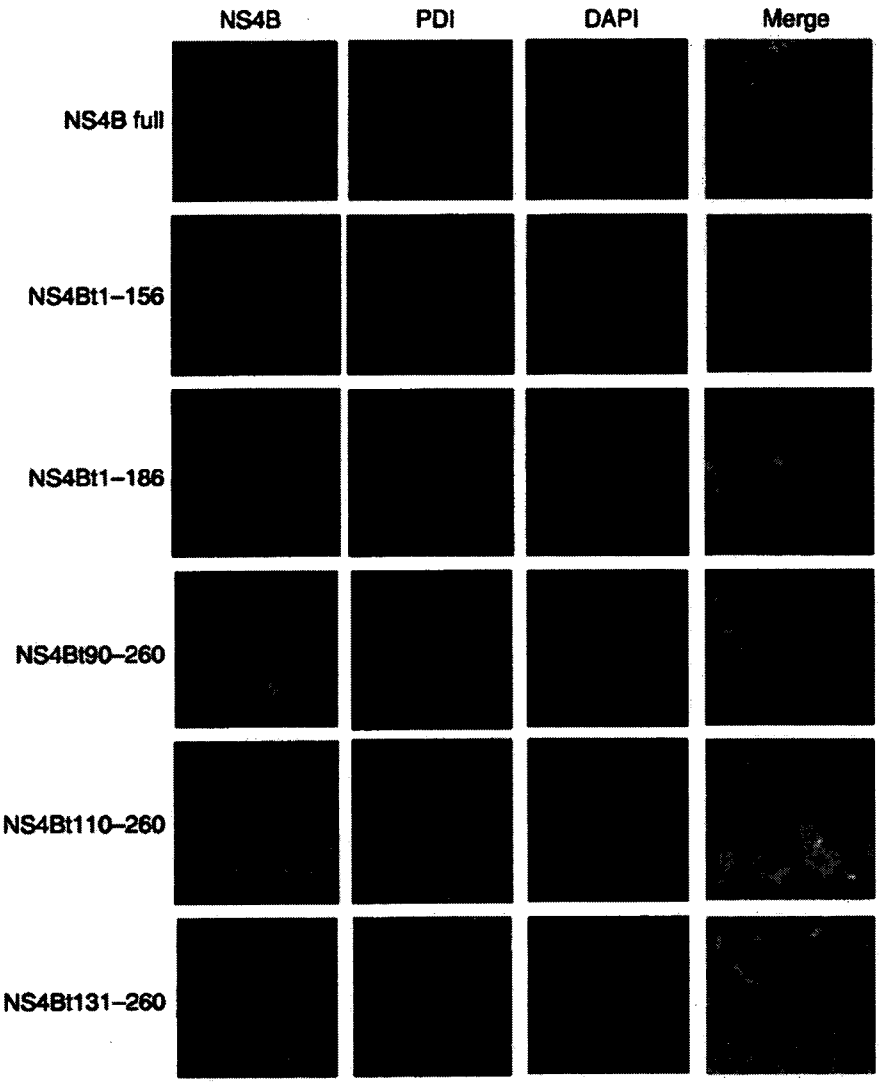

Fig. 7. Indirect immunofluorescence analysis of truncated NS4B proteins. The NS4B constructs indicated were transiently transfected into Huh7 cells. After 48 h, cells were labelled with anti-Myc or anti-PDI antibody. NS4B proteins were immunostained with Alexa Fluor 488-labelled goat anti-mouse IgG, whilst PDI was stained with Alexa Fluor 568-labelled goat anti-rabbit IgG. DAPI staining revealed the nuclear chromatin. Representative immunofluorescence images derived from a number of experiments are shown as four images of a single focal plane of Huh7 cells, showing NS4B proteins (green), PDI (red), DAPI staining (blue) and the superimposed images (merge).