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MAJOR ARTICLE

Detection of Hepatitis C Virus (HCV) in Serum and Peripheral-Blood Mononuclear Cells from HCV-Monoinfected and HIV/HCV–Coinfected Persons

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It has been speculated that hepatitis C virus (HCV) replicates in peripheral-blood mononuclear cells (PBMCs), which, therefore, may be a site for interaction with human immunodeficiency virus (HIV). We used strand-specific real-time polymerase chain reaction to detect HCV RNA in 28 HCV-monoinfected and 20 HIV/HCV–coinfected women. At the first visit, positive-strand HCV RNA was detected in serum samples from 89% of the women, whereas positive-strand HCV RNA was detected in PBMC samples from 32% and 55% of the HCV-monoinfected and HIV/HCV–coinfected women, respectively. After initiation of antiretroviral therapy, the HIV/HCV–coinfected women were significantly more likely to have detectable positive- and negative-strand HCV RNA in the PBMC compartment than were the HCV-monoinfected women. HIV and HCV RNA levels were not correlated. Serum HCV RNA levels were correlated over time; HCV RNA levels in the serum and PBMC compartments were not. These data suggest differential regulation of HCV RNA in the serum and PBMC compartments and may partially explain the limited HCV antiviral response rates observed in coinfected persons.

Hepatitis C virus (HCV) is a positive-strand RNA virus that infects >170 million people worldwide. Because of the inability to infect small animals with HCV and the lack of efficient cell-culture models, much of the current understanding of the HCV life cycle has been inferred from studies that use samples from infected humans. Although hepatocytes are the major site of infection, there is a broad clinical spectrum of disease and extrahepatic complications, including cryoglobulinemia, non-Hodgkin lymphoma, and porphyria cutanea tarda [1].

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Some studies have reported evidence for extrahepatic replication of HCV in peripheral-blood mononuclear cells (PBMCs); however, these studies have typically involved a small number of patients and have often yielded contradictory results [2–7]. Other studies have reported evidence for HCV replication in granulocytes, monocytes/macrophages, dendritic cells, and B lymphocytes, as well as in extrahepatic tissues [8–16]. Because certain amplification methods lack strand specificity, which may influence the reliable detection of replication intermediates (i.e., negative-strand HCV RNA), it has been challenging to definitively demonstrate extrahepatic HCV replication. Recently, modification of the real-time polymerase chain reaction (rtPCR) assay to include the *Tth* enzyme, which has high strand specificity and independent reverse-transcriptase and DNA-dependent polymerase activity, has been used to detect negative-strand HCV RNA in the liver and/or PBMC compartment [7, 9, 17–19].

In the United States, 150,000–300,000 people are coinfected with HCV and HIV [20]. Multiple studies have demonstrated the adverse effects of HIV coinfec-

tion on liver fibrosis, HCV RNA levels, HCV disease progression [21], and treatment response rates [22–24]. The mechanisms by which these 2 viruses interact remain unclear, because no direct virus-virus interactions have been demonstrated to date. However, recent in vitro data suggest that HCV and HIV proteins cooperatively induce hepatic apoptotic pathways [25, 26] and secretion of proinflammatory cytokines [27] without requiring cell infection and viral replication. Thus, it is reasonable to speculate that similar signaling cascades in PBMCs may also permit indirect interactions between HCV and HIV.

We have previously investigated serum HCV diversity in HIV/HCV–coinfected persons initiating antiretroviral therapy (ART) for HIV infection [28]. We found significant evolution of the hypervariable region 1, but not the adjacent envelope 1 region, after ART initiation. However, few studies have addressed the effects of ART on HCV in HIV/HCV–coinfected persons in compartments other than serum. The demonstration of extrahepatic HCV replication in the PBMC compartment would have important implications for transmission of the virus and efficient treatment of HCV infection. Nonetheless, previous studies have not assessed this phenomenon in HIV/HCV–coinfected persons in a longitudinal manner, nor have they addressed it in coinfected persons initiating ART [15, 29, 30]. Therefore, we sought to investigate whether HCV replication could be detected in the serum and PBMC compartments of persons coinfected with HIV and HCV and to assess the effect of ART on extrahepatic HCV replication.

PARTICIPANTS, MATERIALS, AND METHODS

Study population. From April 1993 to February 1995, the HIV Epidemiology Research (HER) Study, a prospective natural-history study of HIV infection, enrolled 871 HIV-infected women and 439 demographically matched HIV-uninfected women [31]. The women participated in clinic visits at 6-month intervals through 1999. By study design, one-half of the women reported injection drug use (IDU), and the other half reported only sexual risk behavior.

As described elsewhere, HCV serostatus was determined by either Abbott HCV EIA (version 2.0) or Ortho HCV ELISA (version 3.0) [32]. Overall, the seroprevalence of HCV was 56.5%, with rates of 48.0% and 60.8% in HIV-uninfected and HIV-infected women, respectively. Of the women who acknowledged prior IDU, 88.3% were HCV seropositive; of these women, 76.9% had detectable HCV RNA [33]. Because the HER Study cohort was formed before the widespread use of combination therapy, only 30% were receiving ART at the beginning of the study. By 1999, 31.3% were still not receiving any ART [34].

HER Study participants were included in the present study if they (1) were HCV seropositive, regardless of their HIV status; (2) had serum and PBMC samples available from at least 2 consecutive study visits conducted at the Providence, RI, site; and (3) were not receiving ART at the beginning of the study (for the HIV-infected women). For the HIV-infected women, study visits corresponded to the visit immediately before ART initiation (denoted "visit A") and the visit immediately after ART initiation (denoted "visit B"). The median intervals between visits were 5.8 months and 6.6 months for the HCV-monoinfected and HIV/HCV–coinfected women, respectively. The drug regimens initiated by the HIV-infected women were as follows: ≥2 nucleoside reverse-transcriptase inhibitors (NRTIs) ($n = 4$); ≥1 NRTI plus ≥1 protease inhibitor (PI) ($n = 11$); ≥2 NRTIs plus 1 nonnucleoside reverse-transcriptase inhibitor (NNRTI) ($n = 4$); and 2 NRTIs plus 1 NNRTI plus 2 PIs ($n = 1$). One HIV/HCV–coinfected woman was missing serum samples at both visits, and 3 HIV/HCV–coinfected women were missing PBMC samples at visit B.

Cellular RNA extraction and strand-specific* Tth *rtPCR. RNA was extracted from serum samples by use of the QIAamp Viral RNA Kit (Qiagen). For PBMC samples, the number of cells available was limited. Because the number of cells varied per sample (range, 1.4–7.6 × 10^6 cells/mL), we normalized all quantitative HCV RNA data on the PBMC compartment to the copy number of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Five hundred microliters of a PBMC suspension was washed with diethopyrocarbonate (DEPC)–treated dH_2O, and cellular RNA was extracted by use of TRIzol (Invitrogen). The resultant RNA was resuspended in 40 μL of DEPC-treated dH_2O and treated 2 times with DNase I (Ambion). Positive- and negative-strand HCV cDNAs were quantified by a validated strand-specific rtPCR assay using SYBR green dye I, as described elsewhere [17, 19]. Extracted RNA was heated at 95°C for 1 min and then incubated at 70°C. A mixture containing 10 pmol/μL HCV-1 antisense primer (5′-TGGATGCACGGTCTACGAGACCTC-3′; nt 342–320, according to the numbering of H77 [35]; GenBank accession number AF009606) for HCV positive-strand synthesis or HCV-2 sense primer (5′-CACTCCCCTGTGAGGAACT-3′; nt 38–56) for HCV negative-strand synthesis, 1× reverse-transcriptase buffer, 1 mmol/L $MnCl_2$, 200 mmol/L each deoxynucleoside triphosphate, and 5 U of *Tth* enzyme (Applied Biosystems) was added. The cDNA reaction consisted of an annealing step for 2 min at 60°C, followed by an extension step for 20 min at 70°C. To inactivate the reverse-transcriptase activity of the *Tth* enzyme, chelating buffer was added after cDNA synthesis. cDNA was purified by use of the High Pure PCR Template Preparation Kit (Roche Diagnostics).

Positive- and negative-strand HCV PCR amplification was performed with 2 μL of purified cDNA in a mixture containing LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics), 4 mmol/L $MgCl_2$, and 5 pmol/L each antisense primer KY78 (5′-CTCGCAAGCACCCTATCAGGCAGT-3′; nt 311–288) and sense primer KY80 (5′-GCAGAAAGCGTCTAGCCA-

Table 1. Characteristics of the study cohort.

Characteristic, parameter	HCV monoinfected	HIV/HCV coinfected	*P*
Risk factor			NS[a]
Injection drug use	24 (86)	18 (90)	
Heterosexual contact	4 (14)	2 (10)	
Age at enrollment, mean ± SD, years	34.4 ± 6.0	35.2 ± 3.9	NS[b]
Race			.008[a]
Black	4 (14)	11 (55)	
White	21 (75)	8 (40)	
Hispanic	3 (11)	1 (5)	
HCV genotype			NS[a]
1	7 (25)	11 (55)	
2	3 (11)	0 (0)	
3	3 (11)	1 (5)	
4	2 (7)	3 (15)	
Unknown	13 (47)	5 (25)	
Visit A			
CD4 cell count, mean ± SD, cells/μL	1139 ± 345	285 ± 144	<.0001[c]
Plasma HIV RNA level, median (IQR), $\log_{10}$ copies/mL	...	4.1 (2.9–4.6)	...
Receipt of ART	...	0 (0)	...
Visit B			
CD4 cell count, mean ± SD, cells/μL	1101 ± 264	376 ± 191	<.0001[c]
Plasma HIV RNA level, median (IQR), $\log_{10}$ copies/mL	...	2.1 (<1.7–3.4)	...
Receipt of ART	...	18 (90)	...

NOTE. Data are no. (%) of women, unless otherwise noted. ART, antiretroviral therapy; HCV, hepatitis C virus; IQR, interquartile range; NS, not significant ($P > .05$).

[a] Fisher's exact test.

[b] Student's *t* test for normal data.

[c] Wilcoxon rank sum test.

TGGCGT-3′; nt 68–91). The PCR consisted of an initial denaturation step for 10 min at 95°C, then 40 cycles under the following conditions: 15 s at 95°C, 5 s at 70°C, and 15 s at 72°C. For generation of GAPDH mRNA, cDNA synthesis was performed with an oligo d(T) primer under standard conditions. For PCR amplification, we used a commercial GAPDH primer set (Roche Search LC), with the conditions recommended by the manufacturer.

For each PBMC sample, we determined the positive- and negative-strand HCV RNA copy numbers and normalized them to the GAPDH copy number, to provide standardized values (i.e., positive-strand HCV RNA copies and negative-strand HCV RNA copies per molecule of GAPDH). Serum HCV quantities were expressed as HCV RNA copies per microliter (extracted from 140 μL of serum). Previous studies have reported very low rates of negative-strand HCV RNA detection in serum [11, 14, 15]; thus, we did not systematically measure negative-strand HCV RNA in this compartment. To avoid potential cross-contamination, samples for each time point and each compartment from an individual were handled separately. Additionally, all rtPCR amplifications included a negative control that contained no template.

Statistical analyses. Demographic and clinical data were compared by Fisher's exact test for categorical variables and either Student's *t* test or the Wilcoxon rank sum test for continuous variables. The Wilcoxon rank sum test was used to compare HCV RNA levels between the HCV-monoinfected and HIV/HCV–coinfected women; values for undetectable levels were set at 0 for serum samples ($\log_{10}$ transformed) and at 0.01 for PBMC samples (untransformed). Spearman's correlation test was used to investigate the linear relationships between CD4 cell count, plasma HIV RNA level, and serum and PBMC HCV RNA levels. All *P* values reported are 2-sided; $P < .05$ was considered to be statistically significant. No adjustments were made for multiple comparisons. All analyses were performed by use of SAS software (version 9; SAS Institute).

RESULTS

Study cohort characteristics. Twenty-eight HCV-monoinfected and 20 HIV/HCV–coinfected women from the HER Study cohort were selected for the present study. These 2 groups of women did not differ with respect to the reporting of IDU as the main risk factor for HCV acquisition, age at enrollment, or HCV genotype; however, HIV/HCV–coinfected women were more likely to be black (table 1). None of the women reported receiving HCV treatment during the visits included in the present study. Mean CD4 cell counts were lower in the HIV/HCV–

Table 2. Strand-specific hepatitis C virus (HCV) RNA detection rates.

Visit, clinical variable	Total	HCV monoinfected	HIV/HCV coinfected	*P*
Visit A				
Serum positive-strand HCV RNA	89 (42/47)	82 (23/28)	100 (19/19)	NS
PBMC positive-strand HCV RNA	42 (20/48)	32 (9/28)	55 (11/20)	NS
PBMC negative-strand HCV RNA	35 (17/48)	32 (9/28)	40 (8/20)	NS
Visit B				
Serum positive-strand HCV RNA	91 (43/47)	93 (26/28)	89 (17/19)	NS
PBMC positive-strand HCV RNA	44 (20/45)	29 (8/28)	71 (12/17)	.01
PBMC negative-strand HCV RNA	38 (17/45)	25 (7/28)	59 (10/17)	.03

NOTE. Data are percentage (no. positive/no. tested) of women, unless otherwise noted. *P* > .05); PBMC, peripheral-blood mononuclear cell.

coinfected women than in the HCV-monoinfected women at both time points (visit A, 285 vs. 1139 cells/μL [$P < .0001$]; visit B, 376 vs. 1101 cells/μL [$P < .0001$]). After ART initiation (between visits A and B), median plasma HIV RNA levels decreased, from 4.1 to 2.1 $\log_{10}$ copies/mL, in the HIV/HCV–coinfected women ($P = .0002$), whereas mean CD4 cell counts increased, from 285 to 376 cells/μL ($P = .4$), in these women.

Strand-specific HCV RNA detection rates. At visit A, positive-strand HCV RNA was detected, by a strand-specific rtPCR assay, in serum from 42 (89%) of 47 women, including 23 (82%) of 28 HCV-monoinfected women and 19 (100%) of 19 HIV/HCV–coinfected women (table 2). At visit B, 43 (91%) of 47 women had detectable levels of positive-strand HCV RNA in serum. Rates of detection in the serum compartment were not significantly different between the HCV-monoinfected and the HIV/HCV–coinfected women at either visit. We did not systematically measure levels of negative-strand HCV RNA in the serum compartment. However, of 47 women tested, 34 (72%) had undetectable or negligible levels (<1000 copies/μL) of negative-strand HCV RNA (data not shown). Furthermore, among those women in whom both strands were detected, the ratio of negative-strand:positive-strand HCV RNA in the serum compartment was <1% in both groups, suggesting that there is a vast excess of positive-strand HCV RNA in the serum compartment (data not shown). In contrast, the proportion of negative-strand HCV RNA relative to positive-strand HCV RNA in the PBMC compartment was significantly higher (particularly in the HIV/HCV–coinfected women), a finding that is consistent with higher rates of HCV replication in the PBMC compartment.

Our findings regarding detection of positive- and negative-strand HCV RNA in the PBMC compartment by the strand-specific rtPCR assay were strongly suggestive of extrahepatic HCV replication. At visit A, positive-strand HCV RNA was detected in the PBMC compartments of 20 (42%) of 48 women, including 9 (32%) of 28 HCV-monoinfected women and 11 (55%) of 20 HIV/HCV–coinfected women. Negative-strand HCV RNA was detected in 17 (35%) of 48 women, including 9 (32%) of 28 HCV-monoinfected women and 8 (40%) of 20 HIV/HCV–coinfected women. At visit B, after the HIV-infected women had initiated ART, positive-strand HCV RNA was still more readily detected in the HIV/HCV–coinfected women than in the HCV-monoinfected women (8/28 [29%] vs. 12/17 [71%]; $P = .01$). The negative-strand HCV RNA detection rate was also significantly different in the HCV-monoinfected and the

Table 3. Median strand-specific hepatitis C virus (HCV) RNA levels.

Visit, clinical variable	HCV monoinfected	HIV/HCV coinfected	*P*
Visit A, median (75th percentile)			
Serum positive-strand HCV RNA level	3.6 (5.0)	5.2 (5.6)	.002
PBMC positive-strand HCV RNA level	0 (31.2)	2.5 (30.7)	NS
PBMC negative-strand HCV RNA level	0 (2.3)	0 (20.0)	NS
Visit B, median (75th percentile)			
Serum positive-strand HCV RNA level	4.4 (5.0)	5.5 (5.9)	.003
PBMC positive-strand HCV RNA level	0 (48.0)	5.4 (38.2)	NS
PBMC negative-strand HCV RNA level	0 (2.3)	0.6 (19.3)	NS

NOTE. Data for serum HCV RNA levels, which are $\log_{10}$ transformed, are no. of HCV RNA copies per microliter (extracted from 140 μL of serum); data for PBMC HCV RNA levels, which are untransformed, are no. of HCV RNA copies per molecule of glyceraldehyde-3-phosphate dehydrogenase. The medians for several of the clinical variables are 0 because of low rates of detection. NS, not significant (*P* > .05); PBMC, peripheral-blood mononuclear cell.

HIV/HCV–coinfected women at visit B (7/28 [25%] vs. 10/17 [59%]; $P = .03$). Negative-strand HCV RNA was detected in the PBMC compartment only when positive-strand HCV RNA was also detected.

Strand-specific HCV RNA levels. Strand-specific HCV RNA levels were determined in the serum and PBMC compartments and, in the latter case, were normalized to the GAPDH copy number (table 3). Using dilutions of serum samples for which HCV RNA levels had previously been determined (by use of the Roche Amplicor Monitor Kit), we determined that the lower level of detection for the strand-specific rtPCR assay was ~260 copies/μL. The HIV/HCV–coinfected women had higher positive-strand HCV RNA levels in serum than did the HCV-monoinfected women, both before and after ART initiation (visit A, 3.6 vs. 5.2 $\log_{10}$ copies/μL [$P = .002$]; visit B, 4.4 vs. 5.5 $\log_{10}$ copies/μL [$P = .003$]). Because of the low rates of detection of HCV RNA in the PBMC compartment, medians could not be defined in several instances; therefore, 75th percentiles are presented in table 3. At visit A, there was no significant difference in either positive- or negative-strand HCV RNA levels in the PBMC compartment between the 2 groups. At visit B, after ART initiation, both positive- and negative-strand HCV RNA levels in the PBMC compartment were higher in the HIV/HCV–coinfected women than in the HCV-monoinfected women, but these differences did not reach statistical significance.

Correlation analyses. We also analyzed potential correlations between specific immunologic parameters and strand-specific HCV RNA levels (table 4). Age and plasma HIV RNA levels were not correlated with either positive- or negative-strand HCV RNA levels in either compartment. CD4 cell count was inversely correlated with positive-strand HCV RNA levels in the serum ($P \leq .0001$), but not in the PBMC, compartment. Positive-strand HCV RNA levels in the serum compartment were consistent over time ($P < .0001$), as were both positive- and negative-strand HCV RNA levels in the PBMC compartment ($P < .0001$). However, in the absence of ART, positive-strand HCV RNA levels in the serum compartment were not correlated with either positive- or negative-strand HCV RNA levels in the PBMC compartment. Between visits, neither positive- nor negative-strand HCV RNA levels in the PBMC compartment were correlated.

Table 4. Correlation between clinical variables and strand-specific hepatitis C virus (HCV) RNA levels.

Comparison	Coefficient[a]	*P*
Serum positive-strand HCV RNA level (A) vs.		
Serum positive-strand HCV RNA level (B)	0.75	<.0001
PBMC positive-strand HCV RNA level (A)	0.02	NS
PBMC negative-strand HCV RNA level (A)	0.01	NS
PBMC positive-strand HCV RNA level (A) vs.		
PBMC negative-strand HCV RNA level (A)	0.89	<.0001
PBMC positive-strand HCV RNA level (B)	0.08	NS
PBMC positive-strand HCV RNA level (B) vs. PBMC negative-strand HCV RNA level (B)	0.90	<.0001
PBMC negative-strand HCV RNA level (A) vs. PBMC negative-strand HCV RNA level (B)	0.06	NS
CD4 cell count vs.		
Serum positive-strand HCV RNA level (A)	−0.54	<.0001
PBMC positive-strand HCV RNA level (A)	−0.10	NS
PBMC negative-strand HCV RNA level (A)	0.02	NS
Plasma HIV RNA level vs.		
Serum positive-strand HCV RNA level (A)	0.11	NS
PBMC positive-strand HCV RNA level (A)	0.31	NS
PBMC negative-strand HCV RNA level (A)	0.18	NS

NOTE. A and B refer to the visit. NS, not significant ($P > .05$); PBMC, peripheral-blood mononuclear cell.

[a] Spearman correlation coefficient for all women.

DISCUSSION

Researchers have long sought to establish whether HCV replicates outside the liver, because detection of HCV RNA in extrahepatic reservoirs has important implications for transmission, disease progression, and effective treatment. Nonetheless, achieving a definitive demonstration of extrahepatic HCV replication has been limited by several biological and technical considerations. Foremost, the lack of a robust cell-culture system has made it exceedingly difficult to compare HCV replication in different cell populations. To date, the dynamics of HCV replication have typically been examined by intensive study of serum-specific or liver-specific HCV RNA; however, viral replication in such extrahepatic reservoirs as PBMCs may not reflect replication in these other compartments. Furthermore, although detection of positive-strand HCV RNA cannot distinguish between nucleic acids participating in replication and those already incorporated into viral particles, detection of replication intermediates, such as negative-strand HCV RNA, is a more biologically relevant measure of active virus replication. Negative-strand HCV RNA is generally present at levels 10–100-fold lower than those of positive-strand HCV RNA [36, 37]; thus, highly sensitive and specific detection assays must be used. Although distinguishing between positive- and negative-strand HCV RNA is critical, not all strand-specific detection methods have high specificity for detection of negative-strand HCV RNA. Here, we have used a validated strand-specific rtPCR assay that includes the *Tth* enzyme. Because this enzyme contains separate reverse transcriptase and DNA-dependent polymerase functions, it is highly specific and is ideal for discriminating between positive- and negative-strand RNA [17, 19].

Although several studies have measured HCV replication in the PBMC compartment, only a subset have used a bona fide *Tth*-based amplification assay to distinguish between positive- and negative-strand HCV RNA [4, 9, 17, 29, 38–41]. Our rate of detection of negative-strand HCV RNA in the PBMC compartment was somewhat elevated, compared with the results of these previous studies. Such differences could reflect minor dis-

crepancies in the amplification assay, study populations, HCV antiviral receipt, and/or sample preparation. However, it has previously been demonstrated that both HIV coinfection and testing of multiple extrahepatic samples are associated with an increased likelihood of detection of negative-strand HCV RNA [29, 38, 41]. For example, Laskus et al. demonstrated the presence of negative-strand HCV RNA in 5 of 14 PBMC samples from HIV/HCV–coinfected patients [29]. The authors also suggested that factors governing HCV replication at hepatic and extrahepatic sites may differ. Thus, one might anticipate increased detection of negative-strand HCV RNA in a population such as ours, because no participant received HCV antiviral therapy, a high prevalence of HIV coinfection existed, and we tested multiple samples for each participant. It is also theoretically possible that our use of an all-female cohort is responsible for increased detection of negative-strand HCV RNA, although sex-specific detection rates have not been reported to date.

Our study design has several distinct advantages over those of previously published studies. First, to date, most studies of extrahepatic replication have been restricted to a small population analyzed in a cross-sectional, rather than a longitudinal, manner. Second, paired serum and PBMC samples have not usually been analyzed, making intercompartment comparisons difficult. Third, despite clinical data suggesting that HIV adversely affects HCV replication, disease progression, and treatment response rates, HCV-monoinfected and HIV/HCV–coinfected persons have not typically been analyzed as distinct groups. Fourth, not all previously published studies used a strand-specific rtPCR assay that had high strand specificity.

The present study design does have several limitations. First, very low levels of negative-strand HCV RNA were detected in the serum compartments of a subset of women. Because "naked" negative-strand HCV RNAs are not known to circulate outside of cells, we suggest that these very low levels of negative-strand RNA likely represent a small amount of contaminating RNA from residual PBMCs that were not completely removed during the initial processing of whole blood. Second, given the limited number of PBMCs available, we were not able to more precisely define the cell population(s) within PBMCs that are responsible for HCV replication. Nonetheless, there is growing evidence that HCV may infect several peripheral-blood cell types, including B lymphocytes, granulocytes, monocytes/macrophages, and dendritic cells [8, 12, 40]; it is, however, important to note that each of these previous studies either excluded persons coinfected with HIV or did not report HIV status.

Results from our pilot study should be interpreted with caution, given its limited sample size. Nonetheless, we here report several novel findings regarding extrahepatic HCV replication. First, rates of detection of HCV RNA in the PBMC compartment were higher for HIV/HCV–coinfected women than for HCV-monoinfected women. Previous studies have suggested that serum HCV RNA levels are higher in HIV/HCV–coinfected persons [21]; however, this phenomenon has not been investigated in the PBMC compartment until now. Importantly, negative-strand HCV RNA, indicative of active viral replication, was detected at higher rates in the PBMC compartments of HIV/HCV–coinfected women, highlighting an important interaction between these 2 viruses in this compartment. Second, there was no correlation between plasma HIV RNA levels and positive- or negative-strand HCV RNA levels in either the serum or PBMC compartment. Moreover, ART initiation appeared to have a minimal effect on HCV detection rates and HCV RNA levels, although, because of the limited number of HIV/HCV–coinfected persons included in the present study, we cannot rule out a possible association. The finding of elevated HCV RNA levels in the serum and PBMC compartments even after ART initiation may suggest that immune reconstitution after suppression of HIV is not sufficient to control HCV replication. Third, there was an inverse correlation between CD4 cell counts and positive-strand HCV RNA levels in the serum, but not the PBMC, compartment (i.e., as the CD4 cell count increased, the serum, but not the PBMC, HCV RNA level decreased). Given that several components of PBMCs may support HCV replication [8, 12, 40], it is provocative to speculate that the PBMC compartment may be a site in which HCV is partially protected from adaptive and/or innate immune responses. Fourth, there was a positive correlation between positive- and negative-strand HCV RNA levels in the PBMC compartment. However, serum and PBMC HCV RNA levels did not correlate with each other. Thus, HCV RNA may be regulated differently in these compartments.

The precise mechanisms by which HIV influences extrahepatic HCV replication have yet to be determined. It is possible that HIV-induced immunosuppression results in less immunologic control of HCV replication, although reproducible correlations between HCV RNA levels in the serum compartment and CD4 cell counts have not been confirmed [30]. Moreover, the presence of replicative viral forms in extrahepatic sites does not correlate with CD4 cell count [29]. Interestingly, in the present study, HCV RNA levels in the PBMC compartment did not correlate with CD4 cell counts, although positive-strand HCV RNA levels in the serum compartment and CD4 cell counts were inversely correlated. These data imply that immunosuppression alone is not the sole driving force behind increased detection of HCV RNA in the PBMC compartment. It is also possible that HIV, through the induction of interferon antagonists, blunts host innate antiviral responses that would otherwise inhibit HCV replication. HIV may also render specific types of PBMCs more susceptible to HCV infection and replication [41].

In summary, low-level HCV replication in the PBMC compartment, as indicated by detection of negative-strand HCV RNA, may adversely influence the effectiveness of HCV anti-

viral therapies [38, 42], particularly in HIV/HCV–coinfected persons. Furthermore, the PBMC compartment may be a privileged site for HCV that is capable of reinitiating viral replication after termination of HCV treatment, when conditions once again become more favorable. Thus, even if clearance of HCV from hepatocytes is achieved by treatment, reinfection from such extrahepatic sites as the PBMC compartment may occur [43]. Future studies of HCV quasispecies diversification in serum and PBMCs may provide additional evidence that HCV replication—and evolution—is distinct in these compartments and may require targeted therapeutic approaches.

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Tracing the History of Hepatitis B Virus Genotype D in Western Japan

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The major hepatitis B virus (HBV) genotypes in Japan are B and C. HBV genotype D (HBV/D), however, is widespread in a small area of Western Japan, where the Gianotti–Crosti syndrome caused by HBV subtype *ayw*, which is suspected to be HBV/D, was endemic in the 1970s. The aim of the study was to elucidate its origin, time of transmission, and spread in this area. Genotyping of HBV-DNA was done in 363 patients with HBV infection. The year of birth was checked in patients with HBV/D. The full genome sequences of 20 HBV/D strains, 2 of which were obtained from a single carrier with a 19-year-interval, were analyzed. An evolutionary rate, the date of the most recent common ancestor, and the effective number of HBV/D infections were calculated. Fifty-two of 363 patients were infected with HBV/D, and 39 were born in 1970s. In a phylogenetic tree, the 20 HBV/D strains produced a definite cluster, and the evolutionary rate was calculated to be 5.4×10^{-5} nucleotide substitutions/site/year. The root of the tree was estimated to be in approximately 1,900 and began to spread from the 1940s, leading to a rapid increase of infected patients in the 1970s. From these results, it is suspected that HBV/D was likely transmitted to the area investigated approximately 100 years ago and then spread widely in the 1970s. From the history of the area and the genetic analysis, HBV/D in this area was speculated to be of Russian origin. ***J. Med. Virol. 78:44–52, 2006.***

KEY WORDS: hepatitis B surface antigen; subtype; evolutionary rate; complete genome sequence; Gianotti–Crosti syndrome; Japanese–Russian war

INTRODUCTION

Hepatitis B virus (HBV) is one of the major causes of liver disease throughout the world, as approximately 350 million people are infected chronically. HBV has approximately 3,200 bases that can be divided into several genotypes by sequence divergence, with 8 genotypes (A–H) reported [Okamoto et al., 1988; Norder et al., 1994; Stuyver et al., 2000; Arauz-Ruiz et al., 2002]. These genotypes have a distinct geographical distribution, with genotypes A (HBV/A) and HBV/D predominant in Europe, Middle East, Central Asia, Siberia, and America, HBV/B and HBV/C in East Asia, and HBV/E in Africa. In addition, HBV/F has been reported in Central America, and HBV/G in the United States and France [Norder et al., 1993; Lindh et al., 1997; Sanchez-Tapias et al., 2002; Chu et al., 2003; Miyakawa and Mizokami, 2003; Deversa et al., 2004; Mulders et al., 2004; Tallo et al., 2004]. In Japan, HBV/C is the most prevalent, followed by HBV/B, while others are encountered very rarely. Although the frequency of HBV/D was reported to comprise only 0.4% of HBV carriers in Japan [Orito et al., 2001], it was found recently that approximately 10% of the HBV carriers in a small geographical area (Ehime Prefecture) in Western Japan were infected with HBV/D [Duong et al., 2004]. In this area, an endemic occurrence of infantile

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papular acrodermatitis (Gianotti–Crosti syndrome), which is known to be related to acute HBV infection [De Gaspari et al., 1970; Gianotti, 1973], emerged in the 1970s, with the subtype (serotype) of the hepatitis B surface antigen (HBsAg) reported to be *ayw* in those patients, whereas that of the majority of other HBV carriers in this area are infected with subtype *adr* [Ishimaru et al., 1976; Toda et al., 1978]. Unfortunately, serum samples from those patients taken in 1970s are no longer available, although that of a girl with this syndrome taken in 1988 was kept and subsequent testing demonstrated that she was infected with HBV/D, serotype *ayw* [Michitaka et al., 2004]. Recently, it was reported that the deduced HBsAg serotype in all HBV/D strains studied in this area was *ayw3* [Duong et al., 2004], in contrast to the majority of HBV/C patients in Japan, who are known to be infected with serotype *adr*. From those results, it is speculated that HBV with subtype *ayw* found in patients with the Gianotti–Crosti syndrome from this area in 1970s was HBV/D, and the endemic occurrence of this disease was related to the spread of HBV/D in this area. Further, it is suspected that the HBV/D was not indigenous, but rather from abroad, since HBV/D is very rare in the surrounding districts.

In the present study, attempts were made to clarify the origin, time of transmission, and time of spread of HBV/D in this area using molecular evolutional analyzes.

MATERIALS AND METHODS

Patients

Three hundred and sixty-three patients (13–86 years old, median 45 years, 213 males and 150 females) with chronic HBV infection living in the Ehime Prefecture who attended hospital between 1997 and 2003 were examined for HBV genotypes and the year of birth. This was done to gain insight on the period of spread of genotype D. Among these patients, 253 patients had normal levels of serum aminotransferase (ALT) and 110 patients had elevated levels of ALT. Four of patients had a past history of Gianotti–Crosti syndrome. The purpose of the study was explained to patients before taking the samples and written informed consent was obtained from all patients.

Materials for Complete Genome Sequence

Twenty complete HBV genome sequences from 19 Japanese patients infected with HBV/D (8 women, 11 men, 13–86 years of age), 16 with chronic and 3 with acute infection, who were born and living in the Ehime Prefecture were analyzed. Among 16 patients with chronic infection, 14 had persistently normal ALT levels, whereas the other 2 had persistent or intermittent elevation of ALT levels. Two of the 20 HBV strains were from a single HBV carrier that was sampled with a 19-year-interval. Seven of the 20 HBV strains were reported previously, while the other 13 were newly sequenced for this study. The newly sequenced strains were selected at random from the patients in this study. Serum samples were stored at −80°C prior to genotyping and sequencing.

HBV Genotyping

The HBV genotype was determined based on the restriction fragment length polymorphism patterns of the *S* gene sequence following amplification by the polymerase chain reaction (PCR-RFLP) [Mizokami et al., 1999].

Complete Genome Sequence

Complete genome sequences were determined by direct sequencing of the PCR-products, the detail of which were described previously [Chen et al., 2003]. Briefly, DNA was extracted from sera and HBV-DNA was amplified by PCR. To obtain the full-length HBV-DNA sequence, 2 amplicons were obtained by PCR, and 1 fragment was 2,936 bases in length (nt 1,994–nt 1,747), and the other 1,080 bases in length (nt 1,399–nt 2,478). Sequencing was done by direct sequencing using a commercially available kit with suitable sequencing primers (BigDye Terminator Cycle Sequencing FS Ready Reaction Kit, Applied Biosystems, Alameda, CA). The accuracy of the sequence was ensured by identification of the sequence data of the complete genome obtained by sense sequencing primers and that obtained by anti-sense sequencing primers.

Estimating Evolutionary Rates and Dating the Origin of HBV

A reconstructed tree was produced using the concatenated non-overlapping regions of the HBV genome. Overlapping regions were excluded, because they are subject to complex evolutionary processes that might increase phylogenetic noise [Bollyky and Holmes, 1999; Fares and Holmes, 2002], resulting in a final alignment of 1,591 bases of the non-overlapping sequences for the phylogenetic analysis. The tree was built on the non-overlapping regions using a heuristic maximum-likelihood (ML) topology search with stepwise-addition and nearest neighbor-interchange algorithms. Tree likelihood scores were calculated using HKY85, with the molecular clock enforced using PAUP version 4.0b8. Using the estimated topology, all possible root positions were evaluated under a single rate dated tips (SRDT) model with the computer software TipDate v1.2 and the root that yielded the highest likelihood was adopted [Rambaut, 2000]. The program provided an ML estimate of the rate and also the associated date of the most recent common ancestor of the sequences, using a model that assumed a constant rate of nucleotide substitution. The molecular clock was tested by a likelihood ratio test between the SRDT model and a general unconstrained branch length model [different rate (DR) model]. To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were also carried out 1,000 times.

Demographic Model

For estimates of demographic history, a non-parametric function N(t), also known as a skyline plot, was obtained by transforming the coalescent intervals of an observed genealogy into a piecewise plot that represented an effective population size through time [Pybus et al., 2001; Pybus and Rambaut, 2002]. A parametric ML was estimated by several models with the computer software Genie v3.0 to build a statistical framework for inferring the demographic history of a population on phylogenies reconstructed from sampled DNA sequences [Pybus and Rambaut, 2002]. This model assumes a continuous epidemic process in which the viral transmission parameters remain constant through time. Model fitting was evaluated by likelihood ratio tests of the parametric ML estimates [Lemey et al., 2003; Pybus et al., 2003]. Approximate 95% confidence intervals for the parameters were estimated using the likelihood ratio test statistics.

RESULTS

Year of Birth

The numbers of patients infected with HBV/A, HBV/B, HBV/C, and HBV/D were 6, 24, 281, and 52, respectively. Figure 1 shows the number of patients infected with HBV/C and HBV/ D in relation to years of birth. Patients with HBV/C were born within a wide spectrum of time (between 1940 and 1980). On the other hand, 39 of 52 patients infected with HBV/D were born in 1970s. All four patients who had a history of the Gianotti–Crosti syndrome were infected with HBV/D, and three were born in 1970s, whereas one was born in 1980s.

Complete Sequences of 20 HBV/D Strains

The 20 serum samples from 19 patients with HBV infection, whose HBV genotype was determined to be HBV/D by PCR-RFLP, were subjected to complete HBV genome sequencing. The accession numbers of the 20 complete HBV genome sequences and additional information regarding the infected patients are shown in Table I. All 20 strains were found to be 3,182 bases in length except 1 with 3,194 bases (AB090269), and the deduced HBsAg serotype was *ayw3* in all 20 strains. No recombinant sequences with other HBV genotypes were detected in any of these 20 HBV complete genomes. Among 19 patients, 1 patient whose HBV sequence was Ehime D5 had the history of sexual contact with 2 patients whose HBV were Ehime D3 and Ehime D4. Other 16 patients had no history of mutual contact.

Phylogenetic Relationship Among Ehime HBV Strains

An un-rooted ML tree for the non-overlapping regions of the HBV genome is represented in Figure 2. The 20 strains from Ehime, which were HBV/D, showed a significant cluster with a high bootstrap value, and some European and Russian (Kamchatka) strains, especially a Swedish strain (AY090453), were found to be closely related. Such a significant cluster is suitable for a coalescent analysis. As the tree topology on the non-overlapping regions was quite similar to that of the complete genomes, the tree on the non-overlapping

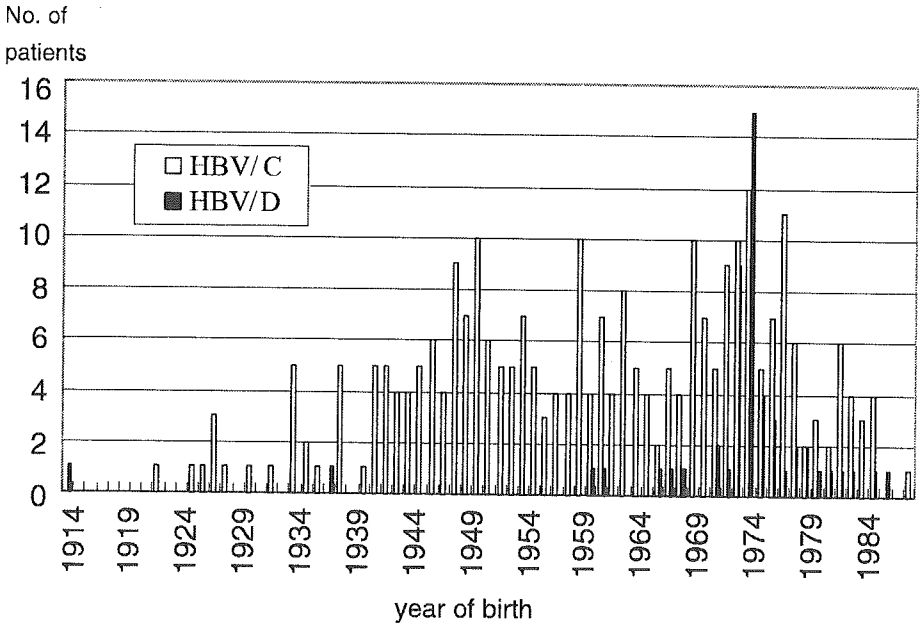

Fig. 1. The years of birth of patients infected with hepatitis B virus (HBV)/C and HBV/D. Numbers of patients infected with HBV/C was shown by white bars, whereas that with HBV/D was shown by black bars.

TABLE I. Hepatitis B Virus (HBV) Genotype D Sequences in Ehime Prefecture Used in This Study

Sequence	Accession no.	Length of genome (bp)	Serotype	Sex	Age	Date of collection	Aminotransferase (ALT)	HBeAg	Diagnosis[a]	Reference
Ehime D1	AB090268	3,182	*ayw3*	M	27	1997	88	+	CH	Duong et al. [2004]
Ehime D2	AB090269	3,194	*ayw3*	M	64	1992	39	+	CH	Duong et al. [2004]
Ehime D3	AB078031	3,182	*ayw3*	F	18	1998	7,620	+	FH	Chen et al. [2003]
Ehime D4	AB078032	3,182	*ayw3*	F	20	2000	189	+	AH	Chen et al. [2003]
Ehime D5	AB078033	3,182	*ayw3*	M	19	1998	42	+	ASC	Chen et al. [2003]
Ehime D6	AB090270	3,182	*ayw3*	M	21	1999	46	+	ASC	Duong et al. [2004]
Ehime D7	AB109475	3,182	*ayw3*	F	64	2001	33	+	ASC	Present study
Ehime D8	AB109476	3,182	*ayw3*	M	70	1997	21	+	ASC	Present study
Ehime D9	AB109477	3,182	*ayw3*	M	24	1997	21	+	ASC	Present study
Ehime D10	AB109478	3,182	*ayw3*	M	24	1998	14	−	ICS	Present study
Ehime D11	AB109479	3,182	*ayw3*	F	25	1997	15	−	ICS	Present study
Ehime D12	AB110075	3,182	*ayw3*	F	86	2001	11	−	ICS	Present study
Ehime D13	AB119251	3,182	*ayw3*	M	28	2002	40	−	ICS	Present study
Ehime D14	AB119252	3,182	*ayw3*	M	27	2002	24	−	ICS	Present study
Ehime D15	AB119253	3,182	*ayw3*	M	26	2000	43	−	ICS	Present study
Ehime D16	AB119254	3,182	*ayw3*	M	28	2003	26	−	ICS	Present study
Ehime D17	AB119255	3,182	*ayw3*	F	28	2001	12	−	ICS	Present study
Ehime D18	AB119256	3,182	*ayw3*	F	23	1997	21	−	ICS	Present study
Ehime D19	AB116266	3,182	*ayw3*	F	13	1987	1,452	−	AH	Michitaka et al. [2004]
Ehime D20	AB120308	3,182	*ayw3*	F	66	1982	378	+	AH	Present study

[a]AH, acute hepatitis; FH, fulminant hepatitis; CH, chronic hepatitis; ASC, asymptomatic HBV carrier; ICS, inactive hepatitis B surface antigen (HBsAg) carrier state [Lok and McMahon, 2001].
Isolates Ehime D12 and Ehime D20 are from the same patient.

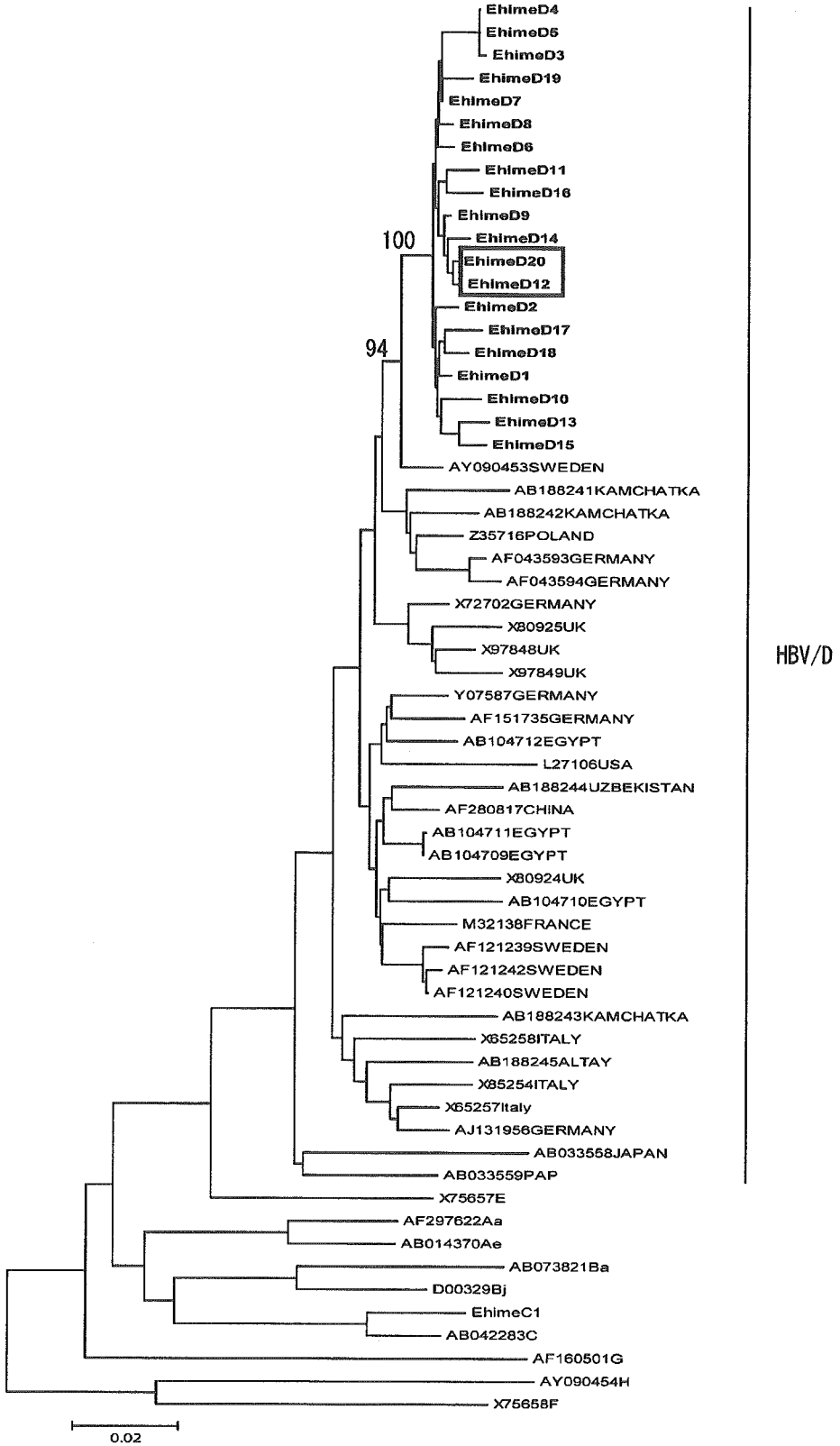

Fig. 2. A phylogenetic tree constructed using non-overlapping sequences of 20 HBV/D strains from Ehime and reference sequences. Reference isolates from the database are identified with accession number, and each country name was added in all HBV/D strains. The Ehime strains in this study were shown in bold. The number in the tree indicates bootstrap reliability. Isolates Ehime D12 and Ehime D20 are from the same patient surrounded by open square.

sequences was used in this study to exclude phylogenetic noise [Bollyky and Holmes, 1999; Fares and Holmes, 2002].

Rates and Demographic History of HBV Evolution

To determine the evolutionary rate of HBV, the 20 HBV/D strains, which included 2 strains (AB110075, AB120308) obtained from the same subject with a 19-year interval, were subjected to molecular evolutionary analyses. The molecular evolutionary rate was estimated by two independent methods. Briefly, direct comparison on non-overlapping sequences with a 19-year interval obtained from the same subject indicated that a molecular evolutionary rate was 5.9×10^{-5} nucleotide substitutions/site/year. Second, TipDate (v1.2) was used to compare the DR model with the single rate (SR) and SRDT models. The SR model was rejected ($P < 0.01$) and the SRDT model provides an adequate fit to the data ($P = 0.15$). Based on the SRDT model, the mean rate of nucleotide substitutions was estimated to be 5.4×10^{-5} nucleotide substitutions/site/year (95% confidence intervals of 4.0×10^{-5} to 7.2×10^{-5}), which was similar to the rate (4.2×10^{-5}) estimated by Fares and Holmes [2002], and resulted in a date estimate of 1902 for the root of the tree (95% confidence intervals of 1,867–1,927).

Based on the phylogenetic tree, the effective number of HBV infections through time, N(t), was analyzed using a skyline plot for the Ehime HBV strains. The parameters for several models in Genie v3.0 were also examined. Time t was then transformed to year using the same rate, assuming the collecting time to be the present. Figure 3 shows the skyline plots and population growth for the HBV patients in Ehime, according to a specific demographic model in Genie v3.0 with three parameters, a piecewise expansion growth model, which was evaluated by likelihood ratio testing [Lemey et al., 2003; Pybus et al., 2003]. Based on this molecular evolution, it was estimated that the divergence time of the most recent common ancestor of HBV/D in Ehime was also estimated to be approximately 1,900. Further, the Ehime HBV/D strains of Ehime began to increase in the 1940s, and the time of spread was estimated to be around 1970 when the spread time was defined temporally as 10% of the present population size of HBV infections (Fig. 3).

DISCUSSION

The HBV/D strains in Ehime were found to have a significant cluster with a high bootstrap value and were clearly distinct from most European strains. Such a significant cluster is suitable for a coalescent analysis. The specific demographic model based on the neutral theory [Pybus et al., 2001, 2003; Lemey et al., 2003], which has a constant size in the past and changes to exponential growth until the present, was applied for investigating the Japanese endemic of HBV/D in Ehime.

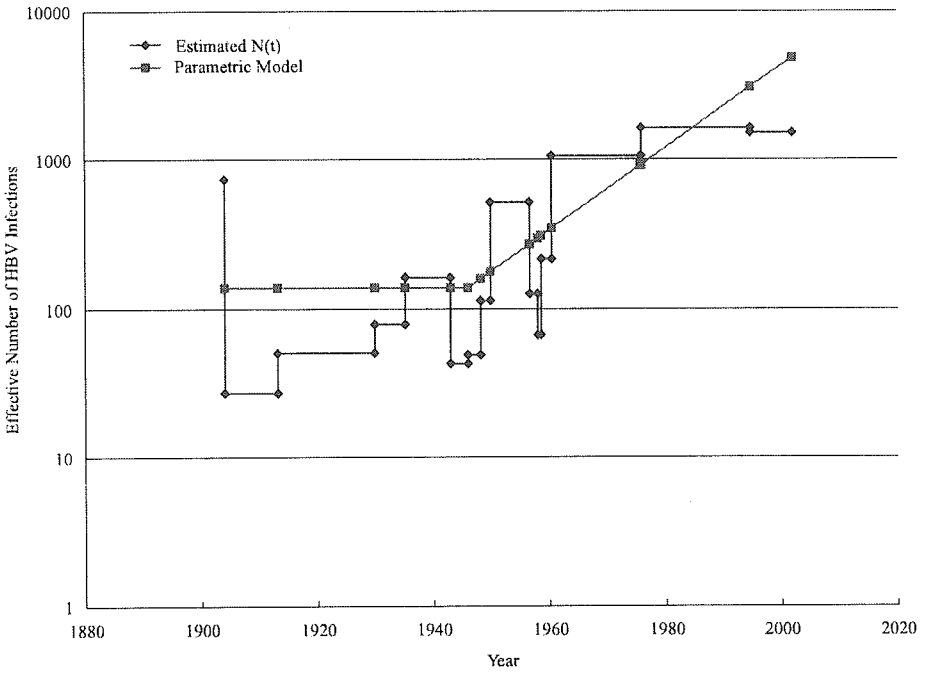

Fig. 3. The maximum-likelihood (ML) estimates of N(t) on the effective number of HBV/D infections in Ehime. The parametric model is indicated by the magenta line and stepwise plots by the blue line, which represent corresponding non-parametric estimates of N(t) (number as a function of time). Genetic distances have been transformed into a time scale of years using estimates of the molecular clock in non-overlapping regions of HBV.

Several historical factors in Japan have probably affected the spread of HBV/D, such as an increase of intravenous drug abuse in the 1940s during and after World War II, and the increase of blood transfusion procedures and the use of non-sterilized medical materials in the 1960s and 1970s. Using molecular evolutionary analyses; the spread of HBV/D in Ehime was determined to have started in the 1940s and rapidly increased around 1970. The endemic occurrence of the Gianotti–Crosti syndrome by HBV with serotype *ayw* in the investigated area emerged in 1970s [Ishimaru et al., 1976], which were close to the estimated spread time of HBV/D in the present study. Many infant patients with the Gianotti–Crosti syndrome in this region were reported to have progressed to a chronic carrier state [Toda et al., 1978]. The fact that majority of the patients infected with HBV/D were born in 1970s indicates a relationship with the endemic of this disease in infants in 1970s because the Gianotti–Crosti syndrome occurred at this time. It was not possible to ascertain the exact time of infection in individual cases, therefore, it is a limitation of this study. However, the time of birth was almost similar, and this circumstantial evidence strongly supports the calculated data from the molecular evolutionary analyses that the time of spread of the infection was 1970s. Although the infectious routes have not been clarified, the use of non-sterilized medical equipment such as injection needles in children may be one of the important routes. It is another problem whether HBV/D with serotype *ayw3* has a character to induce the Gianotti–Crosti syndrome, or whether HBV/D strains from patients with this syndrome have a peculiar motif in their nucleotide or amino acid sequences. This issue should be clarified in the future.

The molecular evolutionary analyses revealed that the time of the root of the HBV/D tree in this area was estimated to be around 1,900. It is of an interest to understand how HBV/D was transmitted to the Ehime area and where it originated from. The history of this region is likely important to solve this issue. Communication between people living in Ehime and those in foreign countries was not frequent prior to the end of the 19th century. However, in the period of time around 1900, Japan became involved in several wars, such as the Japanese–Sino War from 1894 to 1895, the Japanese–Russian War from 1904 to 1905, and World War I from 1914 to 1918. In connection with these international conflicts, many foreigners came to this area, since Matsuyama city in the Ehime Prefecture had a naval port and a large prison camp, in which approximately 100 Chinese prisoners of war were interned from 1894 to 1895, followed by 6,000 Russian prisoners from 1904 to 1906, and 500 German prisoners from 1914 to 1917. These incidents were considered to have played a role in the importation of HBV/D from other countries to this region of Japan. Among the wars noted above, the Japanese–Sino War is thought to have no relation with the spread of HBV/D, because the prevalence of HBV/D in China is very low [Miyakawa and Mizokami, 2003]. Based on the present data that the divergence time of the most recent common ancestor of HBV/D in Ehime to be approximately 1,900, the Japanese–Russian War is the most likely candidate as the initial event that led to HBV/D transmission in Japan.

Four subgenotypes (D1–D4) have been described for HBV/D [Norder et al., 2004]. The 20 isolates in the present study were assigned D2. Two isolates from Kamchatka in Russia shown in Figure 2 (AB188241, AB188242) were also assigned D2. The subtype of HBsAg of the 20 strains was *ayw3*. Several reports have described an association between drug abuse and infection with HBV subtype *ayw3*. van Steenbergen et al. [2002] performed a molecular epidemiological study of acute hepatitis B in Amsterdam, and found that HBV from majority of drug users were genotype D with subtype *ayw3*. Swenson et al. [2001] studied the HBV genotypes and HBsAg subtypes in refugees and injection drug users in the United States, and found that 7 of 15 refugees from former Soviet Union and 17 of 32 drug users were infected with HBV/D with subtype *ayw3*. Further, they described that HBsAg subtype of HBV/D strains from the majority of the drug users showed regular *ayw3* of which HBsAg had Thr 118 and Met 125, whereas that of the seven refugees from Soviet Union showed variant *ayw3* of which HBsAg had Val 118 or Ala 118 and Thr 125. All of the 20 strains in the present study had HBsAg with Val 118 and Thr 125, which was identical with variant *ayw3* of refugees from former Soviet Union in their study. Interestingly, the phylogenetic analysis indicated that the HBV/D strains with subtype *ayw3* in drug users and refugees from former Soviet Union formed a cluster along with the 20 strains in present study and some European strains from database (Fig. 4). Although this phylogenetic tree was not constructed with complete HBV genome, this result supports the speculation that the HBV/D in Ehime would be originated from Russia. As intravenous drug users had been common in 1940s in Japan, indeed, they might have played some role in the spread of HBV/D in this area.

The present study showed that HBV/D has been spreading rapidly in the intervening century. The infectious routes of blood transfusion, non-sterilized medical materials, and maternal transmission are well controlled now, however, sexual transmission, which is the most common infectious route for adults in Japan [Arima et al., 2003], remains uncontrolled. On the other hand, several reports from the metropolitan area in Japan have described that acute hepatitis with HBV/A infection due to sexual transmission has been increasing [Kobayashi et al., 2002; Ogawa et al., 2002]. Those reports together with the present study led to the suspicion that HBV/A and HBV/D, whose main infectious routes are horizontal, might become the dominant genotypes in Japan in the future, rather than HBV/B and HBV/C, whose main infectious route is vertical, if a suitable preventive policy for HBV transmission is not established. Thus, in order to control the spread of HBV, especially HBV/A and HBV/D, additional efforts are

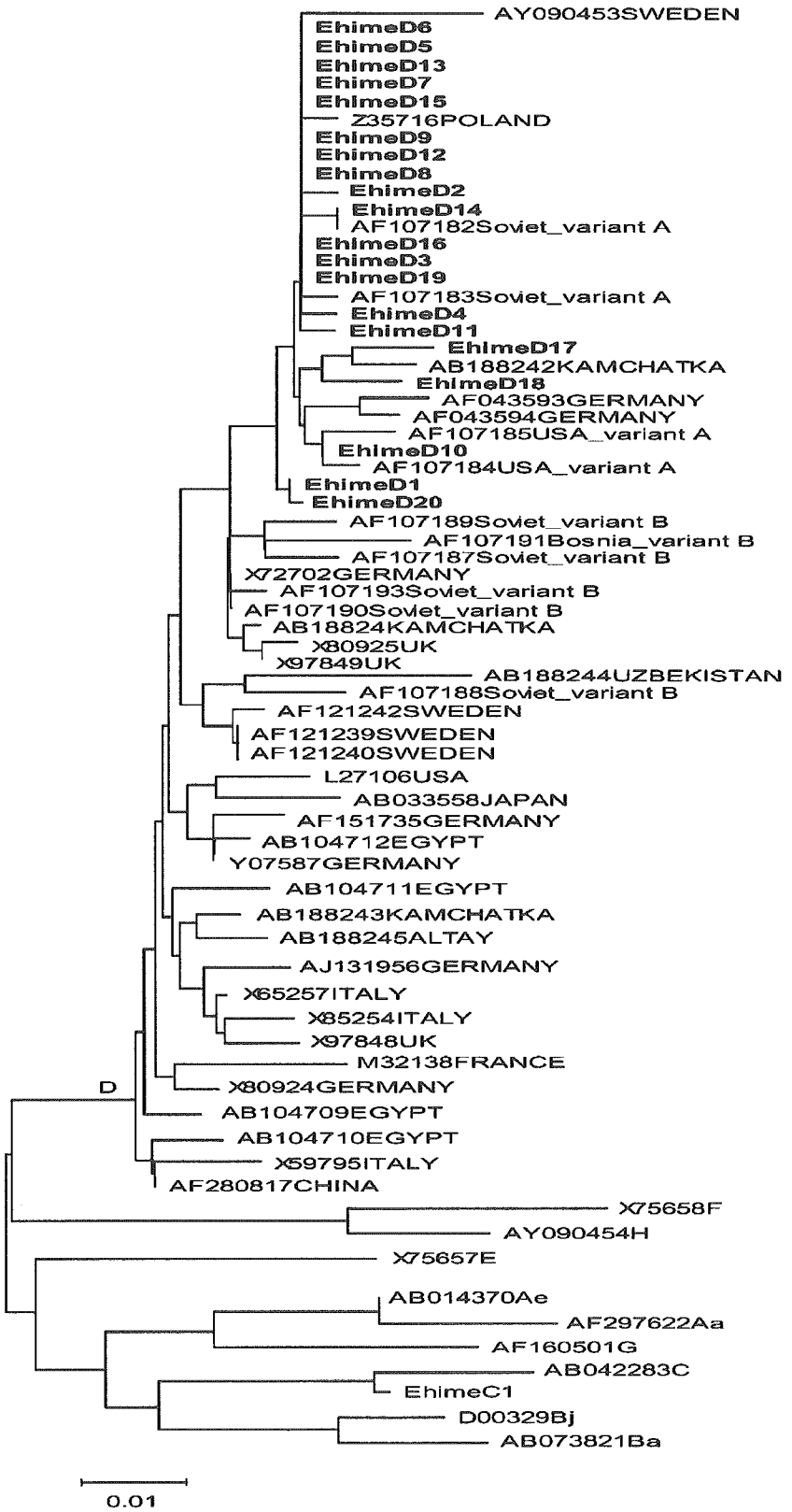

Fig. 4. A phylogenetic tree constructed using small *S* gene (nt 478–774) of 20 HBV/D strains from Ehime and reference sequences.

needed to prevent sexual transmission because universal vaccination against HBV has not yet been introduced in Japan.

In conclusion, HBV/D at the Ehime area in Japan showed a definite cluster, and molecular evolutionary analyses indicate that its root was likely to be around 1,900, followed by a rapid spread in the 1970s.

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Editorial

Optimal timing of interferon treatment for acute hepatitis C

The incidence of acute hepatitis C is declining owing to a near elimination of transfusion associated hepatitis after the initiation of the screening of blood for hepatitis C virus (HCV). However, acute hepatitis C is not totally eliminated. There is still a risk for HCV infection through medical procedures or by accidental needle-stick injury. Since acute hepatitis C is often followed by chronic hepatitis which may eventually progress to liver cirrhosis and hepatocellular carcinoma, establishment of the effective treatment of this disease is still a serious matter.

An appropriate treatment strategy of acute hepatitis C has not been established to date. Several studies have clearly demonstrated the beneficial effect of the interferon (IFN) treatment in the eradication of HCV during acute infection and preventing the progression to chronic hepatitis [1–5]. However, the controversies remain on the following issues: (1) which patients should be treated, (2) when should therapy be started (immediately at the onset of hepatitis or after a period of waiting for spontaneous remission), and (3) what regimen of therapy should be used (whether to use ribavirin combination therapy rather than interferon mono-therapy).

Theoretically, suppression of HCV replication by IFN therapy during the early phase of acute hepatitis may favor the patient's immune systems to clear the virus and prevent the development of chronic infection. In contrast, if HCV replication is not controlled during the early phase due to the delay of the treatment, the immune responses towards HCV during acute hepatitis, which is usually more vigorous compared to chronic hepatitis, may be weakened which lead to the failure of HCV clearance [6–8]. According to this logic, immediate initiation of therapy for acute hepatitis C is desirable before immunologic mechanisms for persistent infection are established. The major disadvantage of the immediate treatment strategy is that exposing patients who may spontaneously clear the virus to unnecessary treatment. In fact, 20–50% of patients clear the virus spontaneously [9–11]. Thus, optimal timing for the IFN treatment remains unresolved.

In this issue of the journal, Ogata [12] found that delay of IFN therapy later than 24 weeks after the onset is associated with a significant decrease in therapeutic efficacy. The rate of sustained clearance of HCV was significantly high when IFN therapy was initiated within 24 weeks compared to later than 24 weeks. On the other hand, as long as the therapy was initiated within 24 weeks, the earlier timing of therapy was not associated with the improved rate of HCV clearance. In other words, the immediate therapy was not associated with improvement in the efficacy. Their results suggest that immediate therapy at the onset of acute hepatitis is not necessary and the initiation of therapy could be delayed after a period of careful waiting for spontaneous clearance of HCV. The critical time point may be 24 weeks. Recent randomized controlled study by Nomura et al. [13] has demonstrated that delaying the initiation of IFN therapy for a period of 12 months lowered the response rates substantially (87–100% in the early treatment (at 8 weeks after the onset) group and 40–53% in delayed-treatment group). Meanwhile, a recent meta-analysis showed that delaying therapy by 8–12 weeks after the onset of acute hepatitis does not compromise the rate of HCV clearance [14]. It is also reported that the spontaneous clearance of HCV is likely to occur within 4–12 weeks of infection [10,11]. These results imply that immediate therapy is too early and waiting for more than 24 weeks is too late. Optimal timing for the IFN treatment may end up within a period of 8–24 weeks after the onset of acute hepatitis.

Besides when to start therapy, controversy also remains on which patients should be treated, since there is no reliable predictors to identify which patients are unlikely to clear the virus spontaneously. If the likelihood of chronicity in individual patients could be predicted, therapy could be started with no delay in high risk patients. It is reported that symptomatic patients [15] or those with jaundice [11] may have more chance of spontaneous clearance of the virus compared to asymptomatic patients. In addition, Ogata [12] depicted that patients with the fluctuation of ALT levels are unlikely to clear the virus spontaneously. From these observations, asymptomatic, non-icteric patients with the fluctuation of ALT levels may be one of the high risk groups for the development of chronic infection and thus therapy should be initiated without delay.

Another important issue is what regimen of therapy should be used. Higher dose of IFN may be preferable [2] but the optimal dose and duration of therapy has not reached a consensus. Recent reports indicate that PEG-IFN monotherapy is equally effective to conventional IFN mono-therapy

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[16,17]. Combination therapy of ribavirin and IFN or PEG-IFN, which is now the standard regimen for chronic hepatitis, may not have additive value over mono-therapy in acute hepatitis since the rate of sustained clearance of HCV is already high with mono-therapy.

Conclusive recommendations on the treatment of acute hepatitis C could not be made due to a lack of a large scale, prospective and randomized study. However, available evidences suggest that IFN therapy should be recommended as a standard therapy in patients with acute hepatitis C. Immediate therapy is not always necessary and a wait and see may be a reasonable strategy since the later therapy with 8–24 weeks of delay does not compromise the rate of sustained clearance of HCV.

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