

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

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

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 antimony 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. Materials 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 *schistosoma* 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-matched patients were also selected from the *Sj*-nonendemic areas excluding influence 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 (Invitrogen 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 (Invitrogen 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 \pm 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).

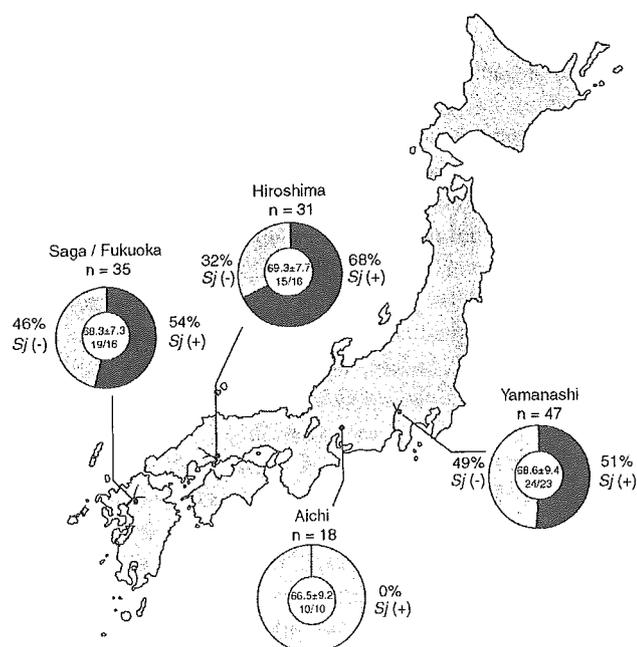


Fig. 1. Geographic distribution of *Schistosoma japonicum* (*Sj*) and characteristics of patients infected with HCV. *Sj* (+) and *Sj* (-) denote, respectively, presence and absence of infection with *Sj* diagnosed by ultrasonographic and/or computer tomographic methods or serological examinations. Pie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

Table 1
Characteristics of patients with and without schistosomiasis

	Schistosoma japonicum		Controls (Aichi) (n = 18)
	Positive (n = 64)	Negative (n = 49)	
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).

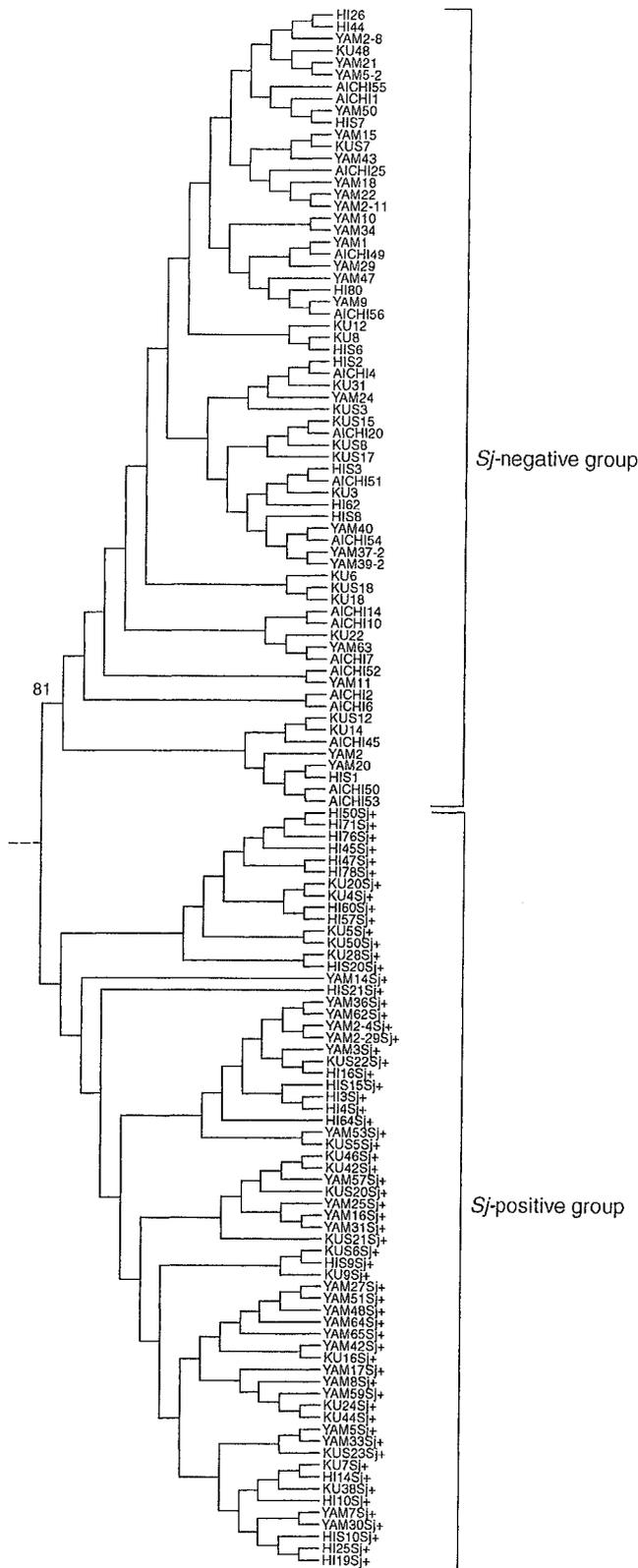


Fig. 2. A phylogenetic tree constructed on NS5B sequences of HCV-1b strains in *Schistosoma japonicum* (*Sj*)-positive ($n=64$) and -negative ($n=67$) groups. The numbers in the tree indicate bootstrap reliability by the interior branch test. *Sj*+ indicates *Sj*-positive strains. YAM; Yamanashi, HI/HIS;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

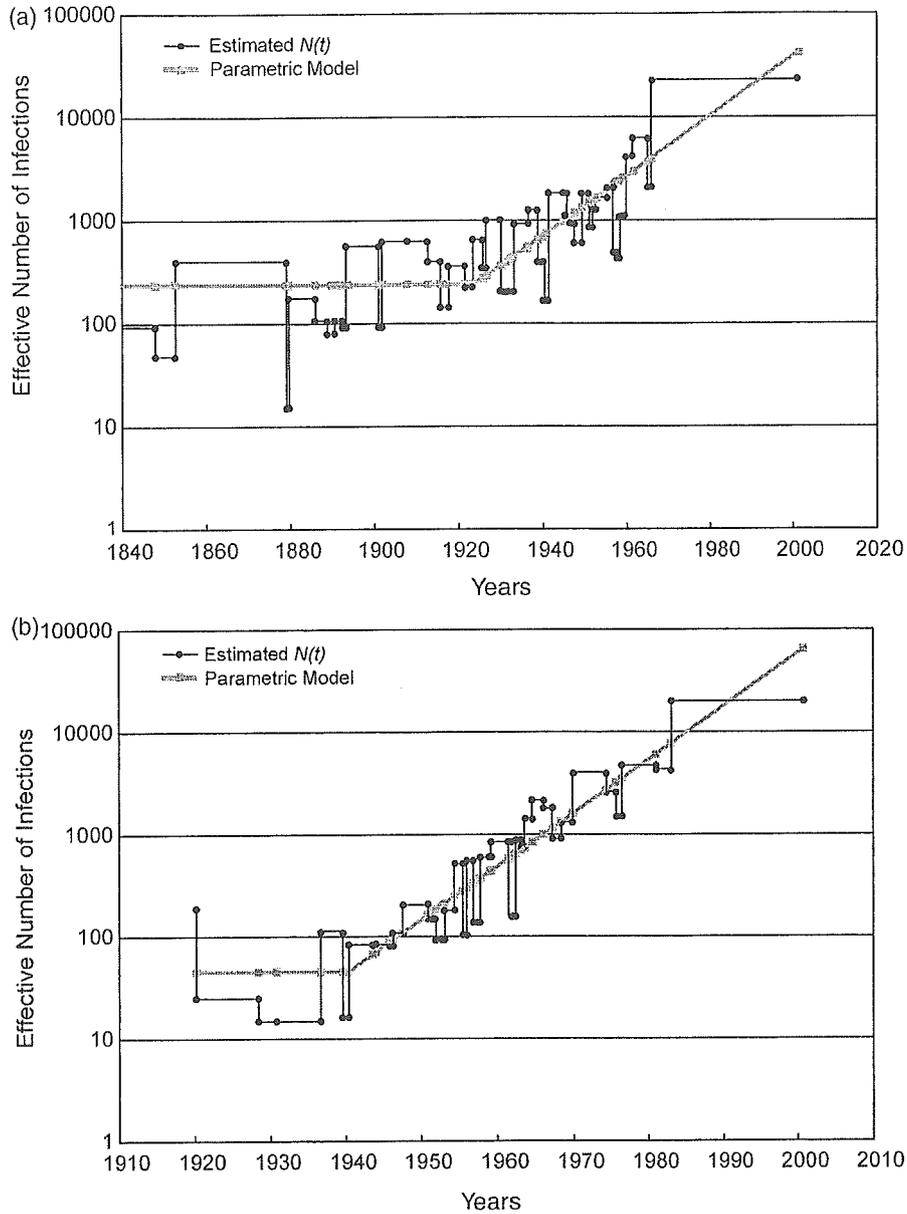


Fig. 3. The maximum-likelihood estimates of $N(t)$ on the effective number of infections with HCV genotype 1b in Japan for *Schistosoma japonicum* (*Sj*)-positive group (a) and *Sj*-negative group (b) separated in the phylogenetic tree (Fig. 2). The parametric model is indicated by the grey line and 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 epidemics, 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 antimony 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 United 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.

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Distinct Geographic Distributions of Hepatitis B Virus Genotypes in Patients With Acute Infection in Japan

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Genotypes of hepatitis B virus (HBV) were determined in 145 patients with acute hepatitis B from various districts in Japan to establish their geographic distribution and evaluating the influence on the clinical illness and outcome. Genotypes were A in 27 (19%) patients, B in 8 (5%), C in 109 (75%) and mixed with B and C in the remaining one (1%). Genotype A was more frequent in metropolitan than the other areas (21/69 (30%) vs. 6/76 (8%), $P < 0.001$). On phylogenetic analysis, seven of the nine (78%) HBV/A isolates selected at random clustered with those from Europe and the United States, while the remaining two with those of subgroup A' prevalent in Asia and Africa. Maximum ALT levels were lower (2069 ± 1075 vs. 2889 ± 1867 IU/L, $P = 0.03$) and baseline HBV DNA titers were higher (5.90 ± 1.45 vs. 5.13 ± 1.36 log genome equivalents (LGE)/ml, $P = 0.002$) in patients infected with genotype A than C. Hepatitis B surface antigen persisted longer in patients infected with genotype A than C (1.95 ± 1.09 vs. 1.28 ± 1.42 months, $P = 0.02$). HBV infection became chronic in one (4%) patient with genotype A and one (1%) with genotype C infection. Fulminant hepatic failure developed in none of the patients with genotype A, one (13%) with genotype B and five (5%) with genotype C. The point mutation in the precore region (A1896) or the double mutations in the basic core promoter (BCP) region

(T1762/A1764) were detected in none of the patients with genotype A, two (25%) with genotype B and 27 (26%) with genotype C. In conclusion, genotype A is frequent in patients with acute hepatitis B in metropolitan areas of Japan, probably reflecting particular transmission routes, and associated with longer and milder clinical course than genotype C. **J. Med. Virol. 77:39–46, 2005.** © 2005 Wiley-Liss, Inc.

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INTRODUCTION

The clinical outcome in patients with acute hepatitis B varies widely. Although hepatitis is self-limited in most patients, the clinical features range from asymptomatic to fulminant hepatic failure, while some patients become carriers of hepatitis B virus (HBV) [Chan HL and Lok, 1999; Chan HLY, 1999]. Factors that determine the clinical outcome remain unknown.

Viral nucleotide (nt) mutations have been shown to influence the clinical outcome of acute hepatitis B. Mutations in the precore region (A1896) and the basic core promoter (BCP) region (T1762/A1764) are common in patients with fulminant hepatic failure [Carman et al., 1991; Kosaka et al., 1991; Liang et al., 1991; Omata et al., 1991; Hawkins et al., 1994; Sato et al., 1995; Baumert et al., 1996; Chu et al., 1996]. Viral factors other than these mutations may influence the clinical outcome of acute hepatitis B.

Eight genotypes of HBV have been identified by sequence divergence greater than 8% in the entire genome, and they are designated by capital alphabet letters from A to H [Okamoto et al., 1988; Norder et al., 1994; Stuyver et al., 2000; Arauz-Ruiz et al., 2002]. Furthermore, recombinant HBV strains consisting of two different genotypes have been reported [Bollyky et al., 1996; Morozov et al., 2000]. Genotype distribution is different in different countries and even in distinct areas of the same country [Orito et al., 2001a; Kao, 2002; Kato et al., 2002; Miyakawa and Mizokami, 2003]. Therefore, surveys on genotype distribution may be helpful in identifying transmission routes and evaluating clinical relevance.

It has been shown that the clinical outcome of chronic hepatitis B is influenced by HBV genotypes. In Asian patients with chronic hepatitis B, genotype C is associated with later seroconversion of hepatitis B e antigen (HBeAg) and more severe liver damage than genotype B [Kao et al., 2000; Orito et al., 2001b; Chu et al., 2002; Ding et al., 2002; Sugauchi et al., 2002a]. Likewise, a study from India has shown that genotype D is associated with more severe liver disease than genotype A [Thakur et al., 2002]. Genotype A is peculiar in that A1896 in the precore region occurs infrequently, because it causes instability of the stem-loop structures of the pregenome encapsidation signal [Li et al., 1993; Lok et al., 1994]. These reports suggest that HBV genotypes also influence the clinical characteristics of acute hepatitis. Recent studies on small numbers of patients with acute hepatitis B suggest that the clinical course may differ among infections with distinct HBV genotypes [Mayerat et al., 1999; Kobayashi et al., 2002; Ogawa et al., 2002]. However, the association between viral genotype and severity of liver disease remains uncertain in acute HBV infection.

To evaluate the effect of HBV genotypes on the clinical characteristics of acute hepatitis B, a multi-center study on 145 patients was conducted in Japan.

MATERIALS AND METHODS

Patients

During 1992 through 2001, serum samples were collected from 147 patients diagnosed with acute hepatitis B in our institutions. Only patients from whom sera at the onset of hepatitis were stored were included in this study. Sixty-nine (47%) patients lived in metropolitan areas (Kawasaki, Tokyo and Tokorozawa), while the others in Kurume, Ube, Osaka, Gifu, Nagoya and Sapporo. Criteria for the diagnosis of acute hepatitis B were: (1) Acute onset of liver injury without a history of liver dysfunction and detection of hepatitis B surface antigen (HBsAg) in serum; and (2) IgM antibody to HBV core (anti-HBc) in high titer. Co-infection with hepatitis A virus or hepatitis C virus was excluded by serological tests.

Among the 147 patients, acute hepatitis B in six (4%) was complicated by hepatic encephalopathy and prolonged prothrombin time for the diagnosis of fulminant hepatic failure. Other two (1%) patients remained positive for HBsAg for longer than 6 months, and they were considered to have acquired chronic infection.

Sera from the 147 patients with acute hepatitis B were examined virologically, and the results were correlated with clinical and demographic characteristics. Informed consent was obtained from each patient for the purpose of this study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and approved by the Ethics Committees of our institutions.

Determination of HBV DNA

Levels of HBV DNA were determined using transcription-mediated amplification (TMA) and hybridization-protection assay (Chugai Diagnostics Science Co., Ltd., Tokyo, Japan) after the protocol as reported [Kamisango et al., 1999]. The range of detection by TMA was from 3.7 log genome equivalents (LGE)/ml ($10^{3.7}$ copies/ml corresponding to 5,000 copies/ml) to 8.7 LGE/ml ($10^{8.7}$ copies/ml). In 16 of 86 studied sera, levels of HBV DNA were under 3.7 LGE/ml and categorized in 3.7 LGE/ml.

Genotyping HBV

HBV genotypes in most samples were determined with commercial enzyme immunoassay kits (HBV Genotype EIA, Institute of Immunology Co. Ltd., Tokyo, Japan) involving monoclonal antibodies to genotype-specific epitopes in the preS2-region, as reported previously [Usuda et al., 1999, 2000; Kato et al., 2001]. Genotypes in 18 (12%) samples were determined by genotype-specific probe assay (Smitest HBV Genotyping Kit, Genome Science, Fukushima, Japan). In brief, DNA extracted from serum was amplified by the polymerase chain reaction (PCR) with three sense primers (s1: 5'-ACC AAC CCT CTG GGA TTC TTT CC-3', s2: 5'-ACC AAT CCT CTG GGA TTC TTC CC-3' and s3: 5'-AGC AAT CCT CTA GGA TTC CTT CC-3' [nt 2902-2924]) and an antisense primer (as1: 5'-GAG CCT GAG GGC TCC ACC C-3' [nt 3091-3073]) biotinylated at the 5'-end;

they were deduced from conserved sequences in the preS1 region of HBV. The biotin-labeled and amplified HBV DNA was denatured in an alkaline solution, and tested for hybridization to probes specific for one or other of the seven genotypes (A–G) immobilized on wells of a 96-well microplate. Thereafter, hybridization was detected by staining with the streptavidine-horseradish peroxidase (HRP) conjugate [Kato et al., 2003].

Subtypes of genotype B, in terms of Ba with the recombination with genotype C and B_j without it were determined by direct sequencing of precore and core regions by the method reported previously [Sugauchi et al., 2002b].

Amplifying and Sequencing HBV DNA of Genotype A Isolates

A subgroup of genotype A is reported with the designation of A' from South Africa, Philippines, Malawi, and Belgium [Bowyer et al., 1997; Kramvis et al., 2002; Sugauchi et al., 2004]. Randomly selected HBV/A samples were classified into genotype A and subtype A' by sequencing the S region. For amplification and sequencing, the entire S region was divided into two fragments, spanning nt 3130–478 and nt 378–878, respectively, and they were amplified by two-stage PCR. The outer primers for amplification of the 1st fragment were: 5'-ACC AAT CGG CAG TCA GGA AG-3' (sense: nt 3121–3140) and 5'-CTG GAA TTA GAG GAC AAA CG-3' (antisense: nt 488–469) and the inner primers were: 5'-CAG TCA GGA AGG CAG CCT ACT-3' (sense: nt 3130–3150) and 5'-AGG ACA AAC GGG CAA CAT AC-3' (antisense: nt 478–459). The outer primers for amplification of the 2nd fragment were: 5'-TGT CCT GGT TAT CGC TGG AT-3' (sense: nt 359–378) and 5'-CAA CGT ACC CCA ACT TCC AA-3' (antisense: nt 909–890) and the inner primers were: 5'-TGT GTC TGC GGC GTT TTA TC-3' (sense: nt 378–397) and 5'-ATG AAG TTT AGG GAA TAA CC-3' (antisense: nt 878–859).

The first stage of amplification was carried out in a thermal cycler for 40 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 1 min) in 100 µl of the reaction mixture containing 200 µM dNTPs, 1.0 µM each of primers and 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂ and 0.001% (wt/vol) gelatin) and 2 U of Ampli-Taq polymerase (Perkin Elmer Cetus Corp., Connecticut). PCR products (2 µl) were subjected to the second stage of amplification under the same conditions as the first stage. Standard precautions to avoid contamination were exercised during PCR, with a negative control serum included in each run.

Amplification products were purified on Wizard PCR preps DNA purification resin (Promega, Wisconsin), and sequenced bidirectionally with the Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, California) using the PCR primers. Sequencing was performed in an automated DNA sequencer (ABI 377; PE Applied Biosystems).

The nucleotide sequences of HBV/A isolates from patients were compared with those of 25 reference HBV/

A strains including subtype A' retrieved from the DDBJ/EMBL/GenBank database, as well as representatives of the other six major genotypes (B–G). Phylogenetic trees were constructed with the mega program version 2.1 using the Kimura two-parameter matrix and the neighbor-joining method [Sugita et al., 1991]. To confirm the reliability of phylogenetic tree analysis, bootstrap resampling, and reconstruction were carried out 500 times.

Detection of Point Mutations in the Precore and BCP Regions of HBV

Mutation in the precore region for A1896 was detected by enzyme-linked minisequence assay (Smitest HBV Pre-C ELMA, Roche Diagnostics, Tokyo, Japan) and mutations in the BCP region for T1762/A1764 were detected by enzyme-linked specific probe assay (Smitest HBV Core Promoter Mutation Detection Kit; Genome Science Laboratory, Tokyo, Japan) according to the manufacturer's instructions, after the principles described previously [Orito et al., 2001b]. The results were recorded as "the wild-type" and "the mutant-type" expressed dominantly by HBV isolates.

Statistical Analysis

Data were analyzed by chi-square test or Fisher's exact test for categorical data and Student's *t*-test or Mann-Whitney *U*-test for continuous variables. *P*-values less than 0.05 were regarded as statistically significant. Logistic regression (backward logistic regression) was used in the multivariate analysis to evaluate the factors associated with differences between genotypes A and C.

RESULTS

Distribution of HBV Genotypes

HBV genotypes were determined in 145 of the 147 (99%) patients with acute hepatitis B; they were untypeable in the remaining two patients (Table I). Genotype A was detected in 27 (19%) patients, B in 8 (5%), C in 109 (75%), and mixed genotypes with B and C in the remaining one (1%). In the 69 patients with acute hepatitis B from metropolitan areas (Tokyo, Kawasaki, and Tokorozawa), genotype A was found in 21 (30%), B in 5 (7%), and C in 43 (63%). In the 76 patients from the other areas in the mainland, by contrast, genotype A occurred in 6 (8%), B in 3 (4%), C in 66 (87%), and mixed genotypes with B and C in one (1%). Thus, genotype A was significantly more frequent in patients with acute hepatitis B from the metropolitan than the other areas (30% vs. 8%, *P* < 0.001).

Demographic and Clinical Differences Among Patients Infected With HBV of Distinct Genotypes

Clinical and demographic backgrounds in patients with acute hepatitis B who were infected with HBV of

TABLE I. Demographic and Clinical Differences Among Patients With Acute Hepatitis Who Were Infected With HBV of Distinct Genotypes

Features	Genotypes of HBV				Differences (A vs. C)	
	A (n = 27)	B (n = 8)	C (n = 109)	B/C (n = 1)	Univariate (<i>P</i> -value)	Multivariate logistic regression (<i>P</i> -value)
Areas					<0.001	0.03
Metropolitan (n = 69)	21 (30%)	5 (7%)	43 (63%)	0		
Others (n = 76)	6 (8%)	3 (4%)	66 (87%)	1 (1%)		
Age (years)	29.3 ± 8.0	35.7 ± 10.1	36.6 ± 13.6	51	0.016	0.152
Male	25 (93%)	7 (88%)	69 (57%)	1 (100%)	0.003	0.018
Transmission routes						
Heterosexual	15 (56%)	3 (37%)	52 (48%)	0	0.197	
Homosexual	5 (19%)	1 (13%)	2 (2%)	0	<0.001	0.133
IV drugs	0	0	8 (7%)	0	0.280	
Unknown	7 (25%)	4 (50%)	47 (43%)	1 (100%)	0.102	
Fulminant hepatic failure	0	1 (13%)	5 (5%)	0	0.582	
ALT (IU/L) ^a	2069 ± 1075	2952 ± 1106	2889 ± 1867	646	0.030	0.084
Bilirubin (mg/dl) ^a	10.7 ± 14.1	10.3 ± 4.9	7.8 ± 6.7	4.8	0.533	
ALP (IU/L) ^a	476 ± 161	501 ± 94	432 ± 116	No data	0.542	
HBeAg	24/26 (92%)	4/8 (50%)	57/93 (61%)	1/1 (100%)	0.357	
Precore and BCP mutations						
Precore (1896A)	0/27	1/8 (13%)	20/102 (20%)	No data	0.250	
BCP (1762T/1764A)	0/27	1/6 (17%)	14/75 (19%)	No data	0.357	
Precore or BCP	0/27	2/8 (25%)	27/102 (26%)	No data	0.096	

^aMaximum data are shown for alanine aminotransferase (ALT), bilirubin and alkaline phosphatase (ALP).

different genotypes are compared in Table I. Patients with genotype A were younger than those with genotype C (29.3 ± 8.0 vs. 36.6 ± 13.6 years, *P* = 0.016). The proportion of male patients was higher in genotype A than C infection (93% vs. 57%, *P* = 0.003). The main route of transmission identified in the patients with acute hepatitis B was extramarital heterosexual contacts. Homosexual activity was more frequent in patients with genotype A than C (5/27 (19%) vs. 2/109 (1.8%), *P* < 0.001).

The maximum ALT levels were lower in patients with genotype A than B or C infection (2069 ± 1075, 2952 ± 1106 and 2889 ± 1867 IU/L, respectively: A vs. B, *P* = 0.02; A vs. C, *P* = 0.03). The maximum bilirubin and alkaline phosphatase levels were no different among patients infected with HBV of different genotypes. Fulminant hepatic failure developed in one (13%) patient with genotype B and five (5%) with genotype C; no patients with genotype A came down with it. Evolution into chronic infection occurred in two patients (one with genotype A and one with genotype C). The remaining 137 (96%) patients ran a non-fulminant and self-limited disease.

HBeAg was found in 24 of the 26 (92%) patients with genotype A, 4 of the 8 (50%) with genotype B and 57 of the 93 (61%) with genotype C; it was no different between genotype A than genotype C infection (*P* = 0.357). Of the six patients with fulminant hepatic failure, only one (17%) had HBeAg.

With logistic multivariate regression analysis, the variables for differences between genotypes A and C were sex (odds ratio (OR), 6.45; 95% confidence interval

(CI), 1.378–30.213; *P* = 0.0018) and area (OR, 0.25; 95% CI, 0.076–0.830; *P* = 0.0024).

Routes of transmission were compared between genotypes A and C in patients with acute hepatitis B from metropolitan areas. Although the mean age was no different, frequently the proportion of male patients was higher in genotype A than C infection (20/21 (95%) vs. 28/43 (65%), *P* = 0.012). Homosexual patients had more frequently genotype A than C infection (5/21 (24%) vs. 1/44 (2%), *P* = 0.012). Additionally heterosexuals with multiple unspecific partners had in genotype A more frequently than C infection (7/12 (58%) vs. 6/26 (23%), *P* = 0.035, respectively). However, with logistic multivariate regression analysis, none of these variables differed between genotype A and C infections.

Figure 1 compares serum HBV DNA levels on admission among patients infected with different genotypes. HBV DNA levels were higher in patients with genotype A than C (5.90 ± 1.45 vs. 5.13 ± 1.36 LGE/ml, *P* = 0.002).

Among the 145 patients whose HBV genotypes could be determined, 54 (A: 15, B: 4, and C: 35) were followed for HBsAg in serum every 2–4 weeks until it disappeared. The time between the first and last detection of HBsAg was defined as the duration of HBsAg, and compared between patients infected with HBV of genotypes A and C (Fig. 2a). The duration of HBsAg was longer in patients with genotype A than C infection (1.95 ± 1.09 (n = 15) vs. 1.28 ± 1.42 months (n = 35), *P* = 0.02). When patients with fulminant hepatic failure were excluded, the mean duration of HBsAg in patients with genotype C became longer, but it was still shorter

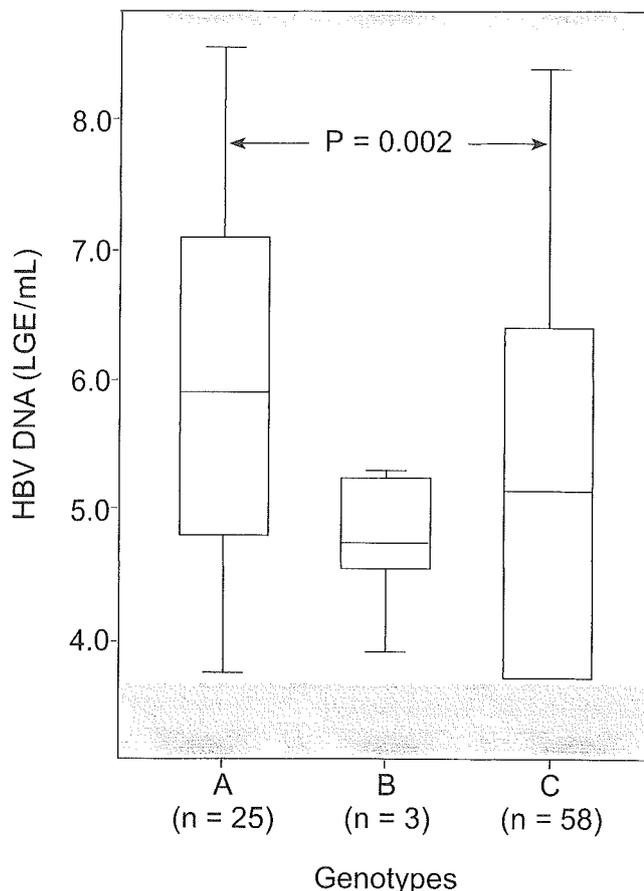


Fig. 1. HBV-DNA levels in patients with acute hepatitis B with genotypes A, B, or C at the presentation. Box plots are given with horizontal lines for the medians, upper and lower edges indicating the 25th and 75th centiles, respectively, and bars represent the extremes without including outliers. Shaded areas are outside the range of detection by the TMA method.

than that in those with genotype A (1.95 ± 1.09 ($n = 15$) vs. 1.41 ± 1.42 ($n = 31$) months, $P = 0.03$).

Subtypes of Genotypes A and B

Among the 27 HBV/A isolates, 9 were selected at random and the entire S region was amplified and sequenced for them. Seven of them were classified into genotype A and the remaining 2 into subgroup A'. The sequence divergence within the seven genotype A isolates ranged from 0.12% to 2.01% in pair-wise comparison, while that between two subgroup A' and seven genotype A isolates spanned from 5.70% to 6.53%.

A phylogenetic tree was constructed on the entire S-gene sequences from these nine sequences along with those from 31 HBV isolates retrieved from the database (Fig. 3). The seven (78%) HBV isolates classified into genotype A clustered with reported HBV/A isolates, while the remaining two isolates classified into subgroup A' (cases 3 and 4) joined the branch of subgroup A'.

Six of the eight HBV/B isolates were available for analysis of subtypes. Two (both from the metropolitan area) were classified as Ba and the remaining four, in-

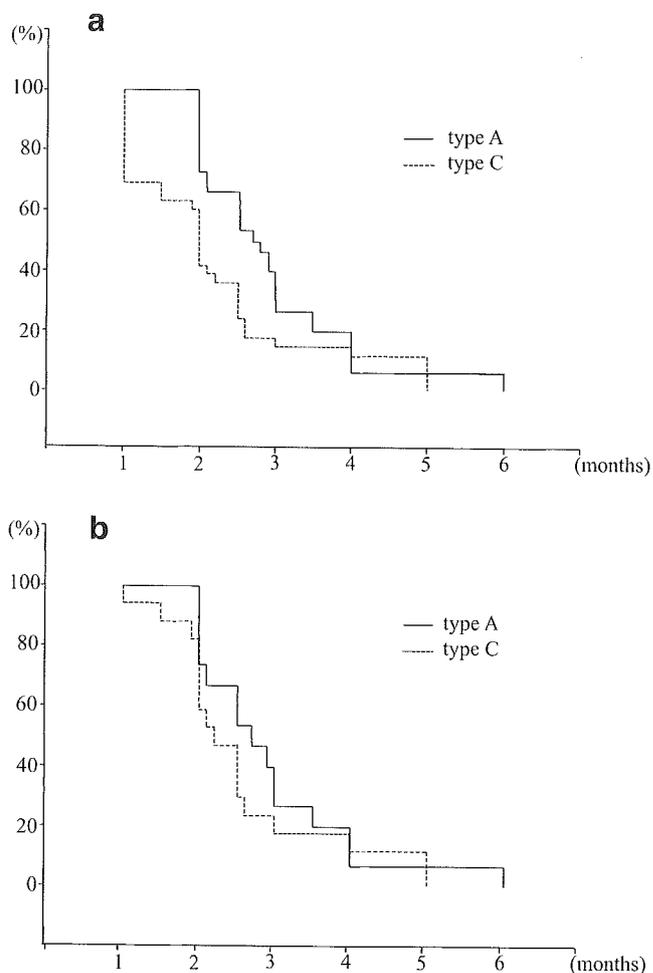


Fig. 2. The duration of HBsAg in patients with acute hepatitis B with genotypes A or C. The results are shown for (a) all patients, and (b) patients with the wild-type sequences both in precore and BCP regions of HBV.

cluding two from Tokyo and two from the other areas, as Bj. One of the four patients infected with subtype Bj developed fulminant hepatic failure, while the remaining three with subtype Bj as well as the two with subtype Ba ran a non-fulminant course.

Point Mutations in the Precore and Basic Core Promoter Regions of HBV

All the 27 HBV isolates of genotype A in which mutations were sought had the wild-type sequences both in the precore and BCP regions. In contrast, of the 102 genotype C isolates whose precore and BCP sequences were examined, 27 (26%) had mutations in the precore or BCP regions ($P = 0.096$). Furthermore, of the four genotype C isolates from patients with fulminant hepatic failure whose genetic mutations could be determined, three had mutations in the BCP region (T1762/A1764) and two had a mutation in the precore region (A1896). Only one isolate had the wild-type sequences both in the precore and BCP regions. Of

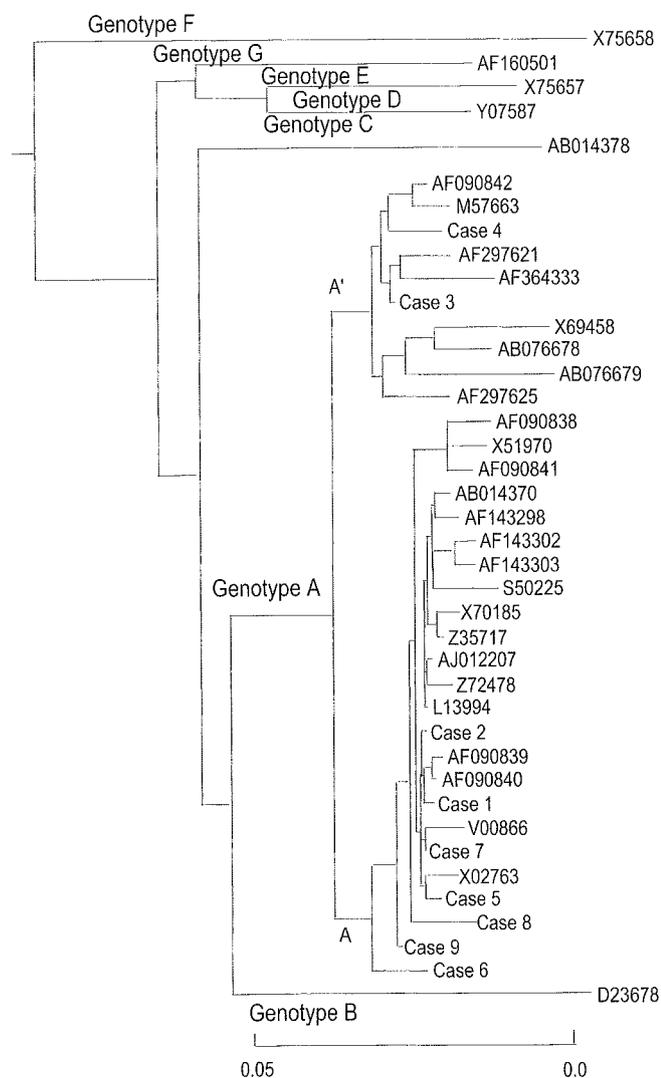


Fig. 3. A phylogenetic tree constructed on HBV DNA sequences spanning the major *S*-gene of all known HBV genomes, including the nine of genotype A. The horizontal bar indicates the number of nucleotide substitutions per site. Accession numbers are shown for the isolates, which have been deposited in the DDBJ/EMBL/GenBank databases. HBV sequences in cases 1–9 were determined in the present study. The HBV/A sequences from cases 1, 2, and 5–9 clustered with the European-American genotype A, while those from cases 3 and 4 clustered with genotype A' that is the African subgroup of genotype A.

the eight genotype B isolates, two (25%) had mutations in the precore or BCP region (Table I).

To examine further differences between genotype A and C infections, patients infected with HBV strains with the wild-type sequences both in precore and BCP regions were compared. The maximum ALT levels were still lower in patients with genotype A than C infection (2069 ± 1075 and 2594 ± 1015 IU/L, respectively, $P=0.02$), but the maximum bilirubin and alkaline phosphatase levels were no different amongst patients infected with HBV of distinct genotypes. There were no differences in the duration of serum HBsAg between patients with genotype A and C infections (1.95 ± 1.09 vs. 1.58 ± 1.24 months, $P=0.35$) (Fig. 2b).

DISCUSSION

The salient finding in this study is that infection with HBV genotype A is frequent in patients with acute hepatitis in Japan, lending support to previous studies [Kobayashi et al., 2002; Ogawa et al., 2002]. Substantial portion of patients with acute hepatitis were infected with genotype A, which is detected rarely among patients with chronic hepatitis in Japan [Orito et al., 2001a; Kobayashi et al., 2002]. Genotype A prevails in North-Western Europe, United States, Central Africa, and India [Kao, 2002; Miyakawa and Mizokami, 2003]. This genotype may be prevalent in countries elsewhere, since the distribution of HBV genotypes has not been examined in many districts of the world. Phylogenetic analysis has shown that seven (78%) HBV/A strains of the nine patients examined with acute hepatitis B were of the European-American type. Although the HBV/A sequences from four, (cases 1, 2, 5, and 7) clustered with those reported previously, those from three (cases 6, 8, and 9) were separated genetically (Fig. 3), which suggests their distinct geographic origin.

Notably, the genotype distribution differed between patients with acute hepatitis B from metropolitan areas and the others including many large cities. As genotype A is seen rarely in patients with chronic hepatitis [Orito et al., 2001a; Kobayashi et al., 2002], it is suspected that genotype A in metropolitan areas has a distinct geographic origin. Many patients with genotype A infection in these areas had a history of extramarital sexual contacts with plural unspecified partners. Such sexual behavior may increase the risk of infection with genotype A. In support of this view, most homosexual people in Tokyo who have human immunodeficiency virus type I are coinfecting with HBV genotype A [Koibuchi et al., 2001]. Taken together, homosexual activity would increase the risk of genotype A infection in metropolitan areas. Further molecular analysis on HBV isolates from transmitters and recipients will verify this hypothesis. With respect to genotype B, both Ba, and Bj subtypes [Sugauchi et al., 2002b] were detected. Although the number of studied patients was small, patients with subtype Ba were found in the Tokyo metropolitan area exclusively. Whether subtype Ba intrinsic to the metropolitan area has a peculiar geographic origin is currently unknown and awaits further analyses.

Another point made in this study is that HBV genotypes influence clinical features and the outcome of acute hepatitis B. It has been shown that the proportion of patients who develop chronic HBV infection is close to 10% in European and American countries [Sherlock S, 1997] but rare in Japan [Kobayashi et al., 2002]. Recent studies suggest that chances for evolution into chronicity may differ among patients acutely infected with HBV of distinct genotypes [Mayerat et al., 1999; Ogawa et al., 2002]. Our study has shown that patients with genotype A had higher HBV DNA and lower ALT levels, as well as a longer duration of HBsAg in serum. Development of chronic hepatitis was seen in one of the 27 (4%) patients with genotype A as against one of the 109 (1%)

with genotype C infection. Although the number of patients studied was not large enough for statistical evaluation, the transition to chronic infection may be more frequent in infection with genotype A than the other genotypes, insofar as higher viral loads can predict chronic infection [Fong et al., 1994]. Further studies on more patients are required to evaluate whether or not viral persistence occurs more often after HBV infection with genotype A than the other genotypes.

Patients with fulminant hepatic failure in the present study were infected with either genotypes B or C; no patient with genotype A developed hepatic failure. As mutations at nt 1896 in the precore and nt 1762/1764 in the BCP regions, which are found frequently in patients with fulminant hepatic failure [Carman et al., 1991; Kosaka et al., 1991; Liang et al., 1991; Omata et al., 1991; Hawkins et al., 1994; Sato et al., 1995; Baumert et al., 1996; Chu et al., 1996], were not detected in patients with genotype A, low frequency of fulminant hepatic failure associated with genotype A infection may be attributed to the lack of these mutations. The high frequency of HBeAg in genotype A infection may also be related to low frequency of fulminant hepatic failure. However, interpretation on this data should be made carefully, because the number of patients studied was small. Further research is necessary to determine if the genotype itself affects the clinical course of acute hepatitis B.

In summary, (1) infection with HBV genotype A is common in patients with acute hepatitis in Japan; (2) patients with genotype A are more frequent in metropolitan areas and may be associated with particular sexual behavior; (3) patients with genotype A have a milder but longer course of infection, which may lead to increased risk of progression to chronic disease.

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Quantitative studies on the impaired filterability of erythrocytes from patients with liver cirrhosis

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Liver cirrhosis (LC) is often associated with several hematologic complications, in which anemia is a common feature. Although the exact mechanism of anemia remains controversial, impaired erythrocyte filterability and subsequent hemolysis have been suspected as the causes of anemia associated with LC. We have investigated erythrocyte filterability in relation to the severity of LC. The severity was assessed according to the Child-Pugh classification (grade A, B, and C). The mean erythrocyte filterability of the entire 31 patients with LC ($64.6 \pm 14.5\%$) was significantly ($P < 0.03$) decreased compared with that in 5 normal controls ($80.5 \pm 1.7\%$). Comparing with the filterability in the controls, the filterability in the LC groups in grade A ($70.9 \pm 9.6\%$) was not significantly decreased ($p = 0.053$, $n = 8$), but the filterability in the LC groups in grade B ($65.9 \pm 11.0\%$; $p < 0.01$, $n = 19$) and in grade C ($45.9 \pm 23.8\%$; $p < 0.02$, $n = 4$) were markedly decreased. We found that the impaired filterability reflects the severity of anemia and liver dysfunctions, suggesting that the impairment is one of the causes of anemia. Further, the present study suggested that the impaired filterability arises from the altered erythrocyte membrane properties and a possible mechanical stress due to the accelerated portal hypertension, thus reflecting disorders of the microcirculation.

Key words: Erythrocytes, Filterability, Anemia, Liver cirrhosis, Child-Pugh classification.

1. Introduction

The deformability of erythrocytes is an important rheologic factor for the maintenance of physiological blood flow in the microcirculation. However, the concept of erythrocyte deformability has no strict definition as a physical quantity, and hence the evaluation of the deformability depends on the measurement techniques used and relative sensitivity of the technique to the

various factors that comprise deformability. Since the filtration of erythrocytes through narrow pores is closely related to the physiological flow system in the microcirculation, filtration techniques are most widely used to assess an indistinct quantity called erythrocyte deformability^{1,2}. In this study, the erythrocyte deformability was quantified as the erythrocyte filterability (whole cell deformability) using a newly devel-

oped nickel mesh filtration apparatus³.

Chronic liver diseases such as liver cirrhosis (LC) are often associated with several hematological complications, in which anemia is a common feature. Although the exact mechanism of anemia remains controversial, anemia has been considered to largely arise from gastrointestinal bleeding due to coagulopathy and from metabolic abnormality such as folic acid deficiency⁴. Another possible etiology of anemia in patients with LC is hemolytic anemia associated with hypersplenism^{4,5}. Also, short erythrocyte longevity⁴, abnormal erythrocyte shape changes such as target cells⁶ and spur cells⁷, and a compensatory increase in the circulating erythropoietin^{8,9} have been reported. This line of evidences supports the possibility that hemolysis is associated with LC^{4,9}. Reportedly, the erythrocyte membrane lipid composition is altered and the erythrocyte membrane rigidity is increased in cirrhotic patients^{10,11}. Furthermore, rheological abnormalities of the erythrocytes in cirrhotic patients have been reported by means of a nuclepore filtration technique^{11,12} or ektacytometry¹². However, it is well known that the nuclepore filtration is neither quantitative nor reproducible^{1,2}, and that sensitivity of ektacytometry is lower than that of the nickel mesh filtration technique^{13,14}.

Therefore, the aim of this study is to investigate the filterability of erythrocytes from patients with LC in a quantitative and reproducible manner using a newly developed nickel mesh filtration apparatus, and to clarify the relation between the cell filterability and the severity of LC. This kind of rheologic study will contribute to the elucidation of the mechanism of anemia observed in LC.

2. Materials and Methods

2.1 Subjects

The present study was performed according to the revised Declaration of Helsinki. We enrolled 31 consecutive patients who were diag-

nosed as having LC (20 men and 11 women, mean age of 67.6 ± 7.8 years, ranging from 45 to 85 years) at Kurume University Hospital and affiliated hospitals during the period of February to July, 2003. The severity of LC was clinically assessed according to the Child-Pugh classification^{15,16}, i.e., Child grade A (n=8), B (n=19) and C (n=4). Of 31 patients, 19 had hepatocellular carcinoma (HCC) as a complication of LC, and two had both HCC and pancreas carcinomas. All patients were positive for the hepatitis C virus antibody (HCV-Ab), but negative for the hepatitis B surface antigen (HBs-Ag). None of the patients had a significant concurrent systemic illness, gastrointestinal bleeding, alcohol abuse, or hematologic diseases. Five healthy volunteers (3 men and 2 women, mean age of 27.1 ± 3.6 years, ranging 23 to 31 years) were included in this study as normal controls. Signed informed consent was obtained from all subjects prior to the commencement of the study.

2.2 Blood samples

The preparation of the erythrocytes suspension has been introduced elsewhere in detail^{17,18}. In brief, venous blood was sampled from the antecubital vein of the subjects in a fasting state for blood cell counting, serum chemistry. Blood cell counting was performed by the autoanalyzer (Hitachi model 7600, Hitachi Co. Ltd., Tokyo, Japan), concurrently detecting morphologic abnormalities of erythrocytes. Chemical examinations of serum were carried out using a routine autoanalyzer (Sysmex model K-4500, Sysmex Co. Ltd., Tokyo, Japan). For preparation of the erythrocyte suspension, venous blood was collected into a disposable, evacuated syringe with a 21-gauge needle, using a 1/10 volume of 3.3% trisodium citrate as an anticoagulant. After centrifugation at $1300 \times g$ for 10 minutes, the plasma and buffy coat were carefully removed and replaced with saline solution buffered with N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic

acid (HEPES) sodium salt (HEPES-Na). The composition of HEPES-buffered saline solution (HBS) was 141mM of NaCl and the 10mM of HEPES-Na. The osmolality and pH of the HBS were 287mOsm/kg · H₂O and 7.4, respectively. Erythrocytes were gently washed three times by repeated resuspension with HBS and centrifugation at 800 x g, 600 x g and 500 x g, for 10 minutes. The hematocrit (Ht) value of the erythrocyte suspension was adjusted to 3.0% for the filtration experiment. Residual leukocytes and concomitant platelet counts in the final suspension were less than 100 / μl and 10³/μl, respectively.

2. 3 Nickel mesh filter

Fig. 1 shows an electron microscopic photograph of a nickel mesh filter that was produced in accordance with our specifications by a photofabrication technique (Dainippon Printing Co. Ltd., Tokyo, Japan). We specified that this filter should have an outer diameter of 13 mm, a diameter in filtration area of 8 mm, a thickness of 11 μm, a pore diameter of 3-6 μm, and an inter-pore distance of 35 μm. The vertical and cylindrical pores are regularly distributed across the filter with no pore coincidence, the pore entrances of which show round and smooth transition into the pore inside. The filter used in this

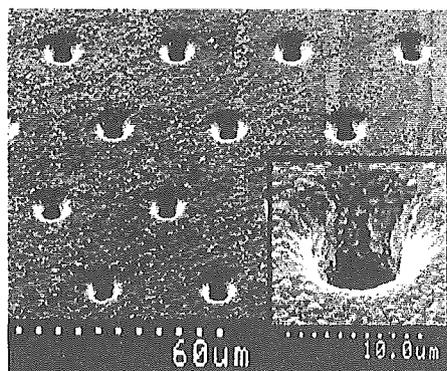


Fig. 1 Electron microscopic photograph of a nickel mesh filter.

study was a nickel mesh filter with a pore diameter of 4.10 μm (Tsukasa Sokken Co. Ltd., Tokyo, Japan).

2. 4 Filtration experiments

Erythrocyte filtration through nickel mesh was performed using a new filtration apparatus³⁾ (Model NOBU-III, Tsukasa Sokken Co. Ltd., Tokyo, Japan). The filtration system consists of a test unit (a vertical glass tube equipped with a nickel mesh at the bottom end), a negative pressure supply unit, and a measurement control and data analysis unit as shown in Fig. 2. The vertical-tube is connected to an air tank or reservoir through electromagnetic valves inside the negative pressure supply unit. An outline of operation of the apparatus follows: the vertical-tube equipped with the nickel mesh is immersed into a sample container filled with a test material. After closing valves 2 and 3 and opening valve 1, a pump evacuates the reservoir until the negative gage pressure reaches around -200mmH₂O. After closing valve 1, by opening valve 2, the test material is sucked into the nickel mesh filter through the action of decreasing negative pressure and rises into the vertical-tube, and then the flow stops at equilibrium. These operations are automatically performed by measurement software installed on a personal computer (PC). The device continually performs sampling measurement of the pressure (P) during a filtration as a time (t) series, performs analog/digital (A/D) conversion, and stores digital data (P-t data) on the PC. Together with the start of the data acquisition process, the software converts the pressure data into the liquid level (height) of the test material, and displays ongoing height and measurement time (h-t) as a graph. When the test has been completed, the measurement software automatically utilizes the h-t data to calculate the quantity of flow per unit of time, and then displays the pressure and flow rate (P-Q) as a

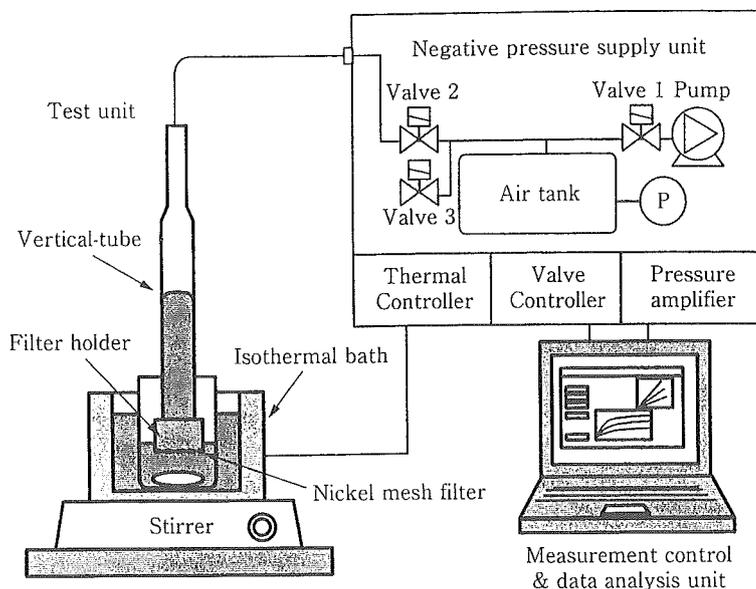


Fig. 2 Schematic illustration of the decreasing, negative pressure-based filtration apparatus³⁾

graph³⁾. Thus, similar to the gravity-based filtration system^{1,2)}, the P-Q relation determines the deformability of the cells. A magnetic stirrer was used to prevent the sedimentation of erythrocytes at 100 rpm (Fig. 2). The filtration experiments were carried out at 36 °C using an isothermal bath (Fig. 2).

2. 5 Statistical analysis

All measurement data are expressed as the mean \pm standard deviation (SD). An unpaired two-tailed Student's t-test was used and a P value less than 0.05 was considered to be statistically significant.

3. Results

3. 1 Clinical characteristics of LC patients

The clinical characteristics of LC patients enrolled in this study are summarized in Table 1 and Table 2. As the clinical severity of LC is advanced from Child-Pugh classification of grade A to grade C, macrocytic anemia and thrombocytopenia were observed (Table 1). Here, the

medical terminology "macrocytic" means that volume index (= MCV value of subject / standard MCV value of 90 μm^3) is larger than 1.1; for example, the volume index of the LC groups in grade C was 1.15. Any discernible erythrocyte shape changes such as acanthocytes and target cells were not observed in these patients (data not shown). In the blood chemistry, values of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin were elevated according to the severity of LC. While values of lecithin cholesterol acyltransferase (LCAT) and total cholesterol decreased according to that. Also, coagulopathy was suspected in the patients by the reduced values of prothrombin time, which was closely parallel to the severity of LC (Table 2).

3. 2 Pressure-flow rate relationships

Fig. 3 shows the representative data of filtration experiments. The filtration system has a data analysis support form installed on the PC, which is an option that allows a graphical display

Table 1 Hematologic examinations of controls and patients with liver cirrhosis

	Control	LC groups in Child A	LC groups in Child B	LC groups in Child C
Hb (g/dl)	13.4±0.8	11.3±1.7*	11.6±1.9*	10.4±1.0**
Ht (%)	39.8±2.9	34.4±4.3*	35.0±5.2	31.0±2.6**
MCV (μm^3)	93.5±3.4	89.9±4.0	96.4±6.0	103.1±7.1*
MCH (pg)	32.2±1.8	29.5±2.3*	31.9±2.0	34.6±2.0
MCHC (%)	34.2±1.3	32.8±1.0*	33.0±1.2	33.6±0.8
Platelet ($10^4/\text{mm}^3$)	19.0±1.5	8.9±3.7***	7.5±6.4***	4.3±1.7***

Hb: hemoglobin, Ht: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, LC: liver cirrhosis. Values are expressed as mean \pm SD. Values of the LC groups were analyzed by Student t-test in comparison with values of normal controls (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$)

Table 2 Serum chemistry in patients with liver cirrhosis

	LC groups in Child A	LC groups in Child B	LC groups in Child C
PT (%)	90±1	70±1	50±0***
AST (IU/l)	46.4±14.8	78.9±40.5*	88.8±46.3*
ALT (IU/l)	28.5±18.8	54.2±27.6**	53.3±25.6
γ GTP (IU/l)	130.5±208.2	93.1±59.6	62.5±31.8
ALP (IU/l)	421.1±247.5	450.6±165.5	455.5±163.4
LDH (IU/l)	218.0±51.4	257.9±62.8	294.3±74.9
T Bilirubin (mg/dl)	0.8±0.3	1.5±0.6**	2.7±1.3**
T Bile Acid ($\mu\text{mol/l}$)	42.1±25.2	72.8±73.8	74.5±49.5
LCAT (UNIT)	52.2±9.1	40.1±12.8*	20.8±6.5**
TC (g/dl)	149.3±14.6	141.8±36.7	109.8±33.8*
CHE (IU/l)	72.1±32.4	58.0±16.3	37.8±15.5
Alb (g/dl)	3.4±0.4	3.2±0.4	3.0±0.2

PT: prothrombin time, AST: aspartate aminotransferase, ALT: alanine aminotransferase, γ GTP: γ -glutamyl transpeptidase, ALP: alkaline Phosphatase, LDH: lactic dehydrogenase, T Bilirubin: total bilirubin, T Bile Acid: Total bile acid, LCAT: lecithin cholesterol acyltransferase, TC: total cholesterol, Alb: albumin, CHE: cholinesterase, LC: liver cirrhosis. Values are expressed as mean \pm SD.

Values of the LC groups in Child B and C were analyzed by Student t-test in Comparison with those in Child A. (*: $p<0.05$, **: $p<0.01$, ***: $P<0.001$)

and representative point sampling of the data acquired during the test to be accessible to Microsoft Excel 2000³⁾; thus, we can easily display a P-Q graph such as that shown in Fig. 3. The P-Q relationships of HBS were linear with the line passing through the origin, indicating the Newtonian behavior of HBS. In contrast, the P-Q relationships of the erythrocyte suspension, the

hematocrit value of which was 3%, displayed smooth curves convex to the abscissa at the low-pressure region, revealing non-Newtonian characteristics of the suspension. Two P-Q relationships of HBS are piled up each other, showing the excellent reproducibility of this filtration experiment. It is evident that the flow rate of the erythrocyte suspension in cirrhotic