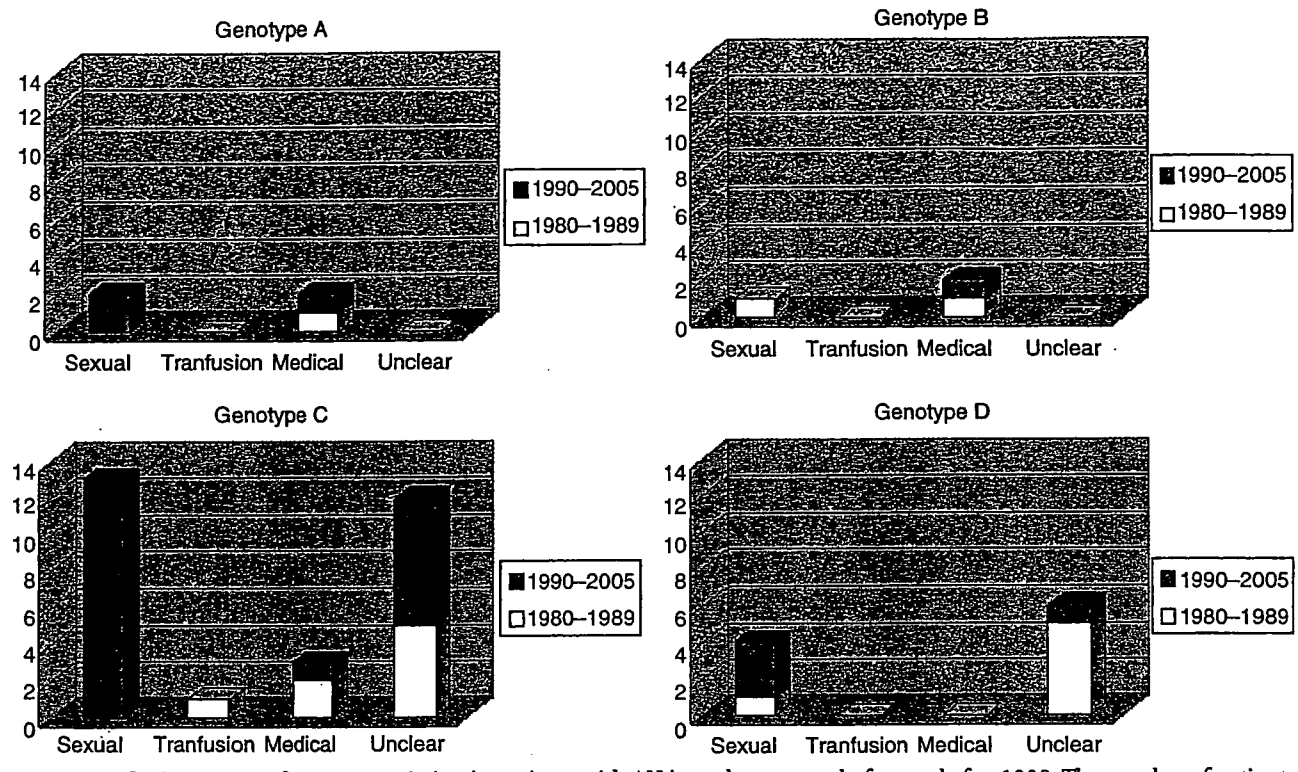

Figure 3 Infectious route of HBV transmission in patients with AH in each genotype before and after 1990. The number of patients of each infectious route in the four genotypes is shown. Before 1990, blood transfusion and medical routes were important infectious routes, whereas the major route of infection after 1990 was sexual transmission among the four genotypes.

The isolate from east Ehime in the present study was also D2. Phylogenetic analysis may indicate that the isolate originated from a common ancestor of HBV/D strains in the central area. Conversely, all patients with AH-B due to HBV/D lived in the central area. This result strongly suggests that all of the infectious sources suspected to be chronically infected with HBV/D lived in central Ehime. These results of both chronically and acutely infected patients indicate that HBV/D is mainly distributed in the central area, and has not yet spread widely to other areas in Ehime. However, a small number of infected individuals have moved to other areas. Of note is the fact that many people born in Ehime, particularly the younger generations, tend to transfer to metropolitan areas such as Tokyo or Osaka. It has been described in a demographic record that 2413 and 2860 people living in Ehime prefecture transferred to the Tokyo and Osaka areas, respectively, in 2004. HBV/D might thus have spread to other areas in Japan. Fixed-point observation of HBV genotypes in several areas in Japan may be useful for planning policies to prevent HBV transmission, not only of HBV/D, but also of other genotypes.

In the present study, the frequency of HBV/D was higher in acute hepatitis than in chronic infection. Frequency of HBV/A was also higher in acute hepatitis than in chronic infection. This tendency was prominent in the central area. This result is supported by several reports that describe a high rate of foreign genotypes among patients with AH, particularly in metropolitan areas,[23,24] as central Ehime is relatively metropolitan compared with the east and south-west areas.

Sexual transmission is a major route of infection among adult patients with AH-B in many countries, including Japan.[24–29] The present study confirmed sexual transmission as the major route of infection in adults, irrespective of HBV genotype. One AH patient with HBV/D in the present study was suspected to be transmitted from a commercial sex worker in central Ehime. The screening of HBsAg and/or HBV genotypes in commercial sex workers may be important to prevent HBV infection, though it has not yet been done in this area.

In the present study, one of the 10 patients (10%) with AH due to HBV/D and one of 29 (3.4%) with HBV/C progressed to chronic infection.[30] The rate of chronicity in patients with AH is reportedly 5–10%,[31] and the rate

in HBV/A is reportedly approximately 20%.[32] Ozasa *et al.* have reported the rate of persistence of HBV infection in patients with acute hepatitis in relation to HBV genotypes and subgenotypes (2/32 in Ae, 0/167 in Ce and 0/3 in D[33]). Rate of chronicity in HBV/D has been suspected to be lower than that in HBV/A.[11] However, the number of patients was still too small to clarify the rate of chronicity in adult patients acutely infected with HBV/D, both in the present study and the literature described above. Further studies are warranted to clarify this issue.

In conclusion, HBV/D has spread in the central area of Ehime prefecture, but has not yet spread widely in other areas, although a small number of infected individuals have moved to other areas. The prevalence of HBV/D (and also with HBV/A) is higher in patients with AH than in chronically infected patients, and discussion of strategies to prevent the sexual transmission of HBV is important.

ACKNOWLEDGMENTS

THIS STUDY WAS supported by a grant from the Ministry of Health and Welfare, Japan and by Grant-in-Aid for Scientific Research no. 17590653 from the Japan Society for the Promotion of Science.

REFERENCES

1 Okamoto H, Tsuda F, Sakugawa H *et al.* Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* 1988; 69: 2575–83.
2 Norder H, Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of hepatitis B virus, four of which represent two new genotypes. *Virology* 1994; 198: 489–503.
3 Araus-Ruiz P, Norder H, Robertson BH, Magnious LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 2002; 83: 2059–73.
4 Lindh M, Andersson AS, Gusdal A. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus – large-scale analysis using a new genotyping method. *J Infect Dis* 1997; 175: 1285–93.
5 Chu CJ, Keeffe EB, Han S *et al.* Hepatitis B virus genotypes in the United States: result of a nationwide study. *Gastroenterology* 2003; 125: 444–51.
6 Orito E, Ichida T, Sata M *et al.* Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* 2001; 34: 590–4.
7 Miyakawa Y, Mizokami M. Classifying hepatitis B virus genotypes. *Intervirology* 2003; 46: 329–38.
8 Chu CJ, Hussain M, Lok AS. Hepatitis genotype B is associated with earlier HbeAg seroconversion compared with hepatitis B virus genotype B. *Gastroenterology* 2002; 122: 1756–62.
9 Furusho N, Nakashima H, Kashiwagi K *et al.* Clinical outcome of hepatitis B virus (HBV) genotypes B and C in Japanese patients with chronic HBV infection. *Am J Trop Med Hyg* 2002; 67: 151–7.
10 Sumi H, Yokoshuka O, Seki N *et al.* Influence of hepatitis B genotypes on the progression of chronic type B liver disease. *Hepatology* 2003; 37: 19–26.
11 Mayerat C, Mantegani A, Frei PC. Does hepatitis B virus (HBV) genotype influence the clinical outcome of HBV infection? *J Viral Hepat* 1999; 6: 299–304.
12 Duong TN, Horiike N, Michitaka K *et al.* Comparison of genotypes C and D of the hepatitis B virus in Japan: a clinical and molecular biological study. *J Med Virol* 2004; 72: 551–7.
13 Michitaka K, Tanaka Y, Horiike N *et al.* Tracing history of hepatitis B virus genotype D in western Japan. *J Med Virol* 2006; 78: 44–52.
14 Gianotti F. Papular acrodermatitis of childhood. An Australia antigen disease. *Arch Dis Child* 1973; 48: 794–9.
15 Ishimaru Y, Ishimaru H, Toda G, Baba K, Mayumi M. An epidemic of infantile papular acrodermatitis (Gianotti's disease) in Japan associated with hepatitis B surface antigen subtype ayw. *Lancet* 1976; 1: 707–9.
16 Toda G, Ishimaru Y, Mayumi M, Oda T. Infantile papular acrodermatitis (Gianotti's disease) and intrafamilial occurrence of acute hepatitis B with jaundice: age dependency of clinical manifestations of hepatitis B virus infection. *J Infect Dis* 1978; 138: 211–16.
17 Norder H, Courouce AM, Coursaget P. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HbsAg subtypes. *Intervirology* 2004; 47: 289–309.
18 Michitka K, Horiike N, Chen Y *et al.* Gianotti–Crosti syndrome caused by acute hepatitis B virus genotype D infection. *Intern Med* 2004; 43: 696–9.
19 Usuda S, Okamoto H, Iwanari H *et al.* Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. *J Virol Methods* 1999; 80: 97–112.
20 Mizokami M, Nkano T, Orito E *et al.* Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett* 1999; 450: 66–71.
21 Chen Y, Michitaka K, Matsubara H. Complete genome sequence of hepatitis B virus (HBV) from a patient with fulminant hepatitis without precore and core promoter mutations: comparison with HBV from a patient with acute hepatitis infected from the same infectious source. *J Hepatol* 2003; 38: 84–90.
22 Japanese Red Cross Non-A, Non-B, Hepatitis Research Group. Effect of screening for hepatitis C virus antibody and hepatitis B virus core antibody on incidence of post-transfusion hepatitis. *Lancet* 1991; 338: 1040–1.

23 Kobayashi M, Suzuki F, Arase Y *et al.* Infection with hepatitis B virus genotype A in Tokyo, Japan during 1976 through 2001. *J Gastroenterol* 2004; 39: 844–50.
24 Yotsuyanagi H, Okuse C, Yasuda K *et al.* Distinct geographic distributions of hepatitis B virus genotypes in patients with acute infection in Japan. *J Med Virol* 2005; 77: 39–46.
25 Arima S, Michitaka K, Horiike N *et al.* Change of acute hepatitis B transmission routes in Japan. *J Gastroenterol* 2003; 38: 772–5.
26 Struve J, Giesecke J, Lindh G, Weiland O. Heterosexual contact as a major route for transmission of acute hepatitis B among adults. *J Infect* 1990; 20: 111–21.
27 Atkins M, Nolan M. Sexual transmission of hepatitis B. *Curr Opin Infect Dis* 2005; 18: 67–72.
28 Hou J, Liu Z, Gu F. Epidemiology and prevention of hepatitis B virus infection. *Int J Med Sci* 2005; 2: 50–7.
29 Chen CJ, Wang LY, Yu MW. Epidemiology of hepatitis B virus infection in the Asia-Pacific region. *J Gastroenterol Hepatol* 2000; 15 (Suppl 2): E3–6.
30 Yamamoto K, Michitaka K, Miyata T. An adult patient with acute hepatitis B who progressed to chronic hepatitis. *Acta Hepatol Jpn* 2002; 43: 406–10.
31 Yim HJ, Lok AS. Natural history of chronic hepatitis B virus infection: what we knew in 1981 and what we know in 2005. *Hepatology* 2006; 43: S173–S181.
32 Suzuki Y, Kobayashi M, Ikeda K *et al.* Persistence of acute infection with hepatitis B virus genotype A and treatment in Japan. *J Med Virol* 2005; 76: 33–9.
33 Ozasa A, Tanaka Y, Orito E *et al.* Influence of genotypes and precore mutations on fulminant or chronic outcome of acute hepatitis B virus infection. *Hepatology* 2006; 44: 326–34.

Hepatology Research 2007; 37: 331–336 doi: 10.1111/j.1872-034X.2007.00052.x

Original Article

Diabetes mellitus reduces the therapeutic effectiveness of interferon-α2b plus ribavirin therapy in patients with chronic hepatitis C

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Aim: Patients with chronic hepatitis C (CHC) often have diabetes mellitus (DM). However, it is unknown whether DM affects patient response to interferon (IFN) plus ribavirin therapy. Therefore, the aim of this study was to examine the influence of DM on the outcome of IFN-α2b plus ribavirin therapy.

Methods: In a cohort of 110 patients with CHC, the outcome of 6 months of IFN-α2b plus ribavirin therapy was evaluated by comparing the patients with and without DM.

Results: There were 46 sustained-responders; 64 patients did not become sustained responders. Higher age ($P = 0.015$), lower platelet counts ($P = 0.036$), hepatitis C virus (HCV) serotype 1 ($P = 0.001$), advanced liver fibrosis ($P = 0.004$), and the presence of DM ($P = 0.007$) were significantly associated with not becoming a sustained-responder. Seventeen CHC (15%) patients had DM. Sex ratio, age, body mass index, alanine aminotransferase levels, HCV-RNA titer, and HCV serotypes did not significantly differ between the patients with and without DM, while fasting plasma glucose, hemoglobin A1c and liver histological staging were significantly different. On multiple logistic regression analysis, HCV serotype 1 (odds ratio 8.743, 95% confidence interval 2.215–34.517; $P = 0.002$) and the presence of DM (odds ratio 8.657, 95% confidence interval 1.462–51.276; $P = 0.014$) were independently associated with not becoming a sustained-responder.

Conclusions: The findings indicate that DM reduces the response to IFN-α2b plus ribavirin therapy in CHC patients.

Key words: chronic hepatitis C, diabetes mellitus, interferon, ribavirin

INTRODUCTION

CHRONIC HEPATITIS C (CHC) has a high prevalence worldwide. Over a period of 20–30 years, CHC progresses to cirrhosis and hepatocellular carcinoma.[1] Interferon (IFN) is often administered to treat chronic hepatitis C virus (HCV) infection, yet many patients do not eliminate the virus. Recently, therapy with IFN combined with ribavirin has been given to patients with a high viral load or who relapse; compared to IFN monotherapy, combination therapy increases the rate of sustained viral response (SVR).[2,3] However, the rate of SVR is still not high enough.

Several factors that contribute to the response to IFN therapy have been identified. A high viral load, viral genotype 1b, and the absence of mutations in the NS5A and NS5B regions in genotype 1b of HCV are associated with a lower rate of HCV clearance in patients receiving antiviral therapy.[4–7] Host factors, including older age, a higher degree of fibrosis, a longer duration of disease, and certain host genetic factors that affect IFN responsiveness, are associated with a poor response to IFN therapy.[8–14] Furthermore, in previous studies, most patients who received combination IFN-α2b plus ribavirin therapy had a high HCV viral load and were serotype 1; thus, there may be other factors that reduce the efficacy of IFN-α2b plus ribavirin therapy.

Diabetes mellitus (DM) has been associated with HCV infection, especially in patients with liver

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Received 5 October 2006; revision 12 December 2006; accepted 20 December 2006.

cirrhosis.[15] Furthermore, DM has been implicated in insulin resistance and obesity, which affect IFN effectiveness.[16–18] However, there is no evidence that DM affects the outcome of IFN-α2b plus ribavirin therapy. Therefore, we explored whether the presence of DM affects the response to IFN-α2b plus ribavirin therapy in patients with CHC.

METHODS

Patients

WE ENROLLED 110 CHC patients (male, 86; female, 24; median age, 51 years; range, 27–74 years) who were given IFN-α2b plus ribavirin therapy from December 2001 to August 2003 at our hospital. All participants were Japanese and unrelated to each other. All patients were positive for both anti-HCV antibody and serum HCV-RNA by polymerase chain reaction (PCR). Ninety-two of the patients had HCV serotype 1, and 18 had HCV serotype 2. The patients were separated based on their viral load determined by the Amplicor-Monitor assay into three groups: more than 700 KIU/mL ($n = 66$); 100–700 KIU/mL ($n = 41$); and less than 100 KIU/mL ($n = 3$). No patients had hepatitis B virus (HBV) infection, alcohol-induced liver diseases, or autoimmune liver diseases. Sixty-two patients had received prior antiviral treatment, while 48 patients had not.

Patients were classified as having DM according to criteria for the diagnosis of type 2 DM established in 1999 by the Japan Diabetes Society, which are similar to the WHO type 2 DM diagnostic criteria. In 46 patients, we evaluated glucose intolerance and insulin resistance using 75-g oral glucose tolerance test.

Liver biopsy specimens were obtained from 108 patients for histological examination: 39 had mild fibrosis; 34 had moderate fibrosis; 23 had severe fibrosis; and 12 had liver cirrhosis. Histological activity was mild in 51, moderate in 56, and severe in one. Specimens were histologically classified according to the criteria of the International Hepatitis Group.[19]

Informed consent was obtained from all patients.

Estimation of HBV and HCV markers and laboratory investigations

The presence of hepatitis B surface antigen (HBsAg) and anti-HCV antibody was determined using enzyme immunoassay kits (Dainabot, Tokyo, Japan; Kokusai-Shiyaku, Kobe, Japan). HCV-RNA was detected using the nested PCR with primers for the 5′ untranslated region of HCV. The HCV-RNA titers immediately before IFN-α2b plus ribavirin therapy that were determined using Amplicor-Monitor (Roche Diagnostics, Branchburg, NJ, USA) are expressed as kilo-international units/mL (KIU/mL).[20] The HCV serotype was determined using an enzyme immunoassay (Ohtsuka Laboratories, Tokushima, Japan). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as reported previously.[21]

Treatment schedule

All patients were treated with combination recombinant IFN-α2b and ribavirin therapy at the Ehime University Hospital from December 2001 to August 2003. Ribavirin (Schering-Plough, Osaka, Japan) was given at a daily dose of 600 or 800 mg, depending on body weight (<60 or ≥60 kg, respectively), in combination with IFN-α2b (Schering-Plough) intramuscularly every day for the first 1–4 weeks, and then three times a week for the following 20–23 weeks (total duration, 24 weeks). The starting doses of IFN-α2b were 10 MU per day in 104 patients and 6 MU per day in six patients. Ribavirin was started at 800 mg per day in 72 patients and 600 mg per day in 38 patients.

During treatment, the IFN dose was decreased in 19 patients (17%), and both IFN and ribavirin were discontinued in 17 patients (15%) due to side-effects; the ribavirin dose was decreased in 35 patients (32%), and ribavirin was discontinued without stopping IFN in three patients. Fifty-five patients (50%) completed treatment without discontinuing or decreasing the dosage of either drug.

Criteria for IFN effectiveness

All patients were followed for at least 6 months after IFN-α2b plus ribavirin therapy. Serum alanine aminotransferase (ALT) and HCV-RNA were assayed monthly during this period. Patients were categorized into two groups. Patients with an SVR (sustained-responders) were those who maintained normal ALT levels and had no detectable HCV-RNA based on PCR assays done during the follow-up period; non-responders were those patients who remained positive for HCV-RNA after IFN-α2b plus ribavirin therapy, irrespective of the HCV-RNA levels or the occurrence of relapse during follow-up.

Statistical analysis

All data are expressed as the medians. For continuous variables, the Mann–Whitney *U*-test was used. The difference in proportions was evaluated using the chi-squared test or Fisher's exact test. We assessed all

variables using a logistic regression model. The model was simplified in a stepwise fashion by removing variables with $P > 0.05$. A value of $P < 0.05$ was considered significant. Calculations were performed using SPSS for Windows, Release 14.0 J (SPSS, Chicago, IL, USA).

RESULTS

Clinical and virological characteristics according to IFN response

FORTY-SIX PATIENTS (42%) were sustained-responders, and 64 (58%) were non-responders. The characteristics of the sustained-responders and the non-responders are shown in Table 1. Sex ratio, body mass index, fasting plasma glucose, hemoglobin (HbA1c), ALT levels, HCV-RNA titer, liver histological activity, and past history of IFN therapy were not significantly different between sustained-responders and non-responders. However, the following were significantly associated with non-response: older age ($P = 0.015$), lower platelet count ($P = 0.036$), HCV serotype 1 ($P = 0.001$), advanced fibrosis ($P = 0.004$), and the presence of DM ($P = 0.007$).

Of the 48 patients identified with fasting plasma glucose and immunoreactive insulin levels, the HOMA-IR was not significantly different between the sustained-responders ($n = 18$; median 2.63, range 0.8–13.3) and the non-responders ($n = 28$; median 2.99, range 0.6–9.5).

Background characteristics of patients with and without DM

To assess the influence of DM, we compared the clinical and virological features of patients with and without DM (Table 2). No patient with DM received drugs that improve insulin resistance. Sex ratio, age, body mass index, ALT levels, liver histological staging, HCV-RNA titer, HCV serotypes, and past history of IFN therapy were not statistically significantly different between the two groups. However, fasting plasma glucose and

Table 1 Clinical and virological characteristics of 110 patients with chronic hepatitis C treated with interferon-α2b plus ribavirin therapy based on therapeutic response

Characteristic	Sustained-responders ($n = 46$)	Non-responders ($n = 64$)	*P*-value
Sex (male/female)	35/11	51/13	NS
Age (years)	46 (32–65)	54 (27–74)	0.015
Body mass index (kg/m^2)	24.1 (17.7–31.7)	23.6 (16.6–32.4)	NS
Fastening plasma glucose (mg/dL)	92 (69–140)	96 (73–209)	NS
HbA1c (%)	4.9 (4.2–6.3)	5.0 (4.2–8.7)	NS
Alanine aminotransferase (IU/L)	68 (20–337)	72 (25–247)	NS
Platelet count (×10^4/mm^2)	16.7 (6.4–35.3)	15.0 (5–32.2)	0.036
HCV serotype			
1	32	60	0.001
2	14	4	
HCV-RNA titer			
<100 KIU/mL	3	0	NS
100–700 KIU/mL	19	22	
>700 KIU/mL	24	42	
Histological fibrosis			
Mild	23	16	0.004
Moderate	8	26	
Severe	12	11	
Cirrhosis	2	10	
Histological activity			
Mild	21	30	NS
Moderate	24	32	
Severe	0	1	
Re-treatment (+/–)	26/20	36/28	NS
Presence of DM (non-DM/DM)	44/2	49/15	0.007

Data expressed as median (range). DM, diabetes mellitus; HbA1c, hemoglobin A1c; HCV, hepatitis C virus; NS, not significant.

Table 2 Clinical and virological characteristics of 110 patients with chronic hepatitis C treated with interferon-α2b plus ribavirin therapy based on the presence of diabetes mellitus

Characteristic	Patients with DM ($n = 17$)	Patients without DM ($n = 93$)	*P*-value
Sex (male/female)	15/2	71/22	NS
Age (years)	55 (39–74)	49 (27–69)	NS
Body mass index (kg/m^2)	23.5 (17.5–32.4)	24.0 (16.6–31.7)	NS
Fastening plasma glucose (mg/dL)	109 (93–209)	92 (69–110)	<0.001
HbA1c (%)	6.7 (5.0–8.7)	4.9 (4.2–5.8)	<0.001
Alanine aminotransferase (IU/L)	93 (39–234)	68 (20–337)	NS
Platelet count (×10^4/mm^2)	14.8 (7.8–32.2)	15.9 (5–35.3)	NS
HCV serotype			
1	14	78	NS
2	3	15	
HCV-RNA titer			
<100 KIU/mL	0	3	NS
100–700 KIU/mL	4	37	
>700 KIU/mL	13	53	
Histological fibrosis			
Mild	3	36	NS
Moderate	4	30	
Severe	6	17	
Cirrhosis	3	9	
Histological activity			
Mild	8	43	NS
Moderate	8	48	
Severe	0	1	
Re-treatment (+/–)	9/8	53/40	NS

Data expressed as median (range). DM, diabetes mellitus; HbA1c, hemoglobin A1c; HCV, hepatitis C virus; NS, not significant.

HbA1c were significantly higher in patients with DM than in those without DM (both $P < 0.001$). Furthermore, SVR was achieved in no patients with an HbA1c ≥6.9%, but was achieved in 11% of patients with an HbA1c <6.9% ($P < 0.05$). The rate of discontinuation of IFN-α2b plus ribavirin therapy due to side-effects did not differ between patients with DM (3/17, 18%) and those without DM (11/93, 12%).

Multiple logistic regression analysis of the factors affecting therapeutic outcome

To evaluate the significance of variables with respect to the outcome of IFN-α2b plus ribavirin therapy, a logistic model to assess factors related to SVR was constructed using all of the variables. The model was refined until it included only the variables independently associated with non-response (Table 3). The two variables were HCV serotype 1 (odds ratio 8.743; 95% confidence interval 2.215–34.517; $P = 0.002$) and the presence of DM (odds ratio 8.657; 95% confidence interval 1.462–51.276; $P = 0.014$).

DISCUSSION

THE PRESENT STUDY offers evidence that DM is one of the independent factors that reduces the effect of 6-month IFN-α2b plus ribavirin therapy in CHC patients. The prevalence of DM is 13–27.6% of CHC patients.[22–26] In some studies, DM patients were shown to have more advanced liver fibrosis than patients without DM,[24,25] and advanced liver fibrosis is a factor

Table 3 Multivariate analysis of the effect of variables on the response to interferon-α2b plus ribavirin therapy

Variable	*P*-value	Multivariate odds ratio (95% CI)†
HCV serotype (1 *vs* 2)	0.002	8.743 (2.215–34.517)
Presence of DM	0.014	8.657 (1.462–51.276)

†Values are the odds of having difficulty becoming a sustained-responder.
CI, confidence interval; DM, diabetes mellitus; HCV, hepatitis C virus.

that influences the ability to achieve SVR. However, in our patients, the liver histological staging of patients with DM tended to be higher than that of patients without DM, but no significant difference was detected. In addition, the presence of DM did not affect the HCV viral load, ALT levels, or liver histological activity. Multiple logistic regression analysis revealed that the presence of DM and HCV serotype 1, but not liver histological staging, was an independent factor affecting the therapeutic efficacy.

The mechanism by which DM interferes with viral elimination in CHC patients remains unclear. Recently, it was reported that insulin resistance impaired the virological response to peg-IFN plus ribavirin treatment;[18] the insulin resistance index has been found to be an independent factor for achieving a sustained response. Insulin resistance has been shown to be caused by increased production levels of tumor necrosis factor (TNF)-α. In fact, the production of TNF-α is increased in chronic liver injury,[27] and TNF-α is one of the causes of DM in CHC patients.[28–30] A study of a mouse model transgenic for the HCV core gene revealed that HCV caused insulin resistance, and the presence of a high TNF-α level was considered to be one of the factors leading to insulin resistance in the transgenic mice.[31] Furthermore, the baseline TNF-α values in sustained-responders have been found to be significantly lower than in non-responders.[32,33] TNF-α inhibits IFN-α signaling by stimulating the expression of the suppressor of cytokine signaling proteins.[34] In this context, TNF-α is important not only for the development of DM but also for interfering with the elimination of HCV in CHC patients. Thus, in our DM patients, TNF-α could have reduced their response to IFN-α2b plus ribavirin therapy. However HOMA-IR did not differ between sustained-responders and non-responders. Our sample size is too small to evaluate the relationship between insulin resistance and the response to IFN-α2b plus ribavirin therapy. However, fasting blood glucose and HbA1c were significantly higher in DM patients than in patients without DM. In particular, no patient with an HbA1c higher than 6.9% became a sustained-responder. Thus, our results indicate that, in addition to insulin resistance, the level of hyperglycemia is an important factor that interferes with HCV elimination. Hyperglycemia causes increased production of advanced glycation end products, which induce oxidative stress and cytokines, such as TNF-α and interleukin, by combining with their receptors.[35–37] In this manner, hyperglycemia may render IFN-α2b plus ribavirin therapy ineffective.

In conclusion, our study revealed that DM and HCV serotype 1 were independent factors that reduced the rate of viral elimination in CHC patients given combined therapy. However, the mechanism responsible for the reduction of viral elimination in DM patients could not be identified. Further studies are needed to determine how the presence of DM affects viral elimination.

Not only hyperglycemic state of DM but also insulin resistance can be improved by suitable diet therapy, exercise, insulin and other hypoglycemic agents. Hepatologists who initiate antiviral therapy should consult with DM specialists and dietitians before or during antiviral therapy of CHC patients with DM. An improvement of hyperglycemia and insulin resistance can lead to good response of antiviral therapy of CHC patients with DM.

REFERENCES

1 Saito I, Miyamura T, Ohbayashi A *et al.* Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci USA* 1990; 87: 6547–9.
2 McHutchison JG, Gordon SC, Schiff ER *et al.* Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; 339: 1485–92.
3 Davis GL, Esteban-Mur R, Rustgi V *et al.* Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; 339: 1493–9.
4 Tsubota A, Chayama K, Ikeda K *et al.* Factors predictive of response to interferon-alpha therapy in hepatitis C virus infection. *Hepatology* 1994; 19: 1088–94.
5 Martinot-Peignoux M, Marcellin P, Pouteau M *et al.* Pretreatment serum hepatitis C virus RNA levels and hepatitis C virus genotype are the main and independent prognostic factors of sustained response to interferon alpha therapy in chronic hepatitis C. *Hepatology* 1995; 22: 1050–6.
6 Enomoto N, Sakuma I, Asahina Y *et al.* Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; 334: 77–81.
7 Horiike N, Michitaka K, Masumoto T *et al.* Relationship between the effect of interferon therapy and the change of hepatitis C virus non-structural 5B gene. *J Gastroenterol Hepatol* 1999; 14: 345–51.
8 Horiike N, Masumoto T, Nakanishi K *et al.* Interferon therapy for patients more than 60 years of age with chronic hepatitis C. *J Gastroenterol Hepatol* 1995; 10: 246–9.
9 Matsushita M, Hijikata M, Matsushita M *et al.* Association of mannose-binding lectin gene haplotype LXPA and LYPB with interferon-resistant hepatitis C virus infection in Japanese patients. *J Hepatol* 1998; 29: 695–700.

10 Edwards-Smith CJ, Jonsson JR, Purdie DM *et al.* Interleukin-10 promoter polymorphism predicts initial response of chronic hepatitis C to interferon alpha. *Hepatology* 1999; 30: 526–30.
11 Hijikata M, Ohta Y, Mishiro S. Identification of a single nucleotide polymorphism in the MxA gene promoter (G/T at nt-88) correlated with the response of hepatitis C patients to interferon. *Intervirology* 2000; 43: 124–7.
12 Yee LJ, Tang J, Gibson AW *et al.* Interleukin-10 polymorphism as predictors of sustained response in antiviral therapy for chronic hepatitis C infection. *Hepatology* 2001; 33: 708–12.
13 Sugimoto Y, Kuzushita N, Takehara T *et al.* A single nucleotide polymorphism of the low molecular mass polypeptide 7 gene influences the interferon response in patients with chronic hepatitis C. *J Viral Hepat* 2002; 9: 377–84.
14 Konishi I, Horiike N, Hiasa Y *et al.* CCR5 promoter polymorphism influences the interferon response of patients with chronic hepatitis C in Japan. *Intervirology* 2004; 47: 114–20.
15 Caronia S, Taylor K, Pagliaro L *et al.* Further evidence for an association between non-insulin-dependent diabetes mellitus and chronic hepatitis C virus infection. *Hepatology* 1999; 30: 1059–63.
16 Martin BC, Warram JH, Krolewski AS *et al.* Role of glucose and insulin resistance in development of type 2 diabetes mellitus: results of a 25-year follow-up study. *Lancet* 1992; 340: 925–9.
17 Bressler BL, Guindi M, Tomlinson G *et al.* High body mass index is an independent risk factor for nonresponse to antiviral treatment in chronic hepatitis C. *Hepatology* 2003; 38: 639–44.
18 Romero-Gomez M, Del Mar Viloria M, Andrade RJ *et al.* Insulin resistance impairs sustained response rate to peginterferon plus ribavirin in chronic hepatitis C patients. *Gastroenterology* 2005; 128: 636–41.
19 Desmet VJ, Gerber M, Hoofnagle JH *et al.* Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994; 19: 1513–20.
20 Lau JY, Davis GL, Kniffen J *et al.* Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. *Lancet* 1993; 341: 1501–4.
21 Bonora E, Formentini G, Calcaterra F *et al.* HOMA-estimated insulin resistance is an independent predictor of cardiovascular disease in type 2 diabetic subjects: prospective data from the Verona Diabetes Complications Study. *Diabetes Care* 2002; 25: 1135–41.
22 Allison ME, Wreghitt T, Palmer CR *et al.* Evidence for a link between hepatitis C virus infection and diabetes mellitus in a cirrhotic population. *J Hepatol* 1994; 21: 1135–9.
23 Mason AL, Lau JY, Hoang N *et al.* Association of diabetes mellitus and chronic hepatitis C virus infection. *Hepatology* 1999; 29: 328–33.
24 Petit JM, Bour JB, Galland-Jos C *et al.* Risk factors for diabetes mellitus and early insulin resistance in chronic hepatitis C. *J Hepatol* 2001; 35: 279–83.
25 Arao M, Murase K, Kusakabe A *et al.* Prevalence of diabetes mellitus in Japanese patients infected chronically with hepatitis C virus. *J Gastroenterol* 2003; 38: 355–60.
26 Chen LK, Hwang SJ, Tsai ST *et al.* Glucose intolerance in Chinese patients with chronic hepatitis C. *World J Gastroenterol* 2003; 9: 505–8.
27 Yoshioka K, Kakumu S, Arao M *et al.* Tumor necrosis factor alpha production by peripheral blood mononuclear cells of patients with chronic liver disease. *Hepatology* 1989; 10: 769–73.
28 Maeno T, Okumura A, Ishikawa T *et al.* Mechanisms of increased insulin resistance in non-cirrhotic patients with chronic hepatitis C virus infection. *J Gastroenterol Hepatol* 2003; 18: 1358–63.
29 Knobler H, Zhornicky T, Sandler A *et al.* Tumor necrosis factor-alpha-induced insulin resistance may mediate the hepatitis C virus–diabetes association. *Am J Gastroenterol* 2003; 98: 2751–6.
30 Aytug S, Reich D, Sapiro LE *et al.* Impaired IRS-1/PI3-kinase signaling in patients with HCV: a mechanism for increased prevalence of type 2 diabetes. *Hepatology* 2003; 38: 1384–92.
31 Shintani Y, Fujie H, Miyoshi H *et al.* Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology* 2004; 126: 840–8.
32 Neuman MG, Blendis LM, Shear NH *et al.* Cytokine network in nonresponding chronic hepatitis C patients with genotype 1: role of triple therapy with interferon alpha, ribavirin, and ursodeoxycholate. *Clin Biochem* 2001; 34: 183–8.
33 Taliani G, Badolato MC, Nigro G *et al.* Serum concentration of gammaGT is a surrogate marker of hepatic TNF-alpha mRNA expression in chronic hepatitis C. *Clin Immunol* 2002; 105: 279–85.
34 Mbow ML, Sarisky RT. What is disrupting IFN-alpha's antiviral activity? *Trends Biotechnol* 2004; 22: 395–9.
35 Yan SD, Schmidt AM, Anderson GM *et al.* Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. *J Biol Chem* 1994; 269: 9889–97.
36 Sheetz MJ, King GL. Molecular understanding of hyperglycemia's adverse effects for diabetic complications. *JAMA* 2002; 288: 2579–88.
37 Rashid G, Benchetrit S, Fishman D *et al.* Effect of advanced glycation end-products on gene expression and synthesis of TNF-alpha and endothelial nitric oxide synthase by endothelial cells. *Kidney Int* 2004; 66: 1099–106.

Immunogenetics (2007) 59:197–209
DOI 10.1007/s00251-006-0185-3

ORIGINAL PAPER

Identification of CTL epitopes in hepatitis C virus by a genome-wide computational scanning and a rational design of peptide vaccine

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Received: 1 October 2006 / Accepted: 22 November 2006 / Published online: 16 January 2007

Abstract Developing a peptide-based vaccine for the highly variable hepatitis C virus (HCV) remains a challenging task. Variant viruses not only escape antigen presentation but also persist in a patient as quasi-species. Such variants are often antagonistic to the responding T cell repertoire. To overcome these problems, we herein propose a cocktail vaccine consisting of a few epitope peptides, which make it possible to outpace the emergence of variant viruses. To design such a vaccine, we developed a way to identify HLA-A*2402-binding peptides efficiently by means of the computational scanning of the whole genome of the pathogen. Most of the predicted peptides exhibited strong binding to the HLA-A*2402 molecule, while also inducing CD8 T cell responses from the patients' peripheral blood mononuclear cells (PBMCs). Peptide-induced T cells were capable of lysing HCV-expressing HepG2 cells which process antigens endogenously. The amount of HCV core antigen in the patients' livers suggested that the lytic activity of the peptide-induced T cells was clearly in a range suitable for therapeutic use. If T cells were activated under optimal conditions by high density peptides, then they tended to be relatively tolerant of single amino acid variations for cytolysis. Finally, an analysis of the viral population isolated in Japan suggested no obvious changes due to immune evasion in the viral genome even in a host population highly biased toward HLA-A*2402.

Keywords Cytotoxic T lymphocyte · Hepatitis C virus · Peptide · HLA · Vaccine

Electronic supplementary material Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s00251-006-0185-3 and is accessible for authorized users.

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Introduction

Hepatitis C virus (HCV) remains a serious threat due to its persistence and the fact that it can also cause liver cirrhosis and cancer. Even the most advanced treatment combining pegylated interferon-α and ribavirin only has a sustained viral clearance rate of just more than 50%, and this falls even further with the HCV genotype 1 and for

patients with other comorbidities (Feld and Hoofnagle 2005; Tsubota et al. 2004). The induction of cytotoxic T lymphocytes (CTLs) specific for HCV-infected cells has been a promising strategy for viral containment. A number of HLA class I-binding peptides have so far been identified (Battergay et al. 1995; Cerny et al. 1995; Kurokohchi et al. 2001; Nakamoto et al. 2003). However, finding HLA-binding peptides and screening them for T cell responses is a laborious, expensive, and time-consuming process. Therefore, HLA-binding has not yet been examined for a number of known T cell epitopes. However, if the affinity is low, even if a high density of exogenously added peptide may stimulate T cells, the infected hepatocytes may not present a sufficient number of peptides to be recognized by such T cells. Therefore, it is important to develop a method to design a peptide-based vaccine more efficiently and effectively.

HCV is an RNA virus with a high rate of mutation due to the absence of any proof-reading activity in its RNA-dependent RNA polymerase. Viral escape from immune attack by epitope changes has been studied by a number of groups and remains a major concern in vaccine development (Ray et al. 2005; Timm et al. 2004; Weiner et al. 1995). The first type of such mutations leads to poor processing of MHC-binding peptides due to an alteration of the amino acid sequences for proteasome cleavage (Kimura et al. 2005; Seifert et al. 2004). The second type leads to poor binding of epitope peptides to the MHC class I molecules (Chang et al. 1997; Cox et al. 2005; Erickson et al. 2001). The third type induces altered responses known as antagonism or anergy in T cells due to a poor recognition by TCR (Chang et al. 1997; Cox et al. 2005; Erickson et al. 2001; Grakoui et al. 2003; Kaneko et al. 1997). The virus not only changes, but it also exists as a quasi-species, i.e., a mixed population of distinct, but closely related, variants. These variants are often antagonistic to the responding T cell repertoire, and thus, annihilate the T cell responses. The quasi-species of viruses are kept in a dynamic yet lasting balance, and thus, effectively suppress the cytolytic activities of a dominant T cell repertoire (Chang et al. 1997). Therefore, the key to a successful vaccine lies in a design that can eradicate the heterogeneous viral population before escaped mutants become prevalent.

Materials and methods

Cells and antibodies

RzM6 is a HepG2 transfectant with a full genome HCV1b isolate AY045702 (Tsukiyama-Kohara et al. 2004). The expression of HCV can be induced by the tamoxifen-induced expression of the doubly transfected Cre that mediates the removal of an intervening sequence in the 5′ promoter region of the HCV genome. C1R was a gift from Dr. P. Cresswell (Edwards et al. 1982). C1R-A24 is a HLA-A*2402-transfected C1R cell line and was kindly provided by Dr. Takiguchi (Karaki et al. 1993). HepG2 was purchased from ATCC. T2-A24 is a TAP-deficient T2 cell line transfected with HLA-A*2402 and was a gift from Dr. A. Tsuboi (Osaka University).

Peptides

The peptides were manually synthesized using Fmoc chemistry and then were purified by HPLC to a purity of >95% using a C18 Microbondasphere column (Japan Waters, Tokyo). The peptides were examined by mass spectrometry using Voyager DE-RP (Applied Biosystems Japan, Tokyo). The concentrations were determined by a MicroBCA assay using bovine serum albumin (BSA) as the standard (Pierce, Rockford, IL).

Peptide-binding assay

The binding of peptides to the HLA-A*2402 molecule was measured by acid stripping and a reconstitution assay as previously described by Zeh et al. (1994) with minor modifications. Briefly, C1R-A24 cells were exposed to pH 3.3 citrate phosphate buffer and then were reconstituted with graded concentrations of peptide and 0.1 μM human β2-microglobulin (Sigma, M-4890, St. Louis, MS) in DMEM containing 0.25% BSA. An FITC-labeled mAb 17A12 (Tahara et al. 1990) was used to detect the properly folded and peptide-bound HLA-A*2402 molecules. The fluorescence intensity was measured by FACScan (Becton-Dickinson Japan, Tokyo). Both high- and low-binding peptides, HER2-63 TYLPTNASL and Met149 RVWE SATPL, respectively, were always included in the assay, and their binding was used to normalize the variations between experiments. The affinity of a peptide was calculated as previously described (Udaka et al. 2000).

Peptide-specific cell lines

The peripheral blood mononuclear cells (PBMCs) from patients or healthy individuals were stimulated weekly with 1 μM peptide in 10% fetal calf serum (FCS) containing 10 U/ml recombinant human IL-2. After five time stimulations, cells were tested for killing activity. The patients had been diagnosed to be suffering from chronic hepatitis with HCV genotype 1b. All the patients were positive for both the anti-HCV antibodies and viral RNA in the serum by polymerase chain reaction (PCR). Informed consent was obtained from all patients. The study protocol

was approved by the Human Research Committee of Ehime University.

^{51}Cr release assay

A peptide-specific cytolysis assay was conducted against C1R-A24 cells or T2-A24 cells in the presence or absence of 1 μM peptide. An HLA-A*2402-binding peptide, HER2-63 (TYLPTNASL, log Ka; 7.3), was used as a negative control. Target cells were labeled with ^{51}Cr-sodium chromate at either 37 or 26°C, respectively, for 1 h, and then, they were loaded with 1 μM peptide before the addition of effector cells. The *E/T* ratio was 10–20. Percent specific lysis during 3.5 h incubation at 37°C was calculated as (experimental release−spontaneous release)/(total release−spontaneous release)×100. The cytolysis of cells that naturally present HCV peptides was measured using tamoxifen-treated RzM6 cells as a target for 3 h at an *E/T* ratio of 10.

Measurement of HCV core protein

Liver tissue specimens from the patients were taken by a biopsy after informed consent was obtained from all the patients. The cell lines and tissue specimens were lysed in Radioimmunoprecipitation assay (RIPA) buffer (1% sodium dodecyl sulfate (SDS), 1% NP40, 10 mM Tris–HCl, pH 8.0 and 0.14 M NaCl) and the supernatant was subjected to the measurement of core protein using an HCV Ag enzyme-linked immunosorbent assay (ELISA) kit (Ortho-Clinical Diagnostics, Tokyo, Japan). The protein concentrations were determined with a DC protein assay (BIO-RAD, Hercules, CA).

Results

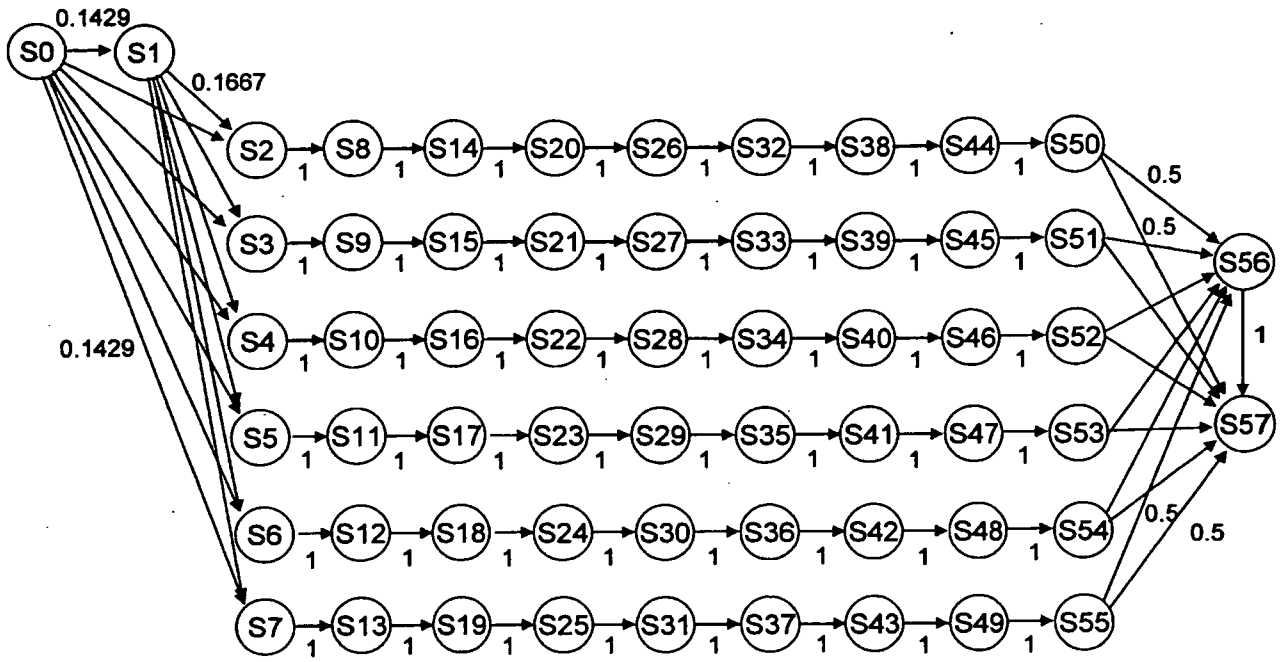
Generation of a program to predict HLA-A*2402-binding peptides

We have previously described a computational method to analyze the specificity of MHC class I-binding peptides (Udaka et al. 2002). The method utilizes a data mining technique, a query learning algorithm based on hidden Markov models (HMMs). This algorithm finds peptides whose binding properties are hard to predict by using a prototype prediction program established with the existing binding data (supplementary material 2). We synthesized such peptides and measured their binding to MHC molecules. By feeding the newly obtained binding data back into the data pool, the prediction program could thus be improved. This cyclic learning that combines data analysis and peptide-binding experiments is repeated until a satisfactory prediction can be achieved. This time, we examined the HLA-A*2402 molecule, the most frequent allele among Asians, e.g., 33% for the Japanese (Tokunaga et al. 1997) and ~10% in the Western countries (Imanishi et al. 1992). The original HMMs employed a cyclic model (Udaka et al. 2002), but this was switched to a more rational parallel model during the learning (Fig. 1a). After 6 rounds of cyclic learning, examining 400 peptides altogether, i.e., 222 before active learning and 178 newly synthesized during learning, a final prediction program was established. However, further learning was still possible when necessary. The binding data for an additional 105 peptides that had been set aside to monitor the progress of the learning were also added to the data pool to generate the final prediction program. This program predicts the binding affinity by approximating the log K_a values, and thus, gives each peptide a score in real numbers. The performance of the program was assessed by 10-fold cross-validation using the binding data on 505 peptides described above. The coefficient of the correlation between the predicted scores and actual affinity was 0.80 (Fig. 1b). Most of the known T cell epitope peptides have an affinity of 5.5 or higher in the log K_a terms. Among the peptides whose predicted scores were 5.5 or higher, 93% (117/126) actually exhibited a log K_a value of 5.5 or higher. Therefore, the accuracy (sensitivity) of the prediction was 93%. On the other hand, among those peptides whose affinity was 5.5 or higher, 60% (113/187) had scored 5.5 or higher. Therefore, the coverage was 60%. If the threshold had been raised to 6.0, then the accuracy would have been 93% (67/72) and the coverage thus would have fallen to 43% (67/156).

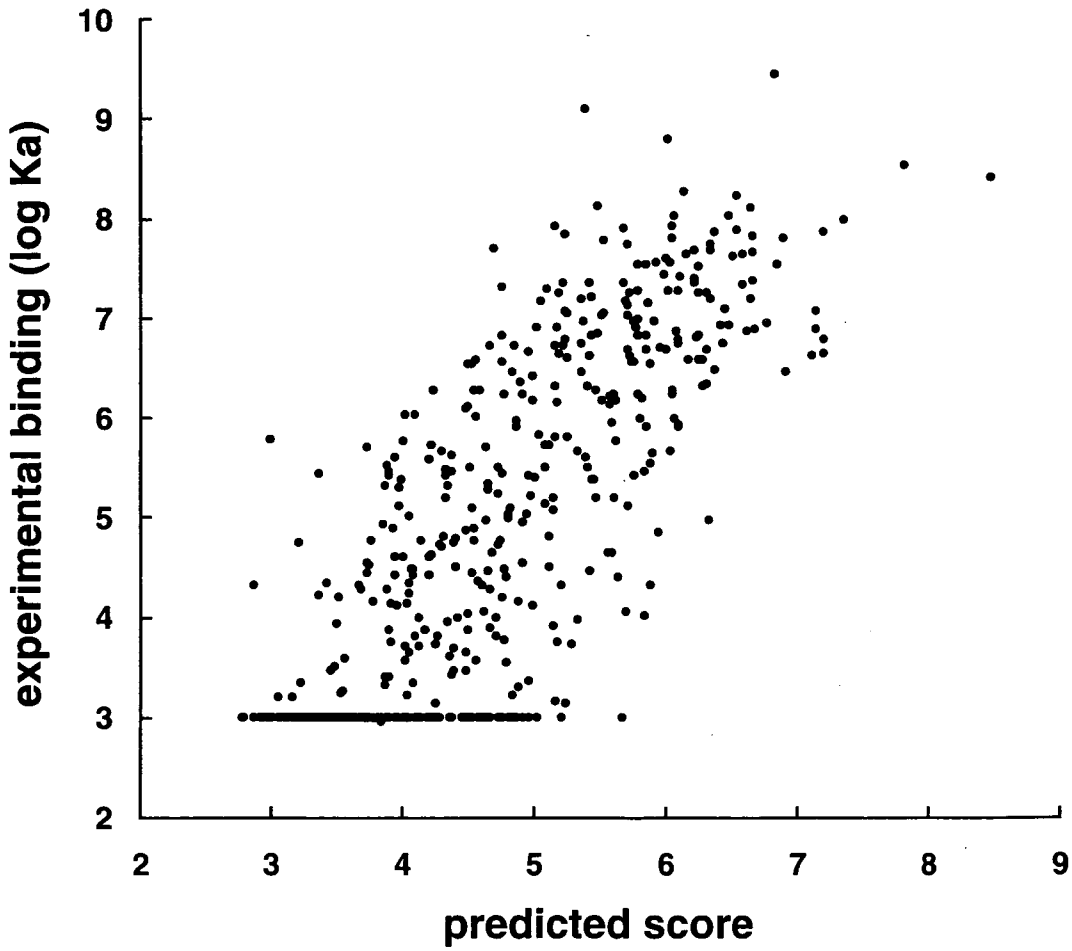
Genome-wide screening of hepatitis C virus for HLA-A*2402-binding peptides

Using the program developed above, we scanned the entire genome of hepatitis C virus (HCV) genotype 1b for HLA-A*2402-binding peptides. We chose a prototype Japanese isolate GenBank D20908 and a subclone of D89815 for the analysis. The latter was chosen due to our initial plan to use cells transduced with that clone as a target. Several amino acids differ between D89815 and its subclone pBRT703′X (Dr. Y. Matsuura, personal communication). The high-scoring peptides were synthesized and subjected to HLA-binding assays. Some peptides (~7% of the peptides synthesized) were hard to synthesize, and thus, were excluded. Several known epitope peptides also scored high, and their binding was examined. The results are shown in Table 1. High-binder peptides (log K_a >5.5) are in bold. The major anchor amino acids identified by Rammensee et al. for HLA-A*2402 are Y/F at P2 and I/L/F at P9 (http://www.syfpeithi.de/). The peptides in italics do not fulfill

a


0.1429
S0
S1
0.1667
S2
S8
S14
S20
S26
S32
S38
S44
S50
0.5
S3
S9
S15
S21
S27
S33
S39
S45
S51
0.5
S56
0.1429
S4
S10
S16
S22
S28
S34
S40
S46
S52
1
S5
S11
S17
S23
S29
S35
S41
S47
S53
S57
S6
S12
S18
S24
S30
S36
S42
S48
S54
0.5
0.5
S7
S13
S19
S25
S31
S37
S43
S49
S55

b


experimental binding (log Ka)
predicted score
10
9
8
7
6
5
4
3
2
2
3
4
5
6
7
8
9

◀ **Fig. 1** The analysis and prediction of HLA-A*2402-binding peptides. **a** A hidden Markov model used for analyzing the specificity of HLA-A*2402-binding peptides. A general description of the model and the data mining algorithm are given in the reference (Udaka et al. 2000) and supplementary material 2. Six independent paths were designed. The first node S0 depicts the initial state and the last node S57 designates the final state. In the states from S1 to S56, the frequency of every amino acid is calculated to give the sum of the probabilities for 20 amino acids to be one in each state. *Arrows* depict transition probabilities. The states S1 and S56 were introduced to accommodate lateral shifts of the binding motif by one amino acid in both directions. Nine states in each row depict amino acid positions in a peptide of nine amino acids starting from the N-terminal amino acid on the left. The initial state can take any path and S1 in the case of an N-terminal shift of the binding motif. The frequency of transition was tentatively given as 0.1429 (=1 / 7) for each *arrow*, but it is replaced by an actual frequency once the model is trained using peptide-binding data. The final state can be reached from any path and from S56. The probability of transition from a row to the final state is tentatively given as 0.5 for each *arrow* in the illustration, but this too is replaced by actual probability. **b** The correlation of the predicted HLA-A*2402-binding scores and experimentally determined binding of peptides. The correlation was examined by a tenfold cross validation using the peptide-binding data obtained during cyclic training of the models

two anchor requirements. The high-binder peptides could also be identified among such peptides (underlined peptides).

Responses of the PBMCs from chronic hepatitis patients

Due to the limits of the cell culture, 15 peptides (log K_a >6) were randomly chosen out of the peptides noted in Table 1 and then were tested for the induction of cytotoxic T lymphocytes (CTLs). PBMCs from patients with chronic hepatitis due to HCV 1b and from healthy individuals were stimulated with peptides and tested for cytolysis by ^{51}Cr release assay. A poor cytolytic activity of CD8 T cells despite a robust production of IFN-γ have been observed among both tumor-bearing patients and patients suffering from chronic viral infections (Appay et al. 2000; Huang et al. 2005; Wherry et al. 2003). Therefore, in this study, with the aim of developing a curative vaccine, we used a cytolysis assay. As shown in Fig. 2a and b, most of the peptide-stimulated cell lines exhibited cytotoxicity. Interestingly, there were several peptides to which many patients responded. These peptides can thus be good targets for immunotherapy. Healthy individuals occasionally exhibited some responses but less frequently than the patients (Fig. 2a,b). Most patients exhibited cytolytic activities, often to several peptides. This strongly indicates that in the patients, a HCV-specific T cell repertoire has expanded and the cytolytic activity can therefore be induced if antigenic peptides are provided in a stimulatory environment in vitro.

Peptide-specific cell lines were further examined for restricting HLA molecules and peptide dependency for

Table 1 Prediction of HLA-A*2402-binding peptides in the HCV 1b genome

Protein	Peptide	Sequence	Predicted score	Actual binding normalized log K_a
Core	C36shpB[a]	***LLPRRGPRL***	5.30	**7.71**
	C77shpB	*AQPGYPWPL*	5.98	5.36
	C169shpB	*LPGCSFSIF*	4.98	4.91
E1	C234sh	**NFSRCWVAL**	5.46	**5.92**
	C360shpB	***AYYSMVGNW***	5.73	**6.47**
E2	C616shpB	***WHYPCTVNF***	5.66	**6.39**
	C666shpB	***LLSTTEWQI***	5.35	**8.34**
	C674sh	***ILPCSFTTL***	6.75	**7.66**
	C688pB	***GLIHLHQNI***	6.00	**5.86**
	C764pB	***GILPFFMFF***	6.26	**7.70**
	C789sh	***ALYGVWPLL***	5.80	**6.98**
	C789pB	**AFYGVWPLL**	6.02	**7.69**
NS2	C834sh	**YYKVFLARL**	5.49	**7.12**
	C838shpB	***FLARLIWWL***	5.24	**6.18**
	C876pB	***LMCAVHPEL***	5.52	**6.59**
	C975sh	**VFSDMETKL**	5.54	**7.31**
	C992shpB	*ACGDIISGL*	5.26	3.91
	C1010shpB	***ILLGPADSF***	5.43	**5.50**
NS3	C1031shpB	**AYSQQTRGL**[b]	5.76	**6.54**
	C1291sh	***ITYSTYCKF***	5.27	**6.98**
	C1291pB	***ITYSTYGKF***	5.16	**6.37**
	C1349pB	***ATPPGSVTF***	6.53	**6.51**
NS4B	C1760shpB	***FWAKHMWNF***[c]	5.93	**8.10**
	C1956shpB	***LLKRLHQWI***	5.20	**6.86**
NS5A	C1976pB	***WLRDVWDWI***	5.27	**6.34**
	C1986pB	***TVLADFKTW***	6.25	**6.52**
	C1987pB	***VLADFKTWL***	5.26	**6.63**
	C2132sh	**RYAPVCKPL**	5.96	**6.15**
	C2132pB	**RYAPACKPL**	5.47	**6.76**
	C2139pB	*PLLRDEVTF*	6.39	5.09
	C2173shpB	***SMLTDPSHI***	5.55	**6.94**
	C2251sh	*VILDSFDPI*	6.05	5.32
	C2251pB	*VILDSFEPL*	6.26	5.00
	C2289shpB	*ARPDYNPPL*	5.47	3.93
NS5B	C2422shpB	**SYTWTGALI**	5.51	**7.13**
	C2593shpB	***ALYDVVSTL***	5.99	**6.39**
	C2841shpB	***RMILMTHFF***[c]	6.01	**7.42**
	C2843shpB	***ILMTHFFSI***	5.57	**7.90**
	C2844shpB	***LMTHFFSIL***	5.20	**5.92**
	C2962shpB	*SQLDLSGWF*	5.66	< 3

High-affinity peptides (log K_a>5.5) are shown in bold. The sequences in italics represent the peptides that do not fulfill two major anchor requirements. The high-affinity peptides that would not have been identified by the criteria of two major anchors are underlined.

[a] The number refers to the position of the first amino acid. The suffix sh stands for a sequence from Genbank D20908, and pB, that from the subclone pBRT703'X of D89815. A substantial number of nucleotide mismatches have been identified between pBRT703'X and D89815 (Matsuura, personal communication).

[b] Epitope reported by Kurokohchi et al. (2001)

[c] Epitopes reported by Nakamoto et al. (2003)

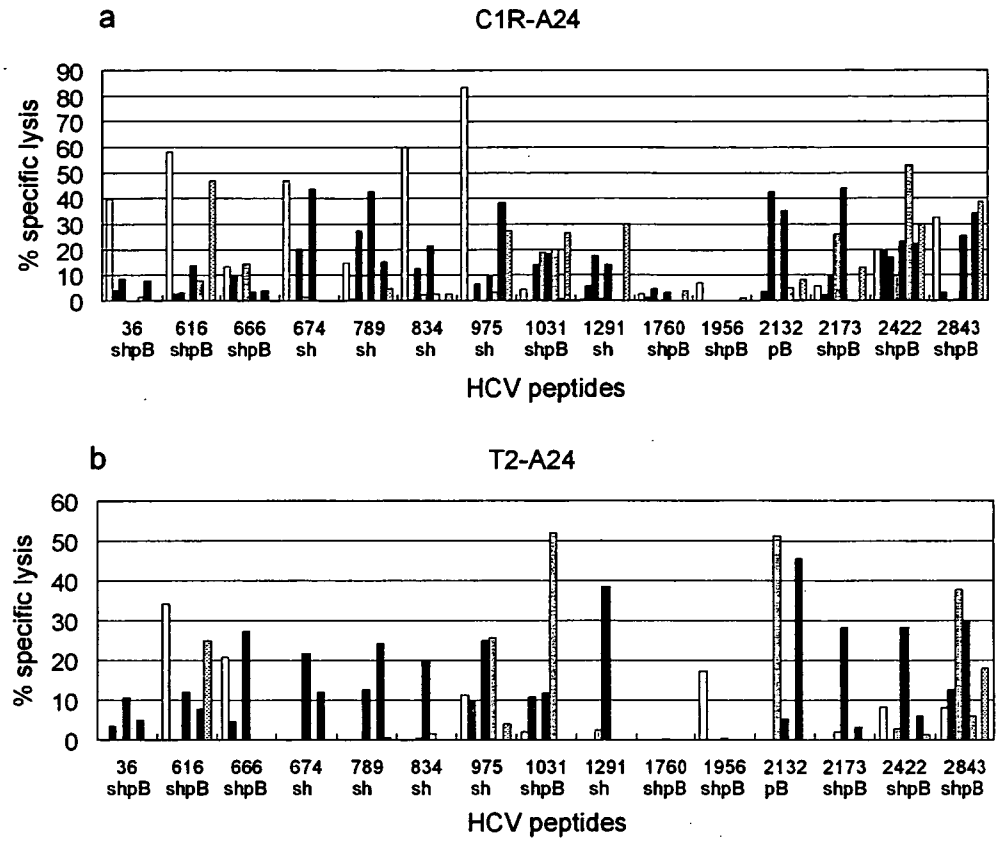


Fig. 2 The cytolytic activities of the peptide-induced cell lines from patients and healthy individuals. Peptide-specific cell lines were established from PBMCs by weekly stimulations with peptides. Each bar demonstrates lytic activity by a cell line from an individual. The assay used allogeneic C1R-A24 in **a** or T2-A24 cells in **b** as a target and was performed in the presence of indicated peptide at a concentration of 1 μM. These target cells exhibited substantial background lysis either without any peptide or with a negative control peptide HER2-63. Therefore, specific lysis is shown as the value from which the background lysis with HER2-63 has been subtracted. Nonoverlapping responders were used for **a** and **b**. The first (*open*) and third (*dark gray*) bars in **a** and the fourth (*closed*) *bar* are cell lines from healthy individuals

recognition. The responding cell lines were predominantly $CD8^+$ T cells as is shown in Fig. 3a. When tested against C1R or C1R-A24 target cells, several cell lines exhibited a stronger cytolytic activity against C1R-A24 than C1R in the presence of peptides (Fig. 3b). It was not possible to perform this assay for all the cell lines shown in Fig. 2a,b due to the limited number of cells. For a few cell lines, the cytolysis of C1R was too high for one to see any peptide dependency. This was most likely due to some alloreactivity or NK-like activity because we used a low level of rIL2 during the cell culture.

Cytolytic activity of the peptide induced cell lines against cells naturally presenting the antigen

Although the peptide-induced T cell lines were highly cytolytic against tumor targets heavily loaded with exogenous peptides, it is not clear whether these effecter cells are also capable of killing target cells that present as endogenous antigens through natural antigen processing. To examine this, we tested a HepG2 transfectant RzM6 (Tsukiyama-Kohara et al. 2004) as a target. RzM6 carries a full-length HCV 1b genome (GenBank AY045702). HepG2 naturally expresses HLA-A*2402. The expression of the HCV gene from the CAG promoter can be conditionally induced by the Cre-mediated removal of the floxed intervening sequence that has been inserted in the 5′ terminal region. The RzM6 cells had been doubly transfected with the *Cre* expression construct whose expression could be induced by tamoxifen. The precise 5′ and 3′ trimming at the ribozyme sequences eventually produces a full-length HCV. As is shown in Table 2, the HLA-A*2402-binding peptides expressed in RzM6 differ by several amino acids from the HCV isolates shown in Table 1. Tamoxifen-treated RzM6 cells were lysed by most of the peptide-specific cell lines established as shown in Fig. 4. This indicated that most of the peptides identified as HLA-A*2402-binders from genomic sequences were actually processed endogenously and then presented on the cell surface. Interestingly, most of the peptide-induced CTL lines could lyse RzM6 cells whose epitope sequences carry amino acid substitution(s) compared with the peptides used for stimulation. Although such variant peptides could be antagonistic to part of the CTL repertoire (Kaneko et al. 1997) once the T cells were optimally activated by high-density peptides, then the responding T cells were also found to be cytolytic for such variant peptides. This is encouraging to those developing a peptide vaccine against highly variable pathogens like HCV. Interestingly, the peptide C616shpB is located in a sequence context which is not ideal for proteasomal cleavage (Nielsen et al. 2005; Nussbaum et al. 2001). However, this peptide also seems to

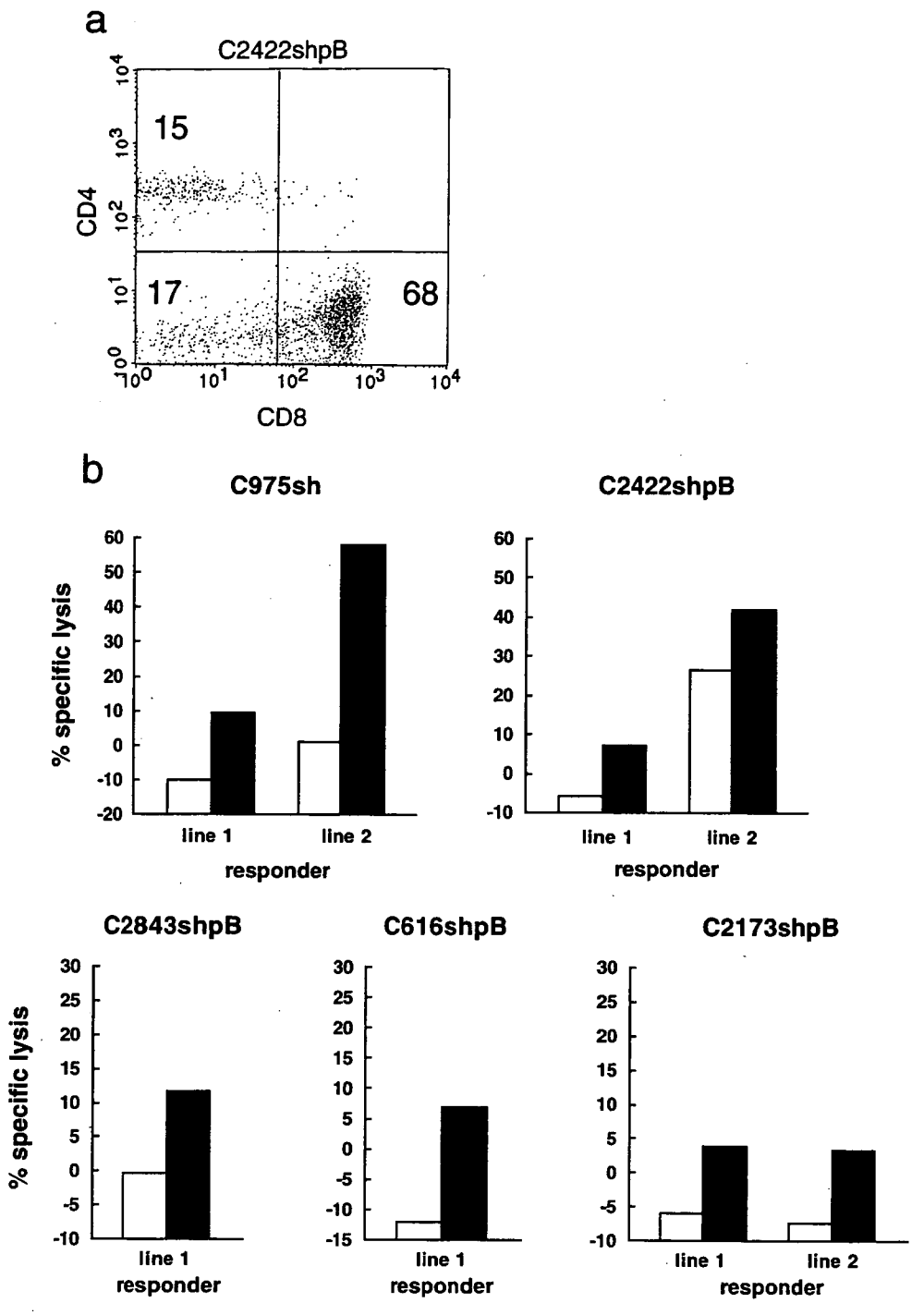


Fig. 3 Peptide-specific cell lines recognize peptides in the context of HLA-A*2402. **a** Cell surface profile of a cell line specific for C2422shpB shown as an example. The enrichment of CD8 cells was obvious for cell lines that exhibited peptide-dependent lysis after 3 to 5 weekly stimulations with HLA-A*2402-binding peptides. **b** Specific lysis of C1R (*open bars*) or C1R-A*2402 (*closed bars*) target cells in the presence of peptides used for the stimulation of the corresponding cell lines. Lysis without peptide has been subtracted

be presented on RzM6. Although inefficient proteasomal cleavage has been reported to be one of the mechanisms for viral escape (Seifert et al. 2004), not all the peptides that are predicted to demonstrate poor cleavage seem to be spared from antigen presentation. Under normal conditions, peptides are in relatively short supply in comparison to the newly synthesized MHC class I molecules in the endoplasmic reticulum (ER) (Lie et al. 1990). Slowly cleaved peptides may still have a chance to bind to empty MHC class I molecules.

HCV peptide presentation in the infected hepatocytes

Importantly, these peptide-specific T cells have, however, not been sufficient to contain viral infections in the patients. The expression of HCV proteins in tamoxifen-treated RzM6 cells (RzM6-Tx) may have been higher than that in the infected hepatocytes of the patients. We, therefore, examined next the expression of core protein as a representative antigen in the patients' livers. As shown in Fig. 5, the expression of core protein of HCV 1b was high

Table 2 Amino acid variations in the epitope sequences of the HVC 1b clones analyzed in this study

Peptides	D20908	pBRT703'X	RzM6
C36	LLPRRGPRL	---------	---------
C616	WHYPCTVNF	---------	------A--
C666	LLSTTEWQI	---------	--------V
C674	ILPCSFTTL	---------	V--------
C789	ALYGVWPLL	-F-------	-F-------
C834	YYKVFLARL	-C-------	---E-----
C975	VFSDMETKL	-A----V	--------V
C1031	AYSQQTAGL	---------	---------
C1291	ITYSTYCKF	------G--	------G--
C1760	FWAKHMWNF	---------	---------
C1956	LLKRLHQWI	---------	------H--
C2132	RYAPVCKPL	----A----	----A----
C2173	SMLTDPSHI	---------	---------
C2422	SYTWTGALI	---------	---------
C2843	ILMTHFFSI	---------	---------

in RzM6-Tx, whereas liver tissues from patients suffering from chronic hepatitis had lower and variable levels of expression. Therefore, the lytic activity of the peptide-specific CTL lines against RzM6-Tx may not necessarily guarantee the lysis of infected hepatocytes. It is not known what proportion of hepatocytes in the patients actually express core antigen. In a report of immunostaining of the liver tissues from chronic hepatitis patients, 1–5% of the hepatocytes express core antigen to a level detectable by specific antibodies (Nouri-Aria et al. 1995). Agnello et al. reported that usually 50% or more, but not all, of the hepatocytes are infected by HCV in chronically infected patients (Agnello et al. 1998). If 50% of the hepatocytes expressed core protein, then the results shown in Fig. 5 would indicate that the expression level of the antigen in individual hepatocytes could be comparable to or within a few fold differences from RzM6-Tx in the patients. If so, there is a good chance that infected hepatocytes are lysed by the peptide-induced CTLs. Therefore, the therapeutic potential of peptide-based vaccine seems to be realistic provided that T cells are activated under optimal conditions with high-density peptides and in an immunostimulatory environment including activated APCs and proper helper activities.

Variability of HCV 1b genomes among Japanese isolates

As HCV is an RNA virus that has a high mutation rate, the sequence variation among HCV isolates is extensive. The

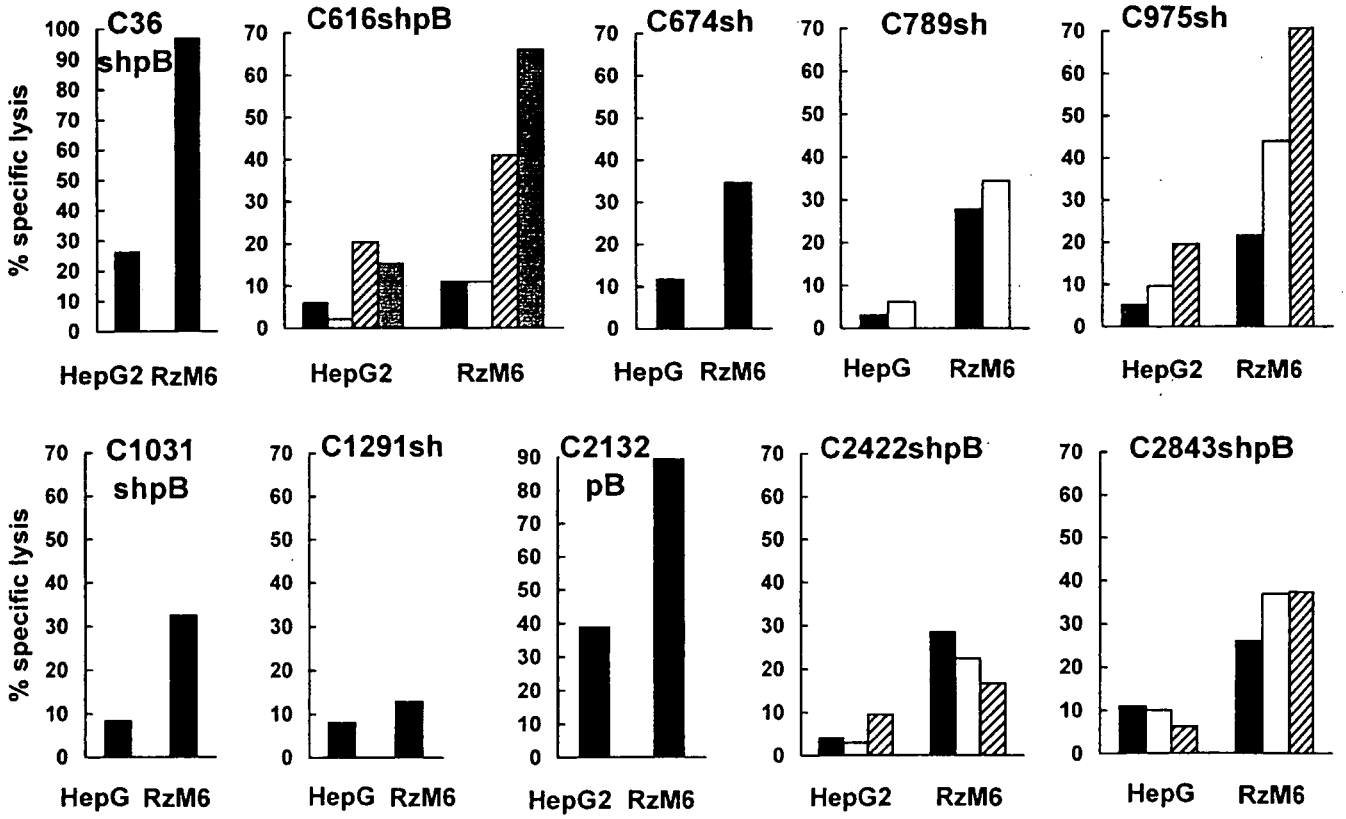


Fig. 4 Lysis of HCV 1b-transfected RzM6 cells by peptide-specific cell lines. The expression of the HCV genome in RzM6 had been induced by tamoxifen-induced Cre expression as described in the Materials and methods section. Peptide-specific cell lines from HCV 1b-infected patients and healthy individuals lysed RzM6 more than the parental HepG2 cells. Individual bars represent the lytic activities of the independent cell lines established from different individuals

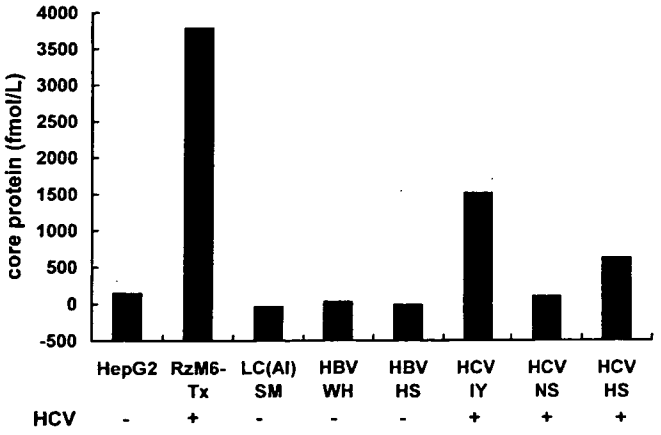


Fig. 5 Core protein expression in a HCV-transfected HepG2 cell line and the liver tissue specimens from HCV-infected patients. The amount of core protein was measured by ELISA in reference to the recombinant core protein. The average value for HCV-negative liver tissues was subtracted as background. The expression of HCV 1b in RzM6 had been induced by tamoxifen (RzM6-Tx). Negative control tissue specimens were from a patient with alcoholic liver cirrhosis; LC (AI) and two HBV-infected patients (WH, HS). Biopsy specimens from three HCV 1b RNA-positive patients (IY, NS, HS) with chronic hepatitis were examined. All samples were measured at a total protein concentration of 0.2 mg/ml

emergence of escape variants during the course of HCV infection has been a subject of serious concern in vaccine development (Chang et al. 1997; Cox et al. 2005; Kaneko et al. 1997; Timm et al. 2004). HLA-A*2402 has a gene frequency of around 30% in the Japanese population (Tanaka et al. 1996; Tokunaga et al. 1997). One-half of all Japanese most likely are at least heterozygous for this allele. Therefore, an HCV virus may thus encounter an HLA-A*2402-bearing host on every other transmissions on average. Such evolutionary pressure over thousands of years may thus have left some footprints on the viral genome. We therefore examined all Japanese isolates of HCV 1b identified to date for whether there is any evidence of amino acid changes in the HLA-A*2402-binding peptides that may have helped the virus escape from immune attack. We analyzed the non-synonymous/synonymous (NS/S) substitution rates (Nei and Gojobori 1986) between HLA-A*2402-binding peptides and the rest of the genome. Hyper-variable regions (HVR) 1 and 2 were analyzed separately because those regions may have been under different evolutionary pressure such as an evasion from antibody responses. The full genome sequence was available for 70 isolates (supplementary material 1).

We first generated an evolutionary tree based on the rapidly changing sequences in HVR 1 and 2 regions (Fig. 6). According to the tree, we calculated and compared the ratio of NS/S substitutions between the HLA-A*2402-binding peptides and then the rest of the genome excluding HVR1 and 2 regions (Table 3). Amino acid substitutions were frequently observed throughout the HCV 1b genome.

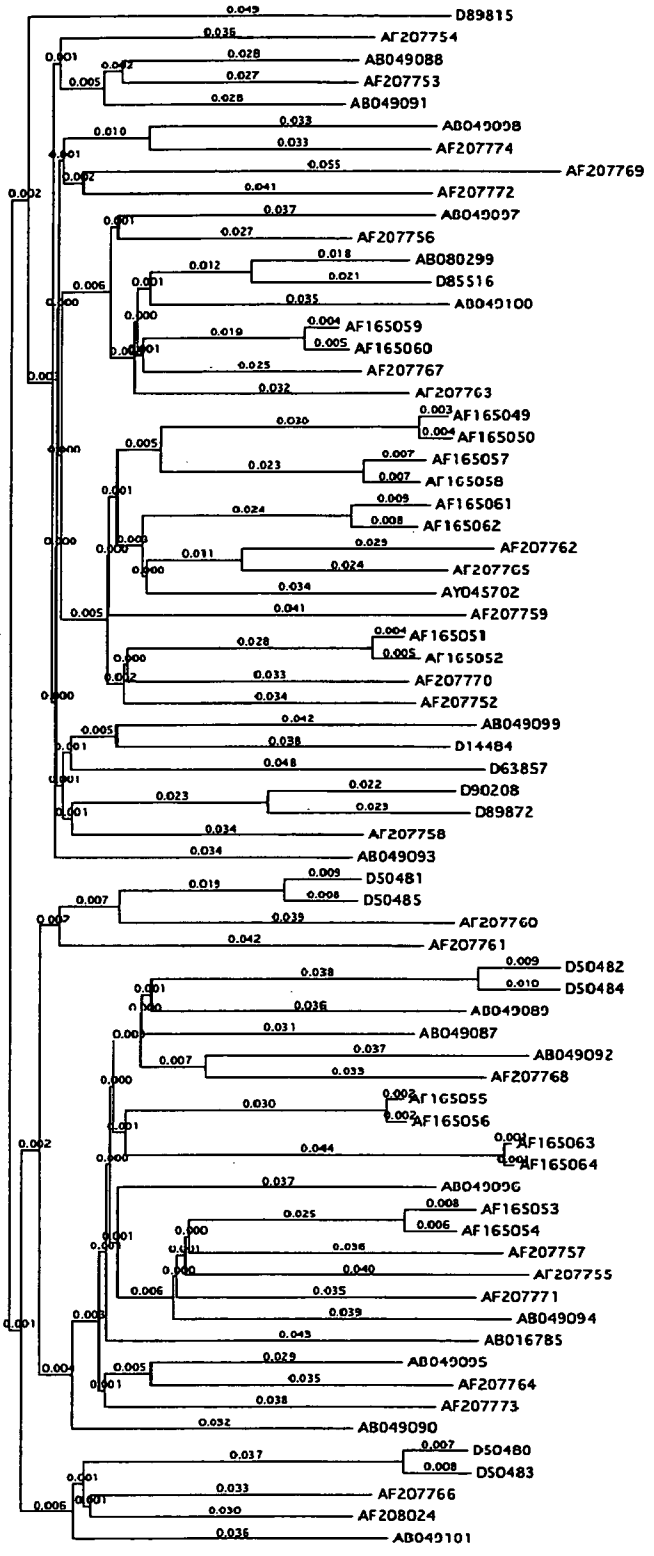


Fig. 6 Phylogenetic tree of the HCV 1b viruses isolated in Japan. An evolutionary relationship among the Japanese isolates of HCV 1b was examined by comparing the sequences in hyper variable regions (HVRs) 1 and 2. The numbers indicate the substitutions per site. The genbank accession numbers of the sequence data are shown

Table 3 Ratio of nonsynonymous versus synonymous substitutions

Region[a]	Nonsynonymous substitutions[b]			Synonymous substitutions[c]			Ratio
	K_N	L_N	d_N	K_S	L_S	d_S	d_N/d_S
Whole	201	6543	0.0307	556	2,418	0.230	0.13
HVR	20.1	58.7	0.342	8.0	22.3	0.359	0.95
ΔHVR	181	6,485	0.0279	548	2,396	0.229	0.12
BS (A24)	12.9	518.4	0.0249	42.2	177.6	0.238	0.11
Non-BS	188	6,025	0.0312	514	2,240	0.229	0.14

[a] *HVR* Hyper Variable Region, *ΔHVR* sequences excluding HVR, *BS(A24)* HLA-A*2402-binding sites, *Non-BS* sequences excluding BS & HVR
[b] K_N, L_N, and d_N stand for the mean number of nonsynonymous substitutions, the mean number of nonsynonymous sites, and the mean number of nonsynonymous substitutions per site, respectively.
[c] K_S, L_S, and d_S stand for the mean number of synonymous substitutions, the mean number of synonymous sites, and the mean number of synonymous substitutions per site, respectively.

HVRs exhibited a marked increase in the NS/S substitution rate, thus, indicating a strong selective pressure for amino acid changes in those regions. In contrast, the NS/S ratio in the HLA-A*2402-binding peptides was not significantly higher than that in other regions of the genome. In several longitudinal studies that followed the changes in individual patients, frequent amino acid substitutions were accumulated in the epitopes for CTLs and antibodies (Cox et al. 2005; Tester et al. 2005; Timm et al. 2004). An elevated NS substitution rate in the HLA class I-binding epitopes (Cox et al. 2005), therefore, suggests that substitutions positively contributed to the survival of the virus. In contrast to longitudinal studies in individual patients, this analysis indicates that such a bias is not obvious in a viral population that has been circulating among a host population which is heavily biased for HLA-A*2402. Ray et al. reported that in the absence of selection, amino acid variation tends to converge toward the consensus sequence, the structure of which is likely better adapted to the function of viral proteins. Such convergence may have rapidly occurred when the virus infected the non-HLA-A*2402-bearing hosts. Judging from the present analysis, it would thus be possible to develop a peptide vaccine for the public where the virus is under continuous selection by one-half of the host population. This is an encouraging result, provided that a strategy is developed to induce immune responses quickly and thoroughly before escape variants emerge.

Discussion

One of the most troublesome features of HCV for vaccine development is its genetic instability. Patients carry quasi-species of variant viruses which can sometimes be antagonistic to each other for virus-specific T cells. One of the authors and her colleagues have previously shown that the antagonistic inhibition of CTL-mediated cytolysis requires the expression of two closely related peptides on the same antigen-presenting cell that should occur only under limited conditions in a natural infection. More problematically, however, an antagonistic peptide expressed alone on a single antigen-presenting cell can effectively impair antigen-induced proliferation (Kaneko et al. 1997) which, therefore, would normally require a stronger engagement of TCRs than cytolysis (Valitutti et al. 1996). This selectively and effectively limits the expansion of agonist-specific CTLs (Kaneko et al. 1997). When designing a peptide vaccine against a variable target like HCV, it has to be kept in mind that the use of a particular peptide always bears a risk of inducing antagonistic responses in the responding T cell repertoire.

In this study, we observed that the peptide induced CTL lines, if properly stimulated, were cytolytic against the HepG2 transfectant that naturally presents HCV peptides. The transfectant carried amino acid substitutions in some of the epitopes, but it was thereafter effectively killed by CTL lines which were stimulated by wild type peptides. It is not known, however, whether this observation is relevant to natural infections because the transfectant may express more HCV antigens than the liver tissue specimens obtained from chronic hepatitis patients. Under suboptimal conditions, variant peptides may act as antagonists, or they could show a poor presentation in the HLA class I molecules. To overcome these problems, T cells have to be stimulated fully, at least, at the local site of immunization. To provide a stimulatory environment for T cells, the following points should be considered: (1) Epitope peptides should be provided at high density to overcome poor responses of the T cell repertoire that may not have an optimal affinity. An exogenously added synthetic peptide has an advantage over a DNA vaccine in this regard. If a DNA vaccine is used, then, it has to be expressed at a high level and compete for MHC class I presentation with cellular

proteins. For a peptide vaccine, choosing peptides with an optimal affinity for HLA class I molecules is a critical step for achieving a high epitope density. The epitope search program presented herein is thus considered to be a powerful tool to help design peptides. Alternatively, in the future, it may be possible to intentionally avoid a high-affinity dominant epitope against which the T cell repertoire may be anergized. In such a case, it may be better to target subdominant epitopes of intermediate affinity. (2) Antigen-presenting cells at a local site of immunization should be activated fully to induce offensive responses against infected hepatocytes, which may not be an optimal antigen-presenting cell. (3) Helper T cells also need to be activated. In patients chronically exposed to HCV antigens, the immune system may have fallen into a state similar to self-tolerance against HCV (Grakoui et al. 2003; Semmo et al. 2005), or it could simply be exhausted due to the high load of viral replicates circulating in a body, i.e., approximately 10^{10} to 10^{12} newly synthesized virions per day (Grakoui et al. 2003). Helper T cell recruitment and their optimal activation thus remains an important issue to be resolved. Whether or not the helper epitopes need to be of HCV origin or they can be from different antigen sources also remains an important question.

Interestingly, viral changes have been reported to be extensive in the acute phase of an infection, but they appear to subside when the disease enters a chronic phase. In addition, the degree of the immune response against the infected hepatocytes seems to have some correlation with the mutation rate (Chang et al. 1997). In the chronic phase, it takes several months before escaped variants form a visible fraction in patients (Chang et al. 1997; Cox et al. 2005; Erickson et al. 2001; Kaneko et al. 1997). Evolutionary studies on viral changes in HCV also suggest that it takes several months before an amino acid change becomes stabilized in the viral population (Cox et al. 2005; Timm et al. 2004). Considering this time scale, we propose a cocktail vaccine containing a few epitope peptides. One amino acid change in an epitope may occur sooner or later, but the incidence would be much lower for a variant virus which happens to have amino acid changes in two epitopes simultaneously. If three peptides could be used for immunization, then the possibility of a variant virus carrying mutations in all three epitopes of developing would be negligible. If the effector CTLs could be recruited in a timely manner, then a chance to eradicate viruses would thus be obtained. The HLA-A*2402 prediction program is a powerful tool for designing multiple peptide vaccines. A more efficient viral eradication by a broad-ranging T cell repertoire, in contrast to the vulnerability of the T cell repertoire directed against a single major epitope to viral escape, has previously been demonstrated in infected patients (Tester et al. 2005).

In this study, we focused on the development of a HLA class I-binding peptide vaccine. However, such peptides alone would usually not be sufficient to induce CTLs. HCV-specific CD8 T cells can be abundantly found in patients especially at the early stage of an infection. Those CTLs become less cytotoxic along the course of chronic transition. A loss of helper T cell activities has been cited by several groups as a cause for the annihilation of the CTL activities (Day et al. 2003; Grakoui et al. 2003; Semmo et al. 2005; Tester et al. 2005; Thimme et al. 2002). In addition, the suppression of the CTL activities by a population of the $CD4^+CD25^+$ regulatory T cells has also been demonstrated (Boettler et al. 2005; Rushbrook et al. 2005; Sugimoto et al. 2003). A strategy is thus needed to overcome these problems. Helper peptides can be processed by APCs from exogenously added proteins. Therefore, the activation of APCs and helper T cells along with CD8 T cells in a more stimulatory environment than the chronically infected liver is thus considered to be a crucial point for developing a curative vaccine for HCV.

Acknowledgements This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (C) Genome Science from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a grant from Formation Program of Research Centers for emerging & reemerging infectious diseases -research & development of life science fields responding to the needs of society. We also would like to express our gratitude to the superb technical assistance of Ms. Satomi Yamanaka. We thank Dr. Yoshiharu Matsuura for providing the HCV 1b clone. We also thank Dr. Masafumi Takiguchi and Dr. Peter Cresswell for providing the cell lines. In addition, the recombinant human IL-2 was a kind gift from Dr. Shinsuke Taki.

References

Agnello V, Abel G, Knight G, Muchmore E (1998) Detection of widespread hepatocyte infection in chronic hepatitis C. Hepatology 28:573–584

Appay V, Nixon D, Donahoe S, Gillespie G, Dong T, King A, Ogg G, Spiegel H, Conlon C, Spina C, Havlir D, Richman D, Waters A, Easterbrook P, McMichael A, Rowland-Jones S (2000) HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. J Exp Med 192:63–75

Battergay M, Fikes J, Di-Bisceglie A, Wentworth P, Sette A, Celis E, Ching W, Grakoui A, Rice C, Kurokohchi K, Berzofsky J, Hoofnagle J, Feinstone S, Akatsuka T (1995) Patients with chronic hapatitis C have circulating cytotoxic T cells which recognize hepatitis C virus-encoded peptides binding to HLA-A2.1 molecules. J Virol 69:2462–2470

Boettler T, Spangenberg H, Neumann-Haefelin C, Panther E, Urbani S, Ferrari C, Blum H, von Weizsaecker F, Thimme R (2005) T cells with a CD4+CD25+ regulatory phenotype suppress in vitro proliferation of virus-specific CD8+ T cells during chronic hepatitis C virus infection. J Virol 79:7860–7867

Cerny A, McHutchinson J, Pasquinelli C, Brown M, Brothers M, Grabsheid B, Fowler P, Houghton M, Chisari F (1995) Cytotoxic T lymphocyte response to hepatitis C virus-derived peptides containing the HLA A2.1 binding motif. J Clin Invest 95:521–530