

Antibody to hepatitis B core antigen is associated with the development of hepatocellular carcinoma in hepatitis C virus-infected persons: A 12-year prospective study

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Abstract. Several studies have reported that antibody to hepatitis B core antigen (anti-HBc) positivity may influence the development of hepatocellular carcinoma (HCC) in chronic hepatitis C patients, but the evidence is still not conclusive. In this study, we examined whether the presence of anti-HBc positive was associated with the development of HCC in hepatitis C virus (HCV)-infected subjects among the residents in an HCV hyperendemic area who were followed up for 12 years. In an HCV hyperendemic area (positive rate of anti-HCV: 23.4%), 509 residents were examined by health screening in 1990. After 12 years of follow-up, we evaluated the risk factors for HCC. The incidence of HCC was compared between anti-HBc positive and anti-HBc negative subjects after 12 years of prospective observation. Univariate and multivariate analyses were conducted to determine risk factors for the development of HCC. The incidence of HCC was significantly higher in the anti-HBc positive group (13 subjects) than in the anti-HBc negative group (0 subjects) ($P=0.012$). Multivariate analysis identified positivity for anti-HBc and HCV RNA, history of icterus, and female gender as independent determinants of the development of HCC. Our findings provide clear evidence in a prospective study that presence of anti-HBc, that is, past hepatitis B virus (HBV) infection, is a risk factor for the development of HCC in HCV-infected people.

Introduction

The number of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection carriers worldwide is estimated at 350 million (1) and 170 million (2), respectively. HBV and HCV

infections include substantial proportions of cases with past infection, asymptomatic carriers, acute hepatitis and chronic hepatitis, and HBV infections may cause fulminant hepatitis. Especially, chronic HBV and HCV infections may lead to cirrhosis and hepatocellular carcinoma (HCC) (1,3). It was reported that the frequency of HCC due to chronic HCV infection is higher in Japan than in any other country (4). Several studies have reported that occult HBV infection may also be one of the causative factors of HCC (5,6). The presence of occult HBV infection is diagnosed based on the fact that HBV DNA still exists in serum and liver tissue after hepatitis B surface antigen (HBsAg) disappears in acute or chronic HBV infection (7-9), or even after antiviral treatment is successful. Although some studies reported that occult HBV infection is associated with HCV-related liver dysfunction (10) or the development of HCC (11-13), these associations have still not been clearly demonstrated in a prospective study.

A higher incidence of HBV DNA is commonly seen in patients with anti-HBc-positive serology than in those with anti-HBc negative serology in coinfections with HBV and HCV (10), and using PCR amplification, most studies have demonstrated the presence of the HBV DNA genome in 22% to 87% of the patients who are HBsAg negative and HCV RNA positive (10,14-18). Some studies showed that HBV infection could occur in recipients of livers donated from subjects with anti-HBc but without HBsAg (19,20). That is, anti-HBc, which was initially considered to be an index for the past HBV infection in which all HBV had been cleared, has emerged as a convincing marker of occult hepatitis B (19,21-23). Also, several studies showed that the anti-HBc positivity was associated with the development of HCC in patients with HCV-associated chronic liver disease (11,24-26), but these associations have not been clearly demonstrated.

Since 1990, we have conducted health screenings of the residents of H town (adult population: 7,389), Fukuoka prefecture in northern Kyushu, Japan (27). This town is known for its high prevalence of liver disease. We previously reported that the town had a high prevalence of HCV carriers, 120/509 (23.6%) in 1990, and that HCV infection was the principal cause of liver dysfunction and HCC (27,28).

In the present study, we analyzed the influence of anti-HBc positivity on the development of HCC in HCV-infected people in the same town during 12 years in a prospective manner.

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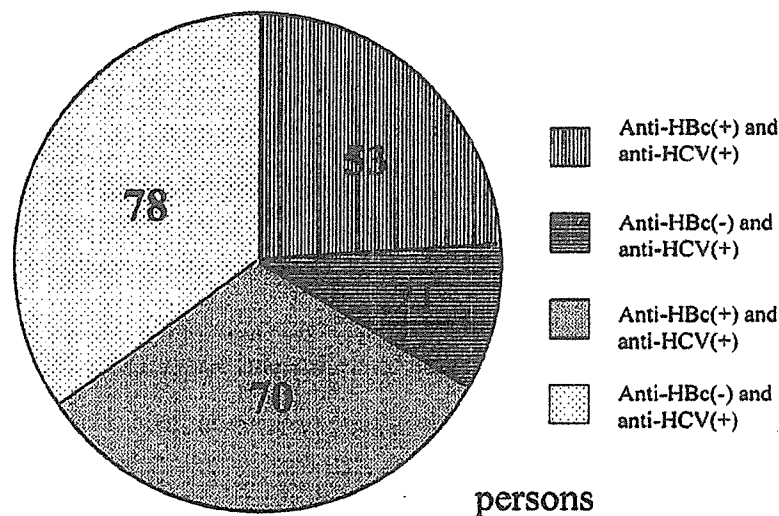


Figure 1. Diagram showing incident of hepatitis virus markers (anti-HCV and anti-HBc) among the 222 inhabitants 12 years ago. Fifty-three inhabitants were anti-HBc positive and anti-HCV positive, 21 were anti-HBc negative and anti-HCV positive, 70 were anti-HBc positive and anti-HCV negative, and 78 were anti-HBc negative and anti-HCV negative.

Subjects and methods

Subjects. In 1990, of a total 9,799 inhabitants, 739 (10%) of the 7,389 inhabitants >20 years old were randomly selected as follows: the names of the residents (as they appeared on their resident cards) were arranged in order according to the Japanese phonetic syllabary. Then every tenth resident was selected. As a result, 509 subjects (6.9% of H town residents) gave their informed consent to participate in the study.

Of 509 participants initially screened in 1990, 69 people had died and 55 people had moved to other regions as of 2002. Thus, 385 of the original 509 residents survived in the area and 139 residents agreed to participate in the medical follow-up survey, while 26 did not agree to participate, and the remaining 220 residents did not declare their intention either way in 2002. For 14 of these remaining 220 inhabitants, the records were obtained from the primary physicians. Consequently, we analyzed the outcome in terms of the liver disease in 222 inhabitants (69+139+14) in 2002.

Information on cigarette smoking, alcohol consumption, and history of icterus, and blood transfusion was obtained at the time of enrollment through interviews by the doctors in charge and experienced public health nurses. Smoking was defined as >10 cigarettes per day for >10 years. Alcohol consumption was defined as a daily intake of ≥ 75 g of ethanol per day for >10 years.

Serological assay. In 1990, sera were collected from all the participants, and conventional liver function tests were performed: serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gammaglutamyl transpeptidase (γ -GTP), total protein (TP), albumin (Alb), total cholesterol (TC), total bilirubin (TB), zinc turbidity (ZTT) were measured. Anti-HCV was measured using HCV PHA 2nd generation kits (Dainabot Co. Ltd., Tokyo, Japan). These results were confirmed using a second generation recombinant immunoblot assay (RIBA II) (Ortho Diagnostic

System, Tokyo, Japan). Measurement of HBsAg and anti-HBc was performed with an enzyme immunoassay kit (Mizuho Medy Co. Ltd., Tosu, Saga, Japan). Titers of anti-HBc yielding >70% inhibition were assessed as positive.

Detection of HCV RNA by RT-PCR. All subjects who were anti-HCV-positive were tested for the presence of serum HCV RNA, which was detected by reverse transcription-nested polymerase chain reaction (RT-nested PCR) using primers based on the sequences of the 5'UTR (untranslated region) of the HCV genome, as described previously (29).

Statistical analysis. Continuous data were expressed as mean \pm SD, minimum and maximum. Categorical data were expressed as frequency and/or percentage. For comparing the background between anti-HBc positive and negative, the χ^2 and Wilcoxon's test were used to analyze quantitative data. Univariate and multivariate analysis were performed by logistic regression to calculate odds ratio and its 95% confidence interval. The SAS (statistical analysis system) computer program (release 8.2; SAS Institute Inc., Cary, NC, USA) was used for the logistic regression. A P-value of <0.05 was considered statistically significant.

Results

In 2002, anti-HCV was detected in 74 of the 222 inhabitants (Fig. 1). HCV RNA was detected in 53 (71.6%), HBsAg in 1 (1.4%), and anti-HBc in 53 (71.6%) of these 74 people. We asked the primary physician of these 74 inhabitants about the diagnosis of liver disease, and found thereby that 8 inhabitants had died of HCC and 5 inhabitants had been treated for HCC (total 13 inhabitants).

The 74 inhabitants were divided into two groups: 53 who were positive and 21 who were negative for anti-HBc, and the clinical characteristics observed in the screening were compared between the two groups. No significant differences

Table I. Characteristics of anti-HCV positive patients with and without anti-HBc.

Characteristics	Anti-HBc positive (N=53)	Anti-HBc negative (N=21)	P-value
Age (year)	62.3±10.9	58.0±16.4	NS
Sex: M:F	23:30	05:16	NS
Smoking (%)	16 (30.2)	4 (19.0)	NS
History of icterus (%)	8 (15.1)	3 (14.3)	NS
Alcohol consumption (%)	3 (5.7)	2 (9.5)	NS
History of blood transfusion (%)	8 (15.1)	4 (19.0)	NS
ALT level (IU/l)	40.6±30.8	27.5±17.9	NS
HBsAg (%)	1 (1.9)	0 (0)	NS
HCV RNA (%)	39 (73.6)	14 (66.7)	NS
HCC (%)	13 (24.5)	0 (0)	0.012

Age and serum ALT level were expressed as mean ± SD. HCC, hepatocellular carcinoma; NS, not significant.

Table II. Univariate analysis of risk factors that influence the development of HCC.

Factors	HCC group (n=13)	non-HCC group (n=61)	Odds ratio	95% CI	P-value
Age (years)	65.3±8.1 (53-82)	60.1±13.4 (23-89)	1.035	0.984-1.088	0.1866
Sex: male (%)	6 (46.2)	22 (36.1)	0.658	0.196-2.205	0.4976
Smoking (%)	4 (30.8)	13 (21.3)	1.641	0.435-6.190	0.4646
Alcohol consumption (%)	5 (38.5)	22 (36.1)	1.108	0.323-3.804	0.8706
History of blood transfusion (%)	3 (23.1)	8 (13.1)	1.988	0.448-8.810	0.3659
History of icterus (%)	5 (38.5)	5 (8.2)	7.000	1.652-29.667	0.0083 ^a
AST (IU/l)	65.5±31.1 (28-131)	33.0±21.9 (13-132)	1.041	1.015-1.068	0.0016 ^a
ALT (IU/l)	57.5±24.8 (20-108)	32.6±27.1 (9-160)	1.028	1.006-1.050	0.0119 ^a
γ-GTP (IU/l)	127.1±195.3 (17-720)	32.4±34.2 (7-196)	1.015	1.003-1.027	0.0158 ^a
Total protein (IU/l)	7.97±0.88 (6.6-10.0)	8.05±0.58 (6.6-9.8)	0.808	0.309-2.107	0.6622
Albumin (g/dl)	3.98±0.49 (3.0-4.9)	4.33±0.31 (3.2-4.8)	0.094	0.017-0.507	0.0060 ^a
Total cholesterol (mg/dl)	160.5±33.1 (111-224)	173.8±32.5 (111-257)	0.987	0.967-1.007	0.1851
Total bilirubin (mg/dl)	1.01±0.50 (0.5-2.3)	0.77±0.27 (0.4-1.3)	7.537	1.170-48.533	0.0335 ^a
ZTT (KU)	15.35±5.76 (1.1-21.7)	11.40±4.86 (2.5-27.4)	1.161	1.026-1.314	0.0183 ^a
Anti-HBc (%)	13 (100)	40 (65.6)	9.150	1.407-	0.0161 ^a
HCV RNA (%)	13 (100)	40 (65.6)	9.150	1.407-	0.0161 ^a

^aP<0.05; HCC, hepatocellular carcinoma; CI, confidence interval. Age, AST, ALT, γ-GTP, total protein, albumin, total bilirubin and ZTT were expressed as mean ± SD (range).

were observed between the two groups in age, sex, smoking, history of icterus or blood transfusion, alcohol consumption, ALT level, HBsAg, or HCV RNA. Significant differences were observed for the incidence of HCC (13 versus 0) between these two groups (P=0.012) (Table I).

Univariate and multivariate analyses of factors that influenced the incidence of HCC. The influence of age, sex, smoking, history of icterus, history of blood transfusion, alcohol consumption, AST, ALT, γ-GTP, TP, Alb, TC, TB, ZTT, anti-HBc and HCV RNA on the development of HCC was analyzed by univariate and multivariate analyses.

Table II shows the basic characteristics of the 74 inhabitants with anti-HCV divided into two groups: a group with HCC (HCC group) and a non-HCC group, and shows the results of univariate analyses. The mean age and sex were not significantly different between the HCC group and non-HCC group. Serum levels of AST, ALT, γ-GTP, TB, and ZTT were significantly higher in the HCC group than in the non-HCC group (P<0.05). The serum level of Alb was significantly lower in the HCC group than in the non-HCC group (P<0.05). The frequency of anti-HBc, HCV RNA, and history of icterus were significantly higher in the HCC group than in the non-HCC group (P<0.05). The frequency of smoking, alcohol

Table III. Multivariate analysis of risk factors that influence the development of HCC.

Factors	Odds ratio	95% CI	P-value
Age (years)	0.987	0.852-1.132	0.8428
Sex: female	190.517	2.157- >999.999	0.0188 ^a
Smoking	40.580	0.656- >999.999	0.0824
Alcohol consumption	5.051	0.163-3.804	0.3644
History of blood transfusion	0.964	<0.001- >999.999	0.9918
History of icterus	311.186	5.066- >999.999	0.0042 ^a
AST (IU/l)	1.013	0.855-1.244	0.8776
ALT (IU/l)	0.974	0.791-1.101	0.7013
γ -GTP (IU/l)	1.006	0.990-1.080	0.6950
Total protein (IU/l)	15.131	0.227- >999.999	0.2035
Albumin (g/dl)	<0.001	<0.001-11.319	0.1236
Total cholesterol (mg/dl)	1.018	0.952-1.106	0.6028
Total bilirubin (mg/dl)	7.911	0.060- >999.999	0.4127
ZTT (KU)	0.695	0.370-1.196	0.1853
Anti-HBc positive	>999.999	1.556-	0.0292 ^a
HCV RNA positive	>999.999	3.767-	0.0063 ^a

^aP<0.05; HCC, hepatocellular carcinoma; CI, confidence interval.

consumption, and history of blood transfusion were not significantly different between the HCC group and non-HCC group.

Multivariate logistic regression analyses identified anti-HBc positivity, HCV RNA positivity, history of icterus, and female sex as independent risk factors for the development of HCC (Table III).

Discussion

Several studies have shown that anti-HBc positivity was associated with the development of HCC in patients with HCV-associated chronic liver disease (11,24-26). However, considering the natural history of all HCV infections, the results of those previous studies have some problems, i.e., the observation period was short and the research was performed in a retrospective manner in patients with chronic hepatitis and liver cirrhosis. Our study was a prospective study that investigated the disease progress after 12 years, and was thought to reflect the natural history of HCV infections, because we did not investigate only HCV-associated chronic liver disease but also covered all HCV infections such as past HCV infection and asymptomatic carriers of HCV (30,31). In this study, we obtained clear evidence that anti-HBc-positivity was a risk factor for the development of HCC in HCV-infected people.

It has been suggested that HBV can induce liver tumor formation by at least two distinct mechanisms. First, HBV DNA sequences are frequently found integrated into chromosomes of hepatocytes that have evolved into HCC, and a direct role of HBV in hepatocarcinogenesis has thus been inferred (32,33). Second, HBV DNA sequences may be caused by disruption of tumor suppressor gene function (34). It

has been shown that HBV DNA sequences can be detected in some of the liver or serum from anti-HBc-positive patients (9,10), and the presence of anti-HBc does not entirely exclude the possibility of chronic HBV infection. Though the presence of anti-HBc has been used as a marker of past HBV infection, the integration of HBV DNA in hepatocytes may cause carcinogenesis, as noted above. That is, anti-HBc-positivity may represent occult HBV infection. The presence of anti-HBc alone, in the absence of HBV DNA testing, has been used in some studies as a marker of occult hepatitis B (19,21-23). Pollicino *et al* provided clear evidence that occult HBV was a risk factor for the development of HCC and showed that the potential mechanisms whereby HBV might induce tumor formation occur in most cases of occult infection (6).

To detect occult HBV infection, it is necessary to examine whether HBV DNA is present. However, serum HBV DNA levels are frequently below the limits of detection in anti-HBc-positive patients, and there is a pronounced risk of false-positive results from contamination (35) or amplification of non-HBV-DNA targets, and the sensitivity of detection is variable (36,37). In a previous study in which serum HBV DNA was tested in 20 anti-HBc positive patients with HCV-associated HCC, HBV DNA was not detected by a real-time PCR assay with a minimum detection limit of $10^{1.7}$ copies/ml (1.7 log copies/ml) (38,39). Considering these results, it might not be possible to detect serum HBV DNA in some anti-HBc-positive subjects. Therefore, if we could examine liver tissues by PCR to examine whether occult HBV infection is present, we could be more certain of the presence of occult HBV infection.

In contrast to our findings, in some studies anti-HBc positivity was not found to be associated with the development

of HCC in patients with HCV-associated chronic liver disease (9,39,40). One study showed that anti-HBc was detected significantly more frequently in blood donors with than without anti-HCV, but the prevalence of anti-HBc was no different between the patients with HCV-associated HCC and anti-HCV-positive blood donors. Therefore, no epidemiological evidence was obtained for a role of past HBV infection in hepatocarcinogenesis in patients infected with HCV in Japan (40). Also, Yano *et al* showed that the clinical features of HCV-associated HCC were unaffected by anti-HBc-positivity (39). In addition, a study in Taiwan suggested that occult HBV infection might have little influence on the clinicopathologic course of chronic HCV infection (9).

It was reported that the frequency of HCC due to chronic HCV infection is higher in Japan compared with any other country (4). If the frequency of HCC due to chronic HCV infection is high, it is necessary to consider the possibility that anti-HBc positivity may be associated with hepatocarcinogenesis. In addition to HBV, other environmental and host factors might also be associated with the pathogenesis of HCC (4,41-43).

We continued carrying out health screenings of the residents of H town and conducted a cohort study of liver disease among the same residents over a 12-year period. The results of this study showed that anti-HBc is associated with the development of HCC in HCV-infected people.

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Simultaneous Hepatic Relapse of Non-Hodgkin's Lymphoma and Hepatocellular Carcinoma in a Patient with Hepatitis C Virus-Related Cirrhosis

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Rituximab · THP-COP

Abstract

We report a 66-year-old man with hepatitis C virus (HCV)-related cirrhosis and simultaneous hepatic relapse of non-Hodgkin's lymphoma (NHL) and of hepatocellular carcinoma (HCC). Although the liver is frequently involved by NHL, hepatic colocalization of NHL and HCC is rarely detected by imaging techniques. HCV has been suggested to be lymphotropic as well as hepatotropic, and therefore has attracted speculation about a causative role in some cases of lymphoma. The patient had a past history of cutaneous diffuse large B cell lymphoma (DLBCL) in concurrence with HCC 32 months previously. Complete remission (CR) had been maintained for both diseases until February 2004, when ultrasonography and computed tomography (CT) showed multiple liver tumors. Two of these, appearing hyperattenuating in the arterial phase of contrast-enhanced CT, were diagnosed histopathologically as HCC, and treated with radiofre-

quency ablation. The other tumors, hypoattenuating in the portal phase CT, were diagnosed histopathologically as DLBCL, and treated with cyclophosphamide, tetrahydropranyl-Adriamycin, vincristine and prednisolone (THP-COP) in combination with rituximab. CR was achieved for both DLBCL and HCC. Given the previously demonstrated immune system tropism and perturbation by HCV, the virus might have contributed to the occurrence of the NHL as well as the HCC.

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Introduction

An Italian study reported B cell malignant diseases as the most frequent neoplasms associated with hepatocellular carcinoma (HCC) [1], but few such cases have involved colocalization of both within the liver. According to the report on the focal liver lesions detected by imaging techniques in 414 patients with non-Hodgkin's lymphoma (NHL) [2], only 1 case presented with simultaneous coexistence with NHL and HCC. We know of only four previously reported similar cases [3–6], all associated with hepatitis B virus (HBV) infection. How HBV

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and development of lymphoma are related is not known, although some reports have suggested a possible pathogenetic role of HBV in the development of hematologic malignant diseases [7, 8].

Hepatitis C virus (HCV) is well known as a causative agent of chronic hepatitis, which often progresses to liver cirrhosis and HCC in the chronically infected patients. On the other hand, HCV has been associated with various extrahepatic autoimmune diseases [9]. HCV, being lymphotropic as well as hepatotropic [10], has attracted speculation about a causative role in some cases of lymphoma [11, 12]. In particular, several investigators have reported an association between HCV and B cell NHL [13–16]. Chronic antigenic stimulation by HCV has been suspected to be related to the development of clonal B cell expansion [12, 17], although the mechanism is not clear. We present a patient with HCV-related cirrhosis who showed simultaneous intrahepatic relapses of HCC and of B cell lymphoma without extrahepatic involvement.

Case Report

A 66-year-old man with HCV-related cirrhosis was referred to our hospital in March 2004 for treatment of newly detected liver tumors. The patient had a 16-year history of HCV-related chronic hepatitis and also a history of treatment of cutaneous diffuse large B cell lymphoma (DLBCL) in concurrence with HCC diagnosed in May 2001. At that time the DLBCL additionally involved the bone marrow, and was assigned to stage IV according to the Ann Arbor staging system. Subsequently complete response (CR) had been maintained after chemotherapy. Further, radiofrequency ablation (RFA) of the HCC located in the left lobe of the liver had been performed successfully; no other lesion had been detected until shortly before the present admission.

In February 2004, ultrasonography and computed tomography (CT) showed multiple tumors in the right lobe of the liver. All tumors except two were hypoattenuating in the portal phase of contrast-enhanced CT (fig. 1a). The other two tumors located in segment 8, were hyperattenuating in the arterial phase (fig. 1b). Histologic examination of a percutaneous needle biopsy specimen obtained from a hypoattenuating tumor showed infiltration by abnormal lymphoid cells with large and sometimes irregularly shaped nuclei (fig. 2a). Immunohistochemical staining indicated that the lymphoid cells were positive for CD20 (fig. 2b), and the tumor was diagnosed as DLBCL.

In March 2004 the patient was admitted to our hospital for treatment. Physical examination on admission disclosed pallor, spider angiomas, and ascites. Laboratory data obtained on admission showed decreases of choline esterase (40 IU/l; normal range 107–233), albumin (3.0 g/dl; normal range 4.0–5.0), and total cholesterol (125 mg/dl; normal range 128–256) as well as an increase of total bilirubin (2.46 mg/dl; normal range 0.0–1.5). Serum concentrations of aspartate aminotransferase, alanine aminotransfer-

ase, and lactate dehydrogenase were normal, as was prothrombin time. Soluble interleukin 2 receptor was increased in serum (1,313 U/ml; normal range 220–530). The serum concentration of α -fetoprotein was normal (7.3 ng/ml; normal range 0–8.7), but PIV-KA-II (protein induced by vitamin K absence or antagonist II) was increased (254 mAU/ml; normal range 0–40). No extrahepatic involvement by DLBCL was detected in CT, ^{67}Ga scintigraphy, or bone marrow examination.

Following the diagnosis of DLBCL, two courses of cyclophosphamide, tetrahydropyran-Adriamycin, vincristine and prednisolone (THP-COP) were given combined with a course of rituximab. As a result, all tumors except two disappeared or decreased greatly in size according to CT (fig. 1c). The two nonresponding tumors were those that were shown as hyperattenuating lesions in the arterial phase of contrast CT (fig. 1d). An abdominal angiogram demonstrated that these two tumors were hypervascular (fig. 3). Histologic examination of a percutaneous needle biopsy specimen obtained from one of these two tumors showed moderately differentiated HCC with a trabecular and pseudoglandular growth pattern (fig. 4). Chemotherapy for DLBCL was suspended, as it had compromised the patient's liver function and exacerbated ascites (fig. 1c). After improvement of liver function, RFA of the two HCC was performed successfully. A CR was attained for both DLBCL and HCC.

Discussion

In a Japanese study concerning extrahepatic primary cancers in 384 patients with HCC, no B lymphocyte-derived neoplasms were detected [18]. On the other hand, an Italian study of 317 patients with HCC found B cell-derived neoplasms to represent the most frequent cancers associated with HCC, accounting for 10 of 35 extrahepatic primary neoplasms, or 28.6% [1]. Disagreement between these two reports concerning the frequency of B cell neoplasms in patients with HCC is likely to involve the difference in ethnicity between study subjects. In Italy, B cell NHL is reported to show a frequent association with HCV. Accordingly, patients with HCV-related HCC are likely to be at increased risk for B cell-derived neoplasms.

As mentioned, the Italian study included 10 patients with B lymphocyte-derived neoplasms associated with HCC. These were varied: 7 cases of NHL, 2 cases of multiple myeloma, and 1 chronic lymphocytic leukemia [1]. These cases also showed a relatively nonspecific distribution pattern, that of double cancers with the B cell neoplasms involving essentially any part of the body. In our patient, DLBCL coexisted with HCC within the liver, with no extrahepatic involvement. According to the report on the focal liver lesions detected by imaging techniques in 414 patients with NHL [2], hepatic lymphomatous involvement was observed in 69 cases, and HCC in

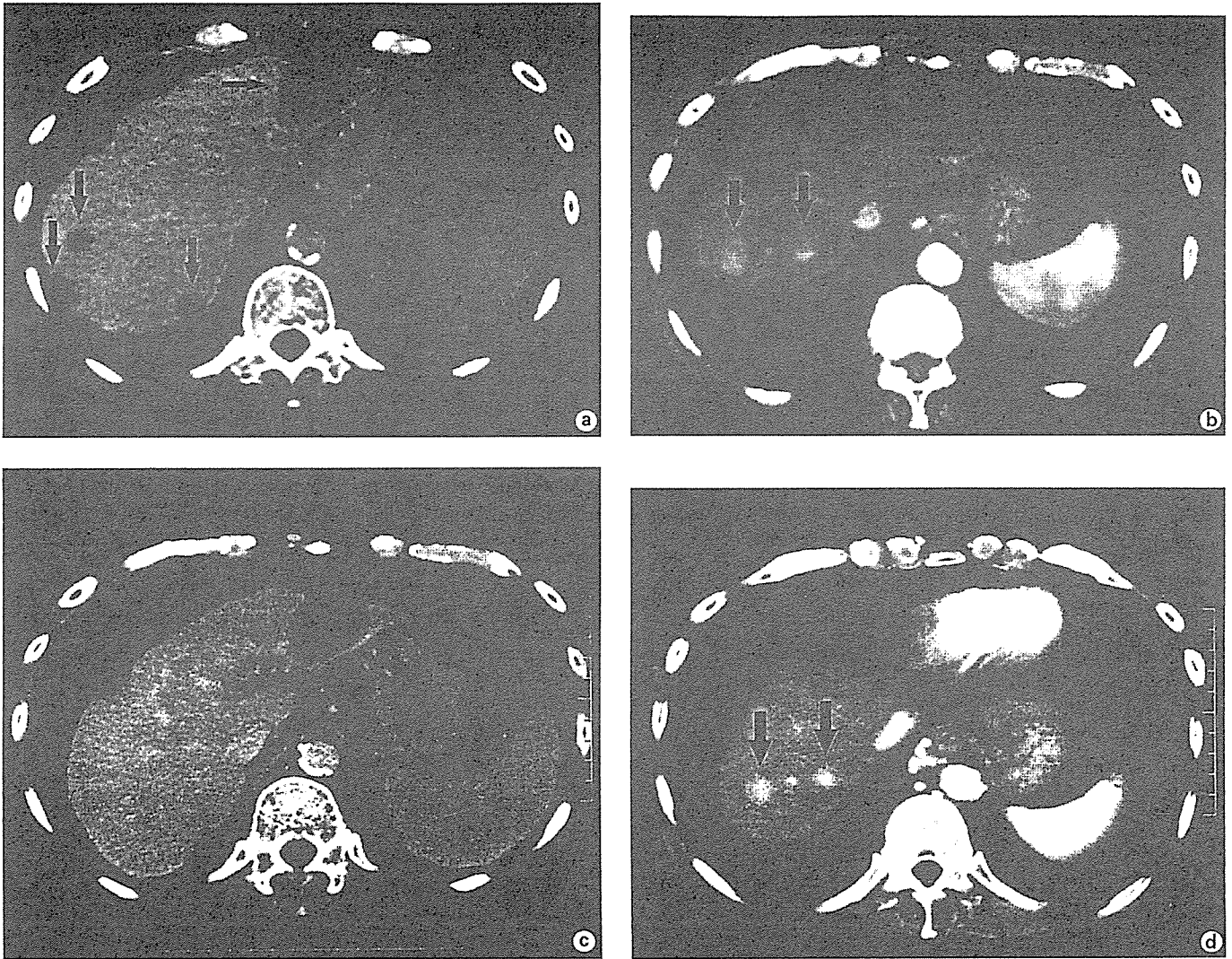


Fig. 1. CT findings during the patient's recent course. **a** Contrast-enhanced CT in the portal phase showed multiple hypoattenuating tumors in the liver (vertical arrows). The horizontal arrow in the left lobe indicates an area of necrosis where RFA had previously been performed. **b** CT in the arterial phase showed two hyperattenuating tumors (arrows) in hepatic segment 8. **c** CT in the portal phase after THP-COP with rituximab showed disappearance or shrinkage of the hypoattenuating tumors shown in **a**. Marked ascites can also be seen. **d** CT in the arterial phase after THP-COP with rituximab showed no shrinkage of the two hyperattenuating tumors shown in **b**.

7 cases, yet only 1 case presented with a simultaneous coexistence of NHL and HCC. That case was described by Cavanna et al. [6]. Although liver is the common site for lymphomatous involvement and occurrence of HCC, we rarely see such a case where the two tumors were simultaneously detected by imaging techniques as distinct hepatic mass lesions without extrahepatic involvement. We know of only 4 previously reported similar cases. Talamo et al. [3] were the first to report a case of simultane-

ous occurrence of primary hepatic lymphoma and HCC. Takeshima et al. [4] reported a patient with hepatic occurrence of mucosa-associated lymphoid tissue lymphoma together with HCC. These 2 cases showed no evidence of extrahepatic involvement by lymphoma, and they are considered to represent primary hepatic lymphoma, defined as confined to the liver with no evidence of lymphomatous involvement in the spleen, bone marrow, or other lymphoid structures. Shikuwa et al. [5] reported a case

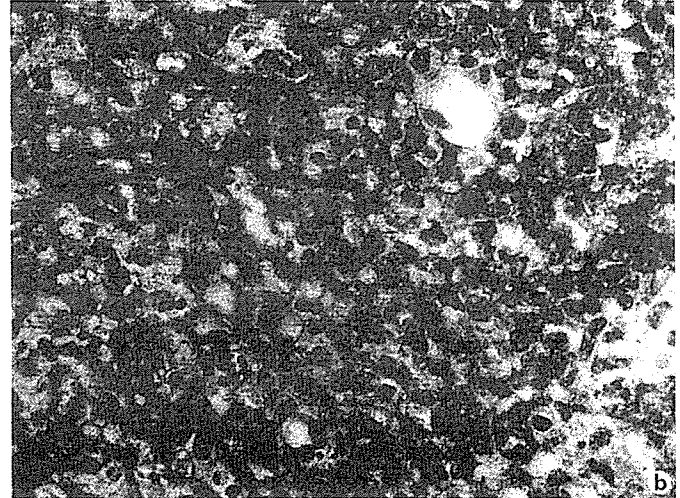
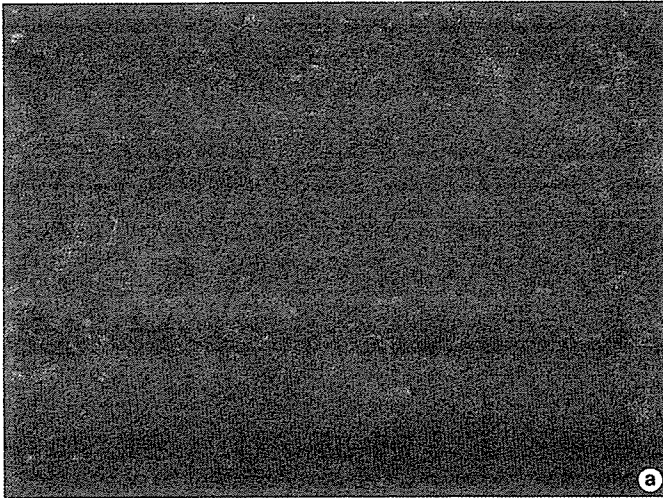


Fig. 2. Microscopic findings in a percutaneous needle biopsy specimen from a hypoattenuating lesion. **a** The liver showed infiltration by abnormal lymphoid cells with large, sometimes irregularly shaped nuclei. Hematoxylin and eosin. $\times 400$. **b** The abnormal lymphoid cells were positive for CD20. Immunohistochemical staining. $\times 400$.

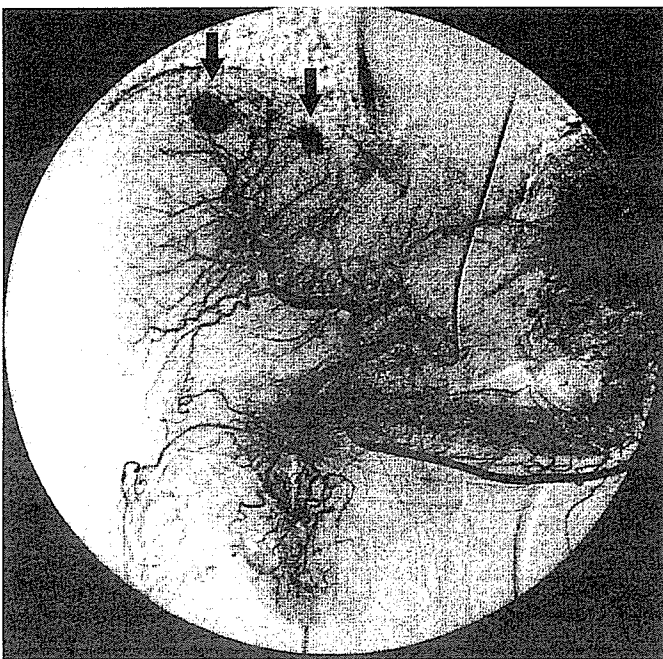


Fig. 3. An abdominal angiogram demonstrated two hypervascular tumors (arrows) in hepatic segment 8.

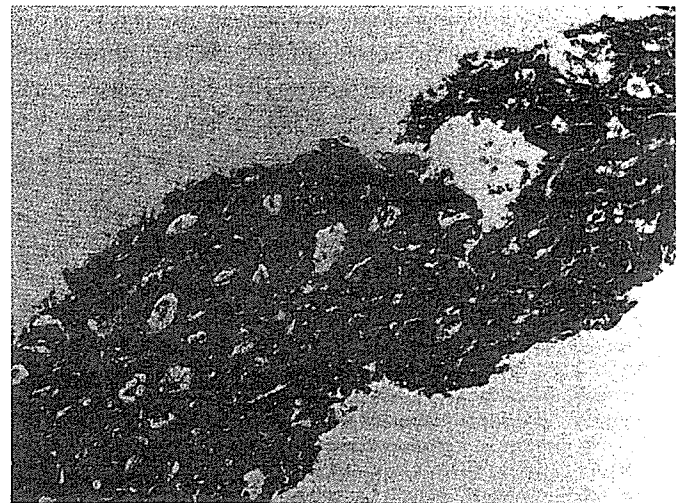


Fig. 4. Histologic findings in a percutaneous needle biopsy specimen from a hyperattenuating, hypervascular lesion showed moderately differentiated HCC with a trabecular and pseudoglandular growth pattern. Hematoxylin and eosin. $\times 100$.

of colocalized HCC and malignant lymphoma, but autopsy demonstrated that the lymphoma involved the bone marrow in addition to the liver. Cavanna et al. [6] reported a case where NHL relapsed in liver without ex-

trahepatic involvement in a patient with HCC. Our patient also presented with relapse, specifically simultaneous relapse of HCC and DLBCL with no extrahepatic involvement. It has been shown that metastatic cell sub-

populations can outgrow their nonmetastatic counterparts within the primary tumor and it was suggested that the metastatic potential of a primary tumor may increase during the course of its growth [19]. Rowbotham et al. [20] referred to the apparent predilection of tumors to invade the liver in patients with acute liver failure secondary to hepatic infiltration and thus suggested the presence of a phenotypic lymphomatous subtype with selective organ invasion, and additionally reported that over one quarter of patients with lymphoma had a history of previous treatment for the same disease. This fact suggests that chemotherapy might change the behavior of the tumors and enhance the properties which preferentially target and invade the liver. The appearance of such chemotherapy-induced lymphoma cell subpopulations as selectively invading the liver and recurrence of HCC may have resulted in the simultaneous colocalization of the two distinct tumors. Our case report demonstrates that it is important to pay attention to patients with HCV or with a previous history of malignant lymphoma at the diagnosis of hepatic mass lesions.

Importantly, all 4 cases reported prior to ours were associated with HBV infection. Although some reports have suggested a possible pathogenetic role of HBV in the development of hematologic malignant diseases [7, 8], a basis for a relationship between HBV and lymphoma occurrence is not clear. In distinction to the other cases, ours is associated with HCV, not HBV, infection. HCV is a well-known cause of chronic hepatitis, which in these

chronically infected patients often progresses to cirrhosis and eventually HCC. On the other hand, HCV has also shown reported associations with various extrahepatic autoimmune diseases, such as mixed cryoglobulinemia, Sjögren's syndrome, renal disease, and neuropathy [9]. As a lymphotropic virus [10], HCV is suspected to contribute to the etiology of B cell NHL [11, 12]. A relationship between HCV and NHL has been demonstrated by many investigators in Italy [13, 14], the United States [15], and Japan [16]. Especially in Italy, a high proportion of HCV positivity has been reported among patients with NHL. Ascoli et al. [21] reported HCV-related extranodal B cell lymphomas of various types. The apparent relationship was supported by a report demonstrating regression in splenic lymphoma with villous lymphocytes in patients with HCV after treatment of the virus with interferon α [22]. In patients with type II mixed cryoglobulinemia, the most common immune disorder related to chronic HCV infection, the paraprotein is a monoclonal IgM rheumatoid factor indicative of clonal B cell proliferation [23]. Thus, chronic B cell stimulation by HCV-related antigens has been proposed as a causative factor in neoplastic transformation [12, 17], although details of the underlying mechanism remain unclear. Our patient had been infected by HCV for over 16 years; indeed, HCV might have caused his malignant lymphoma as well as HCC to result in a unique HCV-related simultaneous hepatic colocalization of HCC and NHL.

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New Epitope Peptides Derived from Hepatitis C Virus (HCV) 2a Which Have the Capacity to Induce Cytotoxic T Lymphocytes in HLA-A2⁺ HCV-Infected Patients

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Abstract: Because cytotoxic T lymphocytes (CTLs) play an important role in the specific immunotherapy of hepatitis C virus (HCV) infection, a series of CTL epitopes has been defined from HCV genotype 1a or 1b protein. Here, we attempted to identify HCV2a-derived epitopes that are capable of inducing HLA-A2-restricted and peptide-specific CTLs. Peripheral blood mononuclear cells (PBMCs) of HLA-A2⁺ HCV2a-infected patients or healthy donors were stimulated *in vitro* with each of the HCV2a-derived peptides, which were prepared based on the HLA-A2-binding motif, and their peptide-specific and HLA-A2-restricted cytotoxicities were examined. The HCV2a 432–441, HCV2a 716–724, and HCV2a 2251–2260 peptides were found to efficiently induce peptide-specific CTLs from the PBMCs of HLA-A2⁺ HCV2a-infected patients. Cytotoxicity was mainly mediated by CD8⁺ T cells in a HLA class I-restricted manner. These results indicate that the HCV2a 432–441, HCV2a 716–724, and HCV2a 2251–2260 peptides might be applicable for peptide-based immunotherapy of HLA-A2⁺ HCV2a-infected patients.

Key words: HCV, Peptide, CTL, Vaccine

Hepatitis C virus (HCV) is a leading cause of liver disease and hepatocellular carcinoma throughout the world. Infection with HCV often progresses to chronic hepatitis and thereby to cirrhosis and hepatocellular carcinoma over several decades (2, 16, 27). HCV is a highly variable virus, and at least six known genotypes are found worldwide (3). The genotype of the virus strongly impacts the success of antiviral therapies, and may affect disease progression (19, 32). HCV genotypes 1b and 2a are the most predominant in Italy and some Asian countries, including China, Japan and Korea (12, 31). Patients with the HCV2a infection usually have better responses to interferon (IFN) therapy

than those with HCV1b infection. However, about 30–40% of HCV2a-infected patients are resistant to IFN treatment (14, 28). Moreover, there are large numbers of HCV2a-infected patients in developing countries that cannot afford the expensive antiviral therapy. Therefore, it is of great importance to find new therapeutic modalities.

CD8⁺ cytotoxic T lymphocytes (CTLs) are known to play an important role in the elimination of HCV (7, 24). Virus-specific CTLs recognize viral antigens on infected cells in a human leukocyte antigen (HLA) class I-restricted manner, and then lyse the cells (9). Many research groups have focused on the identification of epitope peptides that can be recognized by CTLs.

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Abbreviations: CTL, cytotoxic T-lymphocyte; DC, dendritic cells; E/T, effector cells/target cells; HCV, hepatitis C virus; HLA, human leukocyte antigen; IFN, interferon; IL-2, interleukin-2; PBMC, peripheral blood mononuclear cell.

Indeed, a series of CTL-directed epitopes has been identified from proteins of HCV genotypes 1a and 1b (30). However, few HCV genotype 2a-derived CTL-directed epitopes have been identified to date. In the present study, we attempted to identify HCV2a-derived CTL-directed epitopes presented by the HLA-A2 molecule, which is the most major HLA type in the world population (11).

Materials and Methods

Subjects. The Institutional Ethical Review Board of Kurume University approved this study protocol (Protocol # 2244), and informed written consent was obtained from all the blood donors. Ten HLA-A2⁺ HCV2a-infected patients and five HLA-A2⁺ healthy donors were enrolled in this study. HCV2a patients were seropositive for anti-HCV antibodies (Abs) as confirmed by second- or third-generation immunoassay tested by a clinical laboratory company, SRL, Tokyo. The healthy donors were without any symptoms of hepatocellular dysfunction.

Peptide. Nine different kinds of 9- or 10-mer synthetic peptides derived from the protein of HCV genotype 2a, all of which contained an HLA-A2-binding motif, were used in this study (Table 1). Influenza (Flu) A virus matrix protein 1-derived (GILGFVFTL), Epstein-Barr virus (EBV) BMLF1-derived (GLCTLVAML), and HIV-1 gag protein-derived peptides (SLYNTVATL) with the HLA-A2-binding motif were used as controls. All peptides (>90% purity) were purchased from BIO SYNTHESIS (Lewisville, Tex., U.S.A.) or SynPep (Dublin, Calif., U.S.A.), and were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml and stored at -20 C.

Table 1. HCV2a-derived peptides used in this study

Number	Peptides		
	Region	Sequence	Binding score ^a
1	C 35-44	YLLPRRGPR	363
2	E1 284-293	VMLAAQMFIV	1,728
3	E1 285-293	MLAAQMFIV	646
4	E2 432-441	SLHTGFLASL	186
5	E2 716-724	YIVRWEWVV	135
6	NS2 888-897	VVFDITKWLL	361
7	NS5 2251-2260	VVLDLDPMV	156
8	NS5 2850-2858	WLGNIQYA	289
9	NS5 3012-3021	RLLLLGLLLL	181

^aThe peptide binding scores were calculated based on the predicted half-lives of dissociation from HLA-A0201 molecules, as obtained from a Website (Bioinformatics and Molecular Analysis Section, Computational Bioscience and Engineering Laboratory, Division of Computer Research & Technology, NIH).

Cell lines. The HLA-A2-expressing cell lines, T2 and HEK 293-A2, were cultured in RPMI-1640 medium (Gibco BRL, Grand Island, N.Y., U.S.A.) supplemented with 10% FCS (Gibco BRL).

RNA synthesis. The plasmid pSGR-JFH1 (15), which contained the consensus sequence of NS3-NS5 of HCV2a subgenomic JFH-1, was kindly provided by Dr. Takaji Wakita (Tokyo Metropolitan Institute for Neuroscience, Tokyo). The plasmid was linearized by *Xba*I digestion and further treated with mung bean nuclease (New England Biolabs, Beverly, Mass., U.S.A.) to remove four nucleotides and leave the correct 3' end of the HCV cDNA. Digested plasmid DNAs were purified and used as templates for *in vitro* RNA synthesis using the MEGAscript™ T7 kit (Ambion, Austin, Tex., U.S.A.). Synthesized HCV subgenomic RNA was treated with DNase I (RQ1TM RNase-free DNase; Promega, Madison, Wis., U.S.A.) followed by acid phenol extraction to remove any remaining template DNA.

RNA transfection. Trypsinized HEK 293-A2 cells were washed with PBS and resuspended with ice-chilled Cytomix buffer (29) at a concentration of 0.5-1×10⁷ cells/ml; Synthesized replicon RNA (5 µg) was mixed with 400 µl of the cell suspensions, transferred to an electroporation cuvette (Bio-Rad, Hercules, Calif., U.S.A.), and pulsed at 260 V and 950 µF with the Gene Pulser II apparatus (Bio-Rad). Transfected cells were then transferred to RPMI-1640 medium with 10% FCS and cultured in a culture flask. G418 (1.2 mg/ml) (Nacalai Tesque, Kyoto, Japan) was added to the culture medium at 18-24 hr after the transfection, and twice a week the culture medium was replaced with fresh medium supplemented with G418. Three weeks after transfection, cells were diluted with selective medium to a concentration of 1 cell per 200 µl. Cells were then plated on a 96-well plate at 100 µl cells per well and cultured for an additional 2-3 weeks. G418-resistant colonies were collected and expanded until they were 80-90% confluent in 10 cm culture dishes for use in nucleic acid and protein analyses.

RT-PCR. The total RNA of the G418-resistant colonies was isolated with RNA-Bee™ (Tel-Test, Friendswood, Tex., U.S.A.) according to the manufacturer's instructions. cDNA was synthesized from 5 µg total RNA. HCV cDNA was detected by PCR amplification using a set of oligonucleotide primers specific to HCV 2a (forward primer at nucleotide position 7244-7263: 5'-AGGAGGCCAGATTACCAACC-3'; reverse primer at nucleotide position 7376-7395: 5'-AAGGTCTTGATGGCCAGTTG-3'). PCR was performed in 30 cycles (1 min at 95 C, 1 min at 60 C, and 1 min at 72 C) using *Taq* DNA polymerase (Promega).

The PCR product was analyzed on 2% agarose gel.

In vitro induction of CTLs from HCV2a-infected patients with HCV2a-derived peptides. The method used for the detection of peptide-specific CTLs has been reported elsewhere (18). In brief, PBMCs (1×10^6 cells per well) of HLA-A2⁺ HCV2a⁺ patients were incubated with 10 $\mu\text{g/ml}$ of each peptide in a U-bottom 96-well microculture plate (Nunc, Roskilde, Denmark) in a volume of 200 μl of culture medium. The culture medium consisted of 45% RPMI-1640, 45% AIM-V medium (Gibco BRL), 10% FCS, 100 U/ml of interleukin-2 (IL-2), and 40 $\mu\text{g/ml}$ gentamycin. Half of the culture medium was removed and replaced with new medium containing 20 $\mu\text{g/ml}$ of the respective peptide every 3–4 days. On day 15, half of the cultured cells in each well were equally separated into four wells; two wells were further stimulated with the corresponding peptide-pulsed T2 cells, and the other two wells were stimulated with the control HIV peptide-pulsed T2 cells. After 16–18 hr of incubation, the IFN- γ levels of the supernatants were examined by ELISA. The background IFN- γ production in response to the control HIV peptide was subtracted from the value given in the data. The HCV2a peptide-stimulated PBMCs were further cultured with irradiated HLA-A2⁺ buffy coat cells as feeder cells for approximately 2–3 weeks to obtain a sufficient number of cells for cytotoxicity analysis.

Generation of dendritic cells (DC) from blood monocytes. PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation from HLA-A2⁺ healthy donors. After 4–5 washes with PBS, PBMCs were suspended in 10% FCS-PRMI-1640 medium at 4×10^6 cells/ml. The cells were placed in culture dishes (10 ml/dish) and incubated for 60–90 min at 37 C under 5% CO₂. The non-adherent cells were removed by gentle washing, and the remaining adherent monocyte-enriched population was cultured for 7 days in medium consisting of 45% RPMI-1640, 45% AIM-V medium, 10% FCS, 100 U/ml of interleukin-2 (IL-2, Shionogi & Co., Osaka, Japan), 10 ng/ml of GM-CSF (Pepro Tech Ec, London, U.K.), 10 ng/ml of IL-4 (Pepro Tech Ec), and 40 $\mu\text{g/ml}$ gentamycin. No medium was added or removed during this culture period. On day 5, 10 ng/ml of TNF- α (Sigma, St. Louis, Mo., U.S.A.) was added to the culture medium to mature DC. The cells were harvested at day 7 and used as DC.

In vitro induction of CTLs from healthy donors using HCV2a peptide-pulsed DC. On day 0, DC were washed with PBS, resuspended in the culture medium (45% RPMI-1640, 45% AIM-V, 10% FCS, 100 U/ml IL-2) at 1×10^6 cells/ml, and incubated with 3 $\mu\text{g/ml}$ β 2-microglobulin (Sigma) and 10 $\mu\text{g/ml}$ peptide at 37 C for 2 hr. After irradiation (40 Gy), DC were mixed

with non-adherent PBMCs at a ratio of 1:20 and cultured in wells of a 96-well culture plate (1×10^5 cells per well). On days 7, 14 and 21, the PBMC cultures were restimulated with irradiated peptide-pulsed DC (2.5×10^3 cells/well). The cytotoxicity assays were performed on day 28.

Assay of cytotoxicity. The cell-mediated cytotoxicity assays were performed using a standard 6-hr ⁵¹Cr release assay. A total of 1×10^3 ⁵¹Cr-labeled target cells were incubated with various effector cell-to-target cell (E/T) ratios in wells of a U-bottom 96-well plate, and the ⁵¹Cr release into the supernatants was examined in triplicate. To eliminate nonspecific lysis, the cytotoxic activity was tested in the presence of 4×10^4 cells/well of unlabeled K562 cells. In antibody blocking experiments, either anti-HLA class I (W6/32: mouse IgG2a), anti-HLA-DR (L243: mouse IgG2a), or anti-CD14 (H14: mouse IgG2a) mAb was added into the wells at a concentration of 20 $\mu\text{g/ml}$ at the initiation of the assay. The CD8⁺ cells were purified using a CD8 Isolation Kit (DYNAL, Oslo, Norway) in some experiments. L243 mAb is capable of blocking HLA-DRB1- or DRB4-restricted responses, but not HLA-DQ- or -DP-restricted responses, and the effects on DRB3- or DRB5-restricted responses have not yet been clarified.

Statistics. The statistical analyses were performed by a two-tailed Student's *t*-test. *P*-values of less than 0.05 were considered statistically significant.

Results

Determination of T-Cell Epitope Peptides

Putative HLA-A2-binding peptides encoded by HCV2a were analyzed by a BIMAS, U.S.A. software (Bioinformatics and Molecular Analysis Section, NIH). A total of nine peptides were selected for further analyses (Table 1). The BIMAS binding scores used for predicting the half-lives of the peptide-MHC dissociations of the nine peptides were also listed. The peptides were tested for their ability to induce CTLs in the PBMC cultures of ten HLA-A2⁺ HCV2a-infected patients and five HLA-A2⁺ healthy donors. The results of IFN- γ production in response to peptide-loaded T2 cells are shown in Table 2. PBMCs of all patients showed a response to at least one of the epitope peptides tested, with the exception of patients 2 and 7. All of the peptides were able to induce peptide-specific IFN- γ production in the PBMC culture from at least one of the patients. These nine HCV2a-derived HLA-A2-binding peptides also induced peptide-specific CTLs from at least one of the five healthy donors. Among these nine peptides, three HCV2a-derived peptides (HCV2a 432–441, HCV2a 716–724, and HCV2a 2251–2260) induced peptide-spe-

Table 2. Induction of peptide-reactive CTLs from PBMCs of HLA-A2⁺ HCV2a-infected patients and HCV-negative healthy donors

Subject	Peptides									HIV	EBV	Flu
	C 35-44	E1 284-293	E1 285-293	E2 432-441	E2 716-724	NS2 888-897	NS5 2251-2260	NS5 2850-2858	NS5 3012-3021			
	IFN- γ production (pg/ml)											
Pt.1	0	3	39	<u>890</u>	5	3	<u>341</u>	9	0	0	<u>2,000</u>	N.D.
Pt.2	35	N.D.	19	39	N.D.	18	0	N.D.	N.D.	0	28	N.D.
Pt.3	N.D.	39	13	<u>76</u>	26	<u>78</u>	N.D.	0	N.D.	0	N.D.	<u>316</u>
Pt.4	<u>233</u>	0	<u>163</u>	<u>296</u>	N.D.	0	<u>1,255</u>	<u>60</u>	N.D.	N.D.	<u>115</u>	N.D.
Pt.5	0	<u>300</u>	5	<u>429</u>	24	<u>64</u>	<u>118</u>	5	34	N.D.	N.D.	N.D.
Pt.6	<u>107</u>	38	<u>384</u>	N.D.	<u>209</u>	1	47	<u>225</u>	<u>75</u>	0	11	<u>73</u>
Pt.7	5	0	27	7	34	16	2	1	17	N.D.	N.D.	N.D.
Pt.8	0	36	0	21	<u>212</u>	39	9	24	0	N.D.	N.D.	N.D.
Pt.9	<u>206</u>	N.D.	<u>368</u>	<u>127</u>	<u>1,737</u>	36	<u>108</u>	<u>53</u>	<u>246</u>	N.D.	N.D.	N.D.
Pt.10	19	19	52	<u>80</u>	<u>586</u>	0	<u>135</u>	0	<u>164</u>	13	<u>496</u>	<u>2,384</u>
Positive/total	3/9	1/8	3/10	6/9	4/8	2/10	5/9	3/9	3/7			
HD1	4	40	19	<u>52</u>	4	<u>93</u>	0	3	20	0	18	<u>92</u>
HD2	8	0	25	<u>68</u>	10	0	5	34	22	0	15	38
HD3	28	34	7	0	<u>44</u>	5	<u>74</u>	<u>49</u>	36	17	0	<u>70</u>
HD4	<u>90</u>	<u>130</u>	<u>44</u>	<u>45</u>	<u>68</u>	21	0	6	<u>83</u>	4	40	<u>638</u>
HD5	3	0	23	3	<u>45</u>	8	<u>53</u>	23	12	6	0	15
Positive/total	1/5	1/5	1/5	3/5	<u>3/5</u>	<u>1/5</u>	<u>2/5</u>	1/5	1/5			

The PBMCs from patients and HCV-negative healthy donors were tested for their reactivity to peptides after *in vitro* stimulation with each peptide for 2 weeks. Values represent the IFN- γ concentration produced by the effector PBMCs in response to T2 cells prepulsed with each corresponding peptide. Background IFN- γ response to T2 cells prepulsed with the HIV peptide was subtracted. Significant values ($P < 0.05$ by the Student's *t*-test) are underlined. N.D., not determined, Pt., patient; HD, healthy donor.

cific CTLs efficiently, and these peptides could generate peptide-specific CTLs from 67%, 50%, and 56% of the patients tested, respectively. The other six HCV2a-derived epitopes could induce the positive CTL responses at percentages lower than 50%. Meanwhile, the HCV2a 432-441, HCV2a 716-724, and HCV2a 2251-2260 peptides induced peptide-specific CTLs in 3, 3 and 2 of 5 healthy donors, respectively. Thus, these results indicated that the HCV2a 432-441, HCV2a 716-724, and HCV2a 2251-2260 peptides were promising, and the subsequent studies were focused on these peptides.

Cytotoxicity of the HCV2a Peptide-Specific CTLs from the PBMCs of Patients

Then we determined whether these HCV2a peptide-stimulated PBMCs would show any cytotoxicity against corresponding peptide-pulsed T2 cells. The cytotoxicity of the peptide-induced CTLs was further confirmed by a 6-hr ⁵¹Cr-release assay. The CTLs that were induced by each of the three peptides (HCV2a 432-441, HCV2a 716-724, and HCV2a 2251-2260) exhibited significantly higher levels of cytotoxicity against the corresponding peptide-pulsed T2 cells than against the control HIV peptide-pulsed T2 cells. Representative results (patients 1, 4, 5, 6, 9, and 10) are shown in Fig. 1.

Cytotoxicity of the HCV2a Peptide-Specific CTLs Induced from the PBMCs of Healthy Donors

Because of the limited availability of PBMCs of HCV2a-infected patients, we attempted to induce peptide-CTLs from the PBMCs of HLA-A2⁺ healthy donors using the HCV2a peptide-loaded DC for further analysis. Representative results are shown in Fig. 2. Peptide-specific CTLs were successfully induced from the PBMCs of healthy donors by the stimulation of HCV2a 432-441, HCV2a 716-724, or HCV2a 2251-2260 peptide-loaded DC (Fig. 2A). Purified CD8⁺ cells exhibited the cytotoxicity against corresponding peptide-pulsed T2 cells. The cytotoxic activity was blocked by the addition of anti-HLA class I (HLA-A, B, C) antibody, but not anti-HLA class II (HLA-DR) or anti-CD14 antibody (Fig. 2B), which indicates that the cytotoxicity of peptide-specific CTLs was dependent on MHC class I-restricted CD8⁺ T cells.

Because the CTLs were generated by *in vitro* stimulation with synthetic peptides, we wanted to make sure that the CTLs recognized the endogenously processed peptides of HCV2a protein. We prepared HCV2a NS3-NS5-expressing HEK293-A2 cells as targets in the ⁵¹Cr-release assay. The presence of the HCV2a NS3-NS5 subgenomic mRNA in transfected HEK293-A2 cells was confirmed by RT-PCR analysis (Fig. 3A). The HCV2a NS5 2251-2260 peptide-induced CTLs

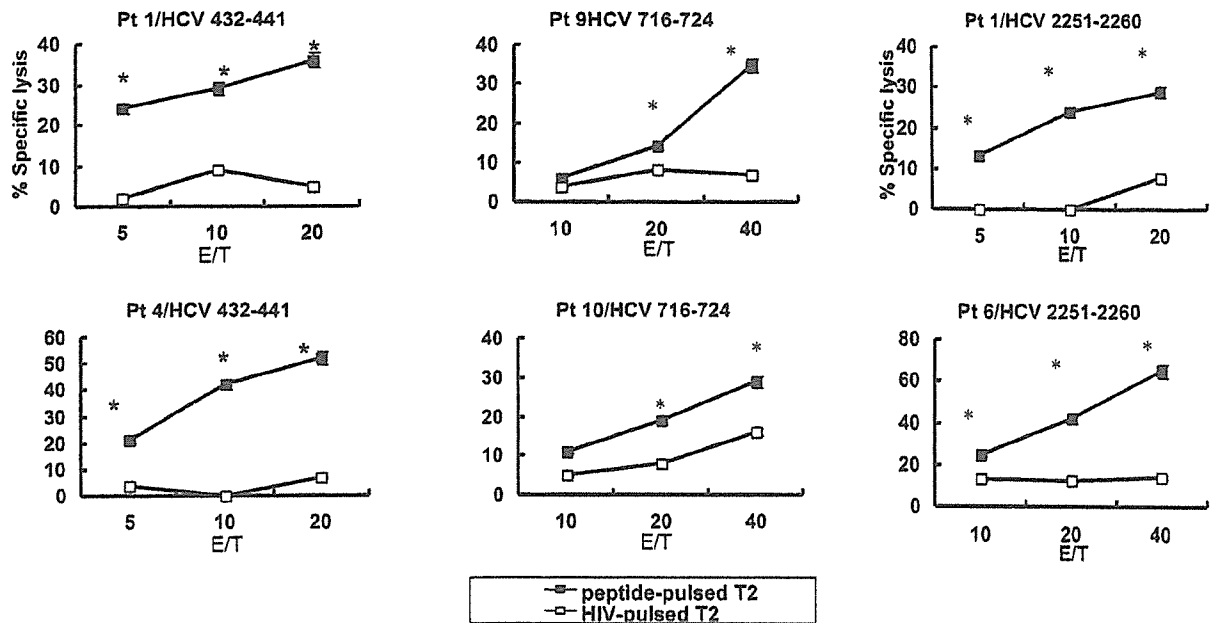


Fig. 1. Cytotoxicity of HCV2a peptide-specific CTLs generated *in vitro* from HCV2a-infected patients. The cytotoxicity against T2 cells pulsed with the corresponding HCV2a peptide or the control HIV-peptide was tested by a standard 6-hr ^{51}Cr -release assay. Representative results in ten experiments using different patient PBMCs are shown. Values represent the mean of triplicate determinations, and statistical analyses were performed by a two-tailed Student's *t*-test (* $P < 0.05$).

lysed the HCV2a NS3-NS5-transfected HEK293-A2 cells significantly, but not the non-transfected HEK293-A2 cells (Fig. 3B). Collectively, these results indicate that three HCV2a-derived epitope peptides (HCV2a 432-441, HCV2a 716-724, and HCV2a 2251-2260) have the potential to induce HLA-A2-restricted CTLs recognizing HCV2a-infected cells, and that the cytotoxicity is mainly mediated by peptide-reactive and CD8⁺ T cells.

Discussion

CD8⁺ T cells have been suggested to play a role not only in chronic hepatitis due to infection by hepatitis B virus (20, 21), but also in the pathogenesis of chronic hepatitis due to HCV infections (13, 17, 25). Rehermann et al. reported that the HCV-specific CTL response in low viral-load patients was stronger than that in high viral-load patients, and suggested that the HCV-specific CTL response might be able to control viral load to some extent in chronically infected patients (23). Moreover, the HCV-specific CTL activity has an impact on the efficacy of interferon therapy, with the patients who show detectable HCV-specific CTL activity developing better or complete responses to IFN treatment (22). Therefore, augmentation of the CTL responses might be useful as therapeutic antiviral strate-

gy, and the identification of T-cell epitopes from HCV protein is a critical step in the development of peptide-based immunotherapy for HCV-infected patients. Actually, a number of CTL-directed epitopes derived from HCV proteins have been identified (5). However, many of the reports have focused on identification of epitopes in HCV1a or 1b proteins, and few epitopes have been identified in HCV2a proteins, although HCV2a is a predominant genotype in various Asian and European countries (12, 31).

In this study, three peptides (HCV2a 432-441, HCV2a 716-724, and HCV2a 2251-2260) were found to be immunogenic in more than half of the patients tested. Meanwhile, all of the peptides failed to induce peptide-specific CTLs in two patients (Pt. 2 and 7). At present, we are unsure why peptide-specific CTLs were not induced in these subjects. This finding may be attributable to one or more of the following causes: the absence of an HCV-specific CTL precursor in these individuals; HCV2a infection with a different strain of HCV2a; or the loss of cells present at a low frequency during the *in vitro* culture. Since HCV is a highly heterogeneous virus and, in some circumstances, even a single amino acid variation within CTL epitopes facilitates viral escape from host immunity (1, 8), amino acid mutation in these individuals may be the most plausible explanation. We previously reported that the HCV1b

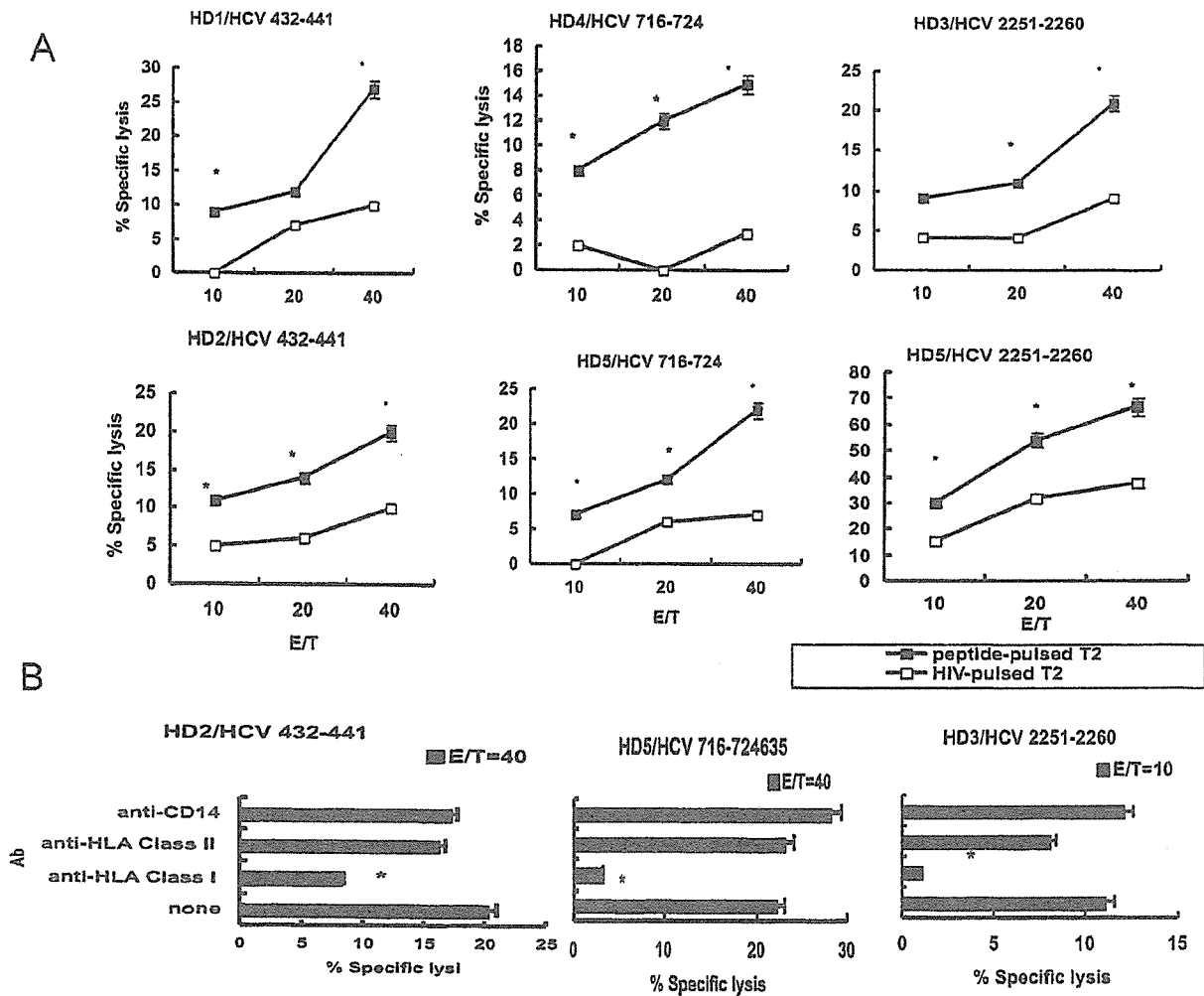


Fig. 2. Cytotoxicity of HCV2a peptide-specific CTLs induced from healthy donors. A: PBMCs of healthy donors were stimulated *in vitro* with HCV2a peptide-loaded autologous DC, and their cytotoxicity was examined by a 6-hr ^{51}Cr -release assay. T2 cells pulsed with the corresponding HCV2a peptide or the control HIV-peptide were used as a target. B: CD8^+ cells purified from the HCV2a peptide-stimulated PBMCs of healthy donors were tested for their cytotoxicity against corresponding HCV2a peptide-pulsed T2 cells in the presence of the indicated mAb. Representative results in three experiments are shown. Values represent the mean of triplicate determinations, and statistical analysis was performed by a two-tailed Student's *t*-test (* $P < 0.05$).

35–44 peptide induced specific CTLs from the PBMCs of HCV1b-infected patients (26). In this study, the HCV2a 35–44 peptide, which has the same amino acid sequence as HCV1b 35–44, was also found to be immunogenic in some patients, but the rate of successful CTL induction was less than 50%.

We wanted to be sure that the CTLs induced by repetitive stimulation with the synthetic peptides actually recognized the endogenously processed peptides, because there are well-established cases of peptides that stimulate CTL responses *in vitro* but are not generated and presented in cells producing the antigen (4). We confirmed that the NS5 2251–2260 peptide-induced

CTLs recognized the endogenously processed peptide expressed on NS3–NS5 gene transfectants. It remains to be determined whether the CTLs specific to the HCV2a 432–441 or the HCV2a 716–724 peptide lysed the HCV2a E2-expressing target cells by using the HCV2a E2 gene. Cerny et al. reported that the HCV1a-derived peptide HCV1a 2252–2260 (ILDSFDPLV), which differs from the present HCV2a 2251–2260 (VVLDSLDPMV) peptide by two amino acids, induced CTLs from PBMCs of HCV1a-infected patients and healthy donors after *in vitro* stimulation (6). In addition, the CD8^+ T cell response directed against this epitope in patients with acute hepatitis C

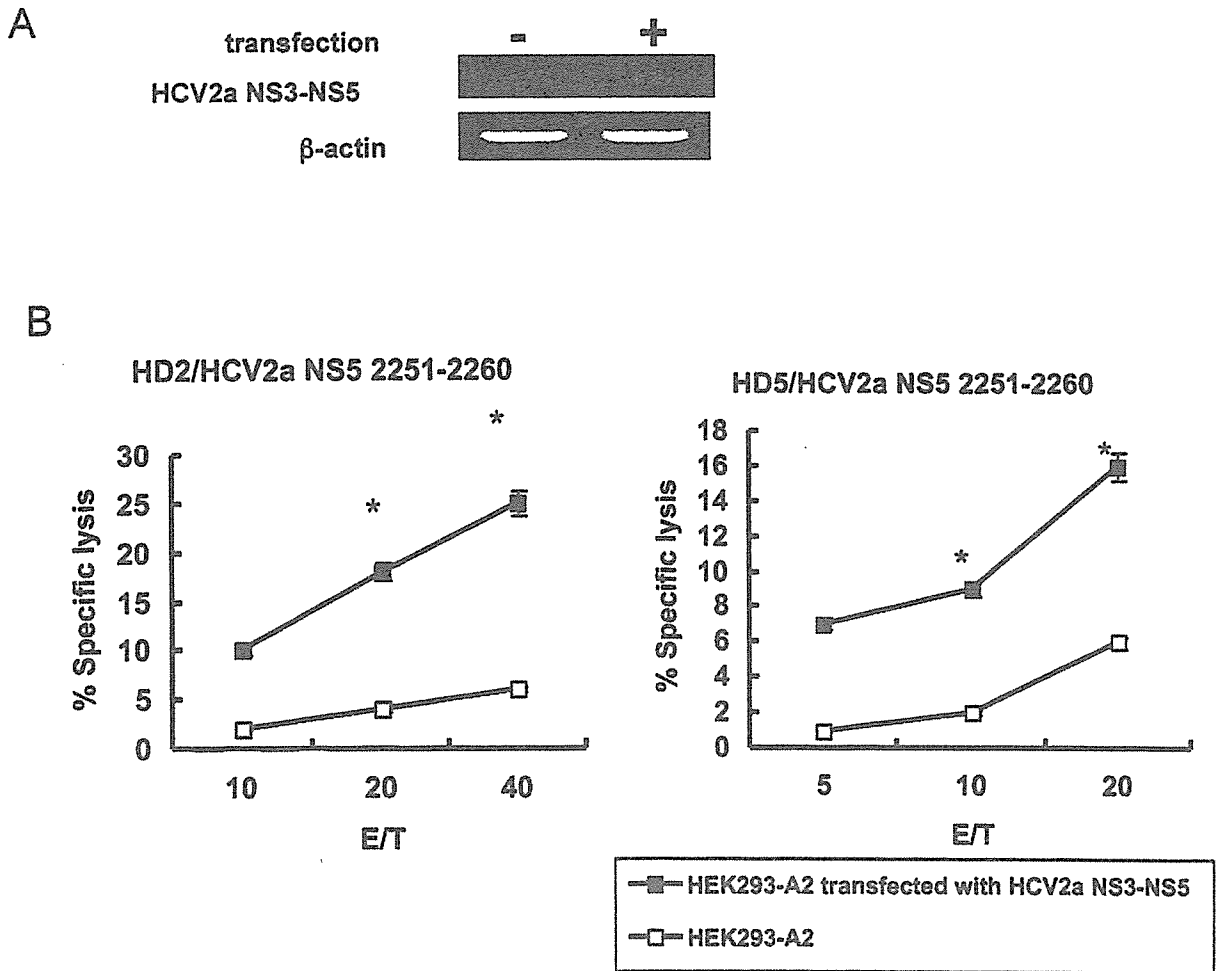


Fig. 3. Recognition of endogenously processed HCV2a peptide by HCV2a peptide-induced CTLs. A: The expression of *HCV2a NS3-NS5* mRNA in the HCV2a NS3-NS5-transfectant and parental HEK293-A2 cells was examined by RT-PCR. β -Actin was used as a control. B: The cytotoxicity of HCV2a NS5 2251-2260 peptide-specific CTLs from two healthy donors against *HCV2a NS3-NS5*-transfected HEK293-A2 cells was examined by a 6-hr ^{51}Cr -release assay. Representative results in three experiments are shown. Values represent the mean of triplicate determinations, and statistical analysis was performed by the two-tailed Student's *t*-test (* $P < 0.05$).

correlates with termination of the disease (10). The effect of HCV2a 2251-2260 peptide-specific CTL activity on the viral clearance or antiviral therapy response remains to be investigated in a future study.

In conclusion, our present study indicates that the HCV2a 432-441, HCV2a 716-724, and HCV2a 2251-2260 peptides are new HLA-A2-restricted CTL epitopes capable of inducing peptide-specific CTLs *in vitro*. If the prevalence of HCV2a in the entire genotypes of HCV-infected people is estimated 10%, 18 million people are infected with HCV2a in the world. In addition, the HLA-A2 is the most frequent allele in the world. Therefore, the identification of HCV2a 432-441, HCV2a 716-724, and HCV2a 2251-2260

peptides would contribute substantially to the development of peptide-based vaccines for HCV-infected patients.

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