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The harmful effect of exercise on reducing taurine concentration in the tissues of rats treated with CCl_4 administration

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Background. We have previously reported that oral taurine administration reduced the frequency of painful muscle cramps in patients with liver cirrhosis, and that skeletal muscle taurine concentration was significantly decreased after exercise. The aim of this study was to examine taurine concentration in various tissues of a liver damaged with fibrosis (LD) in a rat model before and after exercise. ***Methods.*** Rats were divided into normal (NML) and LD groups. The LD group received CCl_4 injection for 10 weeks. Thereafter, both groups were divided into control (NML/CTL, LD/CTL) and exercise (NML/EX, LD/EX) groups, respectively. The rats in the EX groups were subjected to treadmill running. Plasma, liver, brain, heart, and skeletal muscle taurine concentration, as well as plasma and liver lipid peroxidase (LPO) concentration, were measured. ***Results.*** The liver, brain, and skeletal muscle taurine concentration in the LD groups was significantly decreased compared to that in the respective NML groups. Furthermore, the taurine concentration in the heart and skeletal muscles in the LD/CTL group was significantly decreased post exercise. The respective plasma and liver LPO concentration in the LD groups was significantly increased compared to that in the corresponding NML group. Moreover, plasma LPO concentration in the LD/EX group was significantly higher than in the LD/CTL group. ***Conclusions.*** Tissue taurine concentration, particularly in skeletal muscle, was significantly decreased in the LD model rats induced by CCl_4 administration, and furthermore, the significantly decreased concentration, except for liver, was aggravated by exercise, even though at lower intensity.

Key words: amino acid, skeletal muscle, running, carbon tetrachloride, oxidative stress

Introduction

Taurine, 2-amino ethylsulfonic acid, is the most abundant amino acid-like compound in most mammal tissues. The best established function of the taurine is its conjugation with bile acid in the liver. In addition, many biological and physiological functions of taurine have been reported in various tissues and species, including cellular membrane stabilizer,[1] osmoregulator,[2,3] and neurotransmitter.[4,5] Furthermore, it has also been reported that taurine influences skeletal muscle contractions[6,7] as the result of the increased uptake and storage capacity of Ca^{2+} in the sarcoplasmic reticulum vesicles,[8] the promotion of sarcolemmal polarization,[9] the stabilization of the intracellular membrane,[8] and the modulation of nerve excitement.[4]

We, as well as others, have reported that oral taurine administration reduced the frequency of painful skeletal muscle cramps in both hands and legs in patients with liver cirrhosis (LC).[10,11] Moreover, we have reported that the taurine concentration in rat skeletal muscles was significantly decreased after a transient exercise to exhaustion,[12] and that oral taurine administration to rats inhibited the decreased taurine concentration in skeletal muscles after exercise and enhanced exercise performance as a result of prolonging the required running time to exhaustion.[13] Therefore, these results suggest an important relationship between taurine concentration in tissues and respective tissue functions, particularly in the skeletal muscles, in liver disease and/or exercise conditions. Furthermore, taurine has been reported as an antioxidant and a free radical scavenger.[14–16]

Therefore, we proposed, in the present study, to examine the taurine concentration in several tissues including the liver and skeletal muscles as well as to investigate the plasma and liver lipid peroxidase (LPO) concentration as a marker of oxidative stress in the liver damaged with fibrosis (LD) rat model as in-

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duced by carbon tetrachloride (CCl_4) with and without exercise.

Materials and methods

Animal model and exercise protocol

Male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan), 6 weeks of age, were used in this study. After 1 week acclimatization, the rats were randomly divided into two groups: a normal (NML) group and an LD model group. The LD rats were administrated CCl_4 (Wako, Osaka, Japan) as follows: the LD rats received 0.05% phenobarbital (Wako) dissolved in the drinking water for 11 weeks. After the first week, CCl_4 solution (0.1 ml/100 g body weight) was subcutaneously injected into the rat mixed with an equal volume of olive oil twice a week for 10 weeks.[17] The rats in the NML group were maintained without administration of CCl_4 and phenobarbital. All rats were fed a standard diet (MF; Oriental Yeast, Tokyo, Japan). Two days after the last CCl_4 injection, the rats in both the NML and LD groups were divided into control and exercise groups, respectively, as follows: an NML control (NML/CTL) group (n = 7), an NML plus exercise (NML/EX) group (n = 7), an LD control (LD/CTL) group (n = 7), and an LD plus exercise (LD/EX) group (n = 7). The rats in the NML/EX and LD/EX groups were placed on a transient treadmill (KN-73; Nazme, Tokyo, Japan) running at 10 m/min for 90 min. The intensity and duration of the exercise were decided from our previously published data in normal rats[12] and preliminary data in CCl_4 administrated rats. All animals were kept at 21°–25°C under 12-h dark/light cycles, and received humane care in accordance with The Guidelines of the University of Tsukuba for the Care of Laboratory Animals.

Sampling

Two days after the last CCl_4 injection and immediately after the exercise, the rats were anaesthetized with pentobarbital, and blood, liver, brain, heart, and lower leg skeletal muscles were collected. Serum and plasma were obtained for biochemical assays. Skeletal muscles included the soleus, gastrocnemius (GC), and extensor digitorum longus (EDL). The reasons for collecting these muscles are as follows. (1) Skeletal muscle fibers can be classified as fast-twitch and slow-twitch fibers based on energy metabolic and fiber contractile characteristics.[18] Taurine content is higher in the slow-twitch fibers than in the fast-twitch fibers.[7] The skeletal muscles collected in the present study have different muscle fiber composition, i.e., the soleus muscle is slow-twitch fiber dominant type; GC muscle is slow- and fast-twitch fibers mixed type; EDL muscle is fast-twitch fiber dominant type.[19] (2) The three muscles in the lower leg are protagonistic muscles in running exercise. (3) The painful muscle cramps in patients with LC appear frequently in calf muscles. Each tissue was washed in ice-cold physiological saline after removal of the adipose, nerve, and connective tissues, weighed, frozen in liquid nitrogen, and kept at −80°C until the biochemical analyses were preformed.

Biochemical analyses

Serum levels of total bilirubin and albumin were determined as liver function parameters by the vanadic acid oxidation method and the BCG method,[20] respectively. Furthermore, serum levels of lactate and glucose were determined by the lactate oxidase pyruvate oxidase method[21] using a commercially available analysis kit (Determiner LA; Kyowa Medex, Tokyo, Japan) and an automatic analyzer (Hitachi-7170; Hitachi, Tokyo, Japan), and by Trinder's glucose oxidase method[22] using Glucose B-Test kit (Wako), respectively. Plasma and tissues taurine concentration was determined as previously described[12] and using an automatic amino acids analyzer (JLC-300V; JEOL, Tokyo, Japan).[12,17] Plasma and liver LPO concentration was determined as hydroperoxides utilizing a redox reaction with ferrous ions[23] using LPO assay kit (Cayman, Ann Arbor, MI, USA).

Statistical analysis

All data are presented as the mean ± SD. All values on the concentration in tissues are shown as per wet weight. Statistical differences among the groups were assessed between the NML and LD groups, as well as between the control and exercise groups. Significant differences between two groups and among multiple groups were determined by unpaired Student's t test, and one-way ANOVA post hoc Fischer's protected least squares difference (PLSD) analysis, respectively. Statistical significance was set at $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$. The statistical analyses were performed using Stat View (SAS Institute, Cary, NC, USA).

Results

Body weight progress, tissue wet weight, and blood biochemical analyses

Figure 1 shows rat body weight progress in the NML and LD groups during the period studied. From the first week after the start until the end of the CCl_4 injections, body weight gain in the LD group was significant lower than in the NML group. Associated with the significant decrease in body weight, all skeletal muscle wet weights in the LD group were significantly lower than in the

Table 1. Tissue wet weights and ratio per body weight and blood biochemical data

Wet weight (tissue/body)		NML groups	LD groups
Liver	g	17.0 ± 1.3	20.3 ± 4.6*
	(mg/g)	(41.18 ± 10.30)	(54.49 ± 13.2)**
Brain	mg	1192.9 ± 53.5	1157.6 ± 101.1
	(mg/g)	(2.89 ± 0.68)	(3.11 ± 3.11)
Heart	mg	1102.8 ± 90.6	1115.2 ± 146.4
	(mg/g)	(2.69 ± 0.81)	(3.00 ± 0.46)
Soleus	mg	178.7 ± 12.7	153.1 ± 21.0**
	(mg/g)	(0.43 ± 0.12)	(0.41 ± 0.07)
GC	mg	2415.3 ± 219.1	1910.4 ± 431.9**
	(mg/g)	(5.81 ± 1.28)	(5.15 ± 1.31)
EDL	mg	222.3 ± 17.2	191.9 ± 33.4**
	(mg/g)	(0.54 ± 0.13)	(0.52 ± 0.10)
Serum biochemical data			
Bilirubin	mg/dl	0.04 ± 0.02	0.28 ± 0.28**
Albumin	g/dl	3.85 ± 0.51	2.86 ± 0.75***

Values are mean ± SD; the values in parentheses show the ratio of tissue wet weight per total body weight
NML, the normal model group; LD, the liver damaged with fibrosis model group; GC, gastrocnemius muscle; EDL, extensor degitorum longus muscle; bilirubin, total bilirubin
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by unpaired Student's t test

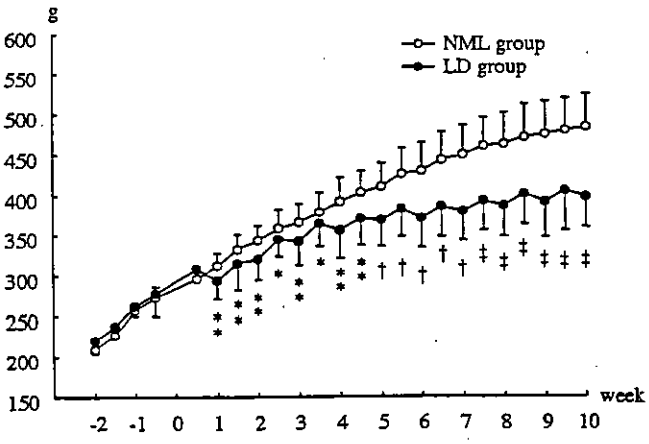

Fig. 1. Total body weight in the NML and the LD groups over the 12-week period. The rats in the LD group were administrated 50% CCl_4 for 10 weeks. Values are expressed as the mean ± SD. *$P \leq 0.05$, **$P \leq 0.01$, †$P \leq 0.001$, ‡$P < 0.0001$ compared to the NML group using unpaired Student's t test. *NML*, the normal model group; *LD*, the liver damaged with fibrosis model group

NML group (Table 1). However, there was no significant difference in the relative ratio of tissue wet weight per body weight for all the skeletal muscles. In contrast, both the liver wet weight alone and the ratio of tissue to body weight were significantly higher in the LD group than in the NML group. Serum albumin and total bilirubin levels in the LD group were significantly decreased and increased compared with those in the NML group, respectively.

Serum lactate, glucose levels, and LPO concentrations in plasma and liver

The serum lactate level in the LD groups was significantly increased compared to that in the respective NML groups, whereas there was no significant difference between the respective CTL and EX groups (Table 2). The serum glucose level in the NML/EX and LD/CTL groups was significantly decreased compared to that in the NML/CTL group and in the LD/EX group compared to that in the NML/EX group.

In the EX of NML and LD groups, the plasma LPO concentration was significantly increased compared to that in the CTL group (Table 2). Moreover, the plasma and liver LPO concentration in the LD groups was significantly increased compared to that in the corresponding NML groups. However, in the liver, there was no significant difference between the CTL and EX groups in both the NML and LD groups.

Plasma and tissue taurine concentrations

Figure 2 illustrates the taurine concentration in plasma and tissues. There was no significant difference in the plasma taurine concentration among the four groups. The liver taurine concentration in the NML/EX group was significantly increased compared to that in the NML/CTL group, whereas there was no significant difference between the LD groups. In the LD groups, the concentration was significantly decreased between the corresponding CTL and EX groups in the NML group. In the brain, there was no significant difference between

Table 2. Lactate and glucose in serum, and lipid hydroperoxide concentration in plasma and liver

	NML/CTL	NML/EX	*a*	LD/CTL	*b*	LD/EX	*a*	*b*	ANOVA
Lactate (mg/dl)	17.8 ± 4.6	22.8 ± 9.2		33.4 ± 10.6	*	37.6 ± 23.5		*	<0.0001
Glucose (mg/dl)	263.7 ± 36.2	176.1 ± 14.8	***	155.4 ± 20.1	****	130.0 ± 24.2		***	0.0343
LPO									
Plasma (nmol/ml)	2.1 ± 1.3	4.3 ± 2.4	**	6.2 ± 1.5	**	8.5 ± 2.0	*	**	0.0343
Liver (nmol/mg)	50.5 ± 12.8	76.6 ± 28.5		205.5 ± 65.8	**	243.3 ± 87.9		**	<0.0001

a and *b* show the significant difference to the corresponding CTL group and to the corresponding respective NML group, respectively
LPO, lipid peroxidate. Data are expressed as the mean ± SD; ANOVA shows one-way ANOVA; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ using one-way ANOVA post hoc test (Fisher's PLSD test)

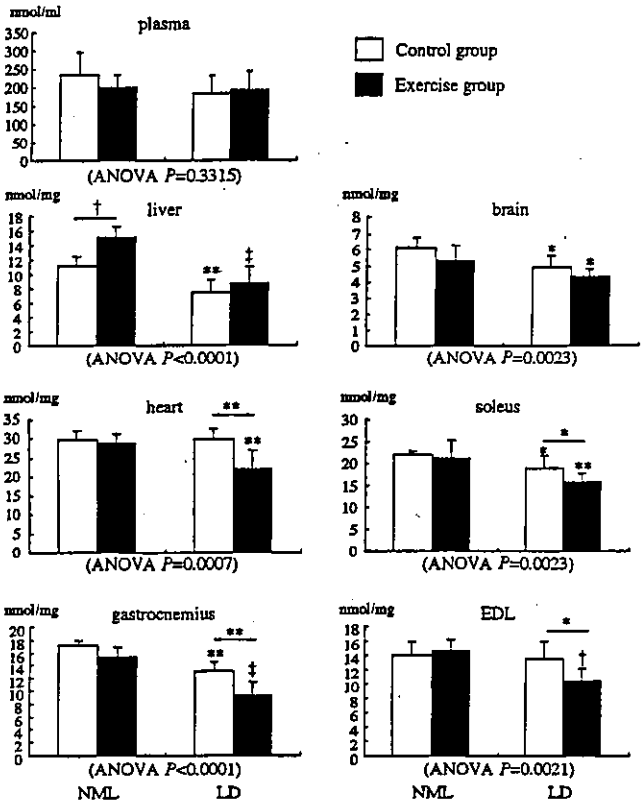

Fig. 2. Taurine concentration in plasma and tissues. Data are expressed as the mean ± SD. ANOVA shows one-way ANOVA *P* value. The marks without bar on the column of the LD group show comparison to the corresponding respective group in the NML group. $*P < 0.05$, $**P < 0.01$, $\dagger P < 0.001$, $\ddagger P < 0.0001$ using one-way ANOVA post hoc test (Fisher's PLSD test). *EDL*, extensor digitorum longus muscle

the CTL and EX groups in both the NML and LD groups. However, the brain taurine concentration in the LD groups was significantly decreased compared to that in the corresponding NML groups. The heart and EDL muscle taurine concentration in the LD/EX group was significantly decreased compared to that in the NML/EX and LD/CTL groups. The soleus and GC muscles taurine concentration in the LD groups was significantly decreased between the corresponding CTL and EX groups in the NML group. Furthermore, taurine concentration in these muscles in the LD/EX group was significantly decreased compared to that in the LD/CTL group.

Discussion

Significant decrease of taurine concentration in the skeletal muscles was shown in the CCl_4-induced LD model rats compared to that in the normal rats. The data in patients with LC published by Montanari et al.[24] are supportive of the results from the present study. Therefore, the taurine concentration in skeletal muscle decreases in both human and rat under liver disease conditions including LC and liver damage induced by CCl_4. Moreover, these results in the present study emphasized further the hypothesis that there might be a close relationship between incidence of muscle cramps in patients with LC and decreased skeletal muscle taurine concentration.

Interestingly, there was no significant difference in heart taurine concentration between the NML and LD groups without exercise, and heart taurine concentration remained higher than in other tissues in both groups. It is well known that cellular taurine uptake requires a specific taurine transporter, which is expressed in most tissues.[25–27] One of the possible explanations for the different tissue taurine concentration might be associated with possible differences in tissue expression or activity of the transporter, i.e., between skeletal and cardiac muscles in LD, as both are striated muscles.

Skeletal muscle taurine concentration was unchanged in the normal rats before and after exercise, whereas our previous study reported that the skeletal muscle taurine concentration was gradually and significantly decreased depending on exercise duration.[12] The discrepancy in the results between the previous and present studies could be explained by the difference in exercise intensity. The running speed (10 m/min) in the present study was slower than in our previous study (25 m/min), and consequently there was no significant

difference in the serum lactate level in the present study between the CTL and EX in the NML groups. However, in the LD group, there was a significantly decreased taurine concentration in the skeletal muscles due to exercise. Furthermore, the heart taurine concentration in the LD/EX group was significantly decreased compared to the NML/EX group. Therefore, the lower exercise intensity in the LD group produces similar metabolic alteration as those observed under the high exercise intensity in the NML group, as far as the alteration of taurine concentration in contractile muscle tissues, i.e., skeletal and cardiac muscles, are concerned.

Liver taurine concentration in both the NML and LD groups was increased after exercise, and this was significant in the NML group. As far as we know, this study is the first to show a change in liver taurine concentration after exercise. Latour et al. have reported that hepatocyte volume was significantly decreased during exercise.[28] Therefore, it can be suggested that taurine is needed during exercise to maintain hepatocyte volume as a function of osmoregulation. Furthermore, it has also been reported that taurine is transported into the blood cells for osmoregulation during endurance exercise.[29] However, several studies have reported that liver perfusion decreases markedly during exercise in the normal state.[30,31] Therefore, it is possible that the conjugation ability of taurine to bile acid might be decreased during exercise due to decreased blood perfusion through the liver tissue. However, the change in liver taurine concentration during exercise will require further investigation.

A number of studies have reported the alteration of plasma taurine concentration in human and rat after exercise. In humans, Cuisinier et al. have reported that plasma taurine concentration was increased after a marathon race, and suggested that the increased plasma taurine concentration might be due to a leakage from skeletal muscle because there was a significant positive relationship between plasma taurine concentration and plasma creatine kinase activity.[32] However, in the present study, there was no significant alteration in plasma taurine concentration after exercise or in the LD state. Similarly, we have also reported that plasma taurine concentration was unchanged in normal rats after a transient exercise.[12] Dietrich et al. have reported that plasma taurine concentration in patients with compensated chronic liver disease was significant lower than in normal subjects, and furthermore the plasma concentration in the patients was unchanged, while in the normal subjects it was significantly decreased, by exercise.[33] Another study has also shown a significant decreased taurine concentration in skeletal muscle while remaining unchanged in the plasma in patients with LC.[24] The results from various studies in rat models support the unchanged plasma taurine concentration in LD and LD plus exercise conditions in the present study. However, there might be some differences regarding the alteration of plasma taurine concentration in exercise or liver disease conditions between the human patients and experimental animal models.

In the present study, the LD model rats were induced by chronic CCl_4 administration. The administrated CCl_4 injures the hepatocytes as the result of conversion to trichloromethyl radicals, and consequently LPO production is enhanced in plasma and tissues.[34,35] Furthermore, it is well known that exercise can also cause oxidative stress,[36–38] and the oxidative stress leads to some muscle fiber damage. In the present study, the increased LPO concentration in plasma and liver was confirmed in the LD and exercise conditions. It has been reported that taurine supplementation to rats decreased liver oxidative stress, and consequently led to some protective effects in LC induced by thioacetamide,[39] in hepatocyte injury by either CCl_4,[14,40,41] galactosamine,[42] or nitric oxide,[43] and in liver LPO production induced by CCl_4.[44,45] Dawson et al.[46] as well as we[13] have reported that the taurine supplement facilitated exercise performance in experimental rats by enhancing the skeletal muscle taurine concentration. Furthermore, Dawson et al. have also shown that the taurine has a cytoprotective role for oxidative stress in skeletal muscles under exercise.[46] Therefore, we suggested that taurine supplementation might improve liver function and reduce oxidative stress, and consequently enhance physical ability in liver disease.

In conclusion, taurine concentration in various tissues but not in plasma was decreased in the liver-damaged condition and the decreased concentration in those tissues, except for liver, particularly in skeletal muscle, was aggravated post exercise even though exercise intensity was low.

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Identification of novel hepatitis C virus-specific cytotoxic T lymphocyte epitopes by ELISpot assay using peptides with human leukocyte antigen-A*2402-binding motifs

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The human leukocyte antigen (HLA)-A*2402 is common in Asians. The authors attempted to identify epitopes for HLA-A*2402-restricted, hepatitis C virus (HCV)-specific $CD8^+$ T cells by an enzyme-linked immunospot (ELISpot) assay using peripheral blood $CD8^+$ T cells from HLA-A*2402-positive hepatitis C patients and synthetic HCV peptides based on HLA-A*2402-binding motifs and the amino acid sequence of type 1b HCV. Ten novel epitopes were identified in five of seven HLA-A*2402-positive patients with acute or short-term chronic HCV infection (<3 years), but in none of four with longer-term chronic infection (>10 years). Only one of the ten epitopes proved to be definitely HLA-A*2402-restricted. Another epitope was identified in one of two HLA-A*2402-negative acute hepatitis C patients. In two of the six patients with positive $CD8^+$ T cell responses, the targeted epitopes were multiple. The same epitope was targeted in two patients. When patients with unresolved acute HCV infection were treated with alpha interferon, peripheral blood HCV-specific $CD8^+$ T cells decreased with resolution of the hepatitis. In conclusion, $CD8^+$ T cell responses to HCV infection are heterogeneous. One definite HLA-A*2402-restricted and ten probably non-HLA-A*2402-restricted epitopes were identified. Patients with short-term HCV infection are suitable for searching for novel HCV epitopes, but peripheral blood HCV-specific $CD8^+$ T cells decrease markedly after loss of antigenic stimulation.

INTRODUCTION

$CD8^+$ cytotoxic T lymphocytes (CTLs) are thought to play an important role in elimination of hepatitis C virus (HCV), while also contributing to pathogenesis in this infection (Chisari, 1997; Cerny & Chisari, 1999; Rehermann & Chisari, 2000). Virus-specific CTLs recognize viral antigens on the infected cells in a human leukocyte antigen (HLA) class I molecule-restricted manner, then lysing the cells (Gotch *et al.*, 1987; Cannon *et al.*, 1988). Although reports concerning HCV-specific CTL epitopes are accumulating (Ward *et al.*, 2002), which epitopes are most active in viral clearance or immunopathogenesis *in vivo* remains to be determined. In HLA-B44-positive patients with chronic HCV infection, we previously demonstrated an inverse relationship between virus loads and peripheral blood CTL activities specific for HCV nucleoprotein amino acid residues 88–96 (Hiroishi *et al.*, 1997). Accordingly, the identification of new epitopes recognized by HCV-specific CTLs that play a role in elimination of HCV may suggest new ways to suppress growth of HCV and prevent persistent infection. A universally immunogenic vaccine against HCV infection would require multiple epitopes, preferably from conserved regions of HCV, with recognition by CTLs that is restricted by HLA molecules occurring commonly in the population to be immunized.

HLA-A24 is one of the most common HLA-A antigens in Asians (Chandanayingyong, 1986), occurring in more than 60% of Japanese (Date *et al.*, 1996). To study the immunopathogenesis of HCV infection in Japanese and other Asians and to develop HCV-specific CTL vaccines for these populations, identification of HCV-specific CTL epitopes with recognition restricted by HLA-A24 is therefore important. An HLA-A24 allele in Japanese people is almost exclusively HLA-A*2402 (Date *et al.*, 1996). Yet few HLA-A*2402-restricted, HCV-specific CTL epitopes have been reported, in contrast to more numerous reports concerning HLA-A2.1-restricted, HCV-specific CTL epitopes (Ward *et al.*, 2002), representing the HLA most common in Caucasians.

A recently reported enzyme-linked immunospot (ELISpot) assay provides a rapid, inexpensive and efficient way to define a given HLA class I molecule-restricted, novel virus-specific $CD8^+$ T cell epitope and to characterize the breadth of CTL responses (Altfeld *et al.*, 2000). To identify HLA-A*2402-restricted, HCV-specific $CD8^+$ T cell epitopes, we synthesized 87 peptides derived from the total protein content of HCV and carrying HLA-A*2402-binding motifs (Ibe *et al.*, 1996) and assessed the ability of the peptides to stimulate $CD8^+$ T cells by counting interferon (IFN)-γ-releasing cells in HLA-A*2402-positive patients with acute or chronic hepatitis C using the ELISpot assay. Using the epitopes identified, we then studied the effects of treatment with IFN-α on frequencies of HCV-specific $CD8^+$ T cells in two HLA-A*2402-positive patients with unresolved acute hepatitis C.

METHODS

Subjects. Three HLA-A*2402-positive patients with acute hepatitis C and eight with chronic hepatitis C, as well as two HLA-A*2402-negative patients with acute hepatitis C, were studied (Table 1). As controls, one patient with acute hepatitis B, one with fatty liver and three healthy subjects with HLA-A24 were also studied. All hepatitis C patients had detectable HCV RNA in serum and had elevated serum concentrations of alanine aminotransferase (ALT). Diagnosis of chronic hepatitis was based on continuous elevation of serum ALT for more than 6 months. Diagnosis of acute hepatitis was based on acute clinical onset of hepatitis and confirmation of previously negative anti-HCV antibody. The study was approved by the Ethical Review Committees of Jichi Medical School and the Institute of Clinical Medicine of the University of Tsukuba. Informed consent was obtained from all subjects.

Synthetic peptide library. We synthesized 87 peptides of 8–11 amino acids in length that carried HLA-A*2402-binding motifs (tyrosine or phenylalanine at position 2 and leucine, isoleucine, phenylalanine or tryptophan at the C terminus; Ibe *et al.*, 1996). The peptides were based on the amino acid sequence of the genotype 1b HCV-J strain (accession no. D90208). Peptides were synthesized by and purchased from Mimotopes and were more than 80% pure according to high-performance liquid chromatography. The peptides were grouped into 17 mixtures of five or six peptides each for experimental convenience (Mixtures A–Q; Table 2). Two previously reported HLA-A*2402-restricted CTL epitopes, HCV NS3 amino acid residues 1031–1039 (Kurokohchi *et al.*, 2001) and 1100–1108 (Ito *et al.*, 2001) were contained in mixture E.

Isolation of $CD8^+$ T cells and monocytes. Peripheral blood mononuclear cells (PBMC) were separated from heparinized peripheral blood by gradient centrifugation using Ficoll-Paque (Amersham Pharmacia Biotech). $CD8^+$ T cells were isolated from PBMC by positive selection using antibody-conjugated magnetic

Table 1. Characteristics of the subjects studied

Subject	Phase	Aetiology†	Duration	HLA (serotype or genotype)
1	Acute	HCV-1	3 months	A*2402, A*1101, B*5201, B*3902, Cw*0702, Cw*1202
2	Acute	HCV-1	5 months	A*2402, A*2601, B*5401, B*4006, Cw*0801, Cw*0803
3	Acute	HCV-2	5 months	A*2402, B51, B52
4	Chronic	HCV-1	1 year	A*2402, A*2601, B*3501, B*4002, Cw*0303, Cw*0304
5	Chronic	HCV-2	1 year	A*2402, A*2601, B7, B59, Cw1, Cw7
6	Chronic	HCV-2	2 years	A*2402, A*0206, B*5201, B*5901, Cw*0102, Cw*1202
7	Chronic	HCV-2	2·5 years	A*2402, A*3101, B*4801, B*5101, Cw*0304, Cw*0801
8	Chronic	HCV-1	>10 years	A*2402, A2, B7, B39, Cw7
9	Chronic	HCV-1	>15 years	A*2402, B54, B61, Cw1, Cw3
10	Chronic	HCV-1	>15 years	A*2402, A31, B60, Cw3
11	Chronic	HCV-1	>20 years	A*2402, A2, B7, B61, Cw3, Cw7
12	Acute	HCV-1	2 months	A*2601, A*3101, B*3501, B*5101, Cw*0303, Cw*1402
13	Acute	HCV-1	4 months	A*0201, A*0206, B52, B46, Cw1
14	Acute	HBV	5 months	A24, A31, B56, B61, Cw3, Cw4
15	Chronic	Fatty liver	>5 years	A24, A26, B54, B61, Cw1, Cw3
16	Healthy			A24, A11, B48, B55, Cw1, Cw3
17	Healthy			A24, A26, B60, B61, Cw7, Cw8
18	Healthy			A24, A33, B61, Cw3

†HCV-1 or -2 indicates the HCV genotype of infected individuals.

Table 2. Peptide library for the ELISpot assay

Mixture	HCV peptides
A	E1 213–220, NS3 1541–1548, NS5A 2153–2160, NS5A 2384–2391, NS5B 2594–2601
B	Core 85–93, Core 129–137, Core 173–181, E1 234–242, E1 360–368
C	E2 464–472, E2 488–496, E2 717–725, E2 770–778, E2 790–798
D	NS2 834–842, NS2 837–845, NS2 885–893, NS2 910–918, NS2 975–983
E	NS2 1017–1025, NS3 1031–1039, NS3 1100–1108, NS3 1267–1275, NS3 1292–1300
F	NS3 1374–1382, NS4B 1716–1724, NS4B 1727–1735, NS4B 1767–1775, NS4B 1773–1781
G	NS5A 2132–2140, NS5A 2280–2288, NS5B 2432–2440, NS5B 2635–2643, NS5B 2870–2878
H	Core 135–144, Core 176–185, Core/E1 191–200, E1 284–293, E2 630–639
I	E2 717–726, E2 767–776, NS2 822–831, NS2 910–919, NS2 932–941
J	NS2 947–956, NS3 1031–1040, NS3 1081–1090, NS3 1243–1252, NS3 1443–1452
K	NS3 1463–1472, NS3 1556–1565, NS4B 1759–1768, NS4B 1792–1801, NS4B 1854–1863, NS5A 1990–1999
L	NS5A 2121–2130, NS5A 2132–2141, NS5A 2146–2155, NS5A 2292–2301, NS5B 2521–2530, NS5B 2944–2953
M	Core 34–44, E1 275–285, E2 593–603, E2 611–621, E2 616–626
N	E2 787–797, E2 790–800, NS2 833–843, NS2 847–857, NS3 1130–1140
O	NS3 1158–1168, NS3 1375–1385, NS3 1416–1426, NS3 1520–1530, NS3 1625–1635
P	NS4A 1672–1682, NS5A 2013–2023, NS5A 2089–2099, NS5B 2456–2466, NS5B 2613–2623
Q	NS5B 2694–2704, NS5B 2801–2811, NS5B 2833–2843, NS5B 2866–2876, NS5B 2973–2983

beads according to the manufacturer's instructions (Dynal). Beads were detached from the isolated cells using the DetachaBead system (Dynal). The yield of $CD8^+$ T cells was 5–20% of PBMC. Monocytes were isolated from the $CD8^+$ T cell-depleted PBMC by negative selection using a monocyte negative isolation kit (Dynal). Use of isolated $CD8^+$ T cells as effector cells and monocytes as antigen-presenting cells in an ELISpot assay reduced the number of non-specific spots by removal of IFN-γ-secreting $CD4^+$ T cells and natural killer cells and also increased the sensitivity. Purities of isolated $CD8^+$ T cells and monocytes on flow cytometry were >95% and >80%, respectively.

ELISpot assay. Analysis of anti-peptide immune responses of peripheral blood $CD8^+$ T cells was performed using an IFN-γ-based ELISpot assay kit (Mabtech). Briefly, 10^5 $CD8^+$ T cells, 10^4 monocytes as antigen-presenting cells and a peptide mixture or individual peptides at 10 μg ml^{-1} each were placed in duplicate in 96-well plates with a PVDF membrane at the bottom (MAIP S45; Millipore). Well bottoms were coated with anti-IFN-γ monoclonal antibody (mAb). Cells were cultured for 40 h at 37 °C in a humidified 5% CO_2 atmosphere. No peptide was added to the negative control wells. After culture, IFN-γ spot-forming cells (SFCs) were visualized as described previously (Lalvani *et al.*, 1997). Responses were considered significant when a minimum of five SFCs were present per well, representing at least twice the number of SFCs in negative control wells. In preliminary studies, monocytes did not present IFN-γ SFCs in response to stimulation with HCV peptides.

To enrich peptide-specific $CD8^+$ T cells that might recognize a known HLA-A*2402-restricted epitope, 2×10^6 PBMC pulsed with 10 μg peptide 1031–1039 ml^{-1} (Kurokohchi *et al.*, 2001) were cultured in a 24-well flat-bottom plate for 9 days in RPMI 1640 medium supplemented with 10% human serum AB blood type and 50 U recombinant human interleukin (rhIL)-2 ml^{-1} added on day 2. The cells were harvested after 9 days of culture and $CD8^+$ T cells were isolated for an ELISpot assay.

Generation of HCV-specific CTLs. HCV-specific CTLs were generated as described previously (Hiroishi *et al.*, 2002). Briefly, PBMC were suspended at a cell density of 10^6 cells ml^{-1} in RPMI 1640 medium supplemented with 10% human AB serum and a single peptide was added on day 0. Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere. On day 2, rhIL-2 was added at a final concentration of 20 U ml^{-1}. On day 7, the culture was re-stimulated with the single peptide and irradiated autologous PBMC. Cytotoxic activity of peptide-induced effector cells was assessed on days 14–16.

CTL assay. The cytotoxic activity of peptide-induced effector cells was assessed using a standard 4 h sodium chromate (^{51}Cr) release assay. Briefly, Epstein–Barr virus-transformed B-lymphoblastoid cell lines (B-LCL) were labelled with 100 μCi (3·7 MBq) ^{51}Cr. The ^{51}Cr-labelled B-LCL were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum and incubated overnight at 37 °C with a synthetic peptide or infected with recombinant vaccinia virus (rVV) that endogenously expressed HCV antigens (K. Funatsuki & H. Ishiko, unpublished results). An m.o.i. of 5 was used for an 18 h incubation with rVV. Then, after incubating the effector cells with the target cells for 4 h at 37 °C in a humidified 5% CO_2 atmosphere, supernatants were collected and radioactivity was measured with a gamma counter.

RESULTS

Screening of $CD8^+$ T cell epitopes by an ELISpot assay using the peptide mixtures

When the isolated $CD8^+$ T cells from 13 patients with HCV infection (subjects 1–13) were stimulated with peptide mixtures A–Q composed of HCV peptides with HLA-A*2402-binding motifs, eight of the 17 peptide mixtures elicited significant IFN-γ SFC responses (Table 3). Mixtures C and J each elicited responses from two patients' cells, but the other peptide mixtures each elicited responses in only one patient's cells. IFN-γ SFC responses to the peptide mixtures were observed in cells from two of three HLA-A*2402-positive patients with acute hepatitis C, three of four with chronic hepatitis C lasting for less than 3 years and none of four with chronic hepatitis C for more than 10 years. In two of the five patients who demonstrated a

Table 3. Number of IFN-γ SFCs per 10^5 $CD8^+$ T cells in response to stimulation with the individual peptide mixtures A–Q

Numbers in bold indicate positive IFN-γ SFC responses.

Subject	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	0	2	0	2	2	1	3	0	0	0	2	0	1	3	**94**	0	2
2	0	1	**56**	1	0	0	**13**	**9**	2	2	**51**	2	1	1	1	1	1
3	0	0	1	2	0	1	1	2	2	1	2	0	0	2	1	2	0
4	0	0	1	0	0	0	0	0	8	**49**	0	0	0	0	0	0	1
5	0	0	0	0	0	0	1	0	1	0	1	0	2	0	1	0	1
6	0	1	0	0	2	0	1	0	0	**44**	1	0	0	1	2	0	0
7	1	1	**7**	1	1	0	1	0	1	1	1	0	1	0	0	1	0
8	0	0	1	0	1	0	1	2	0	1	1	0	1	0	0	0	1
9	1	0	1	1	0	1	1	0	0	1	0	0	0	1	1	0	0
10	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1
11	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0
12	1	1	2	2	0	1	0	1	1	2	0	0	0	2	0	**79**	1
13	1	1	1	1	1	0	0	1	0	1	0	0	1	1	0	0	1
14	1	0	1	0	0	0	1	0	0	0	1	1	0	0	0	0	0
15	0	0	1	0	0	0	1	1	1	0	0	0	0	0	1	0	0
16	0	0	2	1	0	0	0	1	0	0	1	0	1	0	1	0	0
17	0	1	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0
18	0	1	0	1	1	0	1	0	0	1	0	0	0	1	0	1	1

positive IFN-γ SFC response to peptide mixtures, a response was observed against more than one peptide mixture. Although peptide mixture E contained two previously reported HLA-A*2402-restricted CTL epitopes, it did not elicit an IFN-γ SFC response from any patient's cells. Unexpectedly, one of two HLA-A*2402-negative patients with acute hepatitis C also demonstrated an IFN-γ SFC response to one of the peptide mixtures. None of the control subjects (subjects 14–18) demonstrated a positive IFN-γ SFC response to any peptide mixture.

Peptide specificity of HCV-specific $CD8^+$ T cells

To identify the peptides that elicited IFN-γ SFC responses, we assessed responses using isolated $CD8^+$ T cells and the individual peptides making up the peptide mixture that had elicited the initial response. The following 11 peptides that elicited a significant IFN-γ SFC response were identified: peptide 1375–1385 from mixture O in patient 1; peptide 790–798 from mixture C, peptide 2280–2288 from mixture G, peptide 284–293 from mixture H and peptides 1759–1768 and 1990–1999 from mixture K in patient 2; peptide 910–919 from mixture I and peptides 947–956 and 1243–1252 from mixture J in patient 4; peptide 1443–1452 from mixture J in patient 6; peptide 790–798 from mixture C in patient 7; peptide 2456–2466 from mixture P in patient 12 (data not shown). Peptide 790–798 elicited a response in both patients 2 and 7 who shared HLA-A*2402 and HLA-Cw*0801 molecules.

The magnitude of IFN-γ SFC responses to single-peptide stimulation ranged from 4 to 139 SFC per 10^5 $CD8^+$ T cells and summed frequencies of the SFCs in patients 2 and 4 were 146 and 193 SFC per 10^5 $CD8^+$ T cells, respectively. Of the 11 peptides, two were 9-mers, seven were 10-mers and two were 11-mers. These epitopes were distributed throughout the entire HCV protein; one epitope was localized in each of the E1 and E2 regions, two in the NS2 region, three in the NS3 region, two in the NS4 region and three in the NS5 region. None of the 11 epitopes had been reported previously.

The individual peptides previously reported as HLA-A*2402-restricted, HCV-specific CTL epitopes did not elicit an *ex vivo* IFN-γ SFC response in cells from any patient studied. The assay was repeated after $CD8^+$ T cells were expanded by stimulating $CD8^+$ T cells from patients 1 and 4 with the known HLA-A*2402-restricted, HCV-specific CTL epitope peptide 1031–1039 (Kurokohchi *et al.*, 2001) for 9 days in the presence of rhIL-2 to enrich $CD8^+$ T cells with specificity for the peptide. Although stimulation of the $CD8^+$ T cells with the epitope peptides identified in the present study enriched peptide-specific $CD8^+$ T cells, peptide 1031–1039-specific $CD8^+$ T cells could not be enriched to attain a detectable level (Fig. 1).

HLA restriction of peptide recognition

Using peptide–HLA-A*2402 dimer staining (Greten *et al.*, 1998), we examined whether recognition by $CD8^+$ T cells of the epitope peptides identified was truly restricted by the HLA-A*2402 molecule. Although all 11 peptides effectively bound to HLA-A*2402 dimer proteins, only HCV NS5A peptide 2280–2288–HLA-A*2402 dimer complexes could stain $CD8^+$ T cells of patient 2, indicating that recognition by $CD8^+$ T cells of the peptides other than the

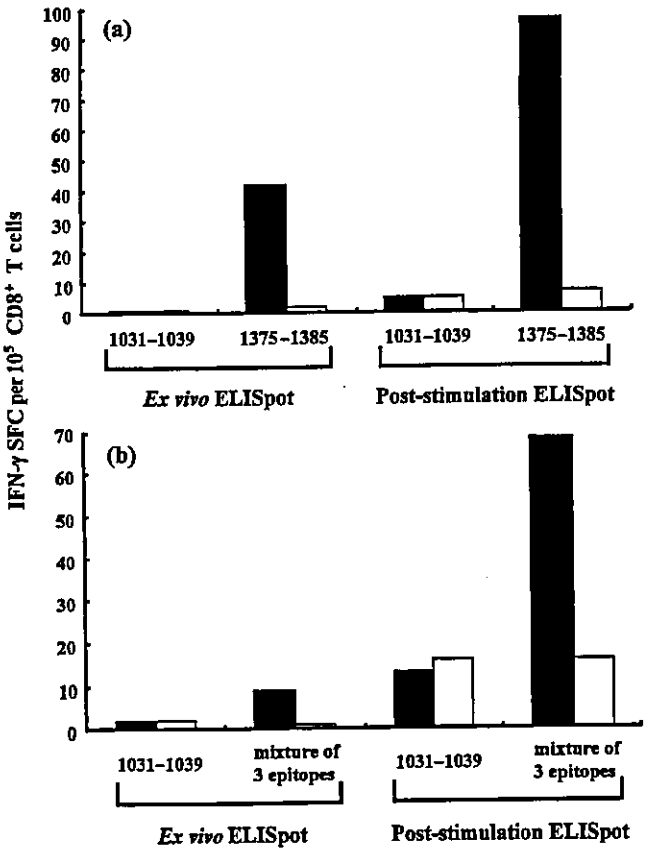

Fig. 1. Frequencies of IFN-γ SFCs in $CD8^+$ T cells assessed by either an *ex vivo* or a post-stimulation ELISpot assay in patients 1 (a) and 4 (b). In a post-stimulation assay, PBMC obtained from patient 1 at 5 months from onset of acute hepatitis and patient 4 at 5 months after the completion of 24 weeks of IFN-α therapy were stimulated with a known HLA-A*2402-restricted epitope, peptide 1031–1039 and cultured in the presence of 50 U rhIL-2 ml^{-1} for 9 days preceding an ELISpot assay. The newly identified epitope peptides (peptide 1375–1385 for patient 1 and a mixture of peptides 910–919, 947–956 and 1243–1252 for patient 4) were used as positive control peptides. Solid bars, Peptide-stimulated SFCs; open bars, non-peptide-stimulated SFCs.

peptide 2280–2288 was probably restricted by HLA class I molecules other than HLA-A*2402 (H. Morita & M. Imawari, unpublished results).

To define the HLA molecules that restricted recognition of peptides by $CD8^+$ T cells other than peptide 2280–2288, we attempted to induce CTL lines by stimulating PBMC from patients with the individual peptides. We could generate CTLs specific for peptides 910–919, 947–956 and 1243–1252 from PBMC of patient 4 and CTLs specific for peptide 1443–1452 from PBMC of patient 6. The peptide-induced CTLs lysed not only peptide-pulsed autologous B-LCL but also B-LCL that had been infected with rVV, resulting in HCV protein expression including the peptide sequence in infected cells (data not shown). HLA restriction of peptide recognition by CTLs was studied using a panel of autologous and allogeneic B-LCL with known HLA haplotypes as target cells. CTLs induced by peptide 910–919 or 1243–1252 selectively lysed B-LCL expressing HLA-Cw3 (HLA-Cw*0303 or HLA-Cw*0304 or both) that had been pulsed with the individual peptides (Fig. 2a and b), indicating that recognition of both HCV NS2 peptide 910–919 and NS3 peptide 1243–1252 was restricted by HLA-Cw*0303 and HLA-Cw*0304 molecules. However, since the NS3 peptide 1243–1252-specific CTL lysis restricted by HLA-Cw*0303 and HLA-Cw*0304 was less than the total lysis for the peptide, the recognition of the peptide also might be restricted by HLA-B*4002 (Fig. 2b). CTL induced by peptide 947–956 selectively lysed peptide-pulsed B-LCL expressing HLA-B61 (HLA-B*4002 or HLA-B*4006; Fig. 2c) indicating that recognition of HCV NS2 peptide 947–956 was restricted by HLA-B*4002 and HLA-B*4006 molecules. CTL induced by peptide 1443–1452 selectively lysed peptide-pulsed B-LCL expressing HLA-A*0206 (Fig. 2d), indicating that the recognition of the peptide 1443–1452 was restricted by an HLA-A*0206 molecule, although the possibility that the CTLs also could recognize the targets in an HLA-A*0207 molecule-restricted manner cannot be ruled out from the data in Fig. 2(d). CTLs induced by peptide 1443–1452 did not lyse peptide-pulsed B-LCL expressing HLA-A*0201 (data not shown).

Although we could not establish peptide-specific CTL lines, HCV E2 protein peptide 790–798 induced an IFN-γ SFC response in cells from patients 2 and 7, who shared HLA class I alleles HLA-A*2402 and HLA-Cw*0801. Since peptide–HLA-A*2402 dimer complexes did not stain PBMC from patient 2 or 7 (H. Morita & M. Imawari, unpublished results), recognition of peptide 790–798 by $CD8^+$ T cells is likely to be restricted by an HLA-Cw*0801 molecule. However, the possibility that HLA-A*2402 restricted the recognition of peptide 790–798 cannot be ruled out completely, since the dimer might not work efficiently.

Recognition of truncated and overlapping HCV peptides by $CD8^+$ T cells

To define further the epitopes within the peptides that elicited an IFN-γ SFC response, truncated and overlapping peptides were synthesized and assayed for their ability to elicit a response from $CD8^+$ T cells obtained from patients 4, 6 and 7 (Table 4).

Peptide 1443–1452 truncated by 1 amino acid at its C terminus (peptide 1443–1451 or GFTGDFDSV by one-letter code) identified in patient 6 evoked 1·5 times as many IFN-γ SFC as the original peptide, although the truncated peptide lost the HLA-A*2402-binding motif. Further truncation at the C terminus to produce peptide 1443–1450 or at the N terminus to produce peptide 1444–1452 led to loss of antigenicity. Thus, peptide 1443–1451 was defined as the minimal and optimal epitope for HCV-specific $CD8^+$ T cells. The amino acid sequence of HCV NS3 protein

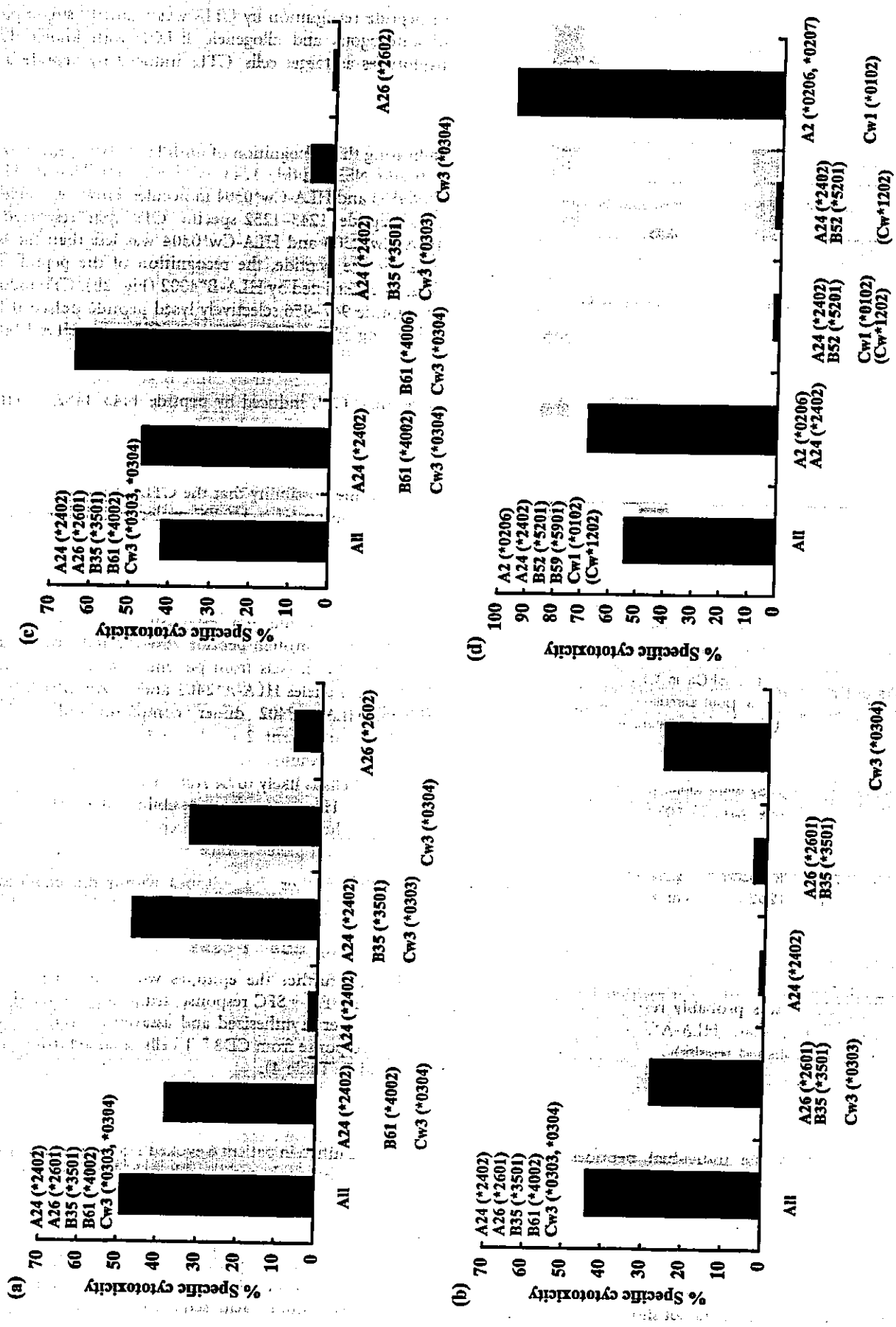
(a)
% Specific cytotoxicity
70
60
50
40
30
20
10
0
A24 (*2402)
A26 (*2601)
B35 (*3501)
B61 (*4002)
Cw3 (*0303, *0304)
All
A24 (*2402)
B61 (*4002)
Cw3 (*0304)
A24 (*2402)
B35 (*3501)
Cw3 (*0303)
A26 (*2602)
Cw3 (*0304)
(b)
% Specific cytotoxicity
A24 (*2402)
A26 (*2601)
B35 (*3501)
B61 (*4002)
Cw3 (*0303, *0304)
All
A26 (*2601)
B35 (*3501)
Cw3 (*0303)
A24 (*2402)
A26 (*2601)
B35 (*3501)
Cw3 (*0304)
(c)
% Specific cytotoxicity
A24 (*2402)
A26 (*2601)
B35 (*3501)
B61 (*4002)
Cw3 (*0303, *0304)
All
B61 (*4002)
Cw3 (*0304)
A24 (*2402)
B61 (*4006)
Cw3 (*0304)
A24 (*2402)
B35 (*3501)
Cw3 (*0303)
A26 (*2602)
Cw3 (*0304)
(d)
% Specific cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
A2 (*0206)
A24 (*2402)
B52 (*5201)
B59 (*5901)
Cw1 (*0102)
(Cw*1202)
All
A2 (*0206)
A24 (*2402)
A24 (*2402)
B52 (*5201)
Cw1 (*0102)
(Cw*1202)
A24 (*2402)
B52 (*5201)
(Cw*1202)
A2 (*0206, *0207)
Cw1 (*0102)

Fig. 2. HLA restriction of peptides 910–919 (a), 1243–1252 (b), 947–956 (c) and 1443–1452 (d) recognition by CTLs induced by the individual peptides. PBMC stimulated with peptides 910–919, 1243–1252 and 947–956 (from patient 4) and PBMC stimulated with peptide 1443–1452 (from patient 6) were assayed for cytotoxicity directed at autologous and allogeneic B-LCL of known HLA class I haplotypes that had been pulsed with the individual peptides. The effector-to-target ratio was 20. Specific cytotoxicity expressed as a percentage was calculated by subtracting cytotoxicity of effector cells to non-peptide-pulsed B-LCL from cytotoxicity to peptide-pulsed B-LCL. HLA molecules of CTLs and those shared by target cells are indicated at the top and bottom, respectively.

residues 1443–1451 is well conserved among members of the same type and among different types of HCV except for the amino acid at position 1444, which can be either phenylalanine or tyrosine. HCV peptide 1443–1452 with tyrosine at position 1444 stimulated IFN-γ production by $CD8^+$ T cells from patient 6 as effectively as HCV peptide 1443–1452 with phenylalanine at position 1444 (data not shown).

Table 4. Recognition of truncated and overlapping peptides by the $CD8^+$ T cells isolated from PBMC of patients 4, 6 and 7

	HCV peptide	Amino acid sequence	SFCs*
Patient 4	910–919	PYFVRAQGLI	35
	910–918	PYFVRAQGL	2
Patient 6	1443–1452	GFTGDFDSVI	40
	1443–1451	GFTGDFDSV	67
	1444–1452	FTGDFDSVI	2
	1443–1450	GFTGDFDS	4
Patient 7	790–798	LYGVWPLLL	15
	790–797	LYGVWPLL	2

*No. of IFN-γ-secreting cells per 10^5 $CD8^+$ T cells in response to the individual synthetic peptides.

Since both peptides 790–798 and 910–919 had an HLA-A*2402-binding amino acid residue at the position next to the C terminus, peptides with truncation of the HLA-A*2402-binding amino acid at the C terminus were synthesized and assayed for antigenicity. Neither of the truncated peptides retained antigenicity, indicating that C-terminal amino acids of peptides 790–798 and 910–919 were essential for antigenicity. The effect of truncation of the N-terminal amino acids was not studied. No studies of effects of truncation were performed for the other eight peptides.

HCV-specific $CD8^+$ T cell epitopes that were identified and their HLA restriction are shown in Table 5.

Sequential analysis of HCV-specific $CD8^+$ T cell responses in two patients with unresolved acute hepatitis treated with IFN-α

To study the effects of IFN therapy on HCV-specific $CD8^+$ T cell responses, we monitored changes in frequency of IFN-γ-releasing $CD8^+$ T cells in the peripheral bloods in patients 4 and 7 by ELISpot assay. The individual HCV epitope peptides were used to carry out assays during and after treatment with IFN-α or consensus IFN (Tong *et al.*, 1997) (Fig. 3). In both patients, frequencies of HCV peptide-specific, IFN-γ-releasing $CD8^+$ T cells in the peripheral blood decreased upon IFN therapy in association

Table 5. Novel HCV-specific $CD8^+$ T cell epitopes identified and their HLA restriction

HCV protein	Amino acid residues	Sequence	HLA restriction	Patient
E1	284–293	VFLVSQLFTF	ND	2
E2	790–798†	LYGVWPLLL	Cw*0801?	2, 7
NS2	910–919†	PYFVRAQGLI	Cw*0303, 0304	4
NS2	947–956	TYVYDHLTPL	B*4002, 4006	4
NS3	1243–1252	AYAAQGYKVL	Cw*0303, 0304	4
NS3	1375–1385	FYGKAIPIEAI	ND	1
NS3	1443–1451‡	GFTGDFDSV	A*0206	6
NS4B	1759–1768	AFWAKHMWNF	ND	2
NS5A	1990–1999	DFKTWLQSKL	ND	2
NS5A	2280–2288	KFPPALPIW	A*2402	2
NS5B	2456–2466	VYSTTSRSASL	ND	12

ND, Not determined.
†Amino acid(s) at the N terminus may be able to be truncated although the amino acid at the C terminus cannot be truncated.
‡Defined as minimal and optimal epitope.

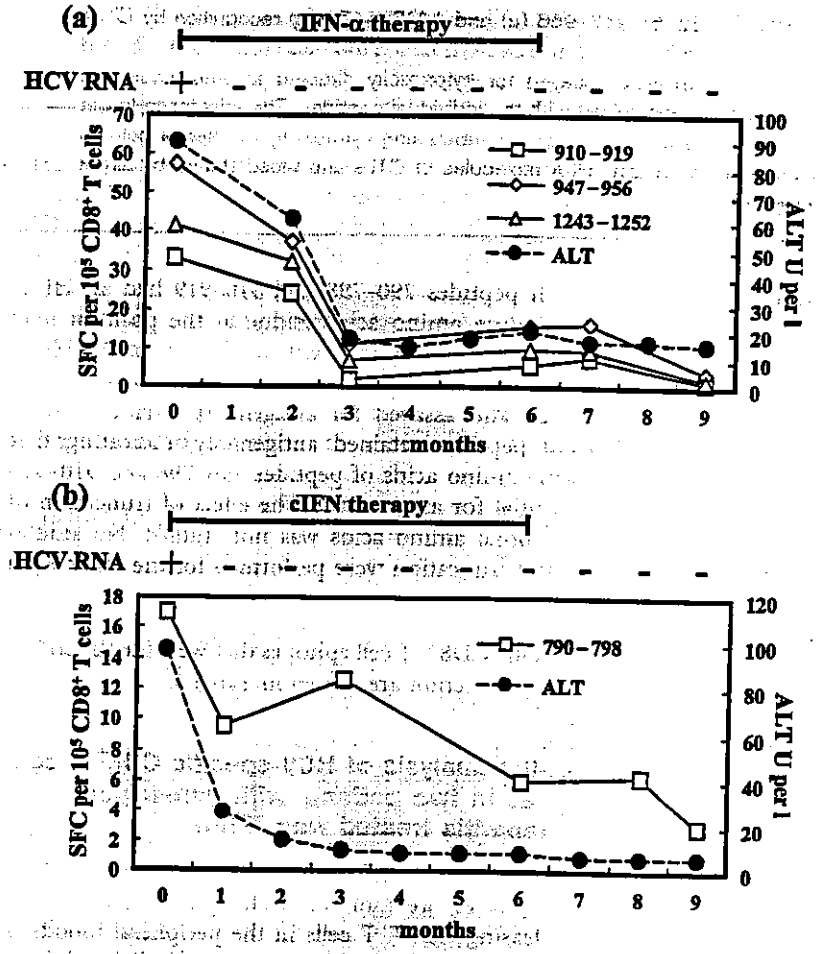

Fig. 3. Sequential analysis of HCV-specific $CD8^+$ T cell responses in two patients with unresolved acute hepatitis who were treated with IFN-α. (a) Patient 4 was treated with 10^7 units IFN-α daily for the first 2 weeks, followed by injection of the same dose of IFN-α three times weekly for the following 22 weeks. (b) Patient 7 was treated with $1{\cdot}8 \times 10^7$ units consensus IFN (cIFN) daily for the first 2 weeks, followed by injection of the same dose of cIFN three times weekly for the following 22 weeks. Both patients showed sustained virological responses.

with disappearance of serum HCV RNA and with ALT normalization. They were nearly undetectable at and after completion of therapy.

DISCUSSION

A proportion of patients with chronic HCV infection remain resistant to antiviral therapies including recently developed treatment modalities such as IFN-α2b plus ribavirin (Poynard *et al.*, 1998) and pegylated IFN (Zeuzem *et al.*, 2000). Such treatment failure may be partly a reflection of insufficient antiviral immune responses. Augmentation of HCV-specific $CD8^+$ CTL responses by therapeutic vaccines could enhance HCV elimination by IFN therapy, leading to a better treatment outcome. Development of a universally immunogenic vaccine would require identification of as many CTL epitopes as possible, especially those recognized by CTLs in association with common HLA class I molecules in the population.

In the present study, we sought to identify HCV-specific, $CD8^+$ T cell epitopes, with recognition restricted by HLA-A*2402, the most frequent HLA class I allele in Japanese and other Asians (Chandanayingyong, 1986). We screened the epitopes by an ELISpot assay based on IFN-γ release by $CD8^+$ T cells obtained from HLA-A*2402-positive patients with acute or chronic hepatitis C in response to peptide stimulation. Eighty-seven peptides were synthesized based on HLA-A*2402-binding motifs and the amino acid sequence of type 1b HCV. We could identify 10 HCV-specific CTL epitopes that induced IFN-γ release by $CD8^+$ T cells from a total of five of seven HLA-A*2402-positive patients with acute or relatively early chronic hepatitis C but not in any of four patients with persisting chronic hepatitis. The findings indicate that the response of HCV-specific CTLs to the panel of peptides is very low in patients with prolonged HCV infection. Consistent with this interpretation, an HLA-B*3501-restricted CTL epitope peptide that induced strong HCV-specific CTL responses in peripheral blood cells in the acute phase of HCV infection reportedly failed to induce CTL responses in seven of seven patients with chronic hepatitis C (Ibe *et al.*, 1998). Frequencies of HLA-B*3501-restricted, HCV-specific CTL also have been reported to be very low in the peripheral blood of patients with chronic hepatitis C, although CTLs were detectable among the PBMC by flow cytometric analysis using HLA-B*3501 tetramers (Sobao *et al.*, 2001). In still other reports, frequencies (Lechner *et al.*, 2000; Rehermann *et al.*, 1996; He *et al.*, 1999) and IFN-γ-production potential (Gruener *et al.*, 2001; Wedemeyer *et al.*,

2002) of antiviral CTLs were low in patients with chronic HCV infection.

Unexpectedly, only one of the ten CTL epitopes identified in HLA-A*2402-positive patients was found to be definitely HLA-A*2402-restricted. In addition, the frequency of $CD8^+$ T cells that responded to stimulation with the epitope was far less than for other CTL epitopes in this patient. The HLA class I molecules that restricted recognition of the other five epitopes by $CD8^+$ T cells were thought to be HLA-Cw*0303 and HLA-Cw*0304 for two epitopes, HLA-B*4002 and HLA-B*4006 for one, HLA-A*0206 for one and probably HLA-Cw*0801 for another. The HLA class I molecules that restricted recognition of the remaining four epitopes by $CD8^+$ T cells have not yet been defined. One more $CD8^+$ T cell epitope was identified using the peptides with HLA-A*2402-binding motifs in one of two HLA-A*2402-negative patients with acute hepatitis C, although the HLA class I molecule restricting recognition of the epitope has not been determined. In two of the six patients with positive $CD8^+$ T cell responses the target epitopes were multiple and only one of the 11 peptides was targeted in more than one patient. These findings indicate that a universally immunogenic HLA-A*2402-restricted, HCV-specific $CD8^+$ T cell epitope may not exist; epitopes with recognition by $CD8^+$ T cells restricted by HLA molecules other than HLA-A*2402 presumably were contained in the synthetic peptides with HLA-A*2402-binding motifs. CTL responses to HCV infection are heterogeneous, as concluded by Lauer *et al.* (2002).

We analysed sensitivity and specificity of previously reported HLA-A*2402-restricted, HCV-specific CTL epitopes (Kurokohchi *et al.*, 2001; Ito *et al.*, 2001) in HLA-A*2402-positive patients with acute and relatively recently acquired chronic hepatitis C. These two epitope peptides did not induce IFN-γ SFC responses, suggesting that their immunogenicity might be low compared with other epitopes. However, HCV NS3 peptide 1031–1039 identified by Kurokohchi *et al.* (2001) has been reported to induce HCV-specific CTLs in three of four HLA-A*2402-positive patients with chronic hepatitis C. It has been reported that *in vitro* expansion of $CD8^+$ T cells by stimulation with known HLA-A2-restricted CTL epitopes and culture in the presence of rhIL-2 revealed the existence of $CD8^+$ T cells specific for the peptide, although IFN-γ SFC responses *ex vivo* could not be induced (Lauer *et al.*, 2002). However, we could not confirm immunogenicity of the 1031–1039 epitope, even after stimulation and expansion with culture in the presence of rhIL-2. The limited number of patients in our study may have happened to lack CTLs responsive to stimulation with peptide 1031–1039; alternatively, the IFN-γ-based ELISpot assay might detect a $CD8^+$ T cell population that is functionally different from the CTLs identified by Kurokohchi *et al.* (2001). Consistent with this speculation, a 'stunned' $CD8^+$ T cell population has been reported to emerge in the acute phase of HCV infection, retaining potent HCV-specific cytotoxicity but only limited capacity for IFN-γ production (Lechner *et al.*, 2000; Thimme *et al.*, 2001). CTLs responsive to the 1031–1039 peptide may belong to such a population.

Reliability of T cell epitope prediction based on HLA-binding motifs or algorithms (Rammensee *et al.*, 1999) has been reported to be limited (Lauer *et al.*, 2002; Anthony *et al.*, 2002; Day *et al.*, 2001). Therefore, establishing that no immunodominant HLA-A*2402-restricted, HCV-specific $CD8^+$ T cell epitope exists would require screening HLA-A*2402-restricted, HCV-specific $CD8^+$ T cell epitopes by an IFN-γ ELISpot assay using overlapping peptides that span the entire HCV protein; such a study, in progress in our laboratory, may identify new HLA-A*2402-restricted, HCV-specific $CD8^+$ T cell epitopes without known HLA-A*2402-binding motifs. This study is intended to define the hierarchy of immunodominance of CTL epitopes in patients with HCV infection. Lauer *et al.* (2002) demonstrated multiple unpredicted specificities of HCV-specific $CD8^+$ T cell epitopes by an ELISpot assay using overlapping peptides that spanned the entire HCV protein, but none of the new epitopes that they found corresponded to those identified in the present study.

Using the $CD8^+$ T cell epitopes currently identified, we sequentially monitored frequencies of $CD8^+$ T cells secreting IFN-γ in response to stimulation with the epitope peptides during and after treatment of two patients with unresolved acute hepatitis C with IFN-α. Although effects of IFN-α therapy on HCV-specific $CD8^+$ T cell responses have been reported from several laboratories (Löhr *et al.*, 1999; Vertuani *et al.*, 2002; Barnes *et al.*, 2002), results are conflicting. Löhr *et al.* (1999) have reported that augmentation of HLA class I-restricted tumour necrosis factor (TNF)-α responses by IFN-α therapy contributes to a better treatment outcome in patients with chronic hepatitis C. The decline of serum HCV RNA during IFN-α therapy has been described as having two phases: a rapid early phase, thought to reflect direct inhibition of HCV replication by IFN-α; and a slower second phase, thought to be mediated by cellular immune responses, especially those of CTLs (Neumann *et al.*, 1998). Augmentation of TNF-α-releasing HCV-specific $CD8^+$ T cell responses by IFN-α may beneficially affect the second phase of HCV RNA decline. However, in our present study, numbers of HCV epitope peptide-sensitized $CD8^+$ T cells in peripheral blood declined in parallel with decreases and disappearance of serum HCV RNA and with ALT normalization. The reason why our results differed from those of Löhr *et al.* (1999) is not known, but it could involve differences in degree of chronicity of disease, decrease rates of serum HCV RNA, doses of IFN-α or ethnicity of patients.

In conclusion, we have newly identified one definite HLA-A*2402-restricted, HCV-specific $CD8^+$ T cell epitope and 10 probably non-HLA-A*2402-restricted epitopes by an IFN-γ-based ELISpot assay using synthetic HCV peptides with HLA-A*2402-binding motifs. We could find HCV-specific CTL epitopes only in patients with acute or relatively

early chronic hepatitis C. $CD8^+$ T cell responses to HCV infection were heterogeneous. The findings indicate a need to identify as many HCV-specific $CD8^+$ T cell epitopes as possible in large numbers of patients with acute or recently acquired chronic hepatitis C to understand better how immune responses eliminate HCV and contribute to pathogenesis. The ultimate aim is development of new strategies for enhancing immune responses for more effective control of HCV infection.

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Marked Elevation of Erythrocyte Ribavirin Levels in Interferon and Ribavirin–Induced Anemia

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Background & Aims: To elucidate the effects of blood ribavirin disposition on ribavirin-induced anemia, the relationship between erythrocyte ribavirin concentration and change in hematologic parameters was examined in interferon and ribavirin combination therapy for HCV eradication. *Methods:* Nine HCV RNA–positive patients were treated with combination therapy including 11.3 ± 1.2 mg · kg^{-1} · day^{-1} of ribavirin. Blood concentrations of ribavirin and its phosphorylated metabolites were measured in plasma as well as erythrocyte. *Results:* Blood ribavirin concentrations gradually increased to steady-state levels of 8.8 ± 1.4 μmol/L and 1389 ± 371 μmol/L in plasma and erythrocytes, respectively, within 3–4 weeks of initiating therapy. Erythrocyte phosphorylated metabolite levels (1215 ± 302 μmol/L) were found to be exceedingly high, i.e., 87% of the measured erythrocyte ribavirin concentration. In contrast, plasma phosphorylated metabolite levels were undetectable. Positive correlation was found to exist between erythrocyte ribavirin concentrations and a decrease in hemoglobin ($r = 0.620$, $P < 0.001$). *Conclusions:* We concluded that marked elevation of erythrocyte ribavirin including its phosphorylated metabolites was associated with hemoglobin reduction, leading to interferon and ribavirin–induced anemia.

Ribavirin, a guanosine-analogue antiviral agent, is widely used for treatment of HCV infection in combination with interferon-α2b.[1] The most common dose-limiting drawback in ribavirin therapy is hemolytic anemia, which is observed shortly after combination therapy is begun.[1] Although the mechanism underlying ribavirin-induced anemia remains unknown, it has been proposed that high ribavirin blood levels impaired erythrocyte integrity.[2] Accordingly, the present study was conducted to determine erythrocyte and plasma ribavirin concentrations early on during interferon and ribavirin combination therapy for patients with chronic hepatitis C.

Patients and Methods

Nine patients with chronic hepatitis C (serum HCV RNA positive) (5 men and 4 women; age, 48.0 ± 18.1 years) were enrolled in the study. The viral load (serum HCV RNA) and liver function (ALT) at baseline were 324 ± 264 KIU/mL and 102 ± 49 IU/mL, respectively. The daily ribavirin dose was 11.3 ± 1.2 mg · kg^{-1} · day^{-1}. Blood samples to determine ribavirin concentrations were collected before the first treatment was administered, as well as 1, 3, 7, 14, 21, and 28 days after starting the combination therapy. Blood drawing was conducted before morning dose of ribavirin in each sample collection day.

The concentrations of ribavirin and its phosphorylated metabolites in both plasma and whole blood were measured by high-performance liquid chromatography (HPLC), as has been previously described.[2] In brief, whole blood samples were precipitated with perchloric acid, and then the supernatant was neutralized by adding potassium hydroxide. The resultant mixture was divided into 2 portions, one for enzyme digestion and the other for treatment without enzyme. Acid phosphatase type IV from sweet potato (Sigma, St. Louis, MO) was used to hydrolyze the ribavirin phosphorylated metabolites. Enzyme-treated samples and nontreated ones, which were added internal standard (3-methylcytidine methosulfate; Sigma), were treated with phenyl boronic acid cartridge (Bond Elute PBA, Varian Harbor City, CA) to extract ribavirin and internal standard. Effluents were analyzed by reverse-phase HPLC developed by us.[2] Plasma concentration was measured by the same procedure with non–enzyme-treated whole blood. The detection limit for ribavirin was as low as 2 μmol/L. Coefficients of variations for intra-assay and interassay were 3–10% and 5–12%, respectively, at the concentration of 20–200 μmol/L.

Erythrocyte ribavirin (Crbc) was calculated with the following formula: $Crbc = [Cw - Cp(1 - Ht)]/Ht$, where Cp and Cw represent the concentrations of ribavirin in plasma and whole blood, respectively.

Data are expressed as the mean ± SD. The statistical significance among the results in the different groups was evaluated by the upaired Student *t* test.

Abbreviations used in this paper: Ht, hematocrit; Hb, hemoglobin; HPLC, high-performance liquid chromatography.

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Results

The change over time in erythrocyte ribavirin concentrations is depicted in Figure 1. Blood concentrations gradually increased to steady-state levels within 3–4 weeks of starting ribavirin administration. Plasma and erythrocyte total ribavirin (free ribavirin + phosphorylated metabolites) steady-state levels were 8.8 ± 1.4 μmol/L and 1389 ± 371 μmol/L, respectively. Although phosphorylated metabolites were undetectable in plasma, 87% of erythrocyte ribavirin was present as phosphorylated metabolites (1215 ± 302 μmol/L). The ribavirin concentration erythrocyte/plasma ratio was 159 ± 45.

Although ALT levels significantly improved during the first week of combination therapy (101.7 ± 49.3 vs. 62.4 ± 20.0 U/L, $P < 0.01$), hemoglobin (Hb) and hematocrit (Ht) levels remained unchanged. Nonetheless, such hematologic parameters significantly decreased after 3–4 weeks of ribavirin therapy, as compared with baseline levels (Hb: 14.0 ± 1.6 vs. 11.6 ± 1.7 g/dL; Ht: 41.3 ± 4.0% vs. 34.1 ± 4.5%; $P < 0.01$), which correlated with erythrocyte ribavirin levels in excess of 1000 μmol/L (Figure 1).

A significant correlation between erythrocyte ribavirin concentrations and a drop in Hb from baseline levels was observed (Figure 2; $r = 0.620$, $P < 0.001$). A similar correlation was observed for plasma ribavirin and Hb, but the correlation coefficient value was smaller than that calculated for erythrocyte ribavirin ($r = 0.588$, $P < 0.001$).

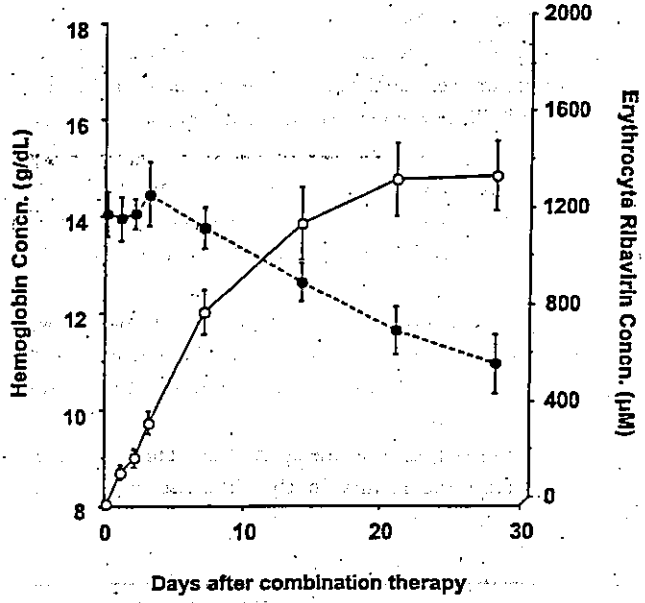

Figure 1. Change in erythrocyte ribavirin (*open circle*) and Hb levels (*closed circle*) early on during interferon and ribavirin combination therapy. The values are indicated as mean ± standard error.

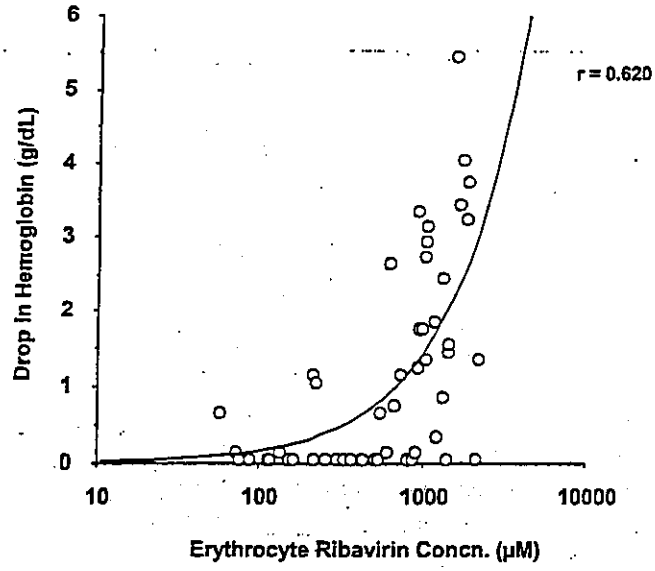

Figure 2. Effects of erythrocyte ribavirin on changes in Hb levels.

Discussion

After active transportation of ribavirin into erythrocytes via nitrobenzylthioinosine-sensitive (es type)–nucleoside transporters, intracellular phosphorylation of ribavirin converts the compound to its phosphate metabolites.[2] Because the phosphorylated metabolites cannot diffuse outside of cells, ribavirin progressively accumulates in erythrocytes during treatment.[2,3] Nonetheless, no data have demonstrated the degree to which ribavirin is concentrated in erythrocytes of patients receiving combination interferon-α and ribavirin. Moreover, it has also not been demonstrated whether intracellular ribavirin levels are associated with changes in hematologic parameters, i.e., decreased Hb and Ht.

As expected, intracellular erythrocyte ribavirin accumulated as phosphorylated metabolites, which were undetectable in plasma. Nonetheless, the fact that erythrocyte concentrations were greater than 1000 μmol/L, and the erythrocyte/plasma ratio was 159 ± 45, was contrary to expected levels based on reported values (3-fold less than values reported in the present work). The discrepancy in erythrocyte/plasma ratios measured in the present work and a previous study[3] probably stems from a difference in the experimental methods. Because the previous study[3] did not include a dephosphorylation procedure during pretreatment of erythrocyte extracts, phosphorylated metabolite levels could not be correctly determined.

Ribavirin blood concentrations, especially erythrocyte levels, are important for predicting the occurrence of anemia. As shown in Figure 2, a marked decrease in Hb was observed when the erythrocyte ribavirin concentra-

tion exceeded 1000 μmol/L. Such concentrations were reached 2–3 weeks after beginning ribavirin treatment, which correlated with initial decreases in Hb levels. Accordingly, ribavirin levels greater than 1000 μmol/L might prove toxic for erythrocyte cell survival. In an in vitro experiment De Franceschi et al.[4] reported that the attainment of a ribavirin concentration of 1000 μmol/L resulted from reduction in erythrocyte ATP levels and Na-K pump activity. Furthermore, they found that ribavirin treatment increased aggregated band 3 on erythrocyte cell surfaces, which accelerates binding with autologous antibody and complement C3 fragments, resulting in removal of injured erythrocytes by erythrophagocytosis in the reticuloendothelial system.[4] The results of the present work support the postulate that blood ribavirin concentrations greater than 1000 μmol/L prove toxic.

Steady-state ribavirin plasma concentrations have been determined to assess the efficacy and toxicity of multidose therapy. Bruchfeld et al.[5] reported that adjusting ribavirin dosages to maintain a plasma level of 10–15 μmol/L allowed prevention of toxicities commonly observed in the first 3 months of combination therapy. Nonetheless, plasma levels did not always correlate with anemia onset, most likely as a result of a lack of knowledge of phosphorylated metabolite levels.

In conclusion, we confirmed that marked elevation of erythrocyte ribavirin including its phosphorylated metabolites was associated with Hb reduction, leading to interferon and ribavirin–induced anemia. Therefore, erythrocyte ribavirin and phosphorylated metabolite levels might represent a suitable marker for predicting interferon and ribavirin–induced anemia. Further study with large sample size will clarify the merit of measuring erythrocyte levels in therapeutic drug concentration monitoring of ribavirin.

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