The examination by the HCVcAg assay undertaken in 246 blood donors infected with various HCV genotypes (1, 2, 3, and 4) was to reveal any genotypic difference. Although the assay seems to be genotype independent, the mean HCVcAg level of genotype 4 among blood donors was significantly lower than that of the other genotypes 1–3. Similarly, in a previous report using the Roche Monitor version 2.0 assay, the mean level of HCV RNA in genotype 4 samples from HCV-infected, PCR-positive blood donors was significantly lower than that of genotype 1 samples [Mellor et al., 1999]. As for reduction of HCVcAg by mutations, it has been reported that one mutation (Thr49Pro) in the core region is responsible for different results of HCVcAg [Tokita et al., 2000]. However, the amino acids alignment of 23 genotype 4 sequences excluded the presence of the mutation in the core region (Fig. 3). The only one common mutation (Thr110Asn) was found in genotype 4 as well as genotype 2a and 3a, however, its role is not confirmed, as there was a significant positive correlation between the HCVcAg and HCV RNA levels in this study. As four independent monoclonal antibodies directed against HCVcAg are used in the HCVcAg assay, the sensitivity was assumed to be stable even though there are a few mutations in the latter regions.

As reported previously [Tanaka et al., 2003], the HCVcAg assay is highly reproducible [coefficients of variation; 0.89–6.92%] and stable (84.8% of the initial level) with incubation of even 25°C for 7 days, compared with the quantitative PCR test. Additionally, it eliminated successfully inhibitors such as heparin from plasma and could be applied to a variety of clinical specimens [Tanaka et al., 2003]. The high sensitivity of the HCVcAg assay reported in this study would widen its use for identification of HCV infection in the diagnostic window period and consequently could improve the safety of blood. Also, the availability of the assay in a quantitative format allows its use in clinical practice to evaluate the effect of antiviral therapy.

In conclusion, the HCVcAg assay is specific, sensitive, and suitable for the detection of viremia in HCV infected subjects with genotypes 1, 2, 3, and 4. It could be a practical alternative to the molecular techniques for detection of viremia among blood donors especially in areas of the world where the nucleic amplification-based methods cannot be implemented. According to our data, we also believe that the quantitation and the monitoring of the HCVcAg level during clinical course would be valuable in HCV-infected patients. The assay is easier, less costly with low risk of contamination compared with RT-PCR assay.

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REFERENCES

Agha S, Sherif L, Allam MA, Fawzy M. 1998. Transplacental transmission of hepatitis C virus (HCV) in human immunodeficiency virus (HIV) negative mothers. Res Virol 149:229–234.

Agha S, Al-Gendy M, El-Fiky A, El-Emshaty W. 1999. Correlation of serum hepatitis C virus RNA titre with aminotransferases and liver histopathological findings in HCV-seropositive cases with end-stage chronic liver disease. Microbes Infect 1:1091–1094.

Amélia R, Ilma A, Olívia B, Andréa M, Edward P, Donald H, Mette S, Edgar M. 2000. Human immune responses to *Schistosoma mansoni* vaccine candidate antigens infection and immunity. Infect Immun 68:2797–2803.

Angelico M, Renganathan E, Gandin C, Fathy M, Profili MC, Refai W, De Santis A, Nagi A, Amin G, Capocaccia L, Callea F, Rapicetta M, Badr G, Rocchi G. 1997. Chronic liver disease in the Alexandria governorate, Egypt: Contribution of schistosomiasis and hepatitis virus infections. J Hepatol 26(2):236–243.

Aoygi K, Ohue C, Iida K, Kimura T, Tanaka E, Kiyosawa K, Yagi S. 1999. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J Clin Microbiol 37(6):1802–1808.

Bouvier-Alias M, Patel K, Dahari H, Beaucourt S, Larderie P, Blatt L, Hezode C, Picchio G, Dhumeaux D, Neumann A, McHutchison J, Pawlotsky JM. 2002. Clinical utility of total HCV core antigen quantification: A new indirect marker of HCV replication. Hepatology 7:211–218.

Icardi G, Ansaldi F, Bruzzone B, Durando P, Lee S, Luigi C, Crovari P. 2001. Novel approach to reduce hepatitis C virus (HCV) window period: Clinical evaluation of a new enzyme-linked immunosorbent assay for HCV core antigen. J Clin Microbiol 39(9):3110–3114.

Laperche S, Le Marrec N, Simon N, Bouchardeau F, Defer C, Maniez-Montreuil M, Levayer T, Zappitelli JP, Lefrere JJ. 2003. A new HCV core antigen assay based on disassociation of immune complexes: An alternative to molecular biology in the diagnosis of early HCV infection. Transfusion 43(7):958–962.

Mellor J, Hawkins A, Simmonds P. 1999. Genotype dependence of hepatitis C virus load measurement in commercially available quantitative assays. J Clin Microbiol 37(8):2525–2532.

Muerhoff AS, Jiang L, Shah DO, Gutierrez RA, Patel J, Garolis C, Kyrk CR, Leckie G, Frank A, Stewart JL, Dawson GJ. 2002. Detection of HCV core antigen in human serum and plasma with an automated chimeluminescent immunoassay. Transfusion 42(3):349.

Ohno T, Mizokami M, Wu RR, Saleh MG, Ohba KI, Orito E, Mukaide M, Williams R, Lau JYN. 1997. New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. J Clin Microbiol 35:201–207.

Ray SC, Arthur RR, Carella A, Bukh J, Thomas DL. 2000. Genetic epidemiology of hepatitis C virus throughout Egypt. J Infect Dis 182:698–707.

Robert GG, Johnson YN. 1997. Hepatitis C virus, eight years old. Viral Hepatitis Rev 3:17–37.

Takeuchi T, Katsume A, Tanaka T, Abe A, Inoe K, Tsukiyama-Kohara K, Kawaguchi R, Tanaka S, Kohara M. 1999. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 116:636–642.

Tanaka E, Ohue C, Aoyagi K, Yamaguchi K, Yagi S, Kiyosawa K, Alter HJ. 2000. Evalution of a new enzyme immunoassay for hepatitis C virus (HCV) core antigen with clinical sensitivity approximating that of genomic amplification of HCV RNA. Hepatology 32:388–393.

Tanaka Y, Takagi K, Fujiwara T, Kitsugi K, Fujiwara K, Hiramatsu K, Takasaka Y, Sakai M, Ito Y, Mizokami M. 2003. Stable enzyme immunoassay for hepatitis C virus core antigen before and after incubation at room temperature. Hepatology Res 26:261–267.

Tokita H, Kaufmann GR, Matsubayashi M, Okuda I, Tanaka T, Harada H, Mukaide M, Suzuki K, Cooper DA. 2000. Hepatitis C virus core mutations reduce the sensitivity of a fluorescence enzyme immunoassay. J Clin Microbiol 38:3450–3452.

Tong MJ, El-Farra NS, Reikes AR, Co RL. 1995. Clinical outcomes after transfusion-associated hepatitis C. N Engl J Med 332:1463–1466.

Veillon P, Payan C, Picchio G, Maniez-Montreuil M, Guntz P, Lunel F. 2003. Comparative evaluation of the total hepatitis C virus core antigen, branched-DNA, and amplicor monitor assays in

determining viremia for patients with chronic hepatitis C during interferon plus ribavirin combination therapy. J Clin Microbiol 41(7):3212–3220.

Widell A, Molnegren V, Pieksma F, Calmann M, Peterson J, Lee SR. 2002. Detection of hepatitis C core antigen in serum or plasma as a maker of hepatitis C viremia in the serological window-phase. Transfusion Med 12:107–113.

Zaaijer HL, Cuypers HT, Reesink HW, Winkel IN, Gerken G, Lelie PN. 1993. Realibility of polymerase chain reaction for detection of hepatitis C virus. Lancet 341:722–724.

Zanetti AR, Romano L, Brunetto M, Colombo M, Bellati G, Tackney C. 2003. Total HCV core antigen assay: A new marker of hepatitis C viremia for monitoring the progress of therapy. J Med Virol 70(1): 27–30.

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Early Dynamics of Hepatitis C Virus in the Circulation of Chimpanzees with Experimental Infection

Junko Tanaka[a] Keiko Katayama[a] Junko Kumagai[a] Yutaka Komiya[a]
Hisao Yugi[b] Shinya Kishimoto[c] Masaaki Mizui[d] Tetsushi Tomoguri[e]
Yuzo Miyakawa[f] Hiroshi Yoshizawa[a]

[a]Department of Epidemiology, Infectious Disease Control and Prevention, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, [b]Department of NAT, Japanese Red Cross Tokyo Blood Center, Tokyo, [c]Research Department, Japanese Red Cross Saitama Blood Center, Saitama, [d]Department of Laboratory Medicine, Japanese Red Cross Hiroshima Blood Center, Hiroshima, [e]Primate Park, Sanwa Kagaku Kenkyusho, Ltd, Kumamoto, and [f]Miyakawa Memorial Research Foundation, Tokyo, Japan

Key Words
Chimpanzees · Chronic hepatitis · Hepatitis C virus · Nucleic acid amplification testing · Transfusion

Abstract
Two chimpanzees were inoculated with hepatitis C virus (HCV) and followed on a daily basis for 12 days. HCV RNA became detectable in their sera on day 5 by polymerase chain reaction with the detection limit of 10^2 copies/ml. Based on an exponential growth observed until 8 or 9 days after inoculation in their sera, the doubling time of HCV in the circulation was estimated at 6.3–8.6 h and log time (time required to grow 10-fold) at 31.3–42.9 h. The exact doubling time of HCV determined in them would help plan an efficient strategy for screening out blood donors in the window period of infection between the exposure and the development of antibody to HCV in serum.

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There still remains a residual risk of contracting hepatitis C virus (HCV) infection after transfusion with blood units without the antibody against it (anti-HCV) detectable by the second- and third-generation immunoassays [1]. Anti-HCV is not raised in the circulation of individuals during the 'window period' after the exposure to HCV that is estimated at an average of 55 days in chimpanzees with experimental infection [2] and 41 days in human beings [3]. In order to identify early HCV infection, nucleic acid amplification testing (NAT) has been introduced to transfusion services [3, 4]. NAT can detect by far the most blood units in the window period of HCV infection, but cannot identify them all on a theoretical basis [5]. Even when 200 μl of serum from a donor is tested by the individual NAT, approximately 10^2 copies/ml of HCV RNA are required to produce a positive result. The sensitivity is reduced further in a mini-pool NAT performed on 50 donors in the current practice, in which a single donor is represented by merely 4 μl of serum. In actuality, HCV infection can occur in the recipient of platelet concentrates from a blood donor testing negative by NAT [6].

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Dr. Hiroshi Yoshizawa
Department of Epidemiology, Infectious Disease Control and Prevention
Hiroshima University Graduate School of Biomedical Sciences
Hiroshima Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551 (Japan)
Tel. +81 82 2575162, Fax +81 82 2575164, E-Mail juntan@hiroshima-u.ac.jp

The doubling time and log time (time required for growing 10-fold) of HCV in the circulation are prerequisite to planning a strategy for efficiently detecting HCV infection in blood donors as well as for understanding the limit of current screening methods based on polymerase chain reaction (PCR). They have not been determined accurately, however, due to the lack of in vitro systems for HCV culture. The documented doubling time of HCV varies widely from 2.4 h (0.1 day) [3] to 14.9 h [7] or 17.3 h (0.72 day) [5].

The dynamics of HCV growth in an early phase of infection were analyzed in the circulation of 2 chimpanzees for determining how soon HCV RNA increases to 10^2 copies/ml that can be detected by the individual NAT. Further, the doubling time and log time of HCV RNA were estimated based on the exponential growth in their sera during an early phase of infection.

Two chimpanzees entered the experimental transmission study – chimp No. 224 (C224: male, 14 years old, weight 59.1 kg) and chimp No. 267 (C267: female, 7 years old, weight 49.0 kg). They both were kept in individual cages and received humane care in accordance with all relevant requirements for the use of primates in an approved facility. Neither of them had serological or molecular virological evidence for past or present HCV infection before the inoculation. They received inocula while they were under anesthesia with ketamine hydrochloride.

Fresh-frozen plasma was obtained from a donor who was in the window period of infection with HCV genotype 1b. It was separated within 6 h after blood collection, and contained HCV RNA at a titer of 8.4×10^6 copies/ml determined by Taq Man PCR (Applied Biosystems Japan, Tokyo, Japan). The plasma was kept frozen at $-80°$ in 1-ml aliquots; the infectious activity of the plasma in chimpanzees decreased >100-fold during these procedures [8]. An aliquot (1 ml) of this plasma was thawed in a 37° bath and injected intravenously to C224. The other chimpanzee (C267) was inoculated with a passage of another fresh-frozen plasma through a chimpanzee that had been inoculated with 1 ml of it and developed viremia [8]; the plasma was donated by an individual in the window period of co-infection with HCV genotypes 1b and 2a. Serum obtained from the chimpanzee 7 weeks after inoculation was aliquoted in 1-ml volumes and snap-frozen in liquid nitrogen. An aliquot was serially diluted 10-fold with self serum of C267, and 1 ml of a $1:10^3$ dilution containing approximately 200 copies of HCV RNA was injected intravenously to C267; it corresponded to 10 times the minimal infectious dose of HCV in chimpanzees [8]. After the inoculation, serum samples were obtained from them daily during the same hour in the morning (9–10 a.m.) for the first 12 days. They had been kept frozen at $-80°$, and were tested for HCV RNA simultaneously in the same assay by Taq Man PCR.

The dynamics of HCV RNA in sera from the 2 chimpanzees during an early phase of infection are illustrated in figure 1. HCV RNA was first detected in serum taken 5 days after the inoculation from them both by Taq Man PCR. Then, HCV RNA titers increased exponentially on the log scale for 8 days after inoculation in C224, and until 9 days in C267. Thereafter, HCV RNA in them deviated from the straight line of exponential growth. Based on the linearity of an initial exponential growth and the coefficient of determination, the doubling time of HCV replication was calculated to be 6.3 and 8.6 h, and the time required to grow 10-fold (log time) to be 31.3 and 42.9 h in C224 and C267, respectively; they were in a remarkably good agreement.

The growth of HCV was closely followed in the circulation of 2 chimpanzees, 1 of whom (C267) had been inoculated with passaged and calibrated HCV from a chimp in the preacute phase of infection [8]. The growth curves of HCV in the 2 chimpanzees were strikingly similar. HCV RNA was not detected for 5 days, then increased exponentially until the 8th or 9th day, and decreased thereafter. The failure in detecting HCV RNA during the initial 4 days would be attributed, in part, to a limited sensitivity of the Taq Man PCR method (10^2 copies/ml). When the linear growth was extrapolated beyond the detection of HCV RNA in serum, however, it converged to day 3 in 1 chimp and a little later than day 2 in the other (fig. 1). Hence, it would be reasonably delineated that HCV would have started circulating in both chimpanzees as early as 2–3 days after infection, a few days before HCV RNA became detectable by PCR 5 days after the inoculation.

The time from HCV transmission to the first detection of viral RNA in the circulation is called the 'eclipse' phase [3], which may vary by the size of inoculated dose and the sensitivity in detecting HCV RNA. The eclipse phase of 5 days observed in the chimpanzees (C267) inoculated with 10 times the minimal infectious dose of HCV [8] was longer than that of 3 days reported for an experimentally transmitted chimpanzee inoculated with 0.5 ml of human serum containing $10^{6.5}$ chimp infectious doses per ml [9]. Since high-dose transmission by transfusions has been excluded by anti-HCV screening [1] and low-dose infection through blood units in the NAT window period is at issue nowadays [3, 4], a longer eclipse phase would be

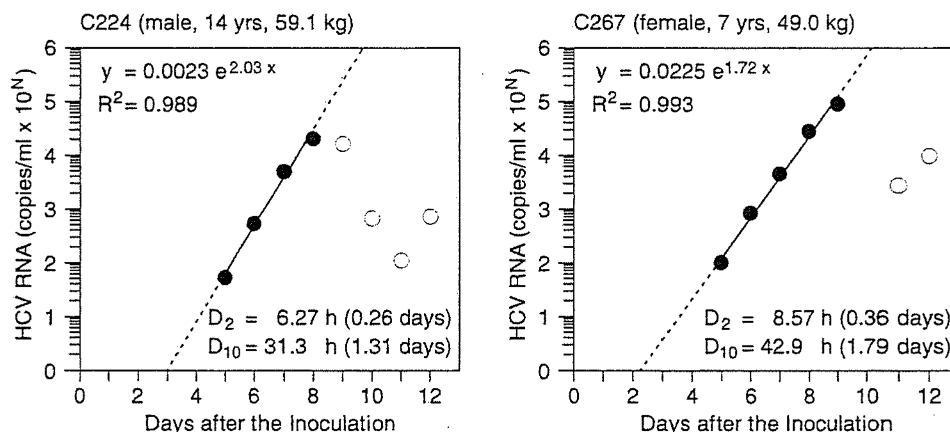

Fig. 1. Exponential growth of HCV during an early phase of HCV infection in 2 chimpanzees experimentally transmitted with pedigreed inocula. HCV RNA was determined by Taq Man PCR. Solid circles and the solid line represent the linear exponential growth of HCV. Dotted lines show an imaginary growth below the detection limit in initial days (to the left) as well as a putative exponential growth beyond the linearity (to the right). Open circles are HCV RNA levels that deviated from the linearity of exponential growth. R^2 = Coefficient of determination; D_2 = doubling time; D_{10} = the log time required for growing 10-fold.

more informative practically. An observed interval between 'true' eclipse phase (2–3 days) and PCR eclipse phase (up to 5 days), however, indicates that NAT would not be able to close the window spanning a few days after the exposure to HCV.

The duration of exponential HCV growth is called the 'ramp-up' phase [5]. Should the ramp-up phase last until all susceptible hepatocytes are infected, the slope of exponential growth would hardly be influenced by the size of HCV dose. Doubling times of HCV in the circulation calculated on the exponential growth of viral RNA in the 2 chimpanzees were 6.23 and 8.36 h, respectively. They were half as long as 14.9 h [7] and 17.3 h [5] reported in human beings. It is not clear how such a big discrepancy has arisen. Although species differences may give an account on it, a rigorous and meticulous design for experimental transmission conducted in chimpanzees would hardly be feasible for HCV infection in the transfusion setting. This issue needs to be looked into and settled, since the doubling time of HCV is crucial in working out measures for increasing the blood safety.

The results obtained in this study would help determine the size of pool in NAT for efficiently screening HCV RNA in blood donors. Due to extremely fast replication of HCV, the merit of reducing the size of pool would have its own limit. Based on the doubling time of 6.3–8.6 h in this study, the window can be narrowed by at most 1.3–1.8 days even by performing the individual NAT, in place of a mini-pool NAT on 50 donors in the current practice. This goes along with the mathematical model of Weusten et al. [5] who calculated the risk of contracting HCV infection to decrease only to one half by performing the individual NAT in comparison with a mini-pool NAT on 50 donors. Despite the doubling time of HCV, which is much shorter than hepatitis B virus (62.4 h) or human immunodeficiency virus type 1 (20.5 h) [10], there would be a limitation in narrowing the window period by performing the individual NAT. For further increasing the safety of blood transfusion, in terms of the risk for HCV infection, the other strategies would need to be considered, such as condensing HCV RNA in more amounts of serum from individual donors before performing a mini-pool NAT.

References

1 Tobler LH, Stramer SL, Lee SR, Masecar BL, Peterson JE, Davis EA, Andrews WE, Brodsky JP, Kleinman SH, Phelps BH, Busch MP: Impact of HCV 3.0 EIA relative to HCV 2.0 EIA on blood-donor screening. Transfusion 2003; 43:1452–1459.

2 Beach MJ, Meeks EL, Mimms LT, Vallari D, DuCharme L, Spelbring J, Taskar S, Schleicher JB, Krawczynski K, Bradley DW: Temporal relationships of hepatitis C virus RNA and antibody responses following experimental infection of chimpanzees. J Med Virol 1992;36: 226–237.

3 Busch MP, Kleinman SH, Jackson B, Stramer SL, Hewlett I, Preston S: Nucleic acid amplification testing of blood donors for transfusion-transmitted infectious diseases: Report of the Interorganizational Task Force on Nucleic Acid Amplification Testing of Blood Donors. Transfusion 2000;40:143–159.

4 Roth WK, Weber M, Seifried E: Feasibility and efficacy of routine PCR screening of blood donations for hepatitis C virus, hepatitis B virus, and HIV-1 in a blood-bank setting. Lancet 1999;353:359–363.

5 Weusten JJ, van Drimmelen HA, Lelie PN: Mathematic modeling of the risk of HBV, HCV, and HIV transmission by window-phase donations not detected by NAT. Transfusion 2002;42:537–548.

6 Schuttler CG, Caspari G, Jursch CA, Willems WR, Gerlich WH, Schaefer S: Hepatitis C virus transmission by a blood donation negative in nucleic acid amplification tests for viral RNA. Lancet 2000;355:41–42.

7 Busch MP, Giachetti C, Gallarda J, Mimms LT, Peddada L, Charles H, Raia S, Fiebig EW, Wright D: Dynamics of HCV viremia during early HCV infection: Implication for minipool vs. individual donation nucleic acid amplification testing (abstract). Transfusion 2000;40: S25.

8 Katayama K, Kumagai J, Komiya Y, Mizui M, Yugi H, Kishimoto S, Yamanaka R, Tamatsukuri S, Tomoguri T, Miyakawa Y, Yoshizawa H: Titration of hepatitis C virus in chimpanzees for determining the copy number required for transmission. Intervirology 2004;47:57–64.

9 Shimizu YK, Weiner AJ, Rosenblatt J, Wong DC, Shapiro M, Popkin T, Houghton M, Alter HJ, Purcell RH: Early events in hepatitis C virus infection of chimpanzees. Proc Natl Acad Sci USA 1990;87:6441–6444.

10 Glynn SA, Kleinman SH, Wright DJ, Busch MP: International application of the incidence rate/window period model. Transfusion 2002; 42:966–972.

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Molecular evolutionary analyses implicate injection treatment for schistosomiasis in the initial hepatitis C epidemics in Japan

Yasuhito Tanaka[1], Kousuke Hanada[2], Etsuro Orito[3], Yoshihiro Akahane[4], Kazuaki Chayama[5], Hiroshi Yoshizawa[6], Michio Sata[7], Nobuo Ohta[8], Yuzo Miyakawa[9], Takashi Gojobori[2], Masashi Mizokami[1,*]

[1]*Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan*

[2]*Center for Information Biology, National Institutes of Genetics, Mishima, Japan*

[3]*Department of Internal Medicine and Molecular Science, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan*

[4]*First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi, Yamanashi, Japan*

[5]*Department of Medicine and Molecular Science, Hiroshima University Graduate School of Biomedical Sciences, School of Medicine, Hiroshima, Japan*

[6]*Department of Infectious Disease and Control, Hiroshima University Graduate School of Biomedical Sciences, School of Medicine, Hiroshima, Japan*

[7]*Second Department of Internal Medicine, Kurume University School of Medicine, Fukuoka, Japan*

[8]*Department of Molecular Parasitology, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan*

[9]*Miyakawa Memorial Research Foundation, Tokyo, Japan*

Background/Aims: The mortality due to hepatocellular carcinoma (HCC) has ranged widely in various areas of Japan since 30 years ago and the incidence was particularly high in once _Schistosoma japonicum_ (_Sj_)-endemic areas. Our aim was to estimate the spread time of hepatitis C virus (HCV) infection in the past with possible relevance to a higher incidence of HCC in once _Sj_-endemic than _Sj_-nonendemic areas.

Methods: During 2001, 131 strains of HCV-1b were obtained from patients in three previously _Sj_-endemic areas, as well as _Sj_-nonendemic areas in Japan and a cross-sectional study was conducted on them with molecular evolutionary analyses.

Results: A phylogenetic tree reconstructed on HCV-1b sequences in the NS5B region disclosed 2 independent clusters for _Sj_-positive and -negative groups with a high bootstrap value. The estimated effective number of HCV-infections indicated a transition from quiescence to rapid exponential growth in the 1920s among patients with schistosomiasis, which is 20 years earlier than that among patients without schistosomiasis.

Conclusions: The estimated spread time in previously _Sj_-endemic areas in Japan coincides with injection treatment for _Sj_ since 1921. A high incidence of HCC there would be attributed to a long duration of HCV infection since 1920s.

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Keywords: Hepatitis C virus; _Schistosoma japonicum_; Molecular evolutionary analysis; Hepatocellular carcinoma

1. Introduction

Recently, the molecular clock has been successfully applied to long-term serial serum samples containing hepatitis C virus (HCV) from the US and Japan and estimated the spread time of HCV in the 1930s in Japan, which is 30 years earlier than that in the US in the 1960s [1]. Insofar as a long duration of HCV infection is the most important factor for the development of hepatocellular

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* Corresponding author. Tel.: +81 52 853 8292; fax: +81 52 842 0021.
E-mail address: mizokami@med.nagoya-cu.ac.jp (M. Mizokami).

Abbreviations HCV, hepatitis C virus; Anti-HCV, antibody to HCV; HCC, hepatocellular carcinoma; *Sj*, *Schistosoma japonicum*.

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carcinoma (HCC), it can be predicted that the incidence of HCC will increase in the US over the next 2–3 decades. Thus, a combination of classical epidemiological approaches and molecular evolutionary analyses would be particularly useful in the study of contagious diseases, typified by HCV infection.

The way how individuals contracted HCV infection has remained unclear in Japan. Recently, a Japanese report (Ministry of Health, Labour and Welfare: Distribution of age-adjusted mortality rate from liver cancer by prefecture between 1971 and 1975, Tokyo, 2001) indicated that the mortality due to HCC has already varied widely in various areas of Japan since 30 years ago; the incidence of HCC was much higher in Saga/Fukuoka, Hiroshima and Yamanashi Prefectures, which were once endemic for schistosomiasis japonica in the long past. Hence, a high incidence of HCC in the 1970s would be related to HCV transmitted by injection treatment for *Schistosoma japonicum* (*Sj*) conducted since 1921 in these areas. In fact, shared needles and syringes for intravenous injection treatment with antimonyl potassium tartrate or sodium antimony tartrate posed a significant risk for HCV transmission in endemic areas [2]. Indeed, the prevalence of antibody to HCV (anti-HCV) is high (36.5; 95% CI = 28.1–44.9%) in patients with chronic schistosomiasis [2] and therefore, HCV infection is considered responsible for the development of HCC in patients with chronic schistosomiasis.

Since, once popular intravenous injection for schistosomiasis was a risk factor for HCV transmission, the spread time of HCV in the areas once endemic for *Sj* in Japan would deserve determination. In this study, molecular evolutionary analyses using principles of both population genetics and mathematical epidemiology [3] were applied to HCV-infected patients with and without a past history of chronic schistosomiasis in once *Sj*-endemic areas.

2. Meterials and methods

2.1. Sample collection

In Japan during 2001, 181 random serum samples positive for anti-HCV were obtained from patients with chronic liver disease in widely separated areas previously endemic for *Sj*, including Kofu in Yamanashi ($n = 75$), Katayama in Hiroshima ($n = 50$) and Chikugo in Saga/Fukuoka Prefectures ($n = 56$). Schistosomiasis was diagnosed by ultrasonographic (US) and/or computer tomographic (CT) modalities or serological examinations [4]. Two kinds of serological tests, which can detect past history of schistosomiasis, were available in this study. In brief, IgG antibodies binding to two different *schistosome antigens*, *Sj* adult worm antigen and *Sj* egg antigen, were detected using an enzyme-linked immunosorbent assay (ELISA). As it is now accepted that ELISA titer of egg antigen-specific IgG is reliable for case-detection rather than IgG for adult worm antigen [4–6], the results based on the egg antigen-specific IgG were accepted in this study. Samples of more than 0.25 of optical density at 415 nm were determined to be positive, as previously confirmed [4–6]. The serum samples were tested for anti-HCV by Lumipulse II Ortho HCV (Ortho-Clinical Diagnostics K.K., Tokyo, Japan). As patients with *Sj* treatments were estimated to be old, relatively older patients were selected in the *Sj*-endemic areas to match age factor that might influence duration of HCV infection or HCC incidence. For a cross-sectional study, 30 serum samples were obtained from patients infected with HCV in Aichi Prefecture where *Sj* has not been endemic. The age- and sex- matced patients were also selected from the *Sj*-nonendemic areas excluding infiuece of these factors on HCC incidence. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by Ethic Committees of institutions. Every patient gave a written informed consent to participate in the virological research of HCV. Information of injection treatment for *Sj* was obtained by means of self-administrated questionnaires or structured interviews. None had been treated with interferon therapy for HCV infection. HCC incidence was estimated by historical information from patients themselves and/or medical records during 2001. HCC was diagnosed by liver biopsy or combination of imaging modalities such as US, enhanced CT and angiography.

2.2. Genotyping and sequencing

Nucleic acids were extracted using a SepaGean RV-R Nucleic acid extracting kit (Sanko Junyaku Co., Ltd., Tokyo, Japan) in accordance with the manufacturer's protocol. They were reverse-transcribed to cDNA using SuperScript II Rnase H^- Reverse Transcriptase (Invirogen Corp., Carlsbad, California, USA) and random hexamer primer (Takara Shuzo Co. Ltd, Tokyo, Japan) by the method described previously [7].

A sequence spanning 339 nucleotides (nt) in the NS5B region was amplified by polymerase chain reaction (PCR) with primers described previously [1]. PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, California, USA) in an ABI 3100 DNA automated sequencer. To reduce the number of artificial substitutions arising in PCR, PLATINUM Pfx DNA Polymerase (Invirogen Corp.) with a very high fidelity was used. The sequences determined were utilized to confirm HCV genotypes and construct phylogenetic trees.

2.3. Test for clustering between Sj-positive and -negative groups

The phylogenetic tree was first constructed to examine the evolutionary history for *Sj*-positive and *Sj*-negative groups by the neighbor joining method [8]. Furthermore, to test whether either *Sj*-positive or *Sj*-negative group have evolved independently or not, we conducted an interior branch test for the neighbor-joining tree [9]. Thereafter, a *t*-test was conducted for the interior branch length and its standard error, which is computed using the bootstrap procedure.

2.4. Demographic model

A reconstructed tree was built on the NS5B sequence of 339 nt by a heuristic maximum-likelihood topology search with stepwise-addition and the nearest neighbor-interchange algorithms. Tree likelihood scores were calculated using HKY85 with the molecular clock enforced by PAUP version 4.0b8.

As estimates of the demographic history, a nonparametric function $N(t)$, known also as the skyline plot, was obtained by transforming coalescent intervals of an observed genealogy into a piecewise plot that represents an effective number of infections through time [3,10]. A parametric maximum-likelihood was estimated by several models with the computer software Genie v3.5 to build a statistical framework for inferring the demographic history of a population on phylogenies reconstructed on sampled DNA sequences [10]. 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 maximum-likelihood estimates [11,12].

2.5. Statistical method

Data for continuous variables were demonstrated as the mean ± standard deviation. The Fishers' exact test, Chi square test with Yates' correction and one-way ANOVA followed by the Scheffe's multiple comparison test were used to evaluate differences in the mean age, sex ratio

and incidence of HCC between groups, respectively. Differences with P values less than 0.05 were considered significant.

3. Results

Of 181 anti-HCV positive samples, 113 were classified into HCV genotype 1b (HCV-1b), which is predominant in Japan. Fifty-two of 181 samples (29%) were negative for HCV RNA or incomplete for sequencing and the remaining 16 samples (9%) of genotype 2a were excluded in this study due to a minor population. Of the HCV-1b strains, 47 were recovered from patients in Yamanashi, 31 in Hiroshima and 35 in Saga/Fukuoka Prefectures. Along with 18 HCV-1b strains in Aichi Prefecture serving as controls, a cross-sectional study was conducted on them with molecular evolutionary analyses. The patients in areas previously endemic for *Sj* revealed a significantly higher prevalence of chronic schistosomiasis [24/47 (51%) in Yamanashi (Kofu area), 21/31 (68%) in Hiroshima (Katayama area) and 19/35 (54%) in Saga/Fukuoka (Chikugo area)] than that in Aichi Prefecture (0/18 [0%], $P<0.0001$). There were no significant differences in the mean age or sex ratio among patients from these four areas (Fig. 1). Although the mean age of *Sj*-positive patients was just higher than that of *Sj*-negative patients in once *Sj*-endemic areas or matched-control patients in Aichi Prefecture, there were also no significant differences between these groups (Table 1).

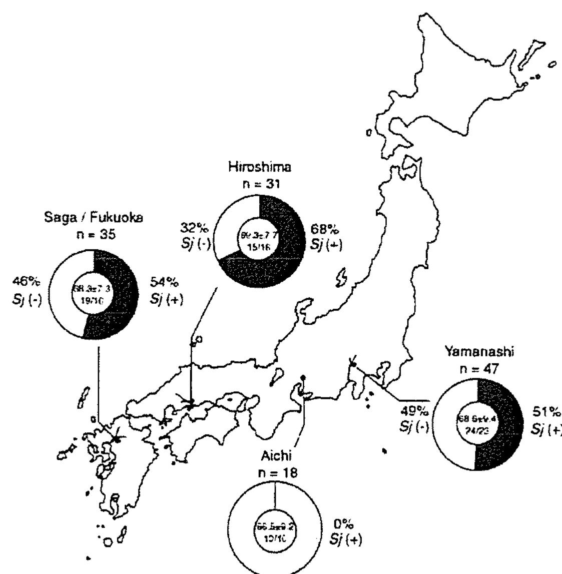

Fig. 1. Geographic distribution of *Schistosoma japonicum* (*Sj*) and characteristics of patients infected with HCV. *Sj* (+) and *Sj* (−) denote, respectively, presence and absence of infection with *Sj* diagnosed by ultrasonographic and/or computer tomographic methods or serological examinations. Pie graphs include the age (mean ± standard deviation) and sex ratio (male/female).

Table 1
Characteristics of patients with and without schistosomiasis

	Schistosoma japonicum		Controls
	Positive ($n=64$)	Negative ($n=49$)	(Aichi) ($n=18$)
Mean age			
Total	69.9 ± 7.7	67.4 ± 8.7	66.5 ± 9.2
Yamanashi	69.9 ± 7.2	67.3 ± 11.2	
Hiroshima	71.2 ± 8.7	67.6 ± 6.5	
Saga/Fukuoka	69.0 ± 7.7	67.5 ± 7.1	
Sex (male/female)			
Total	34/30	24/25	9/9
Yamanashi	13/11	11/12	
Hiroshima	10/11	5/5	
Saga/Fukuoka	11/8	8/8	
Incidence of HCC	25/55 (45%)	11/48 (23%)	3/18 (17%)

The incidence of HCC in *Sj*-positive patients was significantly higher than that in *Sj*-negative patients ($P=0.0226$) or controls ($P=0.0488$).
Abbreviations: HCC, hepatocellular carcinoma.

For cross-sectional study on the viral population size between HCV-infected patients with and without a past history of schistosomiasis, a phylogenetic tree for HCV-1b strains in the *Sj*-positive and -negative patients was constructed with use of the maximum-likelihood method enforced by the molecular clock as introduced in our previous report [1] and an independent study by Pybus et al. [3]; a substitution rate of 5.3×10^{-4} per site per year [1,3] was assumed for HCV. The phylogenetic tree disclosed 2 independent clusters for *Sj*-positive and -negative groups, with a high bootstrap value (81%) by the interior branch testing (Fig. 2), which is comparative with past epidemiological backgrounds in Japan. From distinct evolutionary histories in the two populations, the effective number of HCV-1b infections through time, N (t), were assessed by the skyline plot. The parameters for several models in Genie v3.5 [3,10] were also examined. Time t was then transformed to year using the same rate, assuming the collecting time (year 2001) as the present. Fig. 3 shows the skyline plots and population growth for *Sj*-positive and -negative patients, according to a specific demographic model in Genie v3.5 with three parameters, piecewise expansion growth model, that was evaluated by the likelihood ratio testing [11,12]. Molecular evolutionary results thus obtained supported our previous study in which the divergence time of the most recent common ancestor of HCV-1b in each area in Japan was estimated before 1850 [1]. Our estimates of the effective number of HCV-infections showed a transition from constant size to rapid exponential growth in the 1920s among patients with chronic schistosomiasis in endemic areas, which is 20 years earlier than that among patients without schistosomiasis in the 1940s. Information on HCC was available for 121 of the 131 patients with HCV-1b. Although they were relatively small in number, the incidence of HCC was significantly higher in *Sj*-positive than -negative patients ($P=0.0226$) or controls ($P=0.0488$) (Table 1).

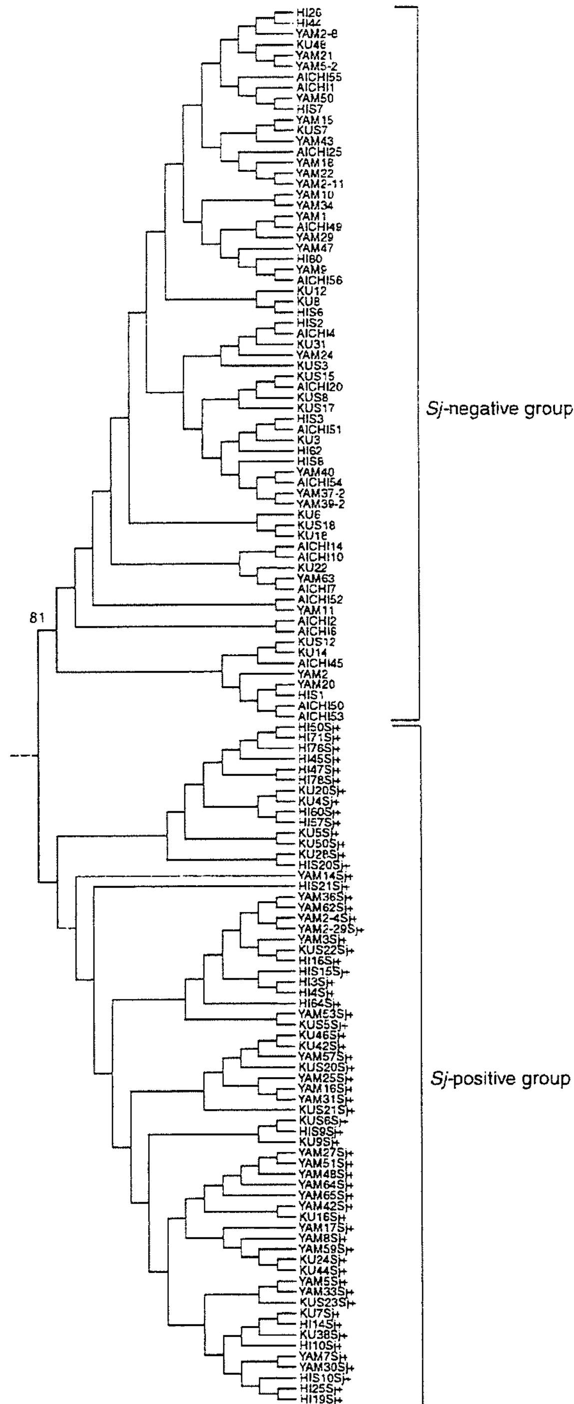

Fig. 2. A phylogenetic tree constructed on NS5B sequences of HCV-1b strains in *Schistosoma japonicum* (*Sj*)-positive ($n=64$) and -negative ($n=67$) groups. The numbers in the tree indicate bootstrap reliability by the interior branch test. *Sj*+ indicates *Sj*-positive strains. YAM; Yamanashi, HI/HIS; Hiroshima, KU/KUS; Saga/Fukuoka, Aichi; control strains.

4. Discussion

The specific demographic model based on the neutral theory [3,11,12], which has a constant size in the past and changes to exponential growth until the present, is applied to investigate the Japanese endemic of HCV. By means of the molecular evolutionary analyses, the spread time of HCV in *Sj*-positive patients was estimated 20 years earlier than that in *Sj*-negative patients from three areas in Japan where *Sj* was previously endemic (Yamanashi, Hiroshima, Saga/Fukuoka Prefectures). The spread time of HCV much earlier in *Sj*-positive than -negative patients indicates that the previous intravenous injection treatment with antimony compounds (antimonyl potassium tartarate or antimony sodium tartarate) on patients with schistosomiasis since 1921 [2] would have been a significant risk factor for HCV transmission in endemic areas through re-used needles and syringes. Indeed, it might be possible that HCV transmission from *Sj*-positive patients to *Sj*-negative patients occurs in the once *Sj*-endemic areas, but we could not find such strains in this study. One of the reasons is that residents in the village around the river, where schistosomiasis had been the most prevalent, might have been isolated from those in the other areas of the same Prefecture in the past due to the endemic disease 'schistosomiasis'. Interestingly, most Japanese strains from *Sj*-nonendemic areas in the database clustered with the *Sj*-negative group of the present study. Hence, factors other than the injection treatment for *Sj*, such as intravenous stimulants popular during and after World War II [13] and medical treatments including transfusion with blood units from paid donors in the past, would have imposed the risk for HCV transmission in most areas in Japan [14]. In addition, there would have been opportunities for HCV transmission through inadequately sterilized needles and syringes in general practices, which have contributed to a large reservoir of chronic HCV infection in Japan during the 1950s [13]. Such inadequately sterilized medical injections were still common in the less-developed world in the 20th century. WHO estimates that unsafe injections result in 2.3–4.7 million new HCV infections worldwide every year [15].

Although the spread time of HCV in *Sj*-positive group was earlier than that in *Sj*-negative group, there was no significant difference of mean age between the 2 groups. Two possibilities are considered. One is a sampling bias; as patients with *Sj* treatments were estimated to be old, relatively older patients were selected in the *Sj*-endemic areas to match age factor that might influence duration of HCV infection or HCC incidence. Second, the ages that patients had been infected with HCV were different between the 2 groups; the treatments for *Sj* in Japan were mainly conducted among relatively younger people including school children after screening of *Sj* [4,16,17], while the

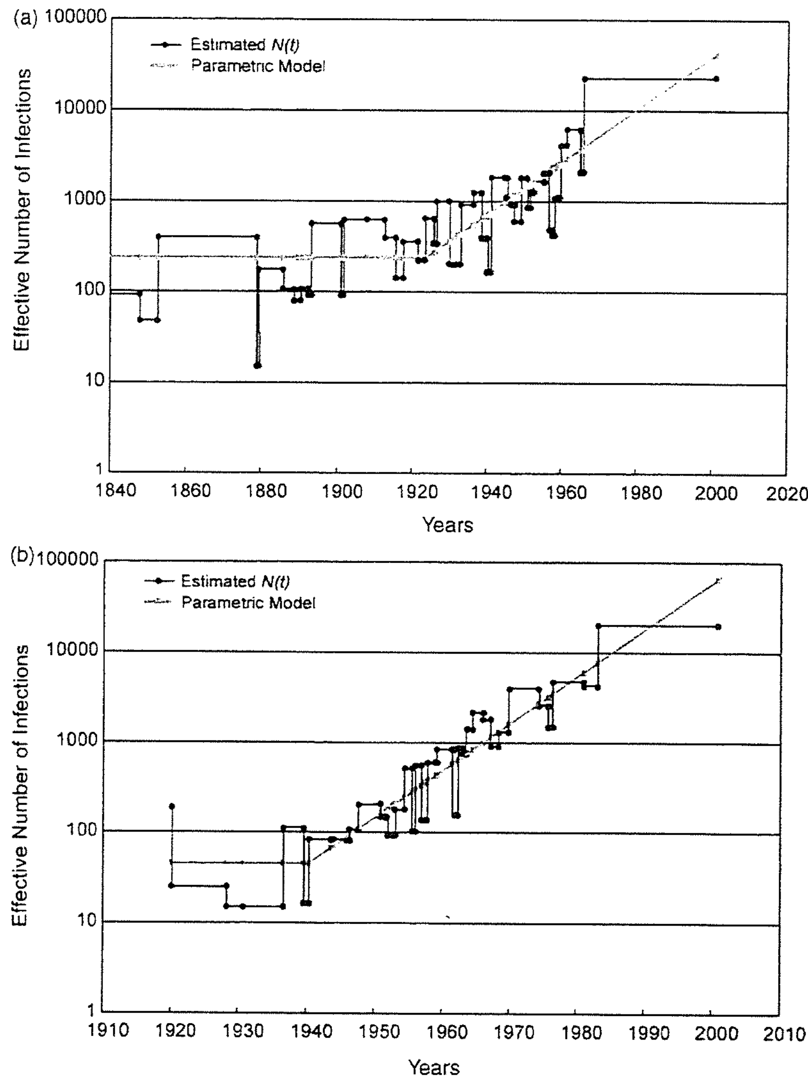

Fig. 3. The maximum-likelihood estimates of N (t) on the effective number of infections with HCV genotype 1b in Japan for *Schistosoma japonicum* (*Sj*)-positive group (a) and *Sj*-negative group (b) separated in the phylogenetic tree (Fig. 2). The parametric model is indicated by the grey line and stepwise plots by the black line that represents corresponding nonparametric estimates of N (t) (number as a function of time). Genetic distances are transformed into a time scale of year using estimates of the molecular clock in the NS5B region.

other risk factors such as blood transfusion were found in older people excluding at least children.

A disease possibly caused by schistosomal infection in Japan is documented in a book written some 300 years ago. In 1847, the clinical picture of this disease was precisely described by Yoshinao Fujii in the book 'Katayama-ki' that documented an endemic disease in Katayama area as Katayama's disease (equivalent to schistosomiasis). Water-borne epidemics of schistosomiasis prevailed in inhabitants around rivers (the tributaries of the Fuji river in Yamanashi, the Takaya river in Hiroshima and the Chikugo river in Saga/Fukuoka) in Japan, mediated by small shellfish (Miyairi-kai) serving as the natural host. More than 200,000 individuals were estimated to have been infected with *Sj* in Yamanashi Prefecture alone during 1965 through 1990 [16] and approximately 1,000,000 patients in the entire Japan since 1920s [17]. To cope with these endemics, more than 10 million intravenous injections with antimony compounds had been given in Japan since 1921 [17]. Thus, Japan would have started ahead of any other countries, in terms of HCV spread in association with schistosomiasis, wherein intravenous drugs were invented. Although acute schistosomal infection has disappeared in Japan since long ago, there are still elderly people with

chronic schistosomiasis in previously endemic areas, some of whom are developing HCC [2,14]. Substantial transmission among regions is supported by the lack of regional clustering of HCV sequences in this study.

A similar situation is reported in the Nile delta in Egypt where schistosomiasis once prevailed mediated by small shellfish [18] and the national campaigns for injection treatment with antimonyl potassium tartarate (tartar emetic) from the 1961 until 1986 are suspected to have given rise to the highest endemicity of HCV in the world ever, involving >20% of the national population there [19]. The prevalence of anti-HCV is extremely high (>70%) in patients with schistosomiasis there [18,20,21]. Highly prevalent HCV infection in the general Egyptian population accounts for most HCC cases in Egypt [22]. A question may arise whether schistosomiasis alone is responsible for the development of HCC. Patients co-infected with HCV and *Schistosoma mansoni* (*Sm*) may have a high incidence of viral persistence, accelerated fibrosis and development of HCC [23,24]. A recent population-based study between two large populations with district histories of *Sm* and hepatitis C infections, however, failed to indicate any interaction between *Sm* infection and the prevalence or severity of hepatitis C [25]. Moreover, no significant histological differences were found between anti-HCV-positive Egyptian patients with and without schistosoma [26]. Hence, the long duration of persistent HCV infection would be a more important factor for the development of HCC than the pathogeneticity of *Sm* itself.

Estimating the effective number of HCV infections has been very informative in looking back epidemic spreads of HCV infection in the Unites States [1] and Egypt [12,27]. In addition, it would also be useful in predicting the population size and extent of HCV infection. Studies to foresee future spreads of HCV would be required to cope with and prevent healthcare problems where de novo infections are increasing. The advantage of molecular evolutionary analyses, its ability to accurately estimate the dynamics of HCV based on a limited number of isolates in particular [3], will extend its application anywhere in the world where clinical sequelae of persistent HCV infection pose increasing burdens on the public health of nations.

In conclusion, the evolutionary analyses indicated that the estimated spread time in previously *Sj*-endemic areas in Japan coincides with injection treatment for *Sj* conducted since 1921. The high incidence of HCC in *Sj*-endemic areas is most likely attributed to long duration of HCV infection there transmitted through injection treatments.

Acknowledgements

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References

[1] Tanaka Y, Hanada K, Mizokami M, Yeo AE, Shih JW, Gojobori T, et al. Inaugural article: a comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. Proc Natl Acad Sci USA 2002;99: 15584–15589.

[2] Iida F, Iida R, Kamijo H, Takaso K, Miyazaki Y, Funabashi W, et al. Chronic Japanese schistosomiasis and hepatocellular carcinoma: ten years of follow-up in yamanashi prefecture, Japan. Bull World Health Organ 1999;77:573–581.

[3] Pybus OG, Charleston MA, Gupta S, Rambaut A, Holmes EC, Harvey PH. The epidemic behavior of the hepatitis C virus. Science 2001;292:2323–2325.

[4] Minai M, Hosaka Y, Ohta N. Historical view of schistosomiasis japonica in Japan: implementation and evaluation of disease-control strategies in yamanashi prefecture. Parasitol Int 2003;52: 321–326.

[5] Matsuda H, Tanaka H, Blas BL, Nosenas JS, Tokawa T, Ohsawa S. Evaluation of ELISA with ABTS, 2-2′-azino-di-(3-ethylbenzthiazoline sulfonic acid), as the substrate of peroxidase and its application to the diagnosis of schistosomiasis. Jpn J Exp Med 1984;54:131–138.

[6] Matsumoto J, Kirinoki M, Kawai S, Chigusa Y, Ilagan EJ, Ducusin BE, et al. Prevalence of schistosomiasis japonica among schoolchildren and animal reservoirs in oriental mindoro, Philippines. Jpn J Trop Med Hyg 1999;27:175–180.

[7] Ohno T, Mizokami M, Wu RR, Saleh MG, Ohba K, Orito E, et al. New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a and 6a. J Clin Microbiol 1997;35:201–207.

[8] Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4:406–425.

[9] Dopazo J. Estimating errors and confidence intervals for branch lengths in phylogenetic trees by a bootstrap approach. J Mol Evol 1994;38:300–304.

[10] Pybus OG, Rambaut A. GENIE: estimating demographic history from molecular phylogenies. Bioinformatics 2002;18:1404–1405.

[11] Lemey P, Pybus OG, Wang B, Saksena NK, Salemi M, Vandamme AM. Tracing the origin and history of the HIV-2 epidemic. Proc Natl Acad Sci USA 2003;100:6588–6592.

[12] Pybus OG, Drummond AJ, Nakano T, Robertson BH, Rambaut A. The epidemiology and iatrogenic transmission of hepatitis C virus in egypt: a Bayesian coalescent approach. Mol Biol Evol 2003;20: 381–387.

[13] Fukui S, Wada K, Iyo M. History and current use of methamphetamine in Japan. Proceedings of Japan–US Scientific

Symposium '90 on Drug Dependence and Abuse; 7–8 September 1990. Tokyo. Japan: Cocaine and methamphetamine behavioral toxicology, clinical psychiatry and epidemiology; 1991 [p. 219–37].

[14] Yoshizawa H. Hepatocellular carcinoma associated with hepatitis C virus infection in Japan: projection to other countries in the foreseeable future. Oncology 2002;62:8–17.

[15] Drucker E. Alcabes PG, Marx PA. The injection century: massive unsterile injections and the emergence of human pathogens. Lancet 2001;358:1989–1992.

[16] Kitani K. Iuchi M. Schistosomiasis japonica: a vanishing endemic in Japan. J Gastroenterol Hepatol 1990;5:160–172.

[17] Okabe K. Epidemiology of schistosomiasis japonica. Kurume Med J 1975;38:11–55.

[18] Kamel MA, Ghaffar YA, Wasef MA, Wright M, Clark LC, Miller FD. High HCV prevalence in Egyptian blood donors. Lancet 1992;340:427.

[19] Cohen J. The scientific challenge of hepatitis C. Science 1999;285: 26–30.

[20] Hibbs RG, Corwin AL, Hassan NF, Kamel M, Darwish M, Edelman R, et al. The epidemiology of antibody to hepatitis C in Egypt. J Infect Dis 1993;168:789–790.

[21] Frank C, Mohamed MK, Strickland GT, Lavanchy D, Arthur RR, Magder LS, et al. The role of parenteral antischistosomal therapy in the spread of hepatitis C virus in Egypt. Lancet 2000;355:887–891.

[22] Hassan MM, Zaghloul AS, El-Serag HB, Soliman O, Patt YZ, Chappell CL, et al. The role of hepatitis C in hepatocellular carcinoma: a case control study among Egyptian patients. J Clin Gastroenterol 2001;33:123–126.

[23] Angelico M, Renganathan E, Gandin C, Fathy M, Profili MC, Refai W, et al. Chronic liver disease in the Alexandria governorate, Egypt: contribution of schistosomiasis and hepatitis virus infections. J Hepatol 1997;26:236–243.

[24] Kamal S, Madwar M, Bianchi L, Tawil AE, Fawzy R, Peters T, et al. Clinical, virological and histopathological features: long-term follow-up in patients with chronic hepatitis C co-infected with S. mansoni. Liver 2000;20:281–289.

[25] Blanton RE, Salam EA, Kariuki HC, Magak P, Silva LK, Muchiri EM, et al. Population-based differences in *Schistosoma mansoni*- and hepatitis C-induced disease. J Infect Dis 2002;185: 1644–1649.

[26] Helal T, Danial M, Ahmed H. The relationship between hepatitis C virus and schistosomiasis: histopathologic evaluation of liver biopsy specimens. Hum Pathol 1998;29:743–749.

[27] Tanaka Y, Agha S, Saudy N, Kurbanov F, Orito E, Kato T, et al. Exponential spread of hepatitis C virus genotype 4a in Egypt. J Mol Evol 2004;58:191–195.

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Hepatitis C Virus Infection in 2,744 Hemodialysis Patients Followed Regularly at Nine Centers in Hiroshima During November 1999 Through February 2003

Junko Kumagai,[1] Yutaka Komiya,[1] Junko Tanaka,[1] Keiko Katayama,[1] Yorimitsu Tatsukawa,[2] Noriaki Yorioka,[3] Yuzo Miyakawa,[4] and Hiroshi Yoshizawa[1]*

[1]*Department of Epidemiology, Infectious Disease Control and Prevention, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan*
[2]*Sanyo Hospital, Fukuyama, Japan*
[3]*Department of Molecular and Internal Medicine, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan*
[4]*Miyakawa Memorial Research Foundation, Tokyo, Japan*

Patients on maintenance hemodialysis (HD) are at increased risk of infection with hepatitis C virus (HCV). A prospective follow-up study on HCV infection from November 1999 to February 2003 was conducted in nine hemodialysis (HD) units in Hiroshima. A total of 2,744 HD patients were surveyed regularly for HCV RNA in serum. The prevalence of HCV RNA decreased from 15.7% (262/1,664) on the first survey to 12.9% (242/1,882) in the last one ($P < 0.05$). This decrease may be attributed to the inclusion of patients with a lower prevalence of HCV RNA compared to patients leaving dialysis centers (111/1,080 [10.3%] vs. 132/862 [15.3%], $P < 0.01$). During the 40 months of this study, 16 de novo HCV infections were documented in the nine HD units corresponding to an incidence of 0.33% per year. These cases included eight new HCV infections, three re-infections, and five infections that presumably occured in the window period when tested during the first survey. Our study shows that the annual incidence of de novo HCV infection during HD was 0.33%, and emphasizes the need for frequent serum HCV RNA testing and for stringent disinfection procedures in order to prevent the transmission of HCV in these settings. ***J. Med. Virol. 76:498–502, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: antibody to hepatitis C virus; chronic hepatitis C; hemodialysis; hepatitis C virus

INTRODUCTION

Hepatitis C virus (HCV) is highly prevalent in hemodialysis (HD) units. The increase in HCV infection among HD patients is directly proportional to the duration of HD and also to the number of transfusions these patients have received in the past. Exclusion of blood units that are positive for antibody to HCV (anti-HCV) has decreased posttransfusion HCV infection in clinical settings [Watanabe et al., 1993]. Nevertheless, HCV infection remains a major global problem in most dialysis centers.

The prevalence of HCV in HD units varies widely from country to country. In a recent study [Fissell et al., 2004], HCV infection in HD units ranged from 2.6% to 22.9%, with an annual incidence varying from 1.2% to 3.9%. It is indeed very difficult to prevent transmission of HCV infection in dialysis units due to lack of concrete evidence for its mode of transmission. Nosocomial transmission has been implicated recently as the major route of HCV infection in HD units [Stuyver et al., 1996; Fabrizi et al., 2000]. Other studies, however, are needed to verify these findings.

In order to prevent de novo HCV infection in HD units, we surveyed 2,744 HD patients prospectively over a period of 40 months for HCV serum markers at frequent intervals. Patients that have developed HCV infection were followed closely. This investigation was

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*Correspondence to: Hiroshi Yoshizawa, Department of Epidemiology, Infectious Disease Control and Prevention, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan.
E-mail: juntan@hiroshima-u.ac.jp

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undertaken to study the prevalence of HCV infection in selected Japanese HD units and to suggest methods to minimize the spread of HCV infection in these units.

MATERIALS AND METHODS

Patients on Maintenance Hemodialysis

In June 1999, seven patients at one of 75 dialysis centers in Hiroshima came down with an HCV infection. A committee was immediately organized to investigate this HCV outbreak. An immediate and thorough HCV virological survey of HD patients at this center was recommended as well as on nine other core dialysis centers in Hiroshima. A prospective study was planned and conducted from November 1999 to February 2003 of all the 2,744 HD patients attending these centers. Sera were collected every 3 months during 14 surveys, and were tested for anti-HCV, HCV RNA, and HCV core antigen utilizing the same procedures and the same source of diagnostic kits by a single laboratory. Routine biochemical tests such as alanine aminotransferase (ALT) were also performed every 3 months and when required.

Serological Markers of HCV Infection

Anti-HCV was determined in serial twofold dilutions of the test serum by passive hemmagglutination (PHA) with currently available commercial kits (Second-generation HCV PHA; Abbott Japan KK, Tokyo, Japan). Results were recorded as the highest twofold dilution that induced hemagglutination; values $\geq 2^3$ were considered as positive. HCV RNA was determined by polymerase chain reaction (PCR) with nested primers deduced from well-conserved areas within the 5′-non-coding region of the HCV genome [Okamoto et al., 1990]. HCV core antigen was determined by enzyme-linked immunosorbent assay of the second generation (Ortho Clinical Diagnostics KK, Tokyo, Japan).

During the collection of sera, the tenets of the Declaration of Helsinki were observed and the study had the approval of our Internal Review Boad.

Statistical Analysis

Differences in the frequency of categorical variables between groups were evaluated by the χ^2 test or the Fisher's exact test, and those of continuous variables by the Student's t test.

RESULTS

Prevalence of HCV Infection in Hemodialysis Patients

During November 1999 through February 2003, 2,744 HD patients at the nine centers in Hiroshima were tested for HCV infection on 14 surveys at a 3-month interval. Among them, men predominated and accounted for 58.8% (1,613/2,744). The mean age was significantly lower in men than women (63.3 ± 13.1 vs. 65.7 ± 13.2 years, $P < 0.01$), while the mean duration on HD was not different between them (6.7 ± 6.4 vs. 6.7 ± 6.5 years). The prevalence of HCV RNA decreased gradually from 15.7% to 12.9% ($P < 0.05$). This is attributed, in part, to entry of patients with a lower prevalence of HCV RNA compared to patients leaving the dialysis centers (111/1,080 [10.3%] vs. 132/862 [15.3%], $P < 0.01$). HCV RNA was found to be more frequent in men than in women (14.5%–16.9% vs. 10.5%–14.4%, $P < 0.01$).

During the survey, 862 (31.4%) patients left the dialysis centers. Some due to sudden death (49.3%) and others due to transfer to other dialysis centers (47.7%). This loss accounted to 97.0% of patients.

Incidence of HCV Infection in Hemodialysis Patients

In this study, HCV RNA positive patients with high level anti-HCV titers were excluded at first and during later surveys. These patients must have acquired their infection before the initiation of HD. This left 2,114 patients who were at risk of HCV infection. These patients were followed for longer than 3 months. Among these patients, 16 (0.8%) were infected persistently with HCV, an incidence of 0.33%. Low-titered anti-HCV ($<2^{12}$) was detected in 125 (5.9%) of these patients. Of the remaining 1,989 patients who were anti-HCV negative, 5 were HCV RNA positive on the first survey. It was concluded that these five patients were in the "window period" of HCV infection. The remaining 1,984 (93.9%) patients were negative for both anti-HCV and HCV RNA.

As shown in Figure 1, three patterns of HCV infection were identified in HD patients. Among these patients, eight became positive for HCV RNA then seroconverted to anti-HCV (Fig. 1a). These patients must have acquired their HCV infection de novo. Among the 125 patients who were HCV RNA negative but with low-titered serum anti-HCV, 3 (2.4%) developed HCV infection and became HCV RNA positive with marked increases in anti-HCV titers (Fig. 1b). It was concluded that these three patients were infected recently with HCV. Another five patients were found to be HCV RNA positive without anti-HCV on the first survey (Fig. 1c). These patients were in the "window period" of HCV infection, since they seroconverted to slow-rising anti-HCV levels on other surveys. HCV core antigen with increasing titers was detected in all 16 patients, an indirect confirmation of a newly acquired HCV infection in these patients (Fig. 1a–c).

Figure 1d illustrates the course of persistent HCV infection in a patient with low anti-HCV titers. This patient was a 71-year-old woman with persistent low anti-HCV titers (2^3–2^5) through 11 surveys. Anti-HCV became undetectable in her serum ($<2^3$) on two occasions. Despite persistent HCV infection, her ALT serum levels remained within the upper limit of normal (<45 IU/L) throughout the observation period.

Figure 2 illustrates both the incidence and the increase in HCV infection during 14 surveys over

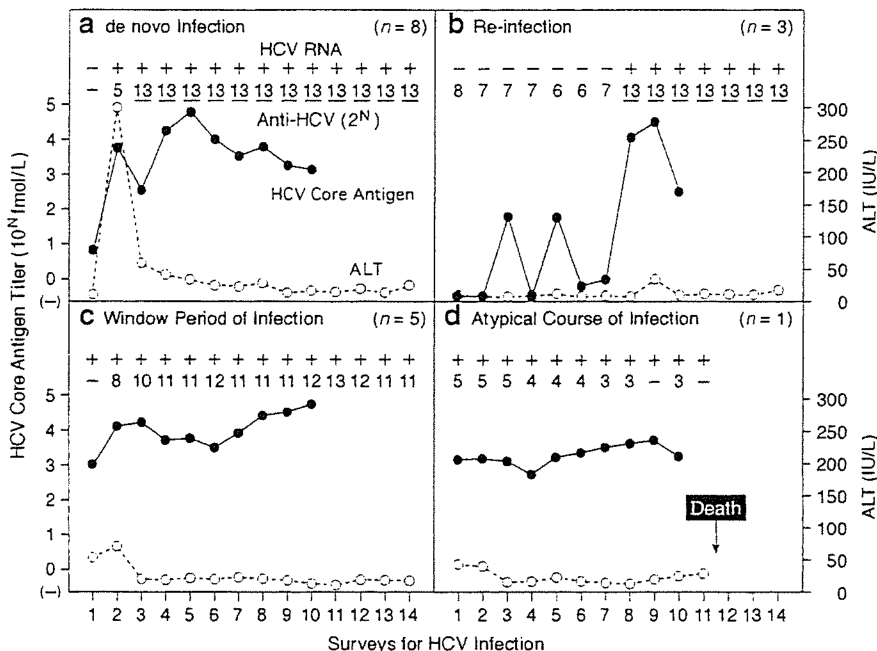

Fig. 1. Clinical courses of the 16 hemodialysis patients who developed HCV infection during the 14 surveys and the single patient with low anti-HCV titers. The course of a patient representative of each course is shown with the number of patients in that category in parentheses for those with de novo HCV infection (**a**), those with re-infection (**b**) and those in the window period of HCV infection (**c**). The course is shown also for the single patient in whom anti-HCV in low titers continued despite persistent infection (**d**). Underlined anti-HCV PHA titers indicate titers ≥13.

40 months. Although five patients were HCV RNA positive on the first survey, they were considered to be in the window period of an HCV infection that they already had acquired before the survey (Fig. 1c). Thereafter, the incidence was lower with only 1 or 2 HCV infections until the 8th survey. No new HCV infections were detected at the 9th, 10th, 13th, and 14th surveys. One patient developed an HCV infection on the 11th survey and two others developed an infection on the 12th survey.

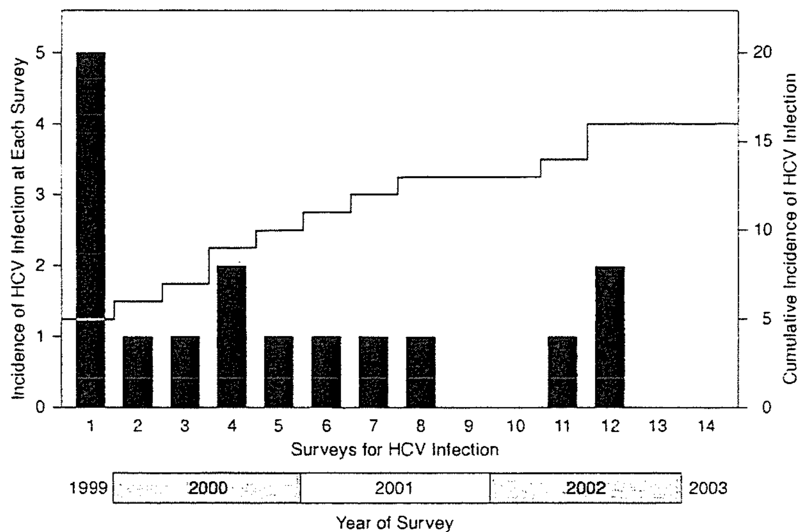

Fig. 2. Incidence of HCV infection during 14 surveys in hemodialysis patients attending the nine centers in Hiroshima, Japan.

TABLE I. HCV Infection in Hemodialysis Patients Stratified by Age and Sex

	Total		Men		Women	
Age (years)	n	HCV RNA	n	HCV RNA	n	HCV RNA
≤29	10	0	5	0	5	0
30–39	72	1 (1.4%)	47	1 (2.1%)	25	0
40–49	208	19 (9.1%)	134	13 (9.7%)	74	6 (8.1%)
50–59	468	65 (13.9%)	287	41 (14.3%)	181	24 (13.3%)
60–69	551	75 (13.6%)	321	51 (15.9%)	230	24 (10.4%)
≥70	573	82 (14.3%)	309	54 (17.5%)	264	28 (10.6%)
Total	1,882	242 (12.9%)	1,103	160 (14.5%)	779	82 (10.5%)

Influence of Age and the Year of Hemodialysis on HCV Infection

The frequency of HCV RNA increased with age in men, but it remained almost constant in women (Table I). Table II compares the prevalence of HCV infection in patients who had been on HD for ≥30 to ≤4 years. HCV infection was found to be very frequent (>44%) in patients who were treated by HD during ≥20 years, but infrequent (about 10%) in those during 15 years or less. There was approximately sixfold difference in the frequency between these patients. During 30 years on HD, the incidence of posttransfusion hepatitis fell dramatically (Table II). The decrease was attributed to anti-HCV screening by the first and second-generation assays that were mandated in 1989 and 1992, respectively. Of note, the prevalence of HCV infection in HD patients paralleled the incidence of posttransfusion hepatitis in the year when these patients started treatment by HD. In addition, the use of recombinant human erythropoietin which was introduced in 1990 decreased the need for transfusion in these patients. HCV infection prevailed, however, in ≥10% of the patients who started HD since 1993 when the incidence of posttransfusion hepatitis was decreased due to mandated screening.

DISCUSSSION

The present survey was carried out in nine core HD centers in Hiroshima. These centers, however, are not representative of other HD centers in Japan. Despite this, key issues have emerged as to current status of HCV infection among HD patients and means to prevent its future spread. During this survey of 3 years duration, the prevalence of HCV RNA in HD patients decreased from 15.7% to 12.9%. As shown in Table II, although the rate of HCV infection increased with the duration of HD, it was higher in those patients who started HD earlier. This is partly attributed to a decrease in the incidence of posttransfusion hepatitis C across the years between 8% and 19% before 1990 to practically negligent levels after 1992 [Yoshizawa, 2002]. Recombinant human erythropoietin that was introduced to patients in 1990 might have decreased further the exposure to HCV through transfusion.

The incidence of HCV infection has decreased since the survey was initiated. The five new infections that were detected on the first survey were attributed to acquisition of HCV before the survey was initiated (Figs. 1a and 2). The overall annual incidence was 0.33%, compared to 3.1% in a previous survey that was carried out in Japanese patients on maintenance HD [Fissell et al., 2004]. These investigatiors, unlike our current study, detected HCV infection by testing for anti-HCV rather than HCV RNA.

A strict adherence to HCV infection control by emphasizing regular and more frequent screening for HCV RNA during this survey would have brought attention of the risks in these nine HD centers concerning nosocomial transmission of HCV.

TABLE II. Prevalence of HCV Infection in Hemodialysis Patients With Reference to the Duration on Hemodialysis and the Year of Start

		HCV RNA				
Duration on hemodialysis (years)	n	n	Odds ratio (95% CI[a])	Differences (P value)	Started on HD[a]	Posttransfusion hepatitis[b]
≥30	5	3 (60.0%)	12.9 (3.2–52.3)	$P < 0.01$	1968–1972	16.2%
25–29	45	20 (44.4%)	6.9 (4.0–11.9)	$P < 0.01$	1973–1977	9.6%
20–24	92	41 (44.6%)	6.9 (4.6–10.5)	$P < 0.01$	1978–1982	19.3%
15–19	123	16 (13.0%)	1.3 (0.7–2.3)		1983–1987	12.3%
10–14	224	17 (7.6%)	0.7 (0.4–1.2)		1988–1992	3.1%
5–9	492	51 (10.4%)	1.0 (0.7–1.4)		1993–1997	Close to 0%
≤4	901	94 (10.4%)	1.0		1998–2002	Close to 0%

[a]Confidence interval. 1989: screening for anti-HCV by the first-generation EIA with c100-3; 1990: recombinant human erythropoietin approved for health insurance; 1992: Screening for anti-HCV by the second-generation immunoassays.
[b]Extracted from data reported by Yoshizawa [2002].

HD patients are at high risk of developing an HCV infection, and most often than not are in the "window period" before the emergence of serum anti-HCV. It has been reported by other investigators [Schroter et al., 1997; Furusyo et al., 2001] that anti-HCV in these patients does not emerge in serum for a period of 6.5–13 months compared to only 2.7 months in patients who acquire posttransfusion HCV infection [Schreiber et al., 1996]. In our study, 5 of 16 (31%) patients with de novo HCV infection were in the "window period" and subsequently developed serum anti-HCV in high titers during the next survey of 3 months later. This is in disagreement with the findings of Dalekos et al. [1998] who reported the absence of HCV RNA in 81 HD patients who were also anti-HCV negative.

Although it is difficult to investigate thoroughly the means of transmission of HCV infection among HD patients in our nine HD units, possibility of a nosocomial route of infection cannot be excluded. This possibility has been reported by others [Stuyver et al., 1996; Fabrizi et al., 2000]. Of interest is the finding that high titers of HCV RNA have been found in the wash of gloves worn by HD nurses [Alfurayh et al., 2000]. Thus it is imperative to take every necessary precaution to prevent HCV infection in such settings [Kellerman and Alter, 1999].

In conclusion, our data supports more frequent testing of HCV RNA in HD units. Based on our experience, testing for HCV RNA on a monthly bases may be necessary to reduce the transmission of HCV in HD units. This is in agreement with the recommendation of Moreira et al. [2003].

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REFERENCES

Alfurayh O, Sabeel A, Al Ahdal MN, Almeshari K, Kessie G, Hamid M, Dela Cruz DM. 2000. Hand contamination with hepatitis C virus in staff looking after hepatitis C-positive hemodialysis patients. Am J Nephrol 20:103–106.

Dalekos GN, Boumba DS, Katopodis K, Zervou E, Sferopoulos G, Elisaf M, Tsianos EV, Siamopoulos KC. 1998. Absence of HCV viraemia in anti-HCV-negative haemodialysis patients. Nephrol Dial Transplant 13:1804–1806.

Fabrizi F, Martin P, Lunghi G, Ponticelli C. 2000. Nosocomial transmission of hepatitis C virus infection in hemodialysis patients: Clinical perspectives. Int J Artif Organs 23:805–816.

Fissell RB, Bragg-Gresham JL, Woods JD, Jadoul M, Gillespie B, Hedderwick SA, Rayner HC, Greenwood RN, Akiba T, Young EW. 2004. Patterns of hepatitis C prevalence and seroconversion in hemodialysis units from three continents: The DOPPS. Kidney Int 65:2335–2342.

Furusyo N, Hayashi J, Kakuda K, Ariyama I, Kanamoto-Tanaka Y, Shimizu C, Etoh Y, Shigematsu M, Kashiwagi S. 2001. Acute hepatitis C among Japanese hemodialysis patients: A prospective 9-year study. Am J Gastroenterol 96:1592–1600.

Kellerman S, Alter MJ. 1999. Preventing hepatitis B and hepatitis C virus infections in end-stage renal disease patients: Back to basics. Hepatology 29:291–293.

Moreira R, Pinho JR, Fares J, Oba IT, Cardoso MR, Saraceni CP, Granato C. 2003. Prospective study of hepatitis C virus infection in hemodialysis patients by monthly analysis of HCV RNA and antibodies. Can J Microbiol 49:503–507.

Okamoto H, Okada S, Sugiyama Y, Tanaka T, Sugai Y, Akahane Y, Machida A, Mishiro S, Yoshizawa H, Miyakawa Y, Mayumi M. 1990. Detection of hepatitis C virus RNA by a two-stage polymerase chain reaction with two pairs of primers deduced from the 5'-noncoding region. Jpn J Exp Med 60:215–222.

Schreiber GB, Busch MP, Kleinman SH, Korelitz JJ. 1996. The risk of transfusion-transmitted viral infections. The Retrovirus Epidemiology Donor Study. N Engl J Med 334:1685–1690.

Schroter M, Feucht HH, Schafer P, Zollner B, Laufs R. 1997. High percentage of seronegative HCV infections in hemodialysis patients: The need for PCR. Intervirology 40:277–278.

Stuyver L, Claeys H, Wyseur A, Van Arnhem W, De Beenhouwer H, Uytendaele S, Beckers J, Matthijs D, Leroux-Roels G, Maertens G, De Paepe M. 1996. Hepatitis C virus in a hemodialysis unit: Molecular evidence for nosocomial transmission. Kidney Int 49:889–895.

Watanabe J, Matsumoto C, Fujimura K, Shimada T, Yoshizawa H, Okamoto H, Iizuka H, Tango T, Ikeda H, Endo N, Mazda T, Nojiri T, Aoyama K, Kanemitsu K, Yamano H, Mizui M, Yokoishi F, Tokunaga K, Nishioka K. 1993. Predictive value of screening tests for persistent hepatitis C virus infection evidenced by viraemia. Japanese experience. Vox Sang 65:199–203.

Yoshizawa H. 2002. Hepatocellular carcinoma associated with hepatitis C virus infection in Japan: Projection to other countries in the foreseeable future. Oncology 62:S8–S17.

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本邦における地域別にみた肝炎ウイルス罹患状況と肝臓がん

Relationship of persistent infection of hepatitis viruses and HCC in Japan

田中　純子　　吉澤　浩司
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永　井　書　店

解　　説

特集

肝臓の臨床最前線

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Relationship of persistent infection of hepatitis viruses and HCC in Japan

田中　純子　　吉澤　浩司*
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Key words：HBV　HCV　キャリア率　推計キャリア数　肝臓がん

わが国における悪性新生物による死亡を部位別にみると「肝」(肝および肝内胆管の悪性新生物，人口動態統計[1])による死亡実数は，1995年に初めて年間3万人を上回り，2002年には死亡数34,637人と，肺がん(56,405人)，胃癌(49,213人)に次いで第3位の位置を占めるに至っている．また，人口10万人あたりの肝がんによる死亡率の推移をみると，1975年から増加の一途を辿り，2002年には全体では27.5人となり，特に男性では38.7人と女性(16.8人)に比べ2倍以上の高い値を示している(図1)．

日本肝癌研究会による調査成績[2]および人口動態統計資料[1]をもとに算出した，成因別にみた肝がん死亡の推移をみると，1978年以降，現在に至るま

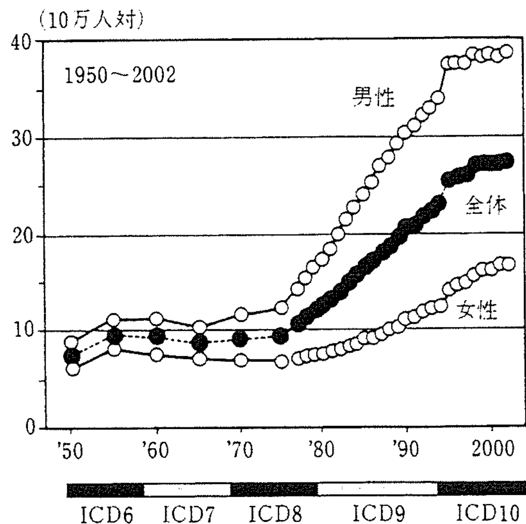

図1　わが国における肝がんによる死亡の推移

広島大学大学院医歯薬学総合研究科疫学・疾病制御学教室　助教授　*教授

0371-1900/05/¥50/頁/JCLS