microplate. Thereafter, hybridization was detected by staining with the streptavidin-horseradish peroxidase (HRP) conjugate.[15]

Amplifying and sequencing the S region of HBV-DNA

The entire aa sequence of MHR in the S region was amplified by two-stage PCR using genotype-specific primers. The outer primers for the amplification of the first fragment were 5′-TTTCCACCAAGCTCTGCAA-3′ (sense: nt 9–28) and 5′-TTCAGGGAATAACCCCATCT-3′ (antisense: nt 872–853) for genotype A, 5′-CTCCA CCACTTTCCA GACT-3′ (sense: nt 1–22) and 5′-CAACTCCCAATTACATATCCC-3′ (antisense: nt 899–879) for genotype B and 5′-TTACAGGCGGGG TTTTTCTT-3′ (sense: nt 70–89) and 5′-TACAGACTT GGCCCCCAATA-3′ (antisense: nt 771–752) for genotype C. The inner primers were 5′-AGAGTCAGGGGCC TGTATTTT-3′ (sense: nt 35–55) and 5′-AGGGAATAA CCCCATCACTTT-3′ (antisense: nt 869–849) for genotype A, 5′-TTCAAGATCCCAGAGTCAGG-3′ (sense: nt 24–43) and 5′-AGGGAATATCCCCACCTTTTT-3′ (antisense: nt 869–849) for genotype B and 5′-CGGGGTT TTCTTGTTGACA-3′ (sense: nt 77–97) and 5′-CCCAAT ACCACATCATCCATA-3′ (antisense: nt 758–738) for genotype C.

The first stage of amplification was carried out in a thermal cycler for 40 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 1 min) in 100 μL reaction mixture containing 200 mM dNTPs, 1.0 mM each of primers and PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$ and 0.001% (wt/vol) gelatin) and 2 U Ampli-Taq polymerase (Perkin Elmer Cetus, Norwalk, CT, USA). PCR products (2 μL) were subjected to the second stage of amplification under the same conditions as those in the first stage. Standard precautions to avoid contamination were taken during PCR, with a negative control serum sample included in each run.

Amplification products were purified on Wizard PCR preps DNA purification resin (Promega, Madison, WI, USA), and sequenced bidirectionally with a Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, USA) using the above-mentioned PCR primers. Sequencing was performed in an automated DNA sequencer (ABI 377; PE Applied Biosystems).

The nucleotide sequences of HBV isolates from the patients were compared with those of three reference HBV strains which are used for vaccine production.[16–18]

Phylogenetic trees were constructed with the Mega Program version 2.1 (Center for Evolutionary Functional Genomics, The Biodesign Institute, Tempe, AZ, USA) using the Kimura two-parameter matrix and the neighbor-joining method.[19] To confirm the reliability of phylogenetic tree analysis, boot-strap resampling, and reconstruction were carried out 500 times.

Hydrophobicity and secondary structure analysis

The hydrophobicity profile of the MHR of the S region was predicted by computer-assisted Kyte-Doolittle analysis (an estimate of hydrophobicity based on the bulk phase partitioning of side chain hydrophobicity alone)[20] with GENETYX-MAC software (version 10.1; Software Development, Tokyo, Japan).

The secondary structures of the amino acids in the same region were predicted by computer-assisted Robson[21] and Chou-Fasman analyses[22] with the GENETYX-MAC software.

Statistical analyses

Data were analyzed by the chi-squared test for categorical data and Student's *t*-test or the Mann–Whitney *U*-test for continuous variables. *P*-values less than 0.05 were regarded as statistically significant.

RESULTS

Distribution and clinical characteristics of HBV genotypes

HEPATITIS B VIRUS genotype was determined in the 48 patients with acute hepatitis B. Genotype A was detected in 11 (23%) patients, genotype B in 11 (23%) and genotype C in 26 (54%).

The clinical and demographic backgrounds of the patients with acute hepatitis B who were infected with HBV of different genotypes are shown in Table 1. The mean ages of all the groups were similar. The proportion of male to female patients was higher in genotype A infection than in genotypes B or C infection (100%, 73% and 64%, respectively: A *vs* B, $P = 0.22$; A *vs* C, $P = 0.01$; B *vs* C, $P = 0.16$). The maximum alanine aminotransferase (ALT) levels were lower in patients with genotype A infection than in patients with genotypes B or C infection (1646 ± 1123, 3085 ± 1119 and 2545 ± 981 IU/L, respectively: A *vs* B, $P = 0.01$; A *vs* C, $P = 0.03$; B *vs* C, $P = 0.89$). The maximum HBV-DNA levels were not significantly different between the

Table 1 Demographic and clinical differences among patients with acute hepatitis infected with HBV of distinct genotypes

Features	Genotypes of HBV			Differences (*P*-value)		
	A ($n = 11$)	B ($n = 11$)	C ($n = 26$)	A *vs* B	A *vs* C	B *vs* C
Age (years)	30.6 ± 7.5	28.1 ± 5.1	31.1 ± 9.1	0.41	0.87	0.33
Gender (M:F)	11:0	8:3	15:11	0.22	0.01	0.16
ALT (IU/L)	1646 ± 1123	3085 ± 1119	2545 ± 981	0.01	0.03	0.89
HBV-DNA (LGE/mL)	6.8 ± 1.7	6.6 ± 2.1	5.2 ± 1.2	0.60	0.23	0.06

ALT, alanine aminotransferase; HBV, hepatitis B virus.

genotypes (6.8 ± 1.7, 6.6 ± 2.1 and 5.2 ± 1.2 LGE/mL, respectively: A *vs* B, $P = 0.60$; A *vs* C, $P = 0.23$; B *vs* C, $P = 0.06$).

Amino acid sequence of the S region

The aa sequence of the S region between aa27 and aa203 was determined in the 48 sequences. Figure 1 shows a phylogenetic tree constructed using the 48 sequences and 15 published sequences (four for genotype A, three for genotype B, three for genotype C, one for genotypes D, E, F, G and H). Among the 48 sequences we studied, 11 were classified into genotype A, 11 into genotype B and 26 into genotype C.

The aa sequence of the region between aa101 and aa163 including MHR (aa111-aa156) was compared among 48 sequences and three HBV sequences (X01587, J02205 and huGK-14) currently used for anti-HBV vaccine production. As shown in Figure 2, the aa sequences of X01587 (used for Bimmugen) and J02205 (used for Heptavax) differed in eight amino acids (i.e. aa110, aa113, aa114, aa126, aa131, aa143, aa160 and aa161). The aa sequence of huGK-14, which is used for the HBV-vaccine Meinyu, differed from that of X01587 in six amino acids and from that of J02205 in two amino acids.

Nine of the 11 isolates classified into genotype A had the same aa sequence as J02205. The remaining two isolates (AB289727 and AB289728) differed from J02205 at aa161 (Fig. 2).

Ten of the 11 isolates classified into genotype B had the same aa sequence as J02205 except for two amino acids (aa114 and aa131). The remaining isolate had another aa substitution at aa112 (Fig. 2).

As shown in Figure 2, 22 of the 26 isolates classified into genotype C had the same sequence as X01587. The remaining four isolates (from patients 10, 24, 30 and 48) had the same sequence as X01587 except for one aa substitution at aa131; the threonine (aa131) of X01587 was substituted with proline for three isolates (AB289714, AB289720 and AB289736) and with alanine for one isolate (AB289701).

Hydrophobicity and secondary structure analysis

As mentioned above, the aa sequences of the MHR from four isolates differed from that of X01587 only at aa131. Furthermore, the aa sequence of the MHR differed between X01587 and J2205 in eight amino acids. We compared the hydropathy and secondary structure of the MHR among J02205, X01587 and two isolates with genotype C (one isolate with proline at aa131 and one with alanine at aa131). The results of Kyte-Doolittle hydropathy analysis based on the hydropathy index are shown in Figure 3. The substitution with alanine-131 was found to alter the patterns on the hydropathy plot, whereas the substitution with proline-131 was found to have little effect. A substitution with alanine-131 could increase the hydrophobicity of the first loop of the MHR, which may affect the antigenicity of HBV.

The secondary structure of our isolate with alanine-131 by Chou-Fasman analysis predicted an α-helix configuration for the region from aa126 to aa135 instead of the β-configuration predicted for the same region of X01587. The predicted secondary structure of our isolate with proline-131 coincided with that of X01587. In contrast, by Robson prediction, the secondary structure of our isolate with alanine-131 coincided with that of X01587; however, that of our isolate with proline-131 was found to have lost a turn structure between aa131 and aa134, which was predicted for X01587.

DISCUSSION

VACCINATION IS THE key to controlling HBV infection. In countries with a high prevalence of HBV infection, universal vaccination is effective not only for controlling viral infections but also for decreasing the incidence of hepatocellular carcinoma.[5,23] Even in

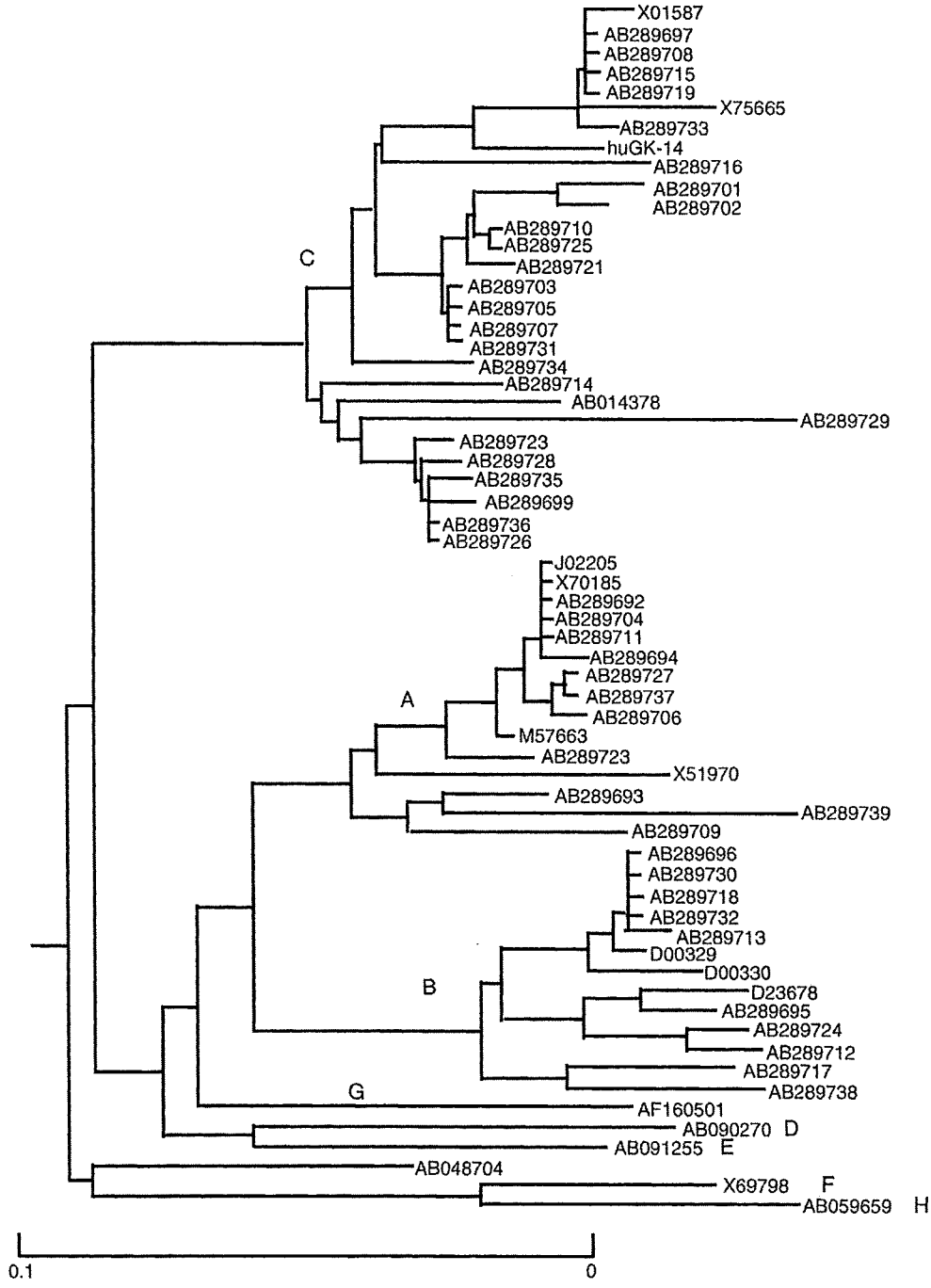

Figure 1 Phylogenetic tree constructed using hepatitis B virus (HBV)-DNA sequences of the *S* gene. The sequences include four with genotype A, four with genotype B, three with genotype C, and those recovered from the serum of 48 patients with acute hepatitis B. J02205 (genotype A) is used for the production of Heptavax and X01587 (genotype C) is used for the production of Bimmugen. The horizontal bar indicates the number of nucleotide substitutions per site. Accession numbers are shown for the isolates that have been deposited in the DDBJ/EMBL/GenBank databases. The accession numbers for the HBV sequences from the 48 patients are also shown.

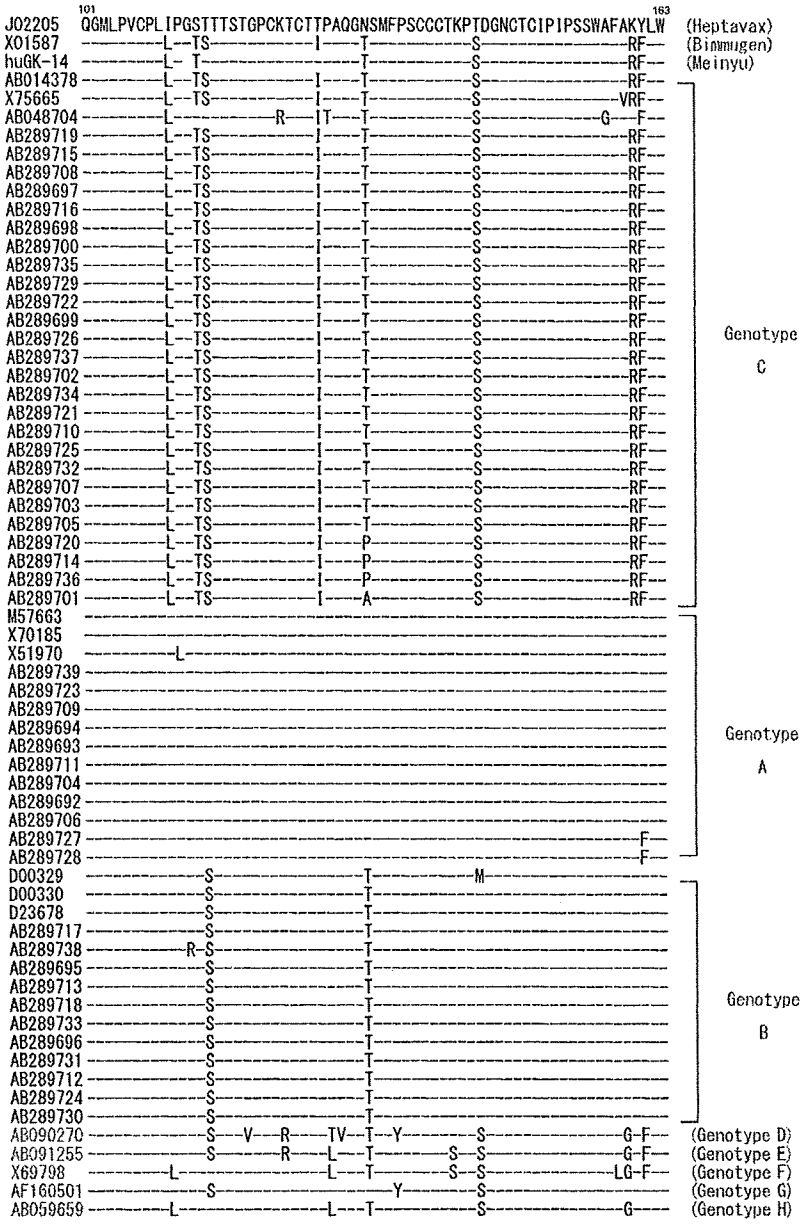

Figure 2 Comparison of amino acid sequences of the major hydrophilic region (MHR) of the *S* gene.

countries with a low prevalence of HBV infection, vaccination is very important for preventing mother-to-child transmission as well as patient-to-staff transmission.

HBV is classified into several genotypes that differ from one another in nucleotide sequence by more than 8% of the entire genome. The aa sequences of their phenotypes also differ among genotypes. The difference in the aa sequence of the 'a' determinant region may alter the three-dimensional structure and antigenicity, and may reduce the protectivity of HBV vaccines.

As mentioned above, the aa sequences of currently available recombinant vaccines differ from each other. J02205 and X01578 differ in eight amino acids (i.e. aa110, aa113, aa114, aa126, aa131, aa143, aa160 and aa161) between aa101 and aa163. Three (i.e. aa126, aa131 and aa143) of them are included in the MHR and may alter the hydropathy and three-dimensional

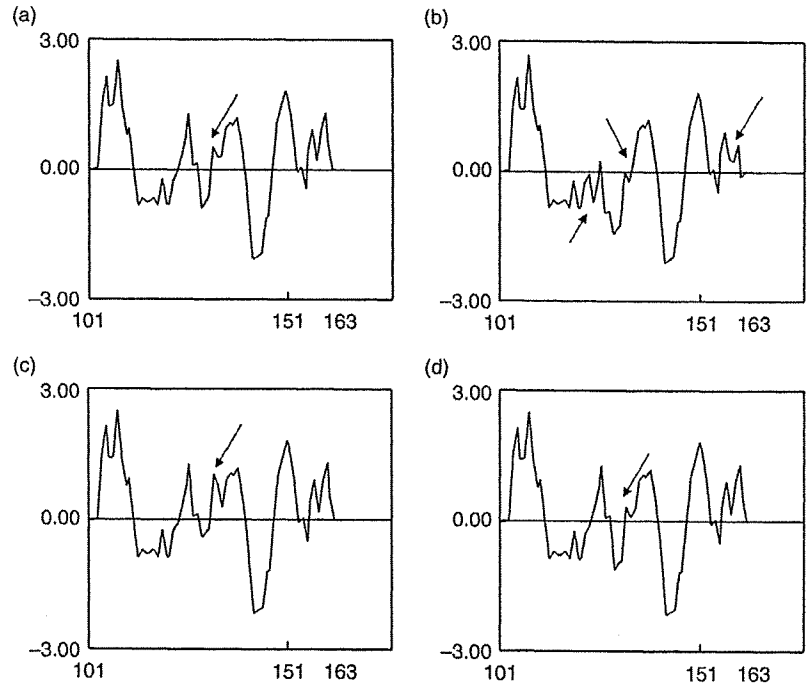

Figure 3 Hydropathy profile of the major hydrophilic region (MHR) of the *S* gene elaborated using the Kyte-Doolittle hydropathy index. Arrows show the positions of amino acids which are different among X01587, J02205, AB289701 (alanine-131) and AB289720 (proline-131). (a) X01587, (b) J02205, (c) AB289701 (alanine-131), (d) AB289720 (proline-131).

structure of the region. Therefore, the antibody produced against J02205 vaccines may not completely neutralize X01578 and vice versa. Indeed, previous studies showed that antibody profiles induced by recombinant vaccines produced from different genotypes are not identical with each other,[12] which suggests that antibodies produced by recombinant vaccines might not protect viral infection with different genotypes.

As shown in Figure 2, the aa sequences of our isolates classified into genotype A are very close to the aa sequence of J02205. Therefore, the transmission of genotype A HBV is prevented by Heptavax which is made from J02205.

The aa sequences of our isolates classified into genotype B are the same as the aa sequence of J02205 except for one substitution at aa131. This aa, which is asparagine and is located in the first stem loop structure of the MHR, was substituted with threonine in our genotype B isolates. Because asparagine and threonine have an uncharged side chain and similar polarity, genotype B HBV infection may be prevented effectively by Heptavax.

The aa sequences of our isolates classified into genotype C were the same as that of X01587 except for four isolates having a substitution at aa131. Bimmugen, which is produced from X01587, may be effective for preventing genotype C HBV infections caused by those four isolates. However, Heptavax may not be effective for preventing genotype C HBV infection because of the difference in eight amino acids as described above.

The four isolates have proline or alanine instead of threonine-131, which has never been reported before. The polarities of threonine and proline/alanine are quite different. The Kyte-Doolittle hydropathy analysis suggests that substituting threonine at aa131 with alanine or proline would increase hydrophobicity, which may then lead to a change in antigenicity. Hou *et al.* reported that some blood donors who were tested negative for serum HBsAg had a substitution of isoleucine for threonine at aa131 in the S region.[24] They suggested that the structure and antigenicity of HBV may be altered by this substitution.

The secondary structure of our isolate with alanine-131 predicted by Chou-Fasman analysis suggested an α-helix configuration instead of a β-configuration in the region from aa126 to aa135. The secondary structure of our isolate with proline-131 predicted by Robson analysis suggested that this change causes the loss of a turn structure between aa131 and aa134. Some changes in the secondary structure can affect the three-dimensional structure of the protein and thus affect antigenicity. These results suggest that the transmission of the four

isolates with an aa substitution at aa131 may not be prevented by either Heptavax or Bimmugen.

However, the protective immunity elicited by HBV vaccines, which is usually polyclonal in nature, may not be totally lost or severely affected *in vivo* by the alteration of only a single amino acid in the 'a' determinant region.[25] Also, antibodies against regions outside the 'a' determinant region may be protective.[26] The protectivity of current vaccines may be elucidated by *in vitro* binding studies using polyclonal antibodies.

It was reported that some individuals immunized with recombinant vaccines are infected with HBV with or without mutations in the 'a' determinant region.[11,27,28] HBV isolates with amino acid substitutions at aa144[29-31] or 145[11,27,28] are known to be transmitted despite vaccination. Indeed, some chronic HBV carriers are reported to have HBV with such amino acid substitutions.[32,33] We were unable to find patients who had these substitutions in the present study. However, large-scale studies are necessary to elucidate the prevalence of 'vaccine-escape mutants' in patients with acute hepatitis B.

In conclusion, we have shown that the aa sequence of the MHR in the *S* gene of HBV is different among isolates from patients with acute HBV infection. Current vaccination may prevent the transmission of these HBV isolates, which should be further investigated.

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The High Incidence of the Emergence of Entecavir-Resistant Mutants among Patients Infected with Lamivudine-Resistant Hepatitis B Virus

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NAGASAKI, F., NIITSUMA, H., UENO, Y., INOUE, J., KOGURE, T., FUKUSHIMA, K. and SHIMOSEGAWA, T. *The High Incidence of the Emergence of Entecavir-Resistant Mutants among Patients Infected with Lamivudine-Resistant Hepatitis B Virus.* Tohoku J. Exp. Med., 2007, **213** (2), 181-186 —— Hepatitis B virus (HBV) infection remains to be one of the most prevailing infection in the world, causing chronic liver diseases. Although lamivudine has been effective to suppress HBV replication, longer durations of administration can lead to the emergence of drug-resistant mutant viruses, followed by reactivation of hepatic inflammation (breakthrough hepatitis). Moreover, the optimal period of administration as well as the effects of anti-viral nucleot(s)ide such as lamivudine, adefovir, and entecavir, has not been established. To evaluate the efficacy of the anti-viral effects of entecavir for lamivudine-resistant HBV, we administered entecavir sequentially in four patients with chronic HBV infection, who demonstrated the emergence of lamivudine-resistant HBV and histological active hepatitis. The antiviral effects were evaluated by the serum viral loads and biochemical laboratory data. After follow-up periods of more than 36 months, we found high incidence in the emergence of entecavir resistant mutants (3/4, i.e., 75%). An additional mutation at the 184th amino acid, different from the previously reported lamivudine-resistant mutations (80th, 180th, and 204th), seemed to have a close relationship with the induction of entecavir-resistant mutants at least for Japanese HBV genotype C. Our observation draws attention to the possibility that the usage of entecavir for lamivudine-resistant HBV could promptly induce entecavir-resistant mutations in addition to lamivudine-resistance. ——— HBV; lamivudine; entecavir; antiviral resistant virus

Chronic hepatitis B virus (HBV) infection remains one of the major serious health problems in the world (Lee 1997). Its clinical manifestation is affected by various factors, particularly the genotype (Maddrey 2000). Eight different genotypes of HBV, designated A to H, have been already determined (Okamoto et al. 1987, 1988; Norder et al. 1992; Stuyver et al. 2000; Arauz-Ruiz et al. 2002). Furthermore, it has been reported that a genotype is composed of various subgenotypes (Norder et al. 2004). For example, genotype B causes seroconversion more frequent-

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ly than genotype C (HBV/C), and those infected with HBV/B appear to have a better prognosis (Kikuchi 2000; Orito et al. 2001). At present, HBV genotype B (HBV/B) has been clustered into five subgenotypes (Nagasaki et al. 2006a).

Lamivudine, the first approved orally administered nucleotide analogue, is well known for its safety and effectiveness in the treatment of HBV infections, including HBV cirrhosis, regardless of the HBV genotype (Yao and Bass 2000; Yao et al. 2001; Yuen et al. 2003). On the other hand, long durations of lamivudine therapy could lead to the evolution of drug-resistant mutants, which can emerge in 15% in one year, and in 50% in three years after the initiation of therapy (Lee 1997; Dienstag et al. 1999; Kapoor et al. 2000; Yao et al. 2001), and could be followed by hepatic failure, i.e., breakthrough hepatitis (Liaw et al. 2000; Santantonio et al. 2000; Lok and McMahon 2001). As we previously reported, some patients could discontinue lamivudine administration successfully (Nagasaki et al. 2006b), but it is usually considered to be very exceptional (Wang et al. 2002).

Recently, other antiviral agents, such as adefovir, entecavir, and tenofovir have revealed usefulness in suppressing the serum HBV-DNA levels. In Japan, the use of adefovir was approved in 2004 and that of entecavir in 2006. However, their long term efficacies have not been established. Especially, the usage of entecavir for lamivudine resistant HBV has been considered questionable since both lamivudine and entecavir show similar mechanisms in the development of resistant mutations.

In this study, we administered entecavir to 4 patients with chronic HBV infection, who had been previously administered lamivudine in whom the emergence of a resistant mutant had been confirmed.

Materials and Methods

We administered entecavir monotherapy treatment for more than 36 months in 4 patients with chronic HBV infection, who had been previously treated with lamivudine 100 mg daily and developed breakthrough hepatitis due to the emergence of lamivudine-resistant mutant virus after the cessation of lamivudine. The inclusion criteria of these patients strictly followed those of the phase III clinical trial by Bristol-Myers Japan Inc. (Tokyo); briefly, both i) the presence of active HBV viremia (> 10^5 copy/mL), and ii) the presence of pathologi-

Table 1. The clinical backgrounds of the four patients.

Patients	1	2	3	4
Sex	M	M	F	M
Age	51	43	51	27
Duration of prior lamivudine administration	31	13	20	16
T. Bil (mg/dL)	0.9	0.5	0.7	0.4
AST (IU/L)	61	34	39	37
ALT (IU/L)	74	62	61	102
HBeAg (RIA)	+	+	+	+
Anti-HBe (RIA)	−	−	−	−
HBV-DNA (Log copy/ml)	7.5	5.4	> 7.6	> 7.6
Genotype (RFLP)	C	C	C	C
YMDD motif	YIDD	YMDD/YIDD	YIDD/YVDD	YIDD

T-Bil, total bilirubin; AST, asparate aminotransferase; ALT, alanine aminotransferase; HBeAg, hepatitis B envelope antigen; Anti-HBe, hepatitis B envelope antibody; RIA, radioimmunoassay; RFLP, restriction fragment length polymorphism; Y, tyrosine; M, methionine; D, aspartic acid; I, isoleucine; V, valine; M, male; F, female.

cally proven chronic hepatitis. The study protocol was approved by institutional review board (IRB) in Tohoku University Hospital. For this clinical trial, 4 patients were initially screened and all of these patients were considered as eligible patients for this study. All of these four patients had been administered entecavir 0.5 or 1.0 mg daily for 52 weeks in a coded manner, and thereafter 1.0 mg daily by the protocol of our present study. Their clinical data are shown on Table 1. The genotype of HBV detected in all 4 patients was C, and the serum HBV-DNA level and alanine aminotransferase (ALT) were 7.5 Log copy/mL and 70.8 IU/L on average, respectively. The quantitative range of HBV-DNA by polymerase chain reaction (PCR)-assay (Roche Ampricor HBV, Roche Japan Inc., Tokyo) was 2.6-7.7 Log copy/mL.

We defined antiviral resistance as a more than 1 log10 increase in the HBV-DNA level in the serum from a patient who had an initial virologic response (Lok and McMahon 2007). We conducted HBV-DNA direct sequence analyses in the polymerase region before the administration of entecavir, and at the time antiviral resistance emerged.

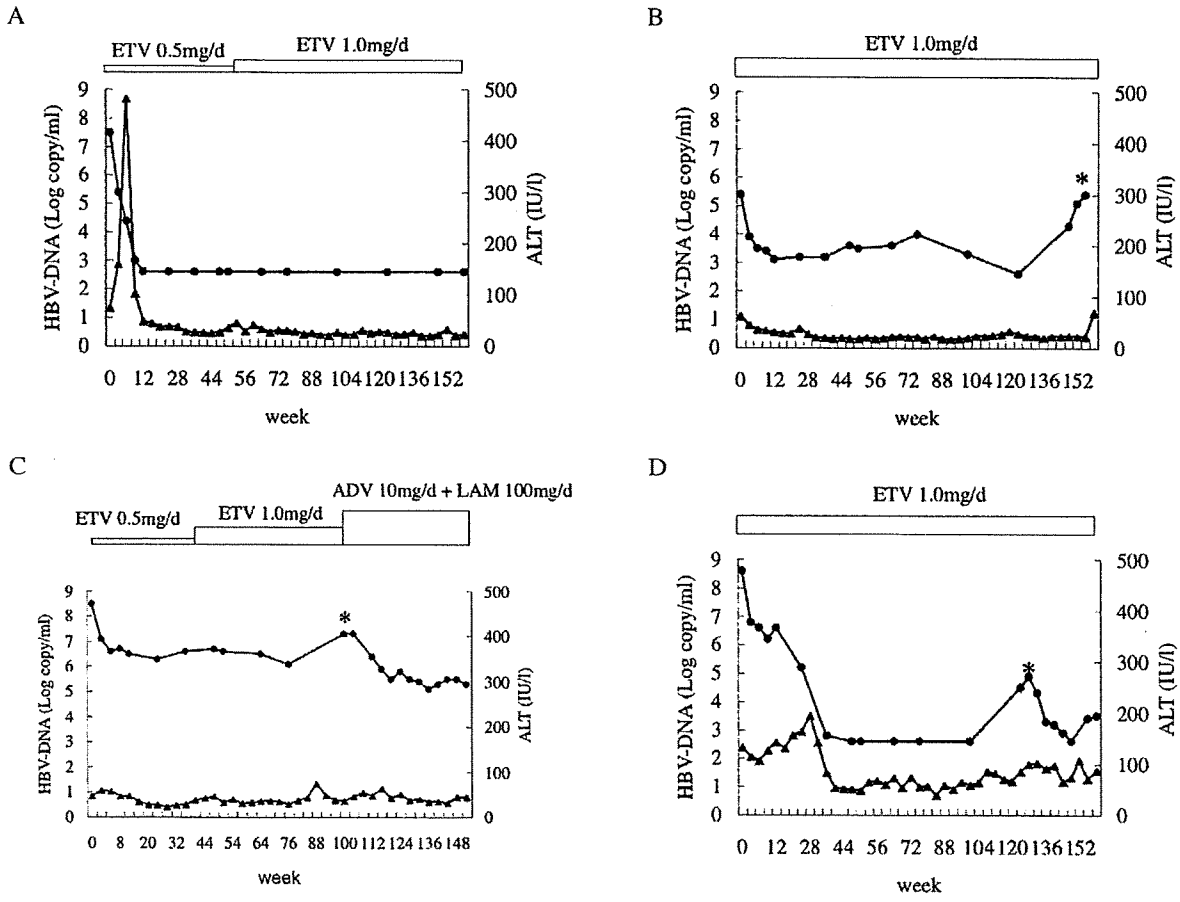

Fig. 1. Clinical course of the four patients treated with entecavir.
A: Clinical course of Patient No. 1. An entecavir-resistant mutant was not found in this patient.
B: Clinical course of Patient No. 2. An entecavir-resistant mutant was detected at the 152nd week (*).
C: Clinical course of Patient No. 3. Entecavir was administered 0.5 mg/day for 52 weeks, thereafter 1.0 mg/day. An entecavir-resistant mutant was detected at the 104th week (*), and the treatment was then changed to adefovir and lamivudine combination therapy.
D: Clinical course of Patient No. 4. An entecavir-resistant mutant was detected at the 130th week (*).
ETV, entecavir; ADV, adefovir; LAM, lamivudine; ALT, alanine aminotransferase.
—●—, HBV-DNA; —▲—, ALT.

Results

Clinical evaluation

Patient No. 1 had a good response to entecavir without the emergence of a resistant mutant virus (Fig. 1A). On the other hand, the other three patients (75%) experienced the emergence of entecavir mutant viruses during the therapy. Although patient No. 2 at first showed continuously suppressed serum HBV-DNA levels of 2.6 to 4.0 Log copy/mL, at the 52nd week the patient showed an increase of the serum HBV-DNA levels followed by an elevation of ALT and was found to have entecavir-resistant mutant viruses (Fig. 1B). Patient No. 3 was administered entecavir 0.5 mg daily and the serum HBV-DNA level decreased from 8.5 Log copy/mL but did not decrease further below 6.0 Log copy/mL even after an increase of the entecavir dosage as shown in Fig. 1C. We changed the entecavir monotherapy to lamivudine and adefovir combination therapy at the 104th week. Thereafter, this patient showed decreases in both the serum HBV-DNA level and ALT. Patient No. 4 had been administered entecavir 1.0 mg daily which resulted in the suppression of HBV-DNA to below a detectable level by PCR assay. At the 130th week, the serum HBV-DNA level increased to 4.9 Log copy/mL (ALT 100 IU/L) and entecavir-resistant mutant viruses were revealed. Later, the serum HBV-DNA level decreased without any change in treatment (Fig. 1D).

The analysis of HBV sequences regarding mutations after entecavir administration

HBV amino acids (aa) in polymerase region analyses from the three patients with entecavir-resistant mutants are shown in Table 2. The HBV nucleotide sequences in the polymerase regions obtained at the initiation of the therapy and during the week entecavir-resistant mutants had emerged were determined and amino acids were analyzed by them. The drug resistant mutations as shown in the table were confirmed as follows. In the three patients the 80th aa in the A domain was substituted to leucine, and the 180th aa in the B domain was methionine. Thus, the lamivudine resistant 80th aa mutation found at the initiation of entecavir treatment had been returned to be wild type. In two patients the 184th aa was leucine, and in one patient there was a mixed pattern of threonine, isoleucine, proline, and leucine. The 204th aa in the C domain of the three patients was valine. Other domains, F, D and E showed no nucleotide and amino acid mutations.

Discussion

We administered entecavir monotherapy for more than 3 years in 4 patients with chronic HBV infection who had previously showed break-

Table 2. The analysis of amino acids in the HBV polymerase region by the nucleotide sequences of HBV detected from three patients with entecavir resistance at the start of the therapy and at the time resistant viruses emerged. Patient No. 1 did not have entecavir resistance and thus sequencing was not performed.

Patient	Weeks when resistant mutation confirmed	HBV-DNA Polymerase region							
		A Domain		B Domain				C Domain	
		80		180		184		204	
(No.)		0w	Mutant	0w	Mutant	0w	Mutant	0w	Mutant
2	152	I/L	L	L	M	T	L	I/M	V
3	104	I/L	L	L	M	T	T/I/P/L	M/I/V	V
4	130	I	L	L	M	T	L	I	V

I, isoleucine; L, leucine; M, methionine; P, proline; T, threonine; V, valine.

through hepatitis induced by prior lamivudine administration. The administration of entecavir for these patients caused the emergence of entecavir-resistant viruses in three of the four patients, although severe liver dysfunction did not yet occur. Breakthrough hepatitis can often be fatal, especially for patients with progressed liver diseases, so we administered entecavir when virologic breakthrough was confirmed.

It was reported that lamivudine-resistant mutant HBV nucleotide sequences showed changes of methionine to isoleucine or valine at the 204th aa, leucine to methionine at the 180th aa, leucine to isoleucine at the 80th nucleotide and valine to leucine at the 173rd aa (Stuyver et al. 2001).

In this study, we determined the HBV nucleotide sequences obtained from three patients, and analyzed the amino acids in the polymerase region both before the administration of entecavir and at the 152nd, 104th and 130th week, respectively. As a result, we found at first that in all of them the resistance to lamivudine was related to the change from leucine to isoleucine at the 80th aa. They then showed the lamivudine resistant mutations after the administration of entecavir monotherapy (180th and 204th aa). It has been reported that susceptibility to entecavir decreases when mutations at both the 180 and the 204th aa emerge (Tenney et al. 2004), which is in accord with our present results.

At the 184th aa, as previously reported concerning its relation to entecavir resistance (Tenney et al. 2004), all of the patients showed the mutation from threonine to leucine, although patient No. 4 showed a mixed pattern.

Additionally, we found a new change at the 80th aa, which showed an inverse pattern of the lamivudine-resistant mutant (Yim et al. 2006). Actually, 80th mutation found at the initiation of entecavir treatment return to be wild type when entecavir-resistant mutants developed (Table 2). The results might suggest that some of the lamivudine-related mutations could disappear with the cessation of lamivudine.

We suppose that the emergence of resistance to antiviral agents in patients with sequential administration of entecavir monotherapy for chronic HBV after the emergence of a lamivudine-resistant mutant virus might have the closest relationship with the 184th aa and, additionally, the 80th aa, at least in HBV genotype C, the most common genotype in Japan.

In addition to lamivudine, adefovir has also been reported to develop resistant mutants (Fung et al. 2005). Sequential therapy for patients with chronic HBV infection could result in the emergence of multi-drug resistant mutants (Yim et al. 2006).

The discontinuation of lamivudine before the emergence of a mutant virus might be useful to prevent breakthrough hepatitis; nevertheless, the reactivation of hepatitis is sometimes fatal, especially for patients with progressed liver diseases, such as liver cirrhosis (Honkoop et al. 2000). These findings underscore the difficulties in determining the best therapy for patients with chronic HBV infection.

The results of our study suggested the possibility that entecavir monotherapy should not be the first choice for the treatment of patients with chronic HBV infection who have developed a lamivudine-resistant virus.

Further studies regarding optimal patient selection, treatment duration, and the dosages of antiviral agents including entecavir are warranted.

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CASE REPORT

Fulminant hepatic failure in a case of autoimmune hepatitis in hepatitis C during peg-interferon-alpha 2b plus ribavirin treatment

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Abstract

A 27-year-old Caucasian female with hepatitis C virus (HCV) infection treated with interferon (IFN) who developed severe autoimmune hepatitis (AIH) is described. The infecting viral strain was of genotype Ib and the pre-treatment HCV viral load was at a high level. The patient was treated with pegylated IFN-alpha 2b and ribavirin, and her HCV-RNA became negative at wk 12, but after that she developed fulminant hepatic failure. The patient recovered after steroid pulse therapy consisting of methylprednisolone 1000 mg/d for three days which was administered twice. A needle liver biopsy revealed the typical pathological findings of AIH.

Key words: Autoimmune hepatitis; Interferon; Ribavirin; Hepatitis C virus; Anti-viral therapyA; Acute liver failure

Kogure T, Ueno Y, Fukushima K, Nagasaki F, Inoue J, Kakazu E, Matsuda Y, Kido O, Nakagome Y, Kimura O, Obara N, Wakui Y, Iwasaki T, Shimosegawa T. Fulminant hepatic failure in a case of autoimmune hepatitis in hepatitis C during peg-interferon-alpha 2b plus ribavirin treatment. *World J Gastroenterol* 2007; 13(32): 4394-4397

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INTRODUCTION

The most effective treatment for hepatitis C virus (HCV) infection is pegylated interferon (peg-IFN) in combination with ribavirin (RBV)[1]. This treatment exerts a variety of immuno-modulatory effects and may unmask the underlying autoimmune diseases such as autoimmune thyroiditis and rheumatoid arthritis[2,3]. In a similar fashion, interferon (IFN) was reported to induce or exacerbate autoimmune hepatitis (AIH)[4-7]. We describe a patient who developed fulminant hepatic failure with AIH during the treatment with peg-IFN and RBV. The patient had no prior history of autoimmune diseases and the severe hepatitis occurred after the HCV RNA level decreased to below the detection limit.

CASE REPORT

Patient

The patient was a 27-year-old Caucasian woman from South Caucasian Country. She had never consumed alcohol. She had not received blood transfusions or undergone surgery. Her sister and mother were receiving treatment for Basedow's disease, but she had no manifestation of Basedow's disease or other autoimmune diseases.

Present illness

She was found to be infected with hepatitis C virus during her pregnancy and was referred to our department in October 2005. She hoped to receive IFN therapy to reduce the risk of transmission to her second baby. The HCV genotype was Ib and the HCV RNA viral load was 4600 kIU/mL (high range) (Table 1). Alanine aminotransferase (ALT) was 47 IU/L (normal < 35 IU/L). The markers of hepatic functional reserve were within normal ranges and ultrasonography of the liver indicated no abnormal findings. After delivery, interferon therapy was commenced with peg-IFN-alpha 2b (Peg-Intron®, Schering-Plough Corp., NJ, USA) and RBV (Rebetol®, Schering-Plough) on January 30, 2006. The clinical course of this anti-viral therapy is illustrated in Figure 1. The initial doses consisted of 100 μg peg-IFN per week and 600 mg RBV per day. This therapy was well tolerated with minimal adverse effects and HCV RNA became negative at wk 12. Around the same time, a rise of transaminase was noted: ALT, 83 IU/L (normal < 35 IU/L); and asparate aminotransferase (AST), 56 IU/L (normal < 30 IU/L). The dosage of peg-

Table 1 Laboratory findings before interferon treatment

WBC	5300 /μL	Total bilirubin	0.6 mg/dL	HCV-Ab	Positive
Hb	11.0 g/dL	Direct bilirubin	0.1 mg/dL	HCV-RNA	4600 kIU/L
Plt	452 × 10^3 /μL	Total protein	7.7 g/dL	HCV genotype	Ib
PT	101.8%	Albumin	4.2 g/dL	HBs-Ag	Negative
		BUN	16 mg/dL	HBs-Ab	Negative
		Creatinine	0.8 mg/dL	ANA	× 80
AST	39 IU/L	Uric acid	3.4 mg/dL	ASMA	Negative
ALT	47 IU/L	Na	140 mEq/L	AMA	Negative
LDH	176 IU/L	K	4.5 mEq/L	IgG	1635 mg/dL
ALP	278 IU/L	Cl	104 mEq/L	IgA	108 mg/dL
γ-GTP	11 IU/L	Total cholesterol	133 mg/dL	IgM	283 mg/dL
ChE	386 IU/L	Glucose	77 mg/dL	HLA-DR allele	DR17/DR13 DRB1*0301/*1302

WBC: white blood cell; Hb: hemoglobin; Plt: platelet; PT: prothrombin time; AST: asparate aminotransferase; ALT: alanine aminotransferase; LDH: lactate dehidrogenase; ALP: alkaline phosphatase; γ-GTP: gamma glutamil transpeptidase; ChE: choline esterase; BUN: blood urea nitrogen; HCV: hepatitis C virus; HBs-Ag: hepatitis B virus surface antigen; HBs-Ab: hepatitis B virus surface antibody; ANA: antinuclear antibody; ASMA: anti-smooth muscle antibody; AMA: anti-mitochondrial antibody; Ig: immunoglobulin; HLA: human leukocyte antigen.

Table 2 Laboratory findings at onset of acute liver failure

WBC	5800/μL	BUN	5.0 mg/dL	ANA	× 160
Hb	12.2 g/dL	Creatinine	0.7 mg/dL	ASMA	Negative
Plt	290 × 10^3 /μL	Uric acid	2.8 mg/dL	LKM-1	Positive
PT	41.4%	Na	138 mEq/L	AMA	Negative
APTT	44.4 sec	K	3.9 mEq/L	IgG	2436mg/dL
		Cl	105 mEq/L	IgA	175 mg/dL
		Total cholesterol	175 mg/dL	IgM	280 mg/dL
AST	311 IU/L	Triglyceride	67 mg/dL		
ALT	280 IU/L	Glucose	127 mg/dL		
LDH	245 IU/L	CRP	0.2 mg/dL	HCV-RNA	13 kIU/l
ALP	571 IU/L			HBs-Ag	Negative
γ-GTP	108 IU/L	Tyrosin	262 μmol/L	HBs-Ab	Negative
ChE	124 IU/L	BCAA	313 μmol/L	HBc-Ab IgM	Negative
Total bilirubin	5.5 mg/dL			HAV	Negative
Direct bilirubin	3.3 mg/dL	TSH	0.499 μIU/mL	EBV	Negative
Total protein	7.4 g/dL	T3	1.66 pg/mL	HSV	Negative
Albumin	3.1 g/dL	T4	0.55 ng/dL	CMV	Negative

APTT: activated partial thromboplastin time; CRP: C-reactive protein; BCAA: branched-chain amino acid; TSH: thyroid-stimulating hormone; LKM-1: antibody to microsomes type 1; HBc-Ab: hepatitis B virus core antibody; HAV: hepatitis A virus; EBV: Epstein-Barr virus; HSV: herpes simplex virus; CMV: cytomegalovirus.

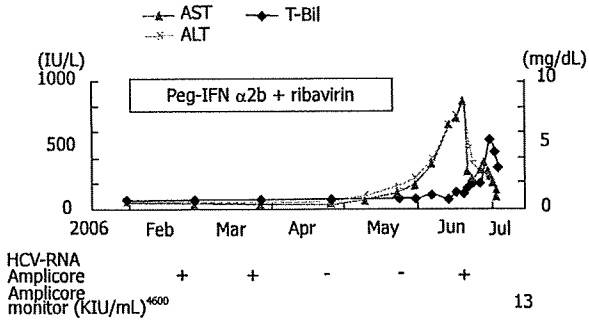

Figure 1 Clinical course of peg-interferon (peg-IFN) plus ribavirin therapy for hepatitis C virus infection. ALT: Alanine aminotransferase; AST: asparate aminotransferase; T-Bil: total bilirubin.

IFN was reduced to 80 μg at wk 17, and 60 μg at wk 18 because of the persistent increase of ALT, and finally, the treatment was discontinued at wk 19. Glycyrrhizinate (Stronger neo-minophagen C®, Minophagen Pharmaceutical Co., Ltd., Tokyo, Japan) was administrated intravenously, and ALT indicated a decreasing tendency (peak ALT was 815 IU/L). After that, she showed jaundice and a prolongation of the prothrombin time (PT) was noted. Her human leukocyte antigen (HLA)-DR serotypes were DR17 and DR13. She was admitted to our hospital on June 30, 2006.

Physical findings on admission

Physical examination revealed: height 167 cm, weight 56 kg, blood pressure 118/64 mmHg, body temperature 37.3℃, and clear consciousness. The bulber conjunctiva was slightly icteric. No peripheral edema, vascular spiders, and flapping tremor were observed.

Clinical course

On admission, elevations of transaminases (ALT 280 IU/L, AST 311 IU/L) (Table 2) and total bilirubin (5.5

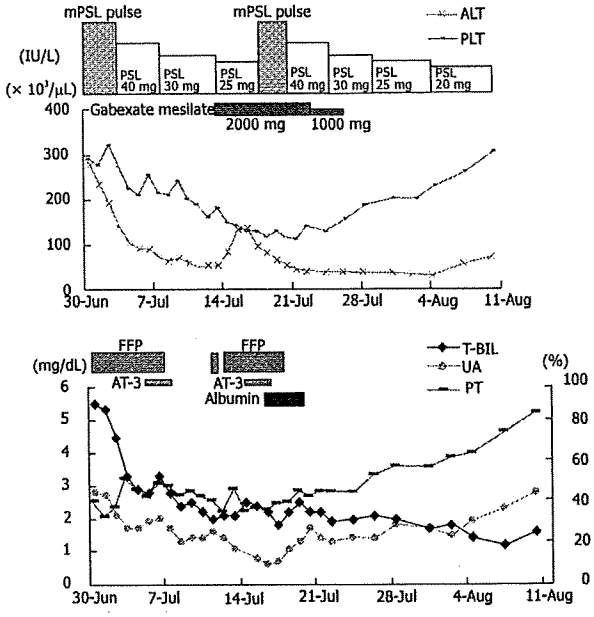

Figure 2 Clinical course of fulminant hepatic failure. PSL: prednisolone; mPSL: methylprednisolone; UA: uric acid; PT: prothrombin time; FFP: fresh frozen plasma; AT-3: antithrombin Ⅲ.

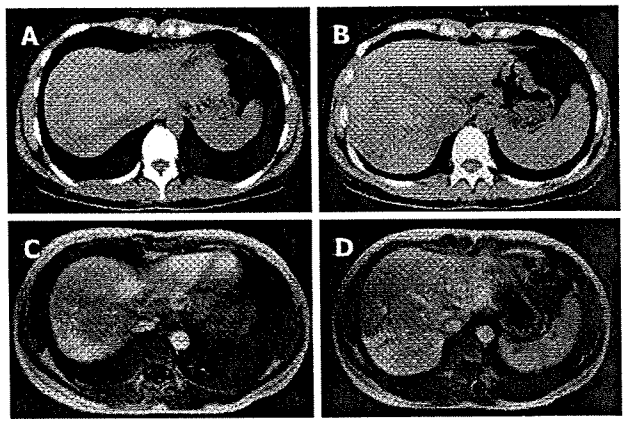

Figure 3 A, B: Abdominal CT at the onset of hepatic encephalopathy; C, D: Enhanced abdominal MRI.

mg/mL) and decrease of blood urea nitrogen (BUN), uric acid (UA), and PT were detected. Hepatitis A, hepatitis B, cytomegalovirus, herpes simplex, and Epstein-Barr virusesl were negative. Anti-nuclear antibody (ANA) was × 160 (normal < × 80) and immunoglobulin G (IgG) 2436 mg/mL (normal < 1695 mg/mL), which were × 80 and 1635 mg/mL before the IFN treatment. Antibody to liver/kidney microsomes type 1 (LKM-1) was positive. Steroid pulse therapy (methylpredonisolone 1000 mg/d for three days) was performed (Figure 2). Nevertheless, hepatic encephalopathy appeared on July 3. Abdominal computed tomography indicated massive necrosis of hepatocytes (Figure 3). Brain magnetic resonance imaging showed no abnormal findings. The findings of her electric encephalogram were typical of metabolic encephalopathy. Total parenteral nutrition was started. The protein load was restricted and lactulose enemas were performed. After

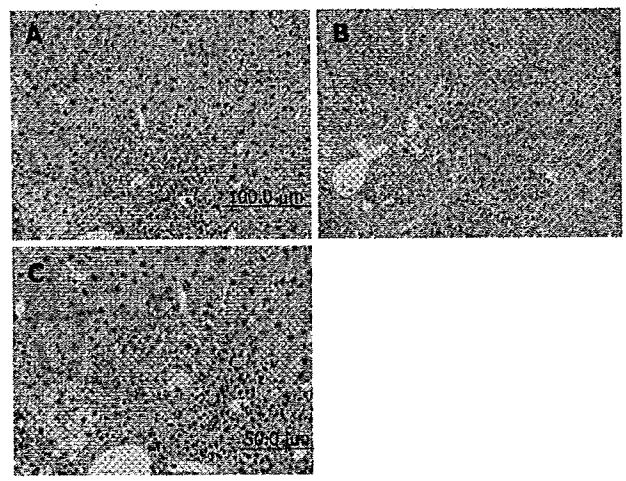

Figure 4 Liver biopsy showing interface hepatitis with infiltration of plasma cells and rosette formations. A, C: Hepatoxyline-Eosin staining; B: Elastica-Masson staining.

that, the hepatic encephalopathy improved gradually. The ALT level rose again on July 14, and steroid pulse therapy was administered again. The ALT showed a decreasing tendency again, after which she started oral intake. PT, BUN, and UA had improved gradually. She was discharged from our hospital on August 10.

A needle liver biopsy was performed on October 16 and it showed the typical pathological features of autoimmune hepatitis. Interface hepatitis with infiltration of inflammatory cells including plasma cells and rosette formation, were found but no biliary change was noted (Figure 4). The serum ALT levels were normalized over the following six months by 10 mg of prednisolone per day.

DISCUSSION

IFN therapy for patients with HCV infection has been reported to induce or exacerbate AIH[4-7]. In the present case, severe hepatitis occurred after HCV RNA had decreased to below the detection limit after treatment with peg-IFN with RBV for 12 wk. The patient presented the typical clinical features of serum aminotransferase elevation, positive ANA and LKM-1, and hypergammaglobulinemia, and responsiveness to glucocorticoid therapy. The pathological findings showed the typical features of AIH, which included interface hepatitis with infiltration of plasma cells, and rossete formation.

One of the explanations for the occurrence of autoimmunity in HCV patients is the loss of self-tolerance due to molecular mimicry between viral proteins and self-antigen[8]. HCV infection is known to be related to autoimmune disease, and chronic hepatitis C patients show autoantibodies such as ANA and LKM-1. HCV core protein has been reported to show homology with cytochrome P450IID6, which could be recognized by LKM-1 antibodies as seen in type 2 AIH[9,10]. Recently, AIH has been found to occur in patients during IFN therapy after liver transplantation for HCV liver cirrhosis[11,12].

In the present case, AIH occurred during IFN therapy and the patient developed fulminant hepatic failure when HCV RNA had decreased to below the detection limit. To our knowledge, there is no report of an HCV patient who developed fulminant hepatic failure after peg-IFN with RBV therapy. Peg-IFN with RBV is an established therapy for chronic hepatitis C patients but AIH should be considered as a potential complication of therapy leading to severe hepatitis. Especially, longer treatment duration (48 wk) and prolonged elevation of serum IFN levels in the pegylated-IFN could contribute to the development of autoimmune phenomenon. Liver biopsy in the early phase of acute liver injury to determine whether immunnosuppression therapy may be required for such patients. In Asians, the development of AIH during antiviral therapy is believed to be rare, although the potential risk should be taken into consideration if female young Caucasian case is treated like this report. However, the treatment of recurrence of HCV after cessation of antiviral therapy due to the emergence of autoimmune hepatitis like our case is a very difficult clinical decision to make. Probably, the interferon based antiviral therapy, the most standard anti-viral agent, could remain as the mainstream regimen for next decade. The balance between antiviral effects and possible autoimmune phenomena could be key factors as described previously[13,14]. Small molecules such as HCV protease inhibitor, either as monotherapy or combined with other small molecules, could be the first choice for the treatment of the current case in future.

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Extracellular Branched-Chain Amino Acids, Especially Valine, Regulate Maturation and Function of Monocyte-Derived Dendritic Cells[1]

Eiji Kakazu,* Noriatsu Kanno,† Yoshiyuki Ueno,[2]* and Tooru Shimosegawa*

The functions of dendritic cells (DCs) are impaired in patients with liver cirrhosis. It is well-known that cirrhotic patients show decreased levels of plasma branched-chain amino acids (BCAA). Although amino acids are associated with maintaining the cell structure and function in many organs, limited data are available regarding the role of amino acids including BCAA in the immune system. We aimed to investigate the roles of BCAA in the function of human monocyte-derived DCs (MoDC). CD14-positive monocytes (CD14 $^+$) were isolated from PBMC from healthy volunteers and hepatitis C virus (HCV) cirrhotic patients. In medium deprived of BCAA or valine, monocytes were able to differentiate into immature, but not into mature, DCs and showed weak expression of CD83. The deprivation of leucine or isoleucine did not affect this process. The MoDC allostimulatory capacity was significantly decreased in medium deprived of BCAA or valine ($p = 0.017$, $p = 0.012$, Bonferroni's analysis, respectively). Annexin V^{FITC}/propidium iodide staining showed that the DC yield and viability were not significantly different under any medium. Immunoblotting demonstrated that depletion of valine or leucine decreased phospho-S6 kinase expression. Valine increased dose-dependently the allostimulatory capacity and IL-12 production of MoDC from both healthy volunteers and HCV cirrhotic patients. An elevated extracellular concentration of valine could improve the DC function in cirrhotic patients. These data provide a rationale for nutrition therapy that could be beneficial to patients with cirrhosis. ***The Journal of Immunology,*** **2007, 179: 7137–7146.**

Hepatitis C virus (HCV)[3] induces chronic liver disease in hosts, which can eventually progress to liver cirrhosis and chronic liver failure. Combination therapy with peginterferon and ribavirin has been shown to result in a sustained virological response in ~50% of patients (1, 2), but others continue to be viremic and progress to advanced fibrosis and liver cirrhosis. The number of advanced liver cirrhotic patients has been increasing, but combination therapy is poorly tolerated under cirrhosis and the response rate is low (3). Bacterial infection, such as spontaneous bacterial peritonitis and pneumonia, is one of the most frequent causes of death in immune-compromised cirrhotic patients. In such cirrhotic patients, a decrease in the levels of plasma branched-chain amino acids (BCAAs) is one of the characteristic features (4, 5). BCAA comprise the three essential amino acids L-leucine, L-isoleucine, and L-valine. BCAA granules (a mixture of L-leucine, L-valine, and L-isoleucine) have been used to effectively reverse the hypoalbuminemia and hepatic encephalopathy in patients with decompensated liver cirrhosis (6, 7), but little is known about the impact of changes in the BCAA levels on the immune system (8). In previous cohort studies, the BCAA-supplemented groups demonstrated elevations in the absolute lymphocyte count (9, 10). In previous in vitro studies, the omission of a single BCAA from the medium of cultured lymphocytes resulted in the complete abolition of protein synthesis or proliferation (11–13). These findings simply reflect the fact that BCAA are essential cell components.

Recently, it has become clear that amino acids are not only important as substrates for various metabolic pathways, but also activate a nutrient-sensitive signaling pathway in synergy with insulin (14). The mammalian target of the rapamycin (mTOR) signaling pathway is one of the most representative pathways, and this pathway has been shown to act as a major effecter of cell growth and proliferation via the regulation of protein synthesis. The pathway is activated by BCAA, especially by leucine (15–17). mTOR was identified and cloned (18–20) shortly after the discovery of the two yeast genes, *TOR1* and *TOR2*, in the budding yeast *Saccharomyces cerevisiae* during a screening for resistance to the immunosuppressant drug rapamycin. Rapamycin, introduced to prevent allograft rejection, has been extensively studied for its effect on T lymphocytes, and is primarily known for its antiproliferative effect (21). Some studies have described that dendritic cell (DC) functions (viability, Ag uptake, cytokine production, and allostimulatory capacity) are impaired by rapamycin (22–24).

DCs are professional APC that initiate and mediate immune responses against pathogens and tumors. Typically, immature DCs capture and process Ags to peptides which are then presented in the context of MHC class II or class I molecules. They migrate to lymphoid tissues and present antigenic peptides to naive T cells. The mature DCs, which characteristically express CD83 (25), can

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[3] Abbreviations used in this paper: HCV, hepatitis C virus; BCAA, branched-chain amino acid; mTOR, mammalian target of rapamycin; DC, dendritic cell; MoDC, monocyte-derived DC; CCM, complete culture medium; FSC, forward light scatter; SSC, side light scatter; PI, propidium iodide; MFI, mean fluorescence intensity.

Table I. *Characteristics of study participants*[a]

Patient Number	Disease	Gender	Age (Years)	AST/ALT	Total Bilirubin	Albumin	PT-INR	PLT	HCV Ab	HCV-RNA	Genotype	Child-Pugh Classification
1	LC-C	F	57	63/96	1.4	4.2	1.12	109	+	500	1b	A
2		F	80	43/31	1.8	2.1	1.31	114	+	+	NA	B
3		M	69	45/44	1.1	3.7	1.12	192	+	170	2b	A
4		F	69	41/37	1.1	3.3	1.05	91	+	1100	1b	A
5		M	42	54/56	3.2	2.9	1.82	29	+	230	1b	C
6		M	30	61/62	0.9	3.6	1.27	129	+	150	1b	A
7		M	78	66/52	1.6	2.8	1.41	58	+	+	NA	B
8		M	56	44/37	3.9	2.9	1.93	46	+	+	1 (serotype)	C
9		F	69	47/25	0.8	3.2	1.61	87	+	+	NA	B
10		F	58	58/53	1.5	3.5	1.28	80	+	3400	1b	B
11		F	69	48/42	2	2.8	1.25	54	+	870	1b	B
12		M	59	127/127	3.5	2.6	1.4	81	+	210	1b	C
13		F	50	79/93	1.5	3.0	1.26	51	+	1700	1b	B
14		M	75	133/103	1.6	2.6	1.39	62	+	2600	1b	B
15	PSC	M	57	72/29	5.7	2.4	1.34	452	−	−		C
16	Non-B, non-C	M	62	28/26	1	4.2	1.19	113	−	−		A
17	Budd-Chiari syndrome	M	60	38/49	2.3	4.3	1.32	66	−	−		B
18	PBC	F	53	90/61	2.7	3.2	1.26	51	−	−		B
19	NASH	F	58	90/99	1	3.8	1.23	118	−	−		A
20	NASH	F	68	76/48	2.2	3.0	1.8	129	−	−		B

[a] LC-C, Liver cirrhosis due to HCV; PSC, primary sclerosing cholangitis; PBC, primary biliary cirrhosis; NASH, nonalcoholic stetohepatitis; NA, not available; PLT, platelet counts ($\times 10^3/\mu l$); PT-INR, prothrombin time-international normalized ratio; AST/ALT, aspartate amino transferase/alanine amino transferase.

rapidly activate other innate immune cells including NK and NKT cells through the production of immunomodulatory cytokines such as ILs IL-10 and IL-12. Human DCs can be generated in vitro from peripheral blood CD14-positive monocytes, termed monocyte-derived DCs (MoDC) (26). The ability of monocytes to differentiate into DCs was originally demonstrated by Sallusto and Lanzavecchia (27), who reported the generation of DCs from human peripheral monocytes after in vitro culture with GM-CSF plus IL-4. Despite in vitro experimental evidence on the potential of monocytes to differentiate into DCs, whether this process occurs under physiological conditions is still controversial. In patients with chronic hepatitis C, the function of MoDC has been studied by various groups. These studies described that MoDC of patients had lower allostimulatory capacity and IL-12 production than MoDC of healthy subjects (28–30). It is considered that HCV proteins impair the hosts' DC functions (31).

In this study, we demonstrated that BCAAs, especially valine, influenced the function of MoDC. Increasing the extracellular concentration of valine could improve the DC function in cirrhotic patients.

Materials and Methods

Patients and healthy volunteers

Liver cirrhotic patients with chronic HCV infection were diagnosed for persistent-positive HCV Ab and the presence of HCV-RNA in the serum. All patients had clinical and laboratory findings compatible with cirrhosis (Table I). Also, six nonviral cirrhotic patients and eight healthy volunteers were recruited to obtain MoDC. Written informed consent was obtained from each individual and the study protocol was approved by the Ethics Committee of Tohoku University School of Medicine (2003-326).

Monocyte isolation and DC generation

PBMC were separated from the peripheral blood of healthy volunteers and patients by centrifugation on a density gradient (Ficoll-Paque Plus; Amersham Biosciences). The CD14-positive monocytes ($CD14^+$) were isolated from PBMC using magnetic microbeads (Miltenyi Biotec). $CD14^+$ were cultured at a density of 3.0×10^6 cells/well in 24-well flat-bottom plates (Falcon) for 5 days in the following culture medium: amino acid-free medium (D-MEM deprived of all amino acids) supplemented with 2 g/L BSA (Sigma-Aldrich), 1000 U/ml GM-CSF (PeproTech), 500 U/ml (human) IL-4 (PeproTech), 3.5 g/L glucose (Sigma-Aldrich), 6 g/L HEPES (Sigma-Aldrich), 1% insuline-transferin-selenium-X (Invitrogen Life Technologies), and variously conditioned amino acids. The culture medium containing all 20 kinds of amino acids was defined as the complete culture medium (CCM). The culture medium in which all amino acids—BCAA, valine, leucine, or isoleucine—were removed was defined as zero, ΔBCAA, ΔVal, ΔLeu, or ΔIle, respectively (Table II). At day 5, 20 ng/ml TNF-α (R&D Systems) and 500 ng/ml LPS (*Escherichia coli* 026:B6; Sigma-Aldrich) were added and the culture was continued for an additional 24 h. Also, CD40L (1 μg/ml) or poly I:C (30 μg/ml) was added at day 5 to evaluate the CD83 expression. The $CD14^+$ monocytes expressed high levels of CD14, HLA-DR, and CD86, and negligible levels of CD83. Immature DCs expressed lower levels of CD14 and CD86, and higher levels of HLA-DR and CD40, but they did not express CD83. Mature DC showed the up-regulation of costimulatory molecules (CD40, CD80, and CD86). These cells were also characterized by the induction of CD83 expression on their cell surface.

Table II. *Concentrations of amino acids in medium*[a]

	Zero	CCM	ΔBCAA	ΔVal	ΔLeu	ΔIle
Glycine	0	400	400	400	400	400
L-Alanine	0	400	400	400	400	400
L-Serine	0	400	400	400	400	400
L-Threonine	0	800	800	800	800	800
L-Cystine 2HCl	0	200	200	200	200	200
L-Methionine	0	200	200	200	200	200
L-Glutamine	0	4000	4000	4000	4000	4000
L-Asparagine	0	400	400	400	400	400
L-Glutamic acid	0	400	400	400	400	400
L-Aspartic acid	0	400	400	400	400	400
L-Valine	0	800	0	0	800	800
L-Leucine	0	800	0	800	0	800
L-Isoleucine	0	800	0	800	800	0
L-Phenylalanine	0	400	400	400	400	400
L-Tyrosine	0	400	400	400	400	400
L-Tryptophan	0	80	80	80	80	80
L-Lysine-HCl	0	800	800	800	800	800
L-Arginine-HCl	0	400	400	400	400	400
L-Histidine HCl-H_2O	0	200	200	200	200	200
L-Proline	0	400	400	400	400	400

[a] CCM contains 20 aa that are relevant to the makeup of mammalian proteins. Zero medium is deprived of all amino acids. ΔBCAA medium is deprived of BCAAs (L-valine, L-leucine, and L-isoleucine). ΔVal medium, ΔLeu medium, and ΔIle medium are each deprived of L-valine, L-leucine, and L-isoleucine, respectively. Amino acid concentrations are expressed in nanomoles per milliliter. We verified that there was no difference between the theoretical value and actual value by HPLC.

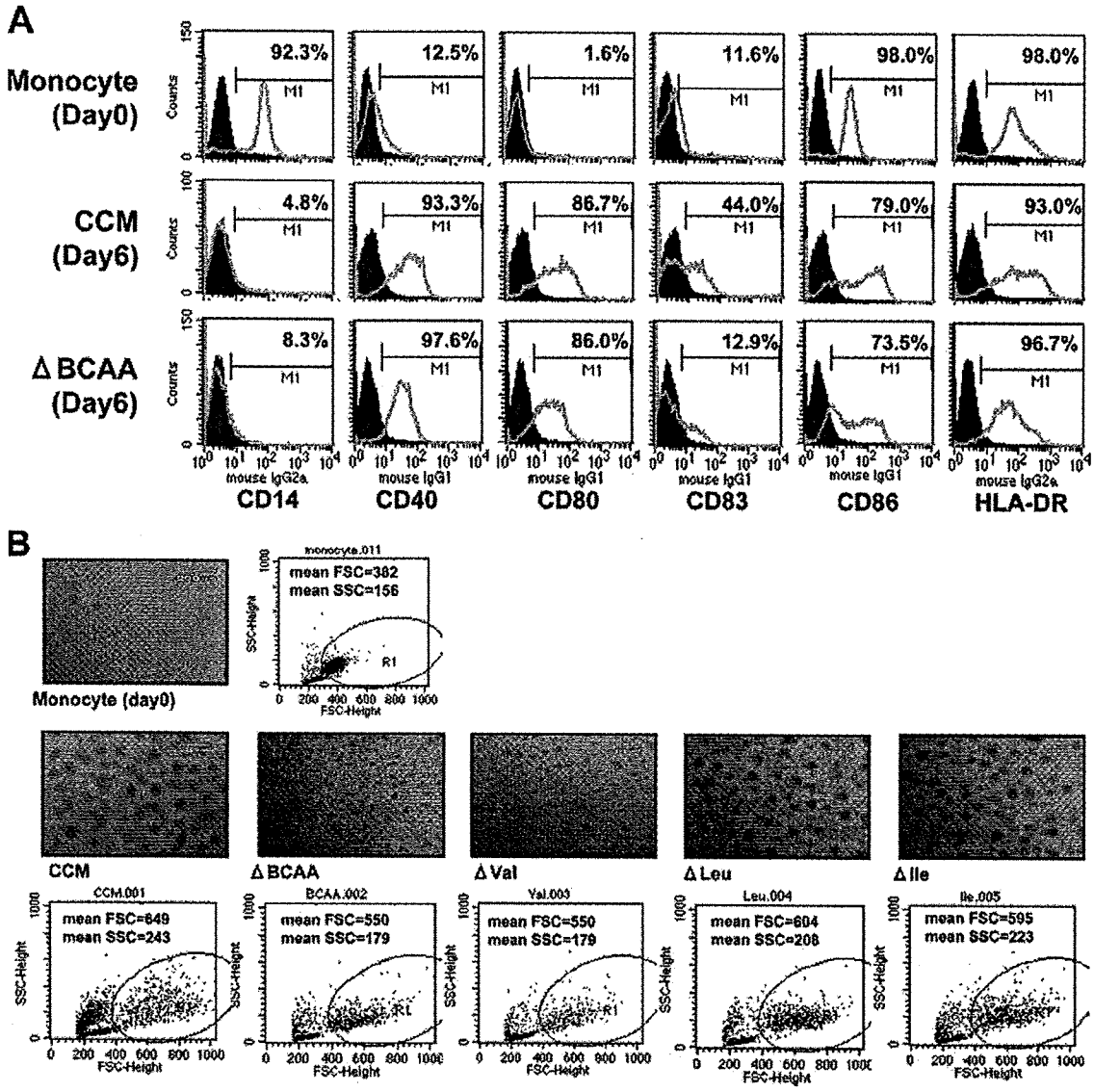

FIGURE 1. MoDC cultured in BCAA-deprived medium express low levels of CD83. We cultured monocytes under CCM or ΔBCAA medium in 24-well tissue culture plates for 5 days and exposed them to LPS and TNF-α for an additional 24 h. *A*, Cells were harvested on day 6 of culture, stained with different mAbs, and analyzed using flow cytometry. Cells were stained with FITC-labeled anti-CD14, -CD40, -CD80, -CD83, -CD86, and -HLA-DR. For the histogram figure, filled traces represent isotype-matched control Ab staining; open traces indicate a marker-specific Ab; percentages indicate positive cells. Results are representative of five experiments from five different donors. *B*, Influence of BCAA on microscopic appearance of monocytes undergoing DC differentiation under serum-free conditions. Day 6, cells in firmly adherent clusters in CCM, ΔLeu, and ΔIle, which are typical of DCs that mature in vitro. In contrast, cells in ΔBCAA and ΔVal formed much smaller clusters. Using a traditional FSC/SSC gate of the FACS plots, the figure expresses the mean FSC and SSC values of the MoDC population.

MoDC surface marker analysis and viability assay

On day 5 or 6 of culture, MoDC were harvested and labeled with FITC- or PE-labeled mAbs (anti-human CD14, CD40, CD80, CD83, CD86 HLA-DR, or the relevant isotype controls: BD Pharmingen), according to the manufacturer's directions. Briefly, cells (1×10^6) were incubated with 20 μl of Ab in a total volume of 100 μl (PBS) for 30 min at 4°C in the dark. Using a FACSCalibur (BD Immunocytometry Systems) flow cytometer, the cells were gated according to their size (forward light scatter (FSC)), granularity (side light scatter (SSC)), and surface marker expression and analyzed using the CellQuest (BD Immunocytometry Systems) program. On day 6, the viability of MoDC was determined using Annexin V^{FITC}, with dead cells identified by propidium iodide (PI) staining (Annexin V^{FITC} Apoptosis Detection kit; BioVision), according to the manufacturer's directions.

MLR and cytokine analysis

$CD14^+$ monocytes isolated by MACS were cultured at a density of 1.0×10^5 cells/well in 96-well round-bottom plates (Falcon) containing 200 μl of various concentrations of valine or leucine in medium supplemented with 1000 U/ml GM-CSF, 500 U/ml IL-4 for the generation of immature DCs. On day 5, immature DCs were induced to mature using 500 ng/ml LPS and 20 ng/ml TNF-α for 24 h. On day 6, the allostimulatory capacity of 5.0×10^4 irradiated DCs (3000 rad) was tested in a one-way MLR with normal, allogeneic T $CD4^+$ lymphocytes (isolated from PBMC using magnetic beads: 1×10^5cells/well) in duplicate or triplicate. Coculture cells were maintained for 4 days at 37°C in a 5% CO_2 humidified atmosphere. The proliferation rate of the cells was measured using an MTS assay (CellTiter 96 aqueous one-solution cell proliferation assay; Promega). Forty microliters of CellTiter 96 aqueous one-solution were added to each well. After 2 h of incubation, the UV absorbance of the solution was measured at a wavelength of 490 nm. All MTS assays were done in triplicate. Supernatants were collected on day 6 and immediately IL-12 (p40 plus p70) and IL-10 were determined by specific cytokine ELISA kits (Bender MedSystems) according to the manufacturer's instructions.