METHODS

Selection of patients. The patients enrolled in the present study were a subset of a well-characterized cohort of 166 patients with haemophilia who had received non-heated plasma-derived coagulation products before 1987 and had been observed regularly since 1995 at Ogikubo hospital (Tokyo, Japan). Plasma samples from patients with known HCV and HIV serological status were stored at –80 °C. Of these patients, 57 were positive and 109 were negative for anti-HIV. After exclusion of HCV-RNA-negative and interferon-treated patients, and those with a mixture or shift in HCV subtypes during the follow-up, 13 HCV-1a-RNA-positive patients (eight HIV-positive and five HIV-negative patients) were selected at random for this study. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the Ethics Committees of each institution. Every patient gave written informed consent to participate in the virological research.

Laboratory tests. Laboratory evaluation included complete blood cell count and serum transaminases [alanine aminotransferase (ALT)]. $CD4^+$ cell counts were examined by fluorescence-activated cell sorting at SRL Inc. (Tokyo, Japan). Serum HCV-RNA levels and HIV-RNA levels were measured by a commercial PCR assay (Amplicor HIV-1 Monitor and Amplicore HCV monitor; Roche Diagnostics). The detection limits of PCR for HCV-RNA and HIV-RNA were 500 IU ml^{-1} [0.5 kilo international unit (KIU) ml^{-1}] and 50 copies ml^{-1}, respectively.

HCV-RNA isolation and amplification from the core, E1 and E2 regions. Nucleic acids were extracted from serum samples using a SepaGene RV-R Nucleic Acid Extracting kit (Sanko Junyaku) in accordance with the manufacturer's protocol. Viral RNA was reverse-transcribed to cDNA using SuperScript II RNase H^- Reverse Transcriptase (Invitrogen) and random hexamer primer (Takara Shuzo) as described previously (Ohno *et al.*, 1997).

Partial core, E1 and E2 fragments were amplified by using PCR with primers as described previously (Tanaka *et al.*, 2002). To reduce the number of artificial substitutions arising during PCR, Platinum *Pfx* DNA Polymerase (Invitrogen) with a very high fidelity was used.

Cloning and sequencing of cDNA. The amplified products were ligated into pCR-Blunt II-TOPO Vector and used to transform DH5-α high-efficiency competent cells according to the manufacturer's protocol (Invitrogen). The plasmid DNA was purified using the QIAprep Spin Miniprep kit (Qiagen) and the presence of the inserts confirmed by digestion with *Eco*RI. Sequencing was performed on more than 10 clones per patient at the baseline (1995–1997) and the end point (2002–2003). All clones were sequenced with Prism Big Dye (Applied Biosystems) in an ABI 3100 DNA automated sequencer.

Construction of phylogenetic trees. Nucleotide sequences of HCV were aligned by using the program CLUSTAL_X and molecular evolutionary analyses were conducted using Molecular Evolutionary Genetic Analysis software (MEGA version 3.0; Kumar *et al.*, 2001). The MEGA algorithms were used to calculate the mean Tamura–Nei pairwise distance for all clones as well as a matrix of Tamura–Nei pairwise distances for each patient. To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 1000 times.

Genetic diversity of HCV over a time course. Two approaches were used to infer the genetic diversity of HCV in each patient. In the first approach, total evolutionary distances among a heterogeneous viral population were compared between the baseline and end point for each patient in the phylogenetic tree. The phylogenetic tree of genetic diversity was constructed by using the maximum-likelihood (ML) method and the ancestral sequence was inferred at every node using the ML method (Yang *et al.*, 1995). As the evolutionary distance in each branch, the number of synonymous substitutions per synonymous site (synonymous distance) was estimated by the modified Nei–Gojobori method. Total synonymous distances were assumed to represent the genetic diversity of a heterogeneous viral population in each patient.

The other approach is the coalescence theory based on estimation of the genetic diversity. A consensus sequence based on the sequences of all HCV clones isolated from each patient was used as an outgroup to locate the position of the root in each phylogenetic tree. The topology of the phylogenetic tree was estimated by the neighbour-joining method (PHYLIP). Based on the topology, we constructed the phylogenetic tree and inferred the evolutionary rate by the ML method under the premise of the molecular clock (TipDate) (Rambaut, 2000). Based on the trees and the evolutionary rates estimated by TipDate, the coalescent analysis of genetic diversity was conducted for each patient using the Genie v3.5 software (Pybus *et al.*, 2001; Pybus & Rambaut, 2002). In brief, time t was transformed to year using the HCV molecular evolutionary rate, assuming the sample-collection time to be the present. Function $N(t)$ (effective numbers of HCV infections through time) was estimated by the ML method to infer the genetic diversity of HCV (Pybus & Rambaut, 2002). Although there are several models to infer $N(t)$, the best-fit model was different among patients. Therefore, we chose a simplified model in which the genetic diversity was assumed to be exponentially increased over time (expansion model).

Identification of positively selective regions. Positively selected regions were identified using the modified method of Suzuki & Gojobori (2001). In brief, a phylogenetic tree of sequences from HCV clones was reconstructed in each patient by the ML method. The ancestral sequence was inferred at every node in the phylogenetic tree using the ML method (Yang *et al.*, 1995). Then, synonymous and non-synonymous substitutions throughout the phylogenetic tree were estimated in each branch for each codon site. Here, to see the differences in selective pressure for HCV between the HIV-positive and -negative groups, we independently summed the total numbers of synonymous (N_s) and non-synonymous (N_n) substitutions occurring at each codon site of the HCV clones from either eight patients infected with HIV or five patients without HIV infection. The mean numbers of synonymous (C_s) and non-synonymous (C_n) sites were calculated for each codon site by the modified Nei–Gojobori method. The genetic distance of synonymous (d_S) and non-synonymous (d_N) was calculated as N_s/C_s and N_n/C_n, respectively. Although the ratio d_N/d_S is usually used for estimating selective pressure, we used $(d_N+0.5)/(d_S+0.5)$ ratio instead in the present study, because no synonymous substitution was found in several codon sites. The ratio was calculated along with the sequence by the sliding-window analysis. Each window size consisted of three codons.

RESULTS

Comparison of clinical characteristics between HCV patients with and without HIV infection

When we compared clinical data between HCV patients with HIV (HIV-positive group) and without HIV (HIV-negative group), there were no significant differences of mean age, sex, putative duration of HCV infection or mean peak ALT levels (116 vs 146) (Table 1). Changes of ALT levels also were not different between these two groups. Mean peaks of HCV-RNA levels in the HIV-positive group (2300 ± 668 KIU ml^{-1}), however, were significantly higher than those in the HIV-negative group (936 ± 423, $P=$

Table 1. Clinical characteristics among HCV patients in this study

All patients are male. HCV genotype of all patients is 1a. LC, Liver cirrhosis; ALT, alanine aminotransferase; NT, not tested; +, positive; −, negative.

ID	Age	Putative date of HCV infection	LC	HIV	AIDS	HIV-RNA (copies ml^{-1})	CD4 at baseline (μl^{-1})	HCV-RNA (KIU ml^{-1})			ALT (U l^{-1})	
								Range	Peak	Increase ×2	Range	Increase ×2
NT211	29	1982	−	+	+	130 000	20	130–2100	2100	yes	20–156	yes
GM248	39	1986	−	+	−	23 000	110	290–1200	1200	yes	34–96	yes
OT33	34	1982	−	+	−	2300	286	170–2000	2000	yes	40–43	no
HH127	33	1980–1982	−	+	−	22 000	270	330–3200	3200	yes	27–168	yes
TA92	32	1984	−	+	−	98 000	271	1300–2900	2900	yes	29–34	no
KY48	31	1980–1983	−	+	−	55 000	242	730–2700	2700	yes	66–213	yes
NK112	28	1982	+	+	+	100 000	27	2300–2600	2600	yes	16–28	no
YY321	27	1987	−	+	+	95 000	35	1200–1700	1700	no	181–186	yes
KK202	19	1987	−	−	−	NT	NT	310–1600	1600	yes	21–41	no
KN201	45	1982	+	−	−	NT	NT	300–710	710	no	38–98	no
TS246	20	1984	−	−	−	NT	NT	230–1000	1000	yes	27–37	no
SH265	20	1985	−	−	−	NT	NT	340–470	470	no	130–470	yes
ST251	26	1984	−	−	−	NT	NT	590–900	900	no	38–83	yes

0.0019), which is consistent with previous reports (Eyster *et al.*, 1994). Additionally, seven of eight patients in the HIV group had HCV-RNA elevation more than twice during follow-up, whereas only two patients in the HIV-negative group had HCV-RNA elevation.

For the eight HCV patients in the HIV-positive group, HIV-RNA and CD4 are shown in Table 1. Three of the patients had already developed AIDS and had very low CD4 counts (20, 27, 35 μl^{-1}), and the remaining five patients with HIV also had relatively low CD4 levels (110–286) at the baseline (1995–1997) before initiating highly active anti-retroviral therapy (HAART). Thereby, all HIV-infected patients studied were considered to be in an immunity-suppressed condition. Four patients with a CD4 count less than 200, including the three AIDS patients, received anti-HIV treatments.

Long-term intra-host diversity of HCV evaluated on distinct genomic regions

It has been shown previously that the genetic diversity of HCV changes in an oscillatory manner during the natural course of the infection (Devereux *et al.*, 1997). Taking into account that the genetic diversity of HCV analysed at a single time point might not accurately reflect the dynamic profile of the population over time, we have examined 26 serum samples collected from 13 patients at two distinct time points with intervals of at least 6 years (6–8 years). At least 11 HCV clones were isolated from a single patient at the baseline (1995–1997) and at the end point (2002–2003) of the follow-up. Overall, 325 HCV clones were thus isolated and analysed. All of them belonged to genotype 1a. Phylogenetic relation of the HCV clones isolated from all patients is shown in Fig. 1. Assuming that HCV is composed of a heterogeneous viral population, which is evolving throughout time in a given host (carrier), we aimed to estimate the size and heterogeneity of the population. Two different methods were used to attain this aim.

First, we directly compared the genetic diversity of a heterogeneous viral population between the two time points. To do that, we estimated totals (for all patients in each of the two groups) of intra-host synonymous distances at each of the two time points. This estimation was done independently in both E1 and E2 genomic regions (Fig. 2). The increased difference from the baseline to the end point between the HIV-positive and HIV-negative groups was tested by the regression analysis, and the genetic diversity of the HIV-positive group is significantly higher than that of the HIV-negative group (P=0.043).

Second, the coalescent analysis of genetic diversity of HCV was conducted for each patient. Further, mean curves of the effective numbers of HCV infections were compared between HIV-positive and -negative groups (Fig. 3). Although the estimated mean number was initially relatively lower in the HIV-positive group, the rapid change to exponential growth, was observed several years after HIV infection in this group, whereas in the HIV-negative group, the effective number was gradually increasing throughout the period of time. The difference of exponential growth is significant (P=0.04). Hence, the result obtained by either method indicated the HIV-positive group to have higher genetic diversity of the heterogeneous viral population than the HIV-negative group, suggesting that this was due to the exposure of HIV infection.

Putative positively selective regions in the E1 and E2 regions

Since the higher genetic diversity of HCV was observed in HIV-positive patients, we further examined genetic evi-

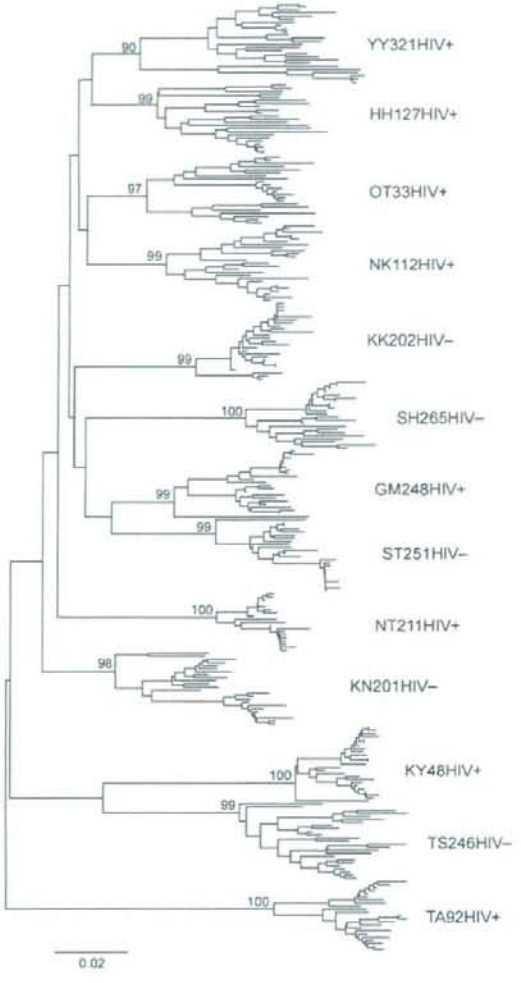

Fig. 1. A phylogenetic tree of E1 and E2 regions of the 325 HCV clones isolated from 13 patients. The significant phylogenetic cluster was observed in each of the eight HIV-positive (HIV+) and five HIV-negative (HIV−) patients. Numbers at nodes indicate bootstrap values of 1000 replications.

dence of the selective immune pressure in both groups. Selective immune pressure was estimated in each, E1 and E2, gene. Some differences were observed between the HIV-positive and -negative groups (Table 2, Fig. 4). Immune epitopes (11 aa segments in the E1 and 5 aa segments in the E2 region) that were observed only in the HIV-negative group might have relatively weak antigenicity. Some of the segments were previously recognized as HCV-specific potential immunogenic targets such as

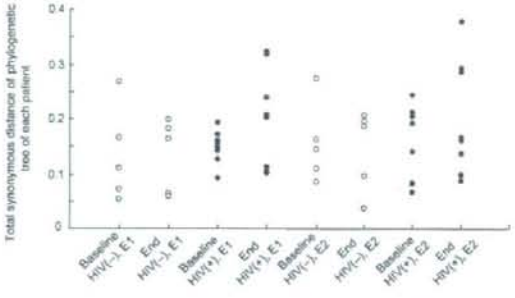

Fig. 2. Total synonymous distances of the phylogenetic tree of each time point. *y*-axis indicates total synonymous distance of the phylogenetic tree constructed by the sequences isolated at each time point. *x*-axis indicates two time points [baseline and end point (End)] and two groups [HIV-positive (HIV+) and -negative (HIV−)]. Each dot represents a patient.

cytotoxic T lymphocyte (CTL) epitopes (URL: http://hcv.lanl.gov/content/immuno/tables/ctl_summary.html), indicating that the positively selected segments estimated in the present study are associated with the immune response. On the other hand, positively selected segments around the hypervariable region (HVR1) regardless of HIV infection should have strong antigenic epitopes, suggesting little influence of the HIV coinfection on the natural immune selection targeting this region.

DISCUSSION

A previous meta-analysis showed a significantly elevated relative risk of severe liver disease in patients coinfected

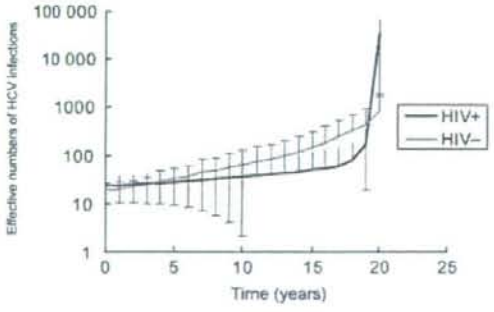

Fig. 3. The mean effective numbers of HCV infections in HIV-positive (HIV+) and -negative (HIV−) groups over the years from the baseline. Two lines indicate the dynamics of the mean effective numbers of HCV infections (*y*-axis) estimated in the E2 region and the bars indicate standard deviations. *x*-axis indicates number of years from the end point.

Table 2. Putative positively selective regions

Unknown epitopes identified only in the HIV-negative group are shown in bold.

Region	PS value*	HCV with HIV	HCV without HIV		
E1	PS>1.0	366–368	**192–195**,	**203–205**,	212–215,
			223–229,	234–237,	**245–247**,
			255–257,	295–299,	**346–349**
			353–358,	**359–361**	
E2	PS>1.0	383–412, 414–418, 434–438	382–410,	418–420,	432–436,
			443–446,	463–465,	**480–482**,
			486–488	496–500	

*PS (positive selection)$=(d_N+0.5)/(d_S+0.5)$.

with HIV and HCV (Braitstein *et al.*, 2004; Graham *et al.*, 2001). Another cohort study of HIV/HCV-coinfected patients also indicated association of low CD4 cell count, alcohol consumption rate and age at HIV/HCV coinfection with acceleration of the liver fibrosis (Benhamou *et al.*, 1999). The increased HCV replication in the HIV/HCV-coinfected patients would induce an intermediate immune response that is large enough to induce hepatic cell destruction and fibrosis but not enough to eradicate the virus from its reservoirs (Lai *et al.*, 2003; Poynard *et al.*, 2003).

In the present study, the genetic diversity of HCV was higher in the HIV-positive group compared with the HIV-negative group, which could be associated with either or both higher rate of HCV replication and HIV-associated immunosuppression, leading to less selective pressure on HCV in the HIV-positive group. The increased HCV replication by immune dysregulation in the HIV/HCV-coinfected patients might damage liver cells through apoptosis and other means such as cytokine disruption as reported previously (Puoti *et al.*, 2001). Although several

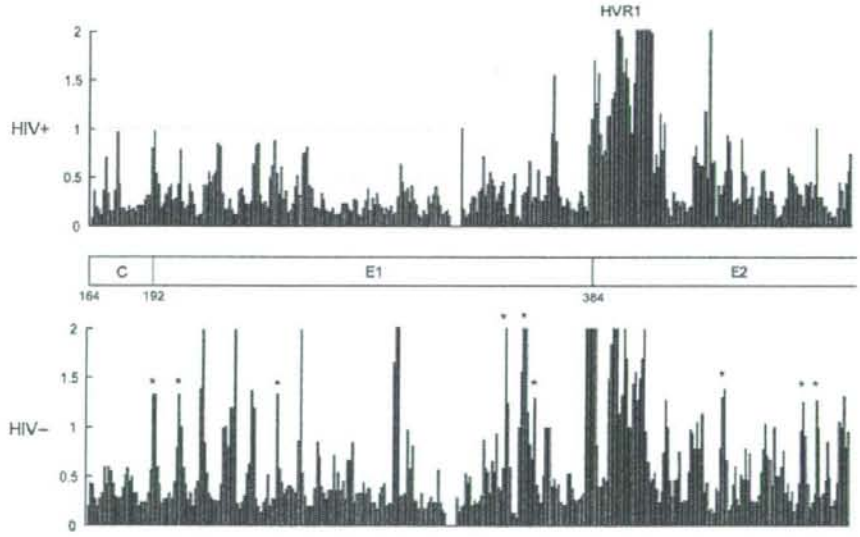

Fig. 4. Comparative histograms of the E1 and E2 regions of the HCV clones from HIV-positive (HIV+) and -negative (HIV−) patients. *y*-axis indicates $(d_N+0.5)/(d_S+0.5)$ ratio, columns exceeding value 1.0 represent the putative positively selective regions. Regions outside of any known CTL epitopes are indicated by asterisks and probably indicate unknown epitopes. *x*-axis indicates the schematic position in the HCV genes: C, core; HVR1, hypervariable region.

studies showed that HCV diversity decreases with the degree of HIV-related immunosuppression (Babik & Holodniy, 2003; Mao *et al.*, 2001; Martell *et al.*, 1992; Qin *et al.*, 2005; Roque-Afonso *et al.*, 2002; Toyoda *et al.*, 1997), this might be associated with the different parameters assessed for the genetic diversity; the conclusions in most previous studies were made by analysing total numbers of HCV clones and overall genetic distances at the amino acid level.

To assess the discrepancy with our results by coalescence-based estimation, we further examined the role of HCV-targeted immune pressure. The HCV nucleotide substitution pattern was compared between subjects with intact immune system versus those with HIV-associated immunosupression using a recently designed approach. Previous reports had used the mean of pairwise synonymous and non-synonymous distances within isolates (Ray *et al.*, 2000; Blackard *et al.*, 2004). Since most isolates do not have an independent evolutionary process, the mean may not represent overall genetic diversity of the heterogeneous viral population. To examine the non-redundant evolutionary process, recent methods have inferred the evolutionary process throughout the phylogenetic tree (Sheridan *et al.*, 2004; Suzuki & Gojobori, 2001; Hanada *et al.*, 2006). In the present study, we have applied a new approach to examine selection of HCV affected by HIV. The approach supported the theory that the diversified population of HIV-positive patients is due to less selective pressure and allows identification of specific regions indicating the presence of positive selection in HIV-negative patients compared with HIV-positive patients with immunosuppression. Although these positively selective segments, which were observed only in the HIV-negative group, might have relatively weak antigenicity, most of them were located inside potential immunogenic targets and others might be somewhat new antigen-recognition regions associated with HCV-specific immune responses. Interestingly, no influence by HIV coinfection was observed in the HVR1, which contains sequence-specific immunological B-cell epitopes that induce the production of antibodies restricted to the specific viral isolate (Kato *et al.*, 1993), indicating that the positively selected segments regardless of HIV infection should have strong antigenic epitopes. Taken together, our findings indicate that defenceless HCV clones that are extinct in usual conditions can survive in HIV-positive patients because of less immune pressure leading to HIV infection. Consequently, the genetic diversity of HCV will be greater in HIV-positive patients. In fact, the diversity of both synonymous and non-synonymous substitutions was larger in HIV-positive patients than HIV-negative patients (data not shown).

One limitation of the present study is the general lack of functional immunological data. In this study, HCV-specific CD4 ELISPOT responses were not detected in all subjects and CD8 cell counts were not measured. Therefore, we chose to use HIV load and CD4 cell counts as surrogate markers of immune suppression. Further investigations of HCV diversity in conjunction with HCV-specific cellular responses will be required when more-sensitive immunological assays are available. Another potential limitation of the study is that only two time points were sampled for each individual. However, as we inferred the evolutionary process based on the phylogenetic tree constructed using the number of clones that were isolated through the long period of follow-up (more than 6 years), we believe that the genetic diversity of the heterogeneous viral population may represent an actual evolutionary process.

In conclusion, HIV-positive patients have more diversified HCV populations than HIV-negative patients, possibly because of reduction of selective pressure from the immune system. The positively selective regions determined in this study might be antigen-recognition regions associated with HCV-specific immune responses.

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Prediction of the Efficacy of Antiviral Therapy for Hepatitis C Virus Infection by an Ultrasensitive RT-PCR Assay

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The efficacy of interferon therapy for hepatitis C virus (HCV) infection improved remarkably. However, virologic relapse occurs in a substantial proportion of patients with virologic response (defined as an HCV RNA level below 50 IU/ml at the end-of-treatment). A highly sensitive RT-nested PCR assay capable of detecting almost a single copy of HCV RNA and a real-time RT-PCR assay to quantify HCV RNA down to 120 copies per ml were developed. The RT-nested PCR assay showed that 1 IU of HCV RNA is equivalent to 12.2 copies. For 28 patients with virologic response (12 relapsers and 16 sustained virologic responders), week-4 and end-of-treatment plasma samples were retested. At week 4, HCV RNA was detected by the RT-nested PCR and qualitative COBAS Amplicor HCV version 2.0 in 8/9 (89%) and 6/9 (67%) samples from relapsers, and in 4/16 (25%) and 2/16 (13%) samples from sustained virologic responders, respectively. End-of-treatment samples with HCV-negative by the qualitative COBAS Amplicor were positive by the present assay in 4/12 (25%) of relapsing patients and 0/16 (0%) of sustained virologic responders. The viral levels detected by the present assay in the Amplicor-negative samples were 3.5–17.3 copies/ml, which is below the detection limit of COBAS Amplicor. In conclusion, the highly sensitive RT-nested PCR assay can predict sustained virologic response at week 4 and virologic relapse at the end-of-treatment more accurately than COBAS Amplicor, suggesting its usefulness in monitoring antiviral therapy for HCV infection. ***J. Med. Virol. 79:1113–1119, 2007.*** © 2007 Wiley-Liss, Inc.

KEY WORDS: hepatitis C virus; ultrasensitive RT-PCR; accurate prediction; residual HCV RNA

INTRODUCTION

Hepatitis C Virus (HCV) chronically infects over 170 million people worldwide. After acute infection, approximately 15% to 25% of infected individuals overcome the virus with resolution of the infection, while the remainders become chronically infected. Of individuals with chronic infection, 10% to 20% progress and thereafter 1% to 5% develop hepatocellular carcinoma [Cohen, 1999]. In Japan, the number of HCV-infected patients is estimated to be 3 million in a population of 120 million. It should be noted that over 90% of hemophilia patients had been infected with HCV and about 40% had been co-infected with human immunodeficiency virus from contaminated blood products in the early 1980s. They are now facing an increasing risk of developing end-stage liver disease.

Recently, treatment for chronic hepatitis C has made remarkable progress. Current protocols of combination therapy of peginterferon plus ribavirin have achieved high rates (40–63%) of sustained virologic response [Manns et al., 2001; Fried et al., 2002; Hadziyannis et al., 2004; Torriani et al., 2004], defined as a serum HCV RNA < 50 IU/ml at week 24 after the end-of-treatment. Since it was reported that patients who did not achieve an early virologic response (EVR), defined as a serum HCV RNA < 50 IU/ml at week 12, did not respond to further therapy [Davis et al., 2003], the consensus guidelines have recommended that therapy be discontinued for patients who did not achieve EVR [European Association for the Study of the Liver, 1999; National Institutes of Health Consensus Development Conference, 2002; Strader et al., 2004]. Moreover, to avoid side effects and save treatment costs, early identification of nonresponse or relapse is increasingly required. Although rapid virologic response, defined as a serum HCV RNA < 50 IU/ml at week 4, was reported to be highly associated with sustained virologic response, virologic relapse occurred at various rates (9–27%)

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among patients who achieved rapid virologic response [Jensen et al., 2006]. This may be explained by the fact that the detection limit of 50 IU/ml is not low enough to identify the patients with strong early virologic suppression that leads to sustained virologic response.

Several studies using highly sensitive HCV assays have shown that virologic relapse can occur in patients with a low level of residual HCV RNA at the end-of-treatment, which cannot be detected by COBAS Amplicor HCV version 2.0 (Roche Molecular diagnostics, Pleasanton, CA) [Sarrazin et al., 2001; Watkins-Riedel et al., 2004; Desombere et al., 2005; Gerotto et al., 2006]. However, early virologic assessment has not been fully evaluated with such highly sensitive assays. To evaluate accurately the relationships between EVR and sustained virologic response, and between the residual HCV RNA at the end-of-treatment and virologic relapse, a highly sensitive reverse transcription (RT)-nested PCR assay that detect almost a single copy of HCV RNA and a real-time RT-PCR assay with a wide quantitation range were developed. These techniques were used to retest plasma samples from patients with relapse or sustained virologic response who had virologic responses at the end-of-treatment.

MATERIALS AND METHODS

Patients and Samples

Plasma samples were collected from 28 patients who had been treated with interferon therapy at Ogikubo Hospital (Tokyo, Japan) and achieved virologic responses at the end-of-treatment; 12 relapsers and 16 sustained virologic responders. All patients were male hemophiliac patients and had been infected with HCV through contaminated blood products. The characteristics of patients are shown in Table I. There were no significant differences in age, sex, HIV-1 co-infection or alanine aminotransferase levels between relapser and sustained virologic responders groups. Although the patients with genotype 1 infection appeared to have a higher rate of relapse, a statistical difference was not obtained ($P = 0.13$). All plasma samples were stored at −60°C until analysis.

RNA Extraction

Total RNA was extracted from 500 μl of plasma using a QIAamp UltraSens Virus Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's recommendations with some modifications. Briefly, plasma was diluted with 500 μl of phosphate buffered saline and centrifuged at 18,000 rpm (35,000*g*) for 60 min. Most of the supernatant was discarded and the residual 30 μl and precipitate were resuspended with 300 μl of Buffer AR containing 5.6 μl of carrier RNA preheated at 60°C. The mixture was incubated for 10 min at 60°C using a mixing incubator. Thereafter, RNA was purified as recommended.

Reverese Transcription-Nested PCR

Primers. Reverse transcription (RT)-nested PCR was performed using two sets of primers targeting a region of the 5′ untranslated region of HCV. Reverse transcription (RT) was carried out with primer KC12, 5′-CTCGCAAGCACCCTATCAGGCAG-3′ (nucleotides (nt) 276–299, identical to primer KY78 designed by Roche Molecular Systems) [Young et al., 1993]. The primer pair for the first-round PCR consisted of forward primer KC11, 5′-CTCCCCTGTGAGGAACTACTGTCT-3′ (nt 24–51), and reverse primer KC12. The primer pair for the second-round PCR consisted of forward primer KC13, 5′-TCCCGGGAGAGCCATAGTG-3′ (nt 115–133) and reverse primer KC14, 5′-TCCAAGAAAGGACCC-3′ (nt 176–196).

RT-nested PCR. RT was carried out in a 20-μl solution of PCR Buffer II (10 mM Tris–HCl, 50 mM KCl), 3 mM $MgCl_2$, 10 mM of DTT, 0.5 mM of each dNTP, 0.5 μM of RT primer (KC12), 0.5 U of RNase inhibitor (Promega, Madison, WI), and 2.5 U of SuperScript III (Invitrogen, Carlsbad, CA). The mixture was incubated for 5 min at 60°C, 5 min at 55°C, 5 min at 50°C, and then 5 min at 45°C. A GeneAmp PCR system 9700 thermocycler and thin-walled MicroAmp reaction tubes (Applied Biosystems, Foster City, CA) were employed for RT and PCR. The first-round PCR was carried out using the whole RT product in a final volume of 50 μl containing 1× PCR Buffer II, 2.5 mM $MgCl_2$, 200 μM

TABLE I. Patient Characteristics

Category	Virologic relapsers	Sustained virologic responders
Number of cases	12	16
Age, median (in years) (range)	35 (24–69)	32 (20–66)
Sex, male/female	12/0	16/0
Genotype[a]		
1	5 (42%)	0 (0%)
2	2 (17%)	2 (40%)
3	5 (42%)	2 (40%)
4	0 (0%)	1 (20%)
HIV co-infection		
HIV positive	6 (50%)	6 (38%)
HIV negative	6 (50%)	10 (62%)
ALT level, median (in U/L) (range)[b]	115 (29–264)	120 (59–708)

[a]Of 16 cases with sustained virologic response, genotype was determined in five cases.
[b]ALT, Alanine aminotransferase.

each dNTP, 1 μM KC11 and KC12 primers, and 1.25 U of AmpliTaq DNA polymerase. The mixture was incubated for 2 min at 97°C to facilitate denaturation, then PCR amplification was performed with 5 cycles of 5 sec at 97°C (denaturation), 15 sec at 48°C (annealing), and 15 sec at 60°C (extension), 25 cycles of 5 sec at 94°C and 15 sec at 60°C, and a final extension step of 5 min at 72°C. One microliter of the first-round PCR product was applied to the second-round PCR in a 50-μl solution containing the same components as the first-round PCR except for primers KC13 and KC14 instead of KC11 and KC12. PCR amplification was performed with preincubation of 2 min at 94°C, 5 cycles of 5 sec at 94°C, 15 sec at 48°C, and 15 sec at 60°C, 25 cycles of 5 sec at 94°C and 15 sec at 60°C, and a final extension step of 5 min at 72°C. The final PCR product was analyzed by electrophoresis in 2% agarose gel containing 0.5 μg/ml ethidium bromide.

Evaluation of Extraction Efficiency

Extraction efficiency was evaluated by comparing the HCV RNA copy counts of seven samples (one sample of genotype 1a, two genotype 1b, one genotype 2a, one genotype 2b, one genotype 3a, and one genotype 4) determined by Poisson quantitation (described below) of HCV RNA in plasma and purified RNA obtained from plasma using the modified RNA extraction protocol.

Poisson quantitation of purified RNA. The HCV RNA concentration was determined by statistical analysis of the results of RT-nested PCR of the sample diluted to near endpoint. First, serial 10-fold dilutions of the extracted RNA solution were assayed with RT-nested PCR as described above. Then, the diluted solution that had conferred the second to last positive band was serially diluted twice and tested with RT-nested PCR. Finally, the RNA solution diluted at the ratio by which the last positive signal was given in a series of twofold dilutions was assayed in 20 replicates. Assuming that a single copy of HCV RNA is detectable, the HCV concentration can be calculated from the proportion of negative reactions (P_0) using the null class equation of the Poisson distribution [Simmonds et al., 1990]. The formula is HCV RNA concentration $= -\ln P_0 / V \times d$ (copies/μl), where V and d are the sample volume (μl) assayed and the dilution ratio, respectively. In these experiments, RNA Diluting Buffer (50% glycerol, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.05% Sarkosyl, 10 μg/ml poly A) was used to ensure no loss of RNA during the dilution procedures. We call the above procedure "Poisson quantitation."

Direct poisson quantitation of plasma RNA. Sample plasma was diluted with Virus Lysis Buffer (50% glycerol, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.05% Sarkosyl, 10 μg/ml poly A, 2 mM DTT, and 0.4 U/μl RNase inhibitor). Diluted samples were quantified by Poisson quantitation without RNA extraction. This method can quantify the absolute copy number of HCV RNA in plasma, since it is not affected by loss of RNA during the purification procedure. It was confirmed that contamination of less than 0.1 μl of plasma in one reaction did not affect quantitation of HCV RNA in this RT-PCR condition.

HCV RNA Real-Time RT-PCR

RT was carried out using a high-capacity cDNA Archive kit (Applied Biosystems) in a final volume of 10 μl containing 5 μl of RNA sample solution and 0.3 μM of KC12 primer. The RT reaction was carried out with a GeneAmp PCR system 9700 for 30 min at 48°C, followed by reverse transcriptase inactivation for 5 min at 95°C. The PCR reaction was carried out in a final volume of 50 μl containing 10 μl of the RT product, TaqMan buffer (Applied Biosystems), 5 mM $MgCl_2$, 400 μM dUTP, 200 μM dATP, dCTP, dGTP (each), 0.025 U/μl AmpliTaq Gold, 0.01 U/μl AmpErase UNG, 0.3 μM forward primer (KC13), and reverse primer (KC14). For signal detection, 0.1 μM MGB probe 5′-CTGCGGAACCGGTG-3′ (KCP) was used. The PCR amplification was performed using Sequence Detection Systems 7700 (Applied Biosystems). The temperature settings were: uracil-*N*-glycosylase incubation for 2 min at 50°C; AmpliTaq Gold activation for 10 min at 95°C; 40 cycles of 15 sec at 95°C; and 1 min at 60°C. Measurements of standard and unknown samples were done in triplicate. After amplification, real-time data acquisition and analysis were performed with the accompanying software. For the standard HCV RNA, we used an RNA solution purified from the plasma of one patient with genotype 1b, which is the most common genotype among Japanese patients and quantified by Poisson quantitation.

Detection and Quantitation of HCV RNA in Clinical Samples

From 28 HCV-infected patients who achieved a viral response with antiviral treatment (12 relapsers and 16 sustained virologic responders), 53 stored plasma samples at week 4 and at the end-of-treatment (9 week-4 samples and 12 end-of-treatment samples from relapsers, and 16 week-4 samples and 16 end-of-treatment samples from sustained virologic responders) were retested by the RT-nested PCR and real-time RT-PCR assays developed in this study. One tenth of the RNA purified from plasma samples as described above was subjected to in-house RT-nested PCR in 10 replicates. Plasma in which HCV RNA was detected in nine or more reactions was quantified by real-time RT-PCR, because a reliable HCV RNA value cannot be obtained for these samples by Poisson quantitation. The results of the RT-nested PCR and COBAS Amplicor HCV v2.0 assays were tested by Fisher's exact probability test with respect of the outcome.

RESULTS

Extraction Efficiency

The efficiency of HCV RNA extraction in the modified protocol of QIAamp UltraSens Virus kit was estimated by comparing the quantities of HCV RNA in seven plasma samples and in their extracted RNA, involving

six different HCV genotypes. The ratios ranged from 50% to 107% with a median of 89%.

Sensitivity of RT-Nested PCR

To evaluate the sensitivity of the RT-nested PCR, the World Health Organization international standard for HCV RNA (NIBSC code 96/798) was quantified by Poisson quantitation. In a total of 4 runs, HCV RNA was detected in 24 reactions of 34 replicates. This result showed that 1 IU was equivalent to 12.2 amplifiable copies (range based on SD, 9.9–15.3 copies).

Linearity, Reproducibility, and Genotype Reactivity of Real-Time RT-PCR

The data obtained for drawing the standard curves in the real-time RT-PCR assay were statistically analyzed to assess the precision within each run (intra-assay) and between runs (inter-assay). The standard curves were obtained with 10-fold dilutions of the RNA standard, ranging from 5 to 5,000 copies per reaction (equivalent to 120–120,000 copies/ml), and analyzed in triplicate in a total of four independent experiments. The results of the analysis are summarized in Table II. The standard deviation of the intra-assay on the basis of C_T values ranged from 0.05 to 0.74 cycles, even including samples with only 5 copies per reaction, and that of the inter-assay ranged from 0.70 to 1.42 cycles.

To overcome the variation of nucleotide sequences of 5′ untranslated region among HCV genotypes, we used the most conserved region for designing PCR primers. To evaluate the genotype reactivity of the real-time RT-PCR, obtained values of HCV RNA were compared with those determined by Poisson quantitation for HCV strains of various genotypes. There was very good linearity between the real-time RT-PCR and Poisson quantitation regardless of genotype (Fig. 1). The coefficient of variation ranged from 7.2% to 30.8% for all quantified points and all genotypes.

Detection and Quantitation of HCV RNA in Stored Plasma

From 28 patients who achieved virologic responses at the end-of-treatment, 53 plasma samples at week 4 or at the end-of-treatment were retested by the RT-nested PCR. The detection rates, statistical tests of week-4 and end-of-treatment samples, and the predictability of sustained virologic response by the undetectability of HCV RNA and relapse by the detection of HCV RNA are shown in Table III. In testing week-4 samples, the RT-nested PCR assay detected HCV RNA in 8 of 9 (89%) available samples from relapsers, and in 4 of 16 (25%) from sustained virologic responders ($P = 0.003$), whereas COBAS Amplicor detected 6/9 (67%) from relapsers and 2/16 (13%) from sustained virologic responders ($P = 0.01$). Among the 13 patients who were HCV RNA-negative at week 4 by the RT-nested PCR assay, 12 (92%) achieved sustained virologic response, compared to 14 of the 17 (82%) who were negative by COBAS Amplicor. In testing end-of-treatment samples, 4 of 12 (33%) Amplicor-negative samples from relapsers were HCV-positive by the RT-nested PCR assay, whereas 0 of 16 end-of-treatment samples from sustained virologic responders were HCV-positive ($P = 0.02$). Among the four patients who were HCV RNA-positive at the end-of-treatment by the RT-nested PCR assay, four (100%) had virologic relapse.

The viral load determined by Poisson quantitation in eight Amplicor-negative samples (six samples from relapsers and two samples from sustained virologic responders) were within 3.5–17.3 copies/ml (Table IV), which was below the detection limit of current highly sensitive assays such as transcription-mediated amplification.

DISCUSSION

This study demonstrates that an ultrasensitive HCV assay can identify both sustained virologic response and relapse in patients with high probabilities. As previously shown, the more sensitive the assay used for the quantitation of HCV RNA, the more predictive the measurements are of virologic relapse after discontinuation of therapy [Sarrazin et al., 2001; Desombere et al., 2005]. The currently developed highly sensitive HCV assay based on RT-nested PCR has an ability to detect almost a single copy of HCV RNA, which was achieved by a stepwise declining temperature in reverse transcription and a lower annealing temperature of 48°C for the first five PCR cycles. To assess the sensitivity of this assay, equivalency between the international unit and the amplifiable copy number of HCV RNA was determined using the WHO international standard. The international unit was first determined in 1998 based on a mean of measurements for the WHO international standard that were quantified by 22 multinational laboratories using various quantitation methods in 1998 [Saldanha et al., 1999], therefore, it is not yet clear how many HCV virions are equivalent to 1 IU.

TABLE II. C_T Values With RNA Dilutions for Standard Curve (Genotype 1b)

Copies/reaction	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Inter-assay (std dev)
5,000	22.20 (0.05)	23.60 (0.23)	22.30 (0.12)	21.98 (0.15)	0.70
500	26.54 (0.28)	27.59 (0.35)	25.90 (0.21)	25.39 (0.12)	0.88
50	30.99 (0.21)	31.35 (0.48)	29.14 (0.16)	28.55 (0.11)	1.26
5	35.46 (0.74)	35.40 (0.64)	33.27 (0.60)	32.60 (0.28)	1.42

Serially diluted samples were analyzed in triplicate in each experiment. Good consistency (intra-assay) and reproducibility (inter-assay) of the real-time PCR are shown in this table.

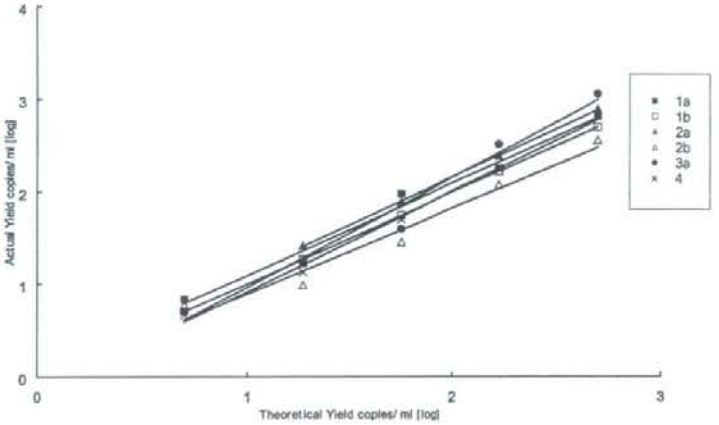

Fig. 1. Linearity of yield with RNA dilutions isolated from genotype 1a, 1b, 2a, 2b, 3a, and 4 samples. A sample from each genotype was serially diluted, and quantified by real-time RT-PCR. Actual yield copy counts were obtained from 10-fold diluted RNA samples by real-time RT-PCR, and theoretical yield copy counts were obtained from the dilution based on Poisson quantification using our RT-nested PCR.

Previously, conversion factors of international unit and amplifiable copy were determined at 2.75 and 5.2 copies/IU by Puig et al. [2002] and Gorrin et al. [2003], respectively. The result of 12.2 copies/IU in this study, which was obtained by analyzing the Poisson distribution of clear positive reactions at endpoint dilution of the WHO international standard, was considerably higher than the previous values. Taken together with a high efficiency (89%) of extraction of HCV RNA from plasma, the present assay is considered to have the ability to detect almost a single copy of HCV RNA in plasma samples.

The present qualitative RT-nested PCR assay was compared with the COBAS Amplicor HCV version 2.0 with a lower detection limit of 50 IU/ml for 53 plasma samples from 28 patients receiving antiviral therapy that were collected at week 4 or the end-of-treatment. While COBAS Amplicor detected HCV RNA in 8 samples, the present assay detected HCV RNA in 16 samples (all of 8 Amplicor-positive samples and 8 of 45 Amplicor-negative samples), providing evidence that the assay is more sensitive for the detection of HCV RNA in clinical samples than COBAS Amplicor version 2.0. Furthermore, regarding prediction of relapse or sustained virologic response after treatment, the present assay is more predictive than COBAS Amplicor at week 4 ($P = 0.003$ vs. $P = 0.02$) as well as at the end-of-treatment ($P = 0.02$ vs. $P = 1$). It is most likely that this difference is explained by a lower detection limit of the present assay. As mentioned above, the RT-nested PCR assay is approaching the ability to detect a single copy of HCV RNA. Therefore, when a 0.5 ml plasma sample is used, the sensitivity of the assay can be calculated from the equation for the Poisson distribution as 5 copies/ml at 95% detection, which is lower than 50 IU/ml for COBAS Amplicor and 25–50 copies/ml for transcription-mediated amplification assay (VERSANT HCV RNA Qualitative Assay) [Sarrazin et al., 2001].

TABLE III. Comparison of the Detection Rates of HCV RNA by Our in-House RT-Nested PCR at Week 4 and the End-of-Treatment, and COBAS Amplicor HCV Version 2.0 at Week 4

	In-house RT-nested PCR			COBAS Amplicor HCV v2.0		
	Relapse	Sustained virologic response		Relapse	Sustained virologic response	
Week 4						
Positive	8	4	Relapse/total positive = 8/12 (67%)	6	2	Relapse/total positive = 6/8 (75%)
Negative	1	12	SVR/total negative = 12/13 (92%)	3	14	SVR/total negative = 14/17 (82%)
P-value	0.003			0.02		
End-of-treatment						
Positive	4	0	Relapse/total positive = 4/4 (100%)	0	0	Relapse/total positive = 12/0
Negative	8	16	SVR/total negative = 16/24 (67%)	12	16	SVR/total negative = 16/28 (57%)
P-value	0.01			1.00		

TABLE IV. Characteristics and Quantified Viral Load of HCV RNA in Plasma Which Was HCV RNA Negative by Cobas Amplicor HCV Version 2.0 (Sensitivity, 50 IU/ml)

Patients	Outcome	Sampling	HCV RNA (copies/ml)
1	Relapse	Week 4	3.5
2	Relapse	Week 4	8.0
3	Relapse	EOT	4.9
4	Relapse	EOT	11.0
5	Relapse	EOT	3.5
6	Relapse	EOT	17.3
7	SVR	Week 4	3.5
8	SVR	Week 4	7.6

EOT, End-of-treatment.

Several clinical studies have shown that early viral assessments provide identification of nonresponders to antiviral therapies [Davis et al., 2003; Jensen et al., 2006], and the consensus guidelines of American Association of Study for Liver Disease (AASLD) [Strader et al., 2004] recommended discontinuation of therapy in patients who do not achieve EVR. Moreover, Jensen et al. [2006] suggested that therapy can be discontinued by 24 weeks in patients who achieve rapid virologic response at week 4, because there was no significant difference in rate of sustained virologic response between patients treated for 24 and 48 weeks. However, virologic relapse occurs at various rates among patients with rapid virologic response (9–27%) [Jensen et al., 2006].

The ratios of sustained virologic response among patients who were HCV negative at week 4 of treatment by the present assay and COBAS Amplicor were 12/13 (92%) and 14/17 (82%), respectively, suggesting that undetectability of HCV RNA at week 4 by the more sensitive assay is more predictive of sustained virologic response. The threshold of plasma viral levels in patients with a strong virologic suppression during the early phase that leads to sustained virologic response may be much below 50 IU/ml. The qualitative transcription-mediated amplification assay, which has a higher sensitivity than COBAS Amplicor (detection limit 25–50 copies/ml), showed a slightly higher detection rate for HCV RNA than COBAS Amplicor (18/21 (86%) by transcription-mediated amplification assay versus 16/21 (76%) by COBAS Amplicor) in the testing of week-4 samples from relapsers [Desombere et al., 2005]. Because the sensitivity of the present RT-nested PCR assay is higher than that of the transcription-mediated amplification assay (5 copies/ml vs. 25–50 copies/ml at 95% detection), the predictability of sustained virologic response by the RT-nested PCR assay may be higher than by the transcription-mediated amplification assay.

Despite of the high sensitivity in detecting a single copy, one of nine samples at week four in relapsers was HCV-negative by the present assay. There are two possible explanations for this result. First, antiviral treatment leads to a complete replication arrest in blood, but small quantities of HCV RNA may persist in the liver [Radkowski et al., 2005]. Second, macrophages and lymphocytes may serve as a replication sites for HCV RNA, resulting in failure of detection of HCV RNA in plasma samples [Laskus et al., 2000; Watkins-Riedel et al., 2004; Radkowski et al., 2005]. On the other hand, detection of HCV RNA at week 4 is not well correlated with virologic relapse; 4 of 16 (25%) and 2 of 16 (13%) sustained virologic responders were HCV positive at week 4 by the present assay and COBAS Amplicor, respectively. It may be too early to identify patients with relapse or nonresponse by the detection of HCV RNA at week 4, because an early decision to discontinue treatment at week 4 would be a serious error in patients with the potential for a sustained virologic response.

In testing end-of-treatment samples, the RT-nested PCR assay detected residual HCV RNA in 4 of 12 (33%) relapsers, and 0 of 16 (0%) sustained virologic responders ($P = 0.02$), all of whom were HCV RNA negative by COBAS Amplicor. Despite of the small number of samples, this result suggests that patients who are HCV-positive by the RT-nested PCR assay at the end-of-treatment will relapse with quite a high probability after treatment. The detection rate of 33% in end-of-treatment samples from relapsing patients by the RT-nested PCR assay is similar to that obtained with the transcription-mediated amplification assay [Sarrazin et al., 2001; Desombere et al., 2005]. By retesting end-of-treatment samples with both the RT-nested PCR assay and the transcription-mediated amplification assay, it was found that the more highly sensitive assays can detect residual HCV RNA at the end-of-treatment from relapsers more frequently, and is suggested that virologic relapse occurs on the basis of replication of remaining HCV virions from a very low level after discontinuation of the antiviral therapy.

As previously reported, the level from which strongly suppressed HCV RNA can lead to virologic relapse is thought to be below 50 IU/ml, which may cause errors in the assessment of the efficacy of antiviral therapies [Sarrazin et al., 2001]. Indeed, the transcription-mediated amplification assay detected residual HCV RNA in the Amplicor-negative plasma from relapsers [Morishima et al., 2006]. However, there has been no report to quantify the minimum level of residual HCV RNA before virologic relapse. This study showed that the quantified HCV RNA concentration of relapsers was in the range of 3.5–17.3 copies/ml (Table IV), which is below the detection limit of the transcription-mediated amplification assay as well as that of the COBAS Amplicor HCV assay.

The assay had several limitations in its data and procedures. First, this study enrolled a limited number of patients. Particularly, the number of week-4 samples from relapsers ($n = 9$) was too small to evaluate the statistical difference in the detection rates between the present RT-nested PCR assay and the COBAS Amplicor HCV assay. Second, in comparison with commercially available kits, RT-nested PCR is labor-intensive, and thus is difficult to use for a large number of samples simultaneously. Therefore, the present assay may not be

suitable for routine use in clinical trials. However, since the threshold level of residual HCV RNA capable of producing a virologic relapse is quite low, the present assay may be useful for distinguishing virologic relapse from sustained virologic response.

In conclusion, a highly sensitive assay, almost capable of detecting a single copy of HCV RNA, was developed for the accurate prediction of the efficacy of antiviral therapy in HCV infection. It has been shown that sustained virologic response can be predicted by the undetectablity of HCV RNA at week 4, and, more strongly, that relapse is associated with detection of HCV RNA at the end-of-treatment.

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Original Article

Individual tissue culture system in a disposable capsule with hypoxic atmosphere

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Abstract

In the present study, a novel system for the hypoxic culture of individual tissues was established for the subculturing of cell lines for research as well as for clinical culture of primary cells. To provide a hypoxic environment throughout the process of tissue handling and culture, we designed a clean bench with CO_2 gas circulation and a hypoxic culture incubator containing disposable capsules. The bench top was covered with an acrylic chamber, and an atmosphere of 5.0% CO_2-air was maintained using a sensor control. The cleanliness class of the chamber could easily be improved to 1 within 5 min of circulative filtration, even though it was found to be 10^5 before the unit was operated. Gas buffer solution (220 ml of 20 mM HEPES, 25 mM $NaHCO_3$, pH 7.4) placed in a 500-ml plastic capsule in the unit stabilized the culture environment by functioning as a heat storage and gas pool. The inflow of air that occurred by the cap of the capsule was opened was excluded by the infusion of purging gas (5.0% CO_2 and 95% N_2); the O_2 level returned to 2.0% within 4 min, after which the gas supply automatically switched to the culture gas (2.0% O_2, 5.0% CO_2, and 93% N_2). If this purging process was omitted, restoration of the O_2 level required 120 min, even though the inner volume was only 280 ml.

Keywords: individual tissue culture, gas circulation clean bench, hypoxic culture, disposable capsule

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Introduction

Tissue culture plays an important role in cell biology, and it is mainly focused towards the subculturing of established cell lines. Culture equipments such as the clean bench and CO_2 incubator have been widely employed, and they provide the appropriate settings for the handling of tissues by supplying filtered fresh air and an atmosphere with saturated humidity and 5.0% CO_2, respectively. It is well known that the oxygen tension in the periphery is substantially lower than that of fresh air. Only some mutant variants that tolerate the given culture conditions such as the atmosphere and culture medium can adapt to long-term subculturing. Although media compositions have been discussed in detail, few efforts have been channeled towards ensuring that the oxygen tension at the original growth environments remains unchanged. The development of a hypoxic culture system will enable the subculturing of many more malignant tissues than what is currently possible. Recent advances in regenerative medicine require the culturing of primary cells or stem cells, and advances with regard to transplantation in humans demand more precise duplication of the original growth environments. Recent reports have indicated the influence of hypoxic culture on some cellular functions. BeWo cells - an in vitro model of human trophoblasts - were cultured in 2% O_2, and RT-PCR conducted after the culture indicated that transcription of the organic cation transporter OCTN2 was higher than that after culture in 20% O_2[1]. Hirao et al.[2] observed that when MC3T3-E1 cells and calvariae from 4-day-old mice were cultured in conditions of 20% or 5% O_2, osteoblastic differentiation and the subsequent transformation to osteocytes were promoted by low oxygen tension.

Some structures and mechanisms of conventional culture equipments are not designed to maintain low oxygen tension in the environment when tissues are being handled and cultured. The present study established a new hypoxic tissue culture system for the culturing of cell lines and primary cells in research and clinical settings, respectively.

Materials and Methods

Measurements with regard to particles in air: The degree of air cleanliness was defined in terms of "cleanliness classes" that are specified by the number of particles of size 0.5 μm or more in 1 cubic foot of air[3]. For in-

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stance, cleanliness class 100 indicates less than 100 particles of the specified size in 1 cubic foot of air. Particle size and number were simultaneously measured using a light-scattering particle counter (KC-03A, Rion, Tokyo, Japan). When an intake air stream is passed through a high-intensity laser beam, the particles in the stream scatter light. The particle sizes were divided across 5 categories in terms of the particle diameter (0.3–0.5 μm, 0.5–1.0 μm, 1.0–2.0 μm, 2.0–5.0 μm, and >5.0 μm), and the number of particles belonging to each category was assessed.

Measurement of temperature and O_2 concentration: The temperature sensor in the device (K470; Techno-Seven, Tokyo, Japan) had a resolution of ± 0.02°C. The O_2 concentration was measured using a galvanic current sensor.

Preparation of premixed gas and accuracy control: In the present study, we used commercially supplied premixed gases for culture (2.0% O_2, 5.0% CO_2, and 93% N_2) and for purging the capsules (5.0% CO_2 and 95% N_2). Pure O_2, CO_2, and N_2 gases were mixed according to their weights of corresponding molar ratios and filled in a container; in a pre-shipment review, their composition was assessed using gas chromatography. Post-receipt accuracy control was performed as follows: when the gases were aerated in a solution containing 20 mM HEPES, 25 mM $NaHCO_3$ at 37°C, and the composition was considered accurate if the pH of the solution remained at 7.4 ± 0.05 after gas equilibration.

Results

1. Clean bench with CO_2 gas circulation

In the present study, a new clean bench with CO_2 gas circulation and a built-in microscope was developed. As shown in Fig. 1, the bench top was covered with an acrylic chamber to prevent leakage of the ambient atmosphere; it resembled an infant incubator. Pure CO_2 was infused using a gas sensor control to maintain the composition at 5.0% CO_2-air, and the temperature was maintained at 30–37°C by using a temperature control (Fig. 2). In addition, if the tissue did not allow exposure to 5.0% CO_2-air, the culture dish was isolated in a small chamber placed on the bench top, and humidified culture gas was supplied to it. While fresh air is filtered only once in the conventional clean bench, the enclosed 5.0% CO_2-air was circulated through HEPA filter every 24 s in the present system. As shown in Fig. 3, the cleanliness class of the air was found to be approximately 4×10^5 before operation of the unit was initiated; particles larger than 0.5 μm were not found, and only 10 particles of size 0.3–0.5 μm were observed in 1 cubic foot of air after 5 min of operation. Repeated filtration easily yielded cleanliness class 1. Thus far, if bench tops were contaminated by some infectious material such as body fluid, they were merely wiped clean. In the present system, however, a disposable clear film is placed as a covering shield on the bench top, and it is discarded after each operation.

2. Hypoxic culture of individual tissues in disposable capsules

We developed a disposable capsule for hypoxic tissue culture (Fig. 4). A 500-ml plastic capsule containing 220 ml of the gas buffer solution (20 mM HEPES, 25 mM $NaHCO_3$) was used; it functioned as a heat storage as well as a gas pool. The culture bath had a 16-well aluminum block for heat storage, and the block and the inner space were maintained at 37°C by using a temperature sensor (Fig. 5). First, the gas buffer solution was equilibrated by infusion of a small amount of the culture gas (10 ml/min) at least overnight. When the door was opened for 10 s, a slight temperature change of 0.1°C was observed in the inner space of the capsule, and it was restored within 7 min.

When conducting tissue culture using this capsule, the inflow of air when the cap is opened should be excluded as soon as possible; The purging gas was flushed (500 ml/min) immediately after the cap was closed, and the O_2 level was restored to 2.0% within 4 min; the gas supply automatically switched to the culture gas, which was continuously infused (10 ml/min) to maintain positive pressure (Fig. 6). If this process was omitted, the restoration of the O_2 level required 120 min, even though the inner volume was only 280 ml. In this system, gas control with a CO_2 sensor was unnecessary, and improper control due to the sensor deterioration was not required to be considered. Gas equilibration in each capsule was roughly estimated by the color of phenol red in the gas buffer solution, and the precise control of the culture environments were monitored by measuring the temperature and pH of the gas buffer solution. Although simultaneous culturing of multiple tissues in a single CO_2 incubator is usually possible, the present method that facilitates the culturing of individual tissues in disposable capsules has some advantages: the individual dish can be easily identified without any confusion, and the culture conditions are not disturbed when the door of the unit is opened.

Discussion

The cleanliness class of room air is generally 10^6–10^5. A low-dust environment with a cleanliness class of 10^2 would be provided when the conventional clean bench is operated under the optimum conditions. However, tissues are handled in fresh air, and under these conditions, O_2 may be dissolved in the medium, and the pH could change due to CO_2 removal. The present clean bench with CO_2-air circulation had overcome the above defects, and the functions had been rather similar with the conventional CO_2 incubator.

Fig. 1 Clean bench with CO_2 gas circulation

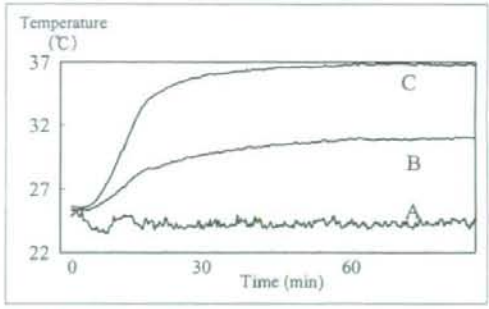

Fig. 2 Temperature of the clean bench
A: fresh air, B: ambient atmosphere, C: bench top

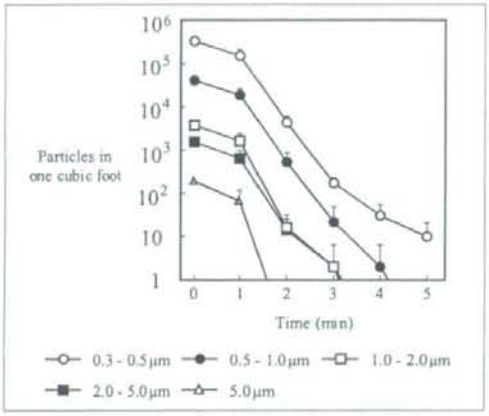

Fig. 3 Change in cleanliness class after beginning of operation
The values are represented as mean ± standard error. They were measured at 5 points on the bench.

Fig. 4 Disposable capsules for hypoxic tissue culture
A maximum of 5 culture dishes (6.0-cm diameter) can be placed on the tray on the stainless steel stand. The gas buffer solution is placed at the bottom of the stand. The 2 tubes protruding from the cap are the gas inlet and outlet.

Fig. 5 Culture bath with a 16-well aluminum block

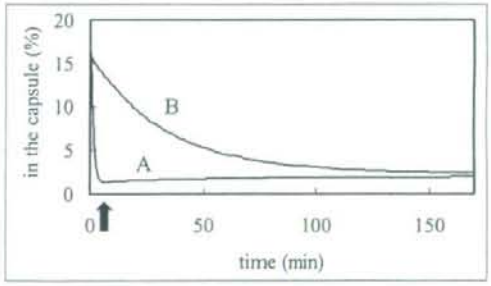

Fig. 6 Effect of gas purging on the restoration of the O_2 level after the cap is closed
A: purging with 5.0% CO_2 and 95% N_2, B: no purging, but 10 ml/min supply of 2.0% O_2, 5.0% CO_2, and 93% N_2. The arrow indicates gas switching.

The conventional CO_2 incubator poses a structural problem if hypoxic tissue culture is to be performed. The inflow of fresh air cannot be avoided when the door is opened. The concentration of CO_2 can be restored by infusing pure CO_2 gas. As shown in Fig. 6, it is very difficult to remove O_2 once it enters the chamber, and the tissues may be exposed to a high concentration of O_2. It is well known that the partial pressure of O_2 in fresh air (159 mmHg) is reduced to 100 and 25 mmHg or less, respectively, in arterial blood and at the periphery. When the saturated vapor pressure at 37°C was estimated to be 47 mmHg, those in 5.0% CO_2-air and the culture gas were calculated to be 142 and 14.3 mmHg, respectively. Numerous authors have shown that reactive oxygen species (ROS) exert various harmful effects such as lipid and protein peroxidation and membrane and DNA damage[4-7], however, only a few reports have addressed the effects of O_2 tension in culture environments[1,2,8]. Tissues and cells in body fluids are protected from O_2 and ROS by physiological antioxidants that are limited in artificial culture media, and an extremely high level of O_2 promotes ROS generation. Since the tolerance of cells to O_2 and ROS differs, the optimum O_2 concentration should be established for each cell line in order to minimize cellular damages and subsequent mutation. In the present system, by changing the composition of the premixed gas, the concentration of O_2 can easily be set, in the range of 0%–95%.

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Studies on the Compatibility of HLA-Class II Alleles in Patient Couples with Severe Pre-Eclampsia Using PCR–RFLP Methods

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Keywords
Compatibility, genotype, gestational hypertension, HLA-class II, PCR–RFLP, pre-eclampsia

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Problem
In this study, we investigated whether or not significant compatibility of human leukocyte antigen (HLA)-class II alleles exists between spouses with severe pre-eclampsia (PE) (including gestational hypertension, GH).

Method of study
The HLA-class II genotypes were determined using a polymerase chain reaction-restriction fragment length polymorphism method. The number of incompatible alleles in 57 patient couples with severe PE (including GH) were determined, and compared with that in 74 control couples.

Results
The number of patient couples and control couples with each number of mismatched alleles of the HLA-DR, -DQ, and -DP genotypes was as follows. The number of patient couples with zero, one, two, three, and four-allele mismatches was 41 (72.9%), and with five and six-allele mismatches was 16 (27.1%). On the other hand, the number of control couples with zero, one, two, three, and four-allele mismatches was 38 (51.4%), and with five and six-allele mismatches was 36 (48.6%). Thus, the number of patient couples with five and six-allele mismatches was significantly lower compared with that in control couples. The same result was obtained as regards the mismatched alleles of the HLA-DR, -DQ, and -DP phenotypes.

Conclusion
These findings suggest that significant compatibility of HLA-class II alleles between spouses is implicated in the genesis of PE (including GH).

Introduction

Pre-eclampsia (PE) (including gestational hypertension, GH) is a major cause of both maternal and perinatal morbidity and mortality, which is diagnosed by the onset of hypertension with or without proteinuria during the latter half of pregnancy. Although both the etiology and pathogenesis of PE/GH are poorly understood, aberrations in the immune response have been suggested as likely causes of pathologies associated with pregnancy in view of the diversity of immunologic responses in pregnant women.[1] It is thought that insufficient maternal recognition of fetal (paternal) antigens is deleterious for the immunologically successful maintenance of pregnancy, which might cause a range of reproductive failures such as sterility, recurrent fetal wastage, and PE/GH.[2–4] Genes

located within the major histocompatibility complex (MHC) have been shown to be associated with a variety of diseases, and have been proposed to regulate reproductive processes in animals and humans. The MHC of human beings, the human leukocyte antigen (HLA), is well known to be useful in examining the immunogenetic basis of some diseases.[5,6] Several studies have attempted to reveal the association between antigens of the HLA system and development of PE/GH;[7–11] especially, the materno-paternal compatibility or disparity of HLA antigens in PE/GH patient couples was investigated by several investigators, although the results were conflicting.[12–16] In this study, we analyzed the HLA-class II alleles, i.e., HLA-DRB1, -DQB1, and -DPB1 genotypes and phenotypes in patient couples with severe PE/GH and normal fertile couples using a polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) method to clarify whether or not there is any relationship between the materno-paternal HLA compatibility and severe PE/GH.

Materials and methods

Patients and Controls

The HLA-DRB1, -DQB1, and -DPB1 genotypes were analyzed in 57 patients with severe PE/GH and their partners after obtaining informed consent. PE/GH was diagnosed according to the clinical criteria defined by the 'International Society for the Study of Hypertension in Pregnancy' (ISSHP).[17] All patients had severe hypertension diagnosed according to the same criteria. Patients who were positive for anti-phospholipid antibodies, such as anti-cardiolipin antibodies, anti-cardiolipin beta-2-glycoprotein I antibodies, and lupus anticoagulant, were excluded from the study. Of 57 patients, 38 were primiparous women, and 19 cases in them had experienced one delivery. Thirteen previous pregnancies in 19 cases with delivery had been complicated with severe PE/GH, and those of remaining six cases had not been complicated with PE/GH.

As a control, 74 women who had experienced at least two normal deliveries without PE/GH and their husbands were examined for the HLA-DRB1, -DQB1, and -DPB1 genotypes also after obtaining informed consent. All individuals were Japanese women. Internal review board approval was obtained before the commencement of the study.

Analyses of HLA-DRB1, HLA-DQB1, and HLA-DPB1 Genotypes

Analyses of HLA-DRB1, HLA-DQB1, and -DPB1 genotypes were performed using the PCR–RFLP method.[18–20] The primers used in this study are listed in Table I, and endonucleases are listed in Table II.

Genomic DNAs, extracted by phenol extraction of sodium dodecyl sulphate-lysed and proteinase K-treated peripheral lymphocytes from each individual, were amplified by the PCR procedure with 2.5 units of *Taq* DNA polymerase (Takara Co., Ltd, Kyoto, Japan). The reaction mixture, which contained 1 μmol/L each of the PCR 3′ and 5′ primers, 1 μg of genomic DNA, 10 μL of dNTP mixture (Takara Co. Ltd), a PCR reaction buffer (10 mmol/L Tris–HCl, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$), and distilled water, to make a total volume 100 μL in a 0.5-mL eppendorf tube, was covered with 50 μL of mineral oil to prevent evaporation and subjected to 30 cycles of 1 min for denaturing, 1 min for annealing, and 2 min for extension in an automated PCR thermal cycler (Thermal Cyclic Reactor; Toyobo Engineering Co., Tokyo, Japan).

For HLA-DRB1 typing, seven group-specific primers, DR1, DR2, DR4, DR7, DR9, DR10, and DRw52-associated (DR3, −5, −6, and −8) antigen specific primers, were used to obtain only the amplified product from the DRB1 gene (Table I). The DR7, −9, −10 alleles, which have no suballeles (DRB1*0701 and 0702 have the same nucleotide sequences in their β1 domain exons), were simply typified by the presence of amplified bands as DRB1*0701 or 0702, DRB1*0901, and DRB*1001 respectively.

For HLA-DQB1 typing, a 241 base pair fragment from the second exon of the HLA-DQB1 gene was amplified using 1 μmol/L each of the PCR primers GH28NL + QB202 (DQ1 group specific primers), and a 237 base pair fragment was amplified using 1 μmol/L each of the PCR primers GH28NL + QB204 (DQ2,3,4 group specific primers).

For HLA-DPB1 typing, a 299 base pair fragment from the second exon of the HLA-DPB1 gene was amplified by using 1 μmol/L each of the PCR primers DPB101N and DPB201.

After amplification, aliquots (6 μL) of the reaction mixture, with an appropriate restriction buffer and restriction enzymes, were incubated for 1–3 hr.

*Ava*II and *Pst*I were used for digestion of the amplified DR1-DRB1, *Fok*I, *Cfr*13I and *Hph*I for

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Table I PCR Primers for Amplification of the DRB1, DQB1, DPB1 Genes

Gene	Primer	Sequences (5' to 3')	Den	Ann	Ext
DRB1 for DR2	5' Primer 5'R2	TTCGTGTGGCAGCCTAAGAGG (261 bp)	94°C	60°C	72°C
DRB1 for DR4	5' Primer 5'R4	GTTTCTTGGAGCAGGTTAAAC (263 bp)	94°C	60°C	72°C
DRB1 for DR1	5' Primer 5'R1	GGTTGCTGGAAAGATGCATCT (206 bp)	94°C	55°C	72°C
DRB1 for DR7	5' Primer 5'R7	AGTTCCTGGAAAGACTCTTCT (206 bp)	94°C	60°C	72°C
DRB1 for DR10	5' Primer 5'R10	GGTTGCTGGAAAGAGGCGTCC (206 bp)	94°C	60°C	72°C
DRB1 for DR3	5' Primer 5'R3568	ACGTTTCTTGGAGTACTCTACG (265 bp)	94°C	60°C	72°C
DR5					
DR6					
DR8					
	3' Primer 3'R[a]	CCGCTGCACTGTGAAGCTCT			
DRB1 for DR9	5' Primer 5'R9	GGACGGAGCGGGTGCGGTATC (193 bp)	94°C	63°C	72°C
	3' Primer 3'R9	CCGTAGTTGTGTCTGCACACGG			
DQB1 for DQ1	5' Primer GH28NL	GCATGTGCTACTTCACCAACG (241 bp)	94°C	55°C	72°C
	3' Primer QB202	CACCTGCAGATCCCGCGGTACGCCACCTC			
DQB1 for DQ2	5' Primer GH28NL	GCATGTGCTACTTCACCAACG (237 bp)	94°C	55°C	72°C
DQ3	3' Primer QB204	CACCTGCAGTGCGGAGCTCCAACTGGTA			
DQ4					
DPB1	5' Primer DPB101N	GTGAAGCTTTCCCCGCAGAGAATTAC (299 bp)	94°C	62°C	72°C
	3' Primer DPB201	CACCTGCAGTCACTCACCTCGGCGCTG			

Den = denaturing; Ann=annealing; Ext = extension.
[a]Common for DRB1 alleles except the DR9 allele.
The time of denaturing is 1 min, that of annealing is 1 min and that of extension is 2 min.

Table II Restriction Endonucleases for Genotyping of DRB1, DQB1 and DPB1 Alleles

Allele	Antigen	Restriction endonuclease
DRB1	DR1	*Ava*II, *Pst*I
	DR2	*Fok*I, *Cfr*13I, *Hph*I
	DR3,5,6,8	*Ava*II, *Fok*I, *Kpn*I, *Hae*II, *Cfr*13I, *Sfa*NI, *Sac*II, *Bsa*JI, *Apa*I, *Hph*I, *Rsa*I
	DR4	*Sac*II, *Ava*II, *Hin*fI, *Hae*III, *Hph*I, *Mnl*I
DQB1	DQw1	*Fok*I, *Apa*I, *Hae*II, *Sta*NI, *Bss*HII, *Hph*I
	DQw2,3,4	*Fok*I, *Bgl*I, *Sac*I, *Acy*I, *Hpa*II
DPB1		*Bsp*1286I, *Fok*I, *Dde*I, *Bsa*JI, *Bss*HII, *Cfr*12I, *Rsa*I, *Eco*NI, *Ava*II

DR2-DRB1, *Sac*II, *Ava*II, *Hin*fI, *Hae*II, *Hph*I and *Mnl*I for DR4-DRB1, *Ava*II, *Fok*I, *Kpn*I, *Hae*II, *Cfr*13I, *Sfa*NI, *Sac*II, *Bsa*JI, *Apa*I, *Hph*I and *Rsa*I for DR3,5,6 and 8-DRB1.

*Fok*I, *Apa*I, *Hae*II, *Sfa*NI, *Bss*HII, *Hph*I, were used to digest the amplified DQB1 genes with DQ1, and *Fok*I, *Bgl*I, *Sac*I, *Acy*I, *Hpa*II were used to digest the amplified DQB1 genes with DQ2,3, and 4 (Table II).

*Bsp*1286I, *Fok*I, *Dde*I, *Bsa*JI, *Bss*HII, *Cfr*13I, *Rsa*I, *Eco*NI, and *Ava*II were used to perform single digestion of the amplified DPB1 genes (Table II).

Samples of the amplified DNAs cleaved by restriction enzymes were subjected to electrophoresis using a 12% polyacrylamide horizontal gel in a minigel apparatus (AE-6450; Atto Corporation, Tokyo, Japan). Cleavage or no cleavage of amplified fragments was detected by staining with ethidium bromide.

Human leukocyte antigen-DRB1 genotypes were determined by comparing the restriction fragment patterns to those of amplified DRB1 genes reported by Ota et al.,[18] and HLA-DQB1 genotypes were determined by comparing the patterns with those of amplified DQB1 genes reported by Nomura et al.[19] HLA-DPB1 genotypes were determined by comparing the patterns of restriction fragment polymorphism obtained in tested individuals with those of the amplified DPB1 gene reported by Ota et al.[20]

Scoring of the Number of Mismatched Alleles

A two-alleles mismatch was scored when both of the paternal alleles at one locus were different from