Table 2 Clinical status, core promoter (CP) and precore (PreC) region mutations in 129 patients with chronic hepatitis B virus (HBV) infection classified by HBV genotype

HBV genotype	No. tested (%)	Clinical status					CP			PreC		
		No. ASC (%)	No. CH (%)	No. LC/HCC (%)	ASC OR	LC/HCC OR	No. wild (%)	No. mutant (%)	Mutant OR	No. wild (%)	No. mutant (%)	Mutant OR
B	42 (100)	35 (83.3)	7 (16.7)	0 (0)	1	1	39 (92.9)	3 (7.1)	1	5 (11.9)	37 (88.1)	1
C	87 (100)	3 (3.4)*	48 (55.2)*	36 (41.4)*	0.007	∞	21 (24.1)	66 (75.9)*	40.9	29 (33.3)	58 (66.7)**	0.27
Total	129 (100)	38 (29.5)	55 (42.6)	36 (27.9)	-	-	60 (46.5)	69 (53.5)	-	34 (26.4)	95 (73.6)	-

ASC, asymptomatic healthy carrier; CH, chronic hepatitis; HCC, hepatocellular carcinoma; LC, liver cirrhosis; OR, odds ratio. Core promoter wild type, nt.1762 A and nt.1764 G; Core promoter mutant type, nt.1762 T and nt. 1764 A. Precore wild type, nt.1896 G; Precore mutant type, nt.1896 A. *$P < 0.0001$; **$P = 0.0175$ *versus* the genotype B (chi-squares test or Fisher's exact test) compared with genotype B. ASC and LC/HCC OR 95% confidence interval (CI), (0.001–0.028); CP mutant OR 95% CI, (11.4–145.9); PreC mutant OR 95% CI, (0.10–0.76).

more in genotype C than B patients ($P < 0.0001$). The mutation in the PreC region was found significantly more in genotype B than in genotype C patients ($P = 0.0175$). From these findings, the pathogenesis of genotype C might be more associated with the mutation of the CP region than with the PreC region.

Relationships between core promoter and precore mutations and clinical status classified by hepatitis B virus genotype

Table 3 shows the relationships between the various CP/PreC combinations and the clinical status classified by HBV genotype in 129 patients. Among genotype B patients, ASC (35/42, 83.3%) was predominant in the clinical status. The combination of CP wild and PreC mutation (28/35, 80.0%) was found most often in ASC patients with genotype B. Among genotype C patients, CH and LC and/or HCC (84/87, 96.6%) were predominant in the clinical status. The combination of CP mutation and PreC mutation was found most often in the 48 CH and the 36 LC and/or HCC patients with genotype C (20/48, 41.7% and 21/36, 58.3%, respectively). However, there were other various combinations of the wild and mutation in the CP and PreC regions among genotype C patients with CH and LC and/or HCC.

Relationships between core promoter and precore mutations and hepatitis B virus markers (HBV) and alanine aminotransferase abnormality classified by HBV genotype

Table 4 shows the relationship between the various CP/PreC combinations and HBeAg positivity, HBV DNA positivity, and ALT abnormality of 129 patients with chronic HBV infection classified by genotype. No combinations including CP mutation were found in the genotype B patients with positive HBV markers and ALT abnormality. Among genotype C patients, the combination of CP mutation and PreC wild (18/35, 51.4%) were predominant in patients who were HBeAg positive. The combination of CP mutation and PreC mutation was found most often in genotype C patients who were HBV DNA positive and those with ALT abnormality (29/66, 43.9% and 28/62, 45.2%, respectively). However, there were other various combinations of the wild and mutation in the CP and PreC regions among genotype C patients classified by HBV markers and ALT abnormality.

Core promoter and precore mutations and hepatitis B early antigen status classified by hepatitis B virus genotype among patients with alanine aminotransferase abnormality

Table 5 shows the various CP/PreC combinations classified by HBV genotype and HBeAg status

Table 3 Relationship between core promoter (CP)/precore (PreC) status and clinical status in 129 patients with chronic hepatitis B virus (HBV) infection classified by HBV genotype

CP/PreC	Genotype B				Genotype C			
	No. tested (%)	No. ASC (%)	No. CH (%)	No. LC/HCC (%)	No. tested (%)	No. ASC (%)	No. CH (%)	No. LC/HCC (%)
W/W	4 (100)	4 (100)	0 (0)	0 (0)	7 (100)	0 (0)	7 (100)	0 (0)
W/M	35 (100)	28 (80.0)	7 (20)	0 (0)	14 (100)	0 (0)	11 (78.6)	3 (21.4)
M/W	1 (100)	1 (100)	0 (0)	0 (0)	22 (100)	0 (0)	10 (45.5)	12 (54.5)
M/M	2 (100)	2 (100)	0 (0)	0 (0)	44 (100)	3 (6.8)	20 (45.5)	21 (47.7)
Total	42 (100)	35 (83.3)	7 (16.7)	0 (0)	87 (100)	3 (3.4)	48 (55.1)	36 (41.4)

ASC, asymptomatic healthy carrier; CH, chronic hepatitis; HCC, hepatocellular carcinoma; LC, liver cirrhosis; W, wild type; M, mutant type. Core promoter wild type, nt.1762 A and nt.1764 G; CP mutant type, nt.1762 T and nt. 1764 A. Precore wild type, nt.1896 G; PreC mutant type, nt.1897 A.

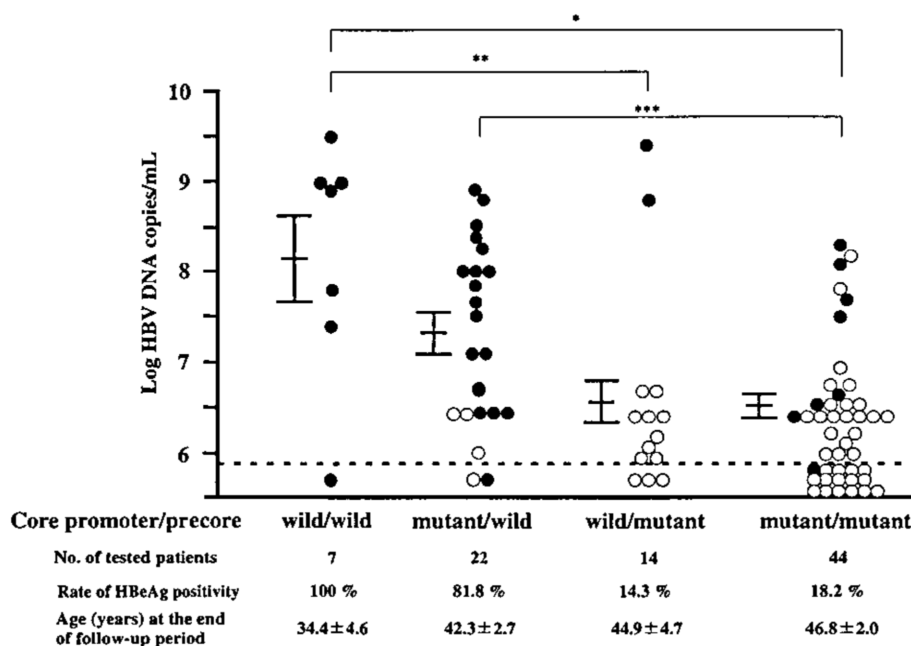

Figure 2 Hepatitis B e antigen (HBeAg) positivity rate and serum hepatitis B virus (HBV) DNA levels in 87 genotype C patients and classified core promoter/precore region status. (○) HBeAg-positive and (●) HBeAg-negative patients. Each value denotes mean ± standard errors of the mean. Statistically significant differences between the groups: $*P < 0.0001$; $**P = 0.0078$; $***P < 0.0001$.

among 67 patients with ALT abnormality. All five genotype B patients with ALT abnormality were HBeAg negative and had the combination of CP wild and PreC mutant. The combination of CP mutation and PreC wild (54.8%) was predominant in 31 genotype C patients with HBeAg positivity and ALT abnormality. The combination of CP mutation and PreC mutant (71.0%) was predominant in 31 genotype C patients who were HBeAg negative. Among genotype C patients, PreC mutation was associated with HBeAg negativity and CP mutation was not associated with HBeAg negativity.

Relationship between hepatitis B early antigen positivity, hepatitis B virus DNA level and core promoter and precore mutations

We investigated the relationship between HBeAg positivity, serum HBV DNA levels and the CP and PreC mutations in 129 patients with chronic HBV infection. Because only three patients were positive for HBV DNA by branched DNA assay out of 42 genotype B patients, we analyzed this issue in 87 genotype C patients (Fig. 2). The HBV DNA level was not significantly

Table 4 Relationships between core promoter (CP) and precore (PreC) mutations, hepatitis B virus (HBV) markers and alanine aminotransferase (ALT) abnormality in 129 patients with chronic HBV infection classified by HBV genotype

	Genotype B				Genotype C			
CP/PreC	No. tested (%)	No. HBeAg+ (%)	No. HBV DNA+ (%)	No. ALT abnormality+ (%)	No. tested (%)	No. HBeAg+ (%)	No. HBV DNA+ (%)	No. ALT abnormality+ (%)
W/W	4 (100)	1 (25.0)	1 (25.0)	0 (0)	7 (100)	7 (100)	6 (85.7)	6 (85.7)
W/M	35 (100)	0 (0)	2 (5.7)	5 (14.3)	14 (100)	2 (14.3)*	11 (78.6)	9 (64.3)
M/W	1 (100)	0 (0)	0 (0)	0 (0)	22 (100)	18 (81.8)**	20 (90.9)	19 (86.4)
M/M	2 (100)	0 (0)	0 (0)	0 (0)	44 (100)	8 (18.2)***,****	29 (65.9)	28 (63.6)
Total	42 (100)	1 (2.3)	3 (7.1)	5 (11.9)	87 (100)	35 (40.2)	66 (75.8)	62 (71.3)

$*P = 0.0003$; $**P < 0.0001$; $***P < 0.0001$; $****P < 0.0001$. ALT abnormality+, number of patients exhibiting positive levels of ALT abnormality; HBeAg+, hepatitis B e antigen.

associated with age of each combination. The HBV DNA levels were 8.2 ± 0.49 Meq/mL in patients with CP wild/PreC wild combination, 7.3 ± 0.21 Meq/mL in patients with mutant/wild combination, 6.6 ± 0.30 Meq/mL in patients with wild/mutant combination, and 6.5 ± 0.16 Meq/mL in patients with mutant/mutant combination. The HBV DNA level was significantly higher in patients with wild/wild combination than in patients with wild/mutant and mutant/mutant combinations ($P = 0.0078$ and $P < 0.0001$, respectively). The HBV DNA level was significantly higher in patients with mutant/wild combination than in patients with mutant/mutant combination ($P < 0.0001$). Also, the HBV DNA level tended to significantly decrease in the absence of HBeAg and the presence of PreC mutation.

DISCUSSION

From recent epidemiological studies about chronic HBV infection, it is a well-known fact that genotype C patients clinically have more serious liver deterioration than genotype B patients.[5,10,11,24,29] There is no clear explanation for the mechanism behind this. The clinical meaning of CP and PreC mutation in HBV infection has been reported.[10,12,16,18,19,22,23,30] Here, we retrospectively investigated CP and PreC mutations in relation to the different HBV genotypic outcomes of Japanese patients with chronic HBV infection.

In accordance with our previous report and those of other investigators,[5,8,9,24,29] we confirmed a higher prevalence of HBeAg positivity, HBV DNA positivity, ALT abnormality, and advanced liver diseases in genotype C patients compared to genotype B patients. The ability of HBeAg secretion and HBV replication was higher in genotype C than in genotype B patients, and this might cause the pathogenesis of genotype C. Our previous studies showed that patients with chronic HBV infection living in Okinawa, where genotype B was predominant, had a higher natural seroconversion rate of HBeAg than those living in Fukuoka, where genotype C was predominant.[5,6] Patients with chronic HBV infection living in Okinawa had the lowest mortality rates of LC and HCC.[31] Both CP and PreC mutation have been reported to be closely associated with the seroconversion of HBeAg and liver deterioration.[10,17–19,21–24] Therefore, it is important to clarify the genotype-related association between PreC and CP mutation and the clinical features.

In the present study, we found that PreC mutation (1896 A) (73.6%) was often observed in both genotypes B and C from Japanese patients with chronic HBV infection in Fukuoka and Okinawa. It is recognized that this PreC mutation increases the stability of the stem-loop structure of the pregenome encapsidation sequence as the opposite nucleotide is 1858T, but that the mutation disrupts a pre-existing C and G pair as the nt 1858 is 1858C.[32] The restriction of 1896 A to specific HBV genotypes accounts for its high prevalence in Asia and the Mediterranean basin (Greece and Italy), where the predominant genotypes B, C and D frequently have T at nt 1858 (1858T), and for its low prevalence in North America and Europe, where the predominant

Table 5 Core promoter (CP) and precore (PreC) mutations among 67 patients with alanine aminotransferase (ALT) abnormality classified by hepatitis B virus (HBV) genotype and hepatitis B e antigen (HBeAg) status

	Genotype B CP/PreC					Genotype C CP/PreC				
	Total	W/W	W/M	M/W	M/M	Total	W/W	W/M	M/W	M/M
HBeAg positive	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	31 (100)	6 (19.4)	2 (6.5)	17 (54.8)	6 (19.4)
HBeAg negative	5 (100)	0 (0)	5 (100)	0 (0)	0 (0)	31 (100)	0 (0)*	7 (22.6)	2 (6.5)**	22 (71.0)**
Total	5 (100)	0 (0)	5 (100)	0 (0)	0 (0)	62 (100)	6 (9.7)	9 (14.5)	19 (30.6)	28 (45.2)

Results are expressed as number (%). *$P = 0.0240$; **$P < 0.0001$ *versus* HBeAg positive group. Core promoter wild type, nt.1762 A and nt.1764 G; Core promoter mutant type, nt.1762 T and nt. 1764 A. Precore wild type, nt.1896 G; Precore mutant type, nt.1897 A. ALT abnormality was defined when an ALT level above 36 IU/L was observed for at least half of each patient's observation period.

genotype A almost always has cytosine (C) at nt1858 (1858C).[33,34] The frequency of our patients' PreC mutation was reflected in the geographical distribution of the HBV genotype.

In the present study, the prevalence of CP mutation (1762T 1764 A) was more often found in genotype C patients than in genotype B patients. The ASC with CP wild/PreC mutant combination was most frequently found in genotype B patients, whereas prevalence of progressive liver disease with the CP mutant/PreC mutant combination was most frequently found in genotype C patients. In genotype C patients, PreC wild type was related to HBeAg secretion, while CP mutation was related to ALT abnormality in HBeAg-positive patients when PreC was wild, and ALT abnormality in HBeAg-negative patients when PreC was mutant. These findings suggest that CP mutation and genotype C might be closely related with clinical features of patients with chronic HBV infection. Our results confirm that CP mutation is reported to be possibly useful as a prognostic marker in genotype C patients.[10]

Taiwanese investigators reported that patients with CP mutation were at increased risk for HCC.[35] The CP plays a central role in HBV replication and morphogenesis[36] and CP mutation is accompanied by a reduced level of HBeAg expression.[37] The HBeAg shares epitopes with hepatitis B core antigen (HBcAg) and this has been postulated to induce immune tolerance against itself, HBcAg or both antigens.[38] The HBcAg may be targeted directly by both the cellular and humoral immune systems,[39] leading to necrosis of hepatocytes and liver damage.[40] This offers one possible mechanism by which CP mutation in genotype C patients may contribute to liver deterioration. Our study retrospectively followed patients with chronic HBV infection and determined CP mutation in the patients in 2000, the last year of the follow-up period. Therefore, our results cannot always determine when the CP mutation developed during the chronic course of infection. A prospective study needs to be carried out to determine the relationship between the disease prognosis and the emergence of CP mutation in patients with chronic HBV infection.

In Taiwanese patients, genotype B was more frequently seen in those with HCC, most of whom were not accompanied by cirrhosis.[29] Conversely, Japanese genotype B patients had good prognosis of chronic HBV infection.[31] There was an interesting report that genotype B isolates having the recombination with genotype C were from most of the non-Japanese carriers in Taiwan, China, Hong Kong, Indonesia, Thailand and Vietnam, but that genotype B strains without the recombination were all observed from carriers in Japan.[41] Even the same genotype had different pathogenesis, possibly because of the genome divergence.

In the natural course of chronic HBV infection, disappearance of HBeAg is usually accompanied by a decrease in HBV replication and remission of liver disease.[2] We reported that HBV genotype C patients continue to show HBV DNA positivity after HBeAg disappearance, but not genotype B patients in the previous study.[5] The present study showed that in genotype B there were many HBeAg negative patients without HBV DNA in combination with PreC mutation, but that in genotype C patients there were many HBeAg negative patients with HBV DNA despite PreC mutation. In fact, the appearance of PreC mutant in our genotype C patients was mainly associated with a reduction in HBeAg and HBV DNA levels, but not so with CP mutation. Taken together, the relationship between HBeAg, PreC mutation and HBV replication may fit in with HBV genotype B, not with genotype C.

In this study, HBV DNA levels tended to be associated with the state of HBeAg and PreC mutation. However, the combination of mutation was not significantly connected with age. In Japan, because it is reported that HBV infection in patients less than 3 years-old causes chronic infection (an immunity tolerant state), age and the duration of infection are linked.[42] We have not determined the relationship between HBV DNA level and mutation with duration of infection.

In conclusion, genotype C might be one of worse prognostic markers in patients with chronic HBV infection because of mutation in the CP region.

REFERENCES

1 Hall AJ, Winter PD, Wright R. Mortality of hepatitis B virus positive blood donors in England and Wales. *Lancet* 1985; **1**: 91–3.

2 Realdi G, Alberti A, Rugge M *et al.* Seroconversion from hepatitis B e antigen to anti-HBe in chronic hepatitis B virus infection. *Gastroenterology* 1980; **79**: 195–9.

3 Bonino F, Rosina F, Rizzetto M *et al.* Chronic hepatitis in HBsAg carriers with serum HBV-DNA and anti-HBe. *Gastroenterology* 1986; **90**: 1268–73.

4 Hadziyannis SJ, Lieberman HM, Karvountzis GG, Shafritz DA. Analysis of liver disease, nuclear HBcAg, viral replication and hepatitis B virus DNA in liver and serum of HBeAg vs anti-HBe positive carriers of hepatitis B virus. *Hepatology* 1983; **3**: 656–62.

5 Furusyo N, Nakahima H, Kashiwagi K *et al.* Clinical outcomes of hepatitis B virus (HBV) genotypes B and C in Japanese patients with chronic HBV infection. *Am. J. Trop. Medical Hyg.* 2002; **67**: 151–7.

6 Noguchi A, Hayashi J, Nakashima K, Hirata M, Ikematsu H, Kashiwagi S. HBsAg subtypes among HBsAg carriers in Okinawa, Japan. Evidence of an important relationship in seroconversion from HBeAg to anti-HBe. *J. Infect.* 1994; **28**: 141–50.

7 Kashiwagi S, Hayashi J, Nomura H *et al.* Age-specific prevalence of hepatitis B e antigen (HBeAg) and antibody to HBeAg (anti-HBe) among asymptomatic hepatitis B surface antigen (HBsAg) carriers in Okinawa and Kyushu, Japan. *Microbiol. Immunol.* 1986; **30**: 675–82.

8 Okamoto H, Tsuda F, Sakugawa H *et al.* Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J. General Virol.* 1988; **69**: 2575–83.

9 Norder H, Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology* 1994; **198**: 489–503.

10 Lindh M, Hannoun C, Dhillon AP, Norkrans G, Horal P. Core promoter mutations and genotypes in relation to viral replication and liver damage in east Asian hepatitis B virus carriers. *J. Infect. Dis.* 1999; **179**: 775–82.

11 Orito E, Mizokami M, Sakugawa H *et al.* A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. *Hepatology* 2001; **33**: 218–23.

12 Li J-S, Tong S-P, Wen Y-M, Virvitski L, Zhang Q, Trepo C. Hepatitis B virus genotype A rarely circulates as an HBe-minus mutant: possible contribution of a single nucleotide in the precore region. *J. Virol.* 1993; **67**: 5402–10.

13 Ding X, Mizokami M, Yao G *et al.* Hepatitis B distribution among chronic hepatitis B virus carriers in Shanghai, China. *Intervirology* 2001; **44**: 43–7.

14 Theamboonlers A, Tangkijvanich P, Pramoolsinsap C, Poovorawan Y. Genotypes and subtypes of hepatitis B virus in Thailand. *Southeast Asian J. Trop. Med. Public Health* 1998; **29**: 786–91.

15 Summers J, Mason WS. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. *Cell* 1982; **29**: 403–15.

16 Okamoto H, Tsuda F, Akahane Y *et al.* Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. *J. Virol.* 1994; **68**: 8102–10.

17 Horikita M, Itoh S, Yamamoto K, Shibayama T, Tsuda F, Okamoto H. Differences in the entire nucleotide sequence between hepatitis B virus genomes from carriers positive for antibody to hepatitis B e antigen with and without active disease. *J. Med. Virol.* 1994; **44**: 96–103.

18 Laskus T, Rakela J, Nowicki MJ, Persing DH. Hepatitis B virus core promoter sequence analysis in fulminant and chronic hepatitis B. *Gastroenterology* 1995; **109**: 1618–23.

19 Takahashi K, Aoyama K, Ohno N *et al.* The precore/core promoter mutant (T1762A1764) of hepatitis B virus: clinical significance and an easy method for detection. *J. General Virol.* 1995; **76**: 3159–64.

20 Bruss V, Gerlich WH. Formation of transmembraneous hepatitis B e-antigen by cotranslational in vitro processing of the viral precore protein. *Virology* 1988; **63**: 268–75.

21 Carman WF, Jacyna MR, Hadziyannis S, McGarvey M, Karayiannis P, Thomas HC. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet* 1989; **2**: 588–91.

22 Okamoto H, Yotsumoto S, Akahane Y *et al.* Hepatitis B virus with precore region defects pre-viral in persistently infected hosts along with seroconversion to the antibody against e antigen. *J. Virol.* 1990; **64**: 1298–303.

23 Honda A, Yokosuka O, Ehata T, Tagawa M, Imazeki F, Saisho H. Detection of mutations in the enhancer 2/core promoter region of hepatitis B virus in patients with chronic hepatitis B virus infection: comparison with mutations in precore and core regions in relation to clinical status. *J. Med. Virol.* 1999; **57**: 337–44.

24 Kobayashi M, Arase Y, Ikeda K *et al.* Clinical characteristics of patients infected with hepatitis B virus genotypes A, B, and C. *J. Gastroenterol.* 2002; **37**: 35–9.

25 Furusyo N, Hayashi J, Sawayama Y, Kishihara Y, Kashiwagi S. Hepatitis B surface antigen disappearance and hepatitis B surface antigen subtype: a prospective, long-term, follow-up study of Japanese residents of Okinawa, Japan with chronic hepatitis B virus infection. *Am. J. Trop. Med. Hyg.* 1999; **60**: 616–22.

26 Usuda S, Okamoto H, Iwanari H *et al.* Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. *J. Virol. Methods* 1999; **80**: 97–112.

27 Usuda S, Okamoto H, Tanaka T *et al.* Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. *J. Virol. Methods* 2000; **87**: 81–9.

28 Kato H, Orito E, Sugauchi F *et al.* Determination of hepatitis B virus genotype G by polymerase chain reaction with hemi-nested primers. *J. Virol. Methods* 2001; **98**: 153–9.

29 Kao JH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. *Gastroenterology* 2000; **118**: 554–9.

30 Gunther S, Piwon N, Iwanska A, Schilling R, Meisel H, Will H. Type, prevalence, and significance of core promoter/enhancer II mutations in hepatitis B viruses from immunosuppressed patients with severe liver disease. *J. Virol.* 1996; **70**: 8318–31.

31 Sakugawa H, Nakasone H, Nakayoshi T *et al.* Preponderance of hepatitis B virus genotype B contributes to a better prognosis of chronic HBV infection in Okinawa, Japan. *J. Med. Virol.* 2002; **67**: 484–9.

32 Lok ASF, Akarca U. Mutations in the pre core region of hepatitis B virus serve to enhance the stability of the sec-

ondary structure of the pre-genome encapsidation signal. *Proc. Natl Acad. USA* 1994; **91**: 4077–81.

33 Rodriguez-Frias F, Buti M, Jardi R *et al.* Hepatitis B virus infection: precore mutants and its relation to viral genotypes and core mutations. *Hepatology* 1995; **22**: 1641–7.

34 Lindh M, Andersson AS, Gusdal A. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus – large-scale analysis using a new genotyping method. *J. Infect. Dis.* 1997; **175**: 1285–93.

35 Kao JH, Chen PJ, Lai MY, Chen DS. Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. *Gastroenterology* 2003; **124**: 327–34.

36 Summers J, Mason WS. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. *Cell* 1982; **29**: 403–15.

37 Okamoto H, Tsuda F, Akahane Y *et al.* Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. *J. Virol.* 1994; **68**: 8102–10.

38 Carman W, Thomas H, Domingo E. Viral genetic variation: hepatitis B virus as a clinical example. *Lancet* 1993; **341**: 349–53.

39 Milich DR, McLachlan A, Stahl S *et al.* Comparative immunogenicity of hepatitis B virus core and E antigens. *J. Immunol.* 1988; **141**: 3617–24.

40 Brunetto MR, Giarin MM, Oliveri F *et al.* Wild-type and e antigen-minus hepatitis B viruses and course of chronic hepatitis. *Proc. Natl Acad. Sci. USA* 1991; **88**: 4186–90.

41 Sugauchi F, Orito E, Ichida T *et al.* Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. *J. Virol.* 2002; **76**: 5985–92.

42 Kashiwagi S, Hayashi J, Ikematsu H *et al.* Transmission of hepatitis B virus among siblings. *Am. J. Epidemiol.* 1984; **120**: 617–25.

Am. J. Trop. Med. Hyg., 70(5), 2004, pp. 571–575
Copyright © 2004 by The American Society of Tropical Medicine and Hygiene

RELATIONSHIP OF GENOTYPE RATHER THAN RACE TO HEPATITIS B VIRUS PATHOGENICITY: A STUDY OF JAPANESE AND SOLOMON ISLANDERS

NORIHIRO FURUSYO, NORIHIKO KUBO, HISASHI NAKASHIMA, KENICHIRO KASHIWAGI, AND JUN HAYASHI
Department of General Medicine, Kyushu University Hospital, Fukuoka, Japan; Department of Environmental Medicine and Infectious Disease, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan

Abstract. The aim of this study was to determine the predominant hepatitis B virus (HBV) genotype in the Solomon Islands and determine if there is any racial correlation between genotype and hepatitis B e antigen (HBeAg) production in Japanese and Melanesian individuals. A total of 403 serum samples from 206 Melanesian HBV carriers in the Solomon Islands and 197 Japanese carriers from Fukuoka (n = 106) and Okinawa (n = 91) living in Japan in 2001 were tested. The HBV genotypes of 206 Melanesian subjects were 114 with genotype C (55.3%) and 92 with genotype D (44.7%). The HBV genotypes of 197 Japanese subjects were 74 with genotype B (37.6%) and 123 with genotype C (62.4%). The total HBeAg prevalence of subjects in Fukuoka (36.8%) was significantly higher than that of subjects in Okinawa (14.3%) ($P < 0.0001$) and subjects in the Solomon Islands (35.0%; $P = 0.0014$, by the Mantel-Haenszel test). The genotype C prevalences were significantly different, ranging from 24.2% in Okinawa, to 54.4% in the Solomon Islands, to 95.3% in Fukuoka (all $P < 0.0001$, by chi-square test). The prevalence of HBeAg positivity was significantly higher in Melanesian genotype C subjects (42.0%) than Melanesian genotype D subjects (26.6%) ($P = 0.0310$). Similarly, the prevalence of HBeAg positivity was significantly higher in Japanese genotype C subjects (36.6%) than Japanese genotype B subjects (9.5%) ($P < 0.0001$). These findings indicate that that HBV was of genotypes C and D in the Solomon Islands, and that the pathogenesis of HBV-infected patients is related to HBV genotype rather than race.

INTRODUCTION

Hepatitis B virus (HBV) infection is a global public health problem. More than 300 million HBV carriers are estimated worldwide, of whom one million die annually from HBV-related liver disease.[1] Asia and the southwestern Pacific area, including Japan and the Solomon Islands, are reported to be hyperendemic for HBV infection.[2–4]

This virus has long been characterized into different antigenic subtypes and more recently into nucleotide divergence-based genotypes. Traditionally, HBV has been classified into four subtypes (adr, adw, ayr, and ayw) based on antigenic determinants of the hepatitis B surface antigen (HBsAg). The HBsAg subtypes show distinct geographic distribution.[5] Early studies reported HBsAg subtype-related clinical differences in HBV infection.[6–8] Recently, in place of the HBsAg subtype classification, HBV genotype has often been used in analysis of the clinical features of HBV infection.[8–11] After the start of phylogenetic analyses based on intergroup divergence of 8% or more over the complete HBV nucleotide sequence, seven different genotypes, arbitrarily designed A-G, have been recognized.[9–11] Several reports have shown geographic distribution of the genotypes, with genotypes A and D predominant in western Europe, B and C in the Far East, and F in South America.[9–13] Moreover, genotype C has been reported to cause more severe liver damage and to have lower rates of hepatitis B e antigen (HBeAg) clearance, which usually indicates cessation of HBV replication and represents a later stage of chronic HBV infection, than genotype B in Japanese and Chinese patients.[14–16] Reports from Spain and France found the prevalence of genotype A to be significantly higher in HBeAg-positive patients, with genotype D more prevalent in patients positive for antibody to HBeAg (anti-HBe).[17,18] We previously demonstrated that the rates and ages of seroconversion from HBeAg to anti-HBe were higher and younger in patients with HBsAg subtype adw (typically genotype B) than in those with adr (typically genotype C) in Japanese patients with chronic HBV infection, suggesting that the HBeAg secretion and HBV replication were higher in genotype C than in genotype B, and that this may be the cause of the more severe pathogenesis of genotype C than genotype B.[7,8,16,19]

We have also reported HBV infection to be highly endemic in the Solomon Islands, which are located in the southwestern Pacific Ocean, probably due to high prevalence of HBeAg.[4] The HBsAg subtype adr has been reported as being predominant in Melanesians, who account for approximately 90% of the original islander population.[4,20] Although there is increasing evidence of clinical differences among HBV genotypes, most studies have compared genotype A with D or genotype B with C. No studies have compared the same genotype in different areas due to the geographic pattern of genotype distribution. The distribution of HBV genotypes has been closely related to ethnic background. However, there is a question as to which factor, viral genotype or ethnicity of the host, plays a more important role in the determination of the clinical course of HBV carriers.

Because no data were available to document the HBV genotype distribution in the Solomon Islands and no reports were found comparing the pathogenesis of the same HBV genotype in different areas, the present study was done to determine the predominant HBV genotype in the Solomon Islands, and to determine if there is any racial correlation between genotype and HBeAg production in Japanese and Melanesian individuals.

MATERIALS AND METHODS

Serum samples. A total of 403 serum samples from 206 Melanesian and 197 Japanese HBV carriers who were without serologic markers of infection with hepatitis C virus or human immunodeficiency virus type 1 in 2001 were tested. The samples were from 110 male and 96 female healthy Melanesian volunteer blood donors in the Solomon Islands (age range = 16–43 years, mean ± SD = 26.0 ± 6.7 years) and 115 Japanese males (60 in Fukuoka and 55 in Okinawa 55) and 82 females (46 in Fukuoka and 36 in Okinawa 36), all residents of Fukuoka and Okinawa Prefectures, Japan (106 subjects, age range = 22–66 years, mean ± SD = 39.5 ± 13.2

years in Fukuoka and 91 subjects, age range = 10–62 years, mean ± SD = 32.5 ± 15.5 years in Okinawa). All were born and raised in these areas and voluntarily participated in the present study in response to our announcement of free health examinations (Table 1). The study protocol was reviewed and approved by the Ethics Committee of Kyushu University, and informed consent to participate in the study was obtained from all adult participants and from the patients or legal guardians of minors.

The Solomon Islands carrier group includes 206 islanders of Melanesian ancestry from Guadalcanal Island, which is principal one in the Solomon Islands. The Solomon Islands, located in the tropical southwestern Pacific Ocean (5–12°S, 156–170°E), are part of the great island chain that extends eastward from southeast Asia north of Australia to New Caledonia and are comprised of seven provinces made of the principal islands and islands groups. Ethnographic and demographic data were determined by interviews conducted in the native language or in Melanesian pidgin, as described previously.[4]

The Japanese carrier group includes 106 participants from Fukuoka Prefecture and 91 from the Yaeyama District of Okinawa Prefecture. Fukuoka Prefecture is located in the northern part of Kyushu Island, the most southern of the main islands of Japan.[16] Okinawa is located in the subtropical zone approximately 1,000 km south of the main islands of Japan, which lie in the temperate zone. The Yaeyama District, with nine inhabited islands, is situated in the southwest part of Okinawa and is close to Taiwan.[8,16,19,21]

Serologic assays. All serum samples were separated and stored at -20°C until testing for HBsAg, HBeAg, HBsAg subtype, and HBV genotype. The HBsAg subtype was determined by enzyme immunoassay (EIA) using monoclonal antibodies (HBsAg Subtype EIA; Institute of Immunology, Tokyo, Japan). The presence of HBeAg was determined by a radioimmunoassay (RIA) (HBeAg RIA; Abbott Laboratories, North Chicago, IL).[8]

Genotyping of HBV. The HBV genotypes were determined by an enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies (mAb). The HBV genome contains many important promoter and signal regions necessary for viral replication. The four open reading frames for transcription are controlled by four promoter elements (preS1, preS2, core, and X) and two enhancer elements (Enh I and Enh II).The mAbs were raised against genotype-specific epitopes in the preS2-region product of HBV and labeled with horseradish peroxidase using commercial kits (HBV Genotype EIA, Institute of Immunology, Tokyo, Japan).[22,23]

TABLE 1
HBV carriers from the Solomon Islands and Japan by age*

Age group (years)	Melanesians (Solomon Islands) No. tested	Japanese (Fukuoka) No. tested	Japanese (Okinawa) No. tested
10–19	42	0	6
20–29	99	16	12
30–39	56	24	23
40–49	9	28	22
50–59	0	29	20
60	0	9	8
Total	206	106	91

* HBV = hepatitis B virus.

Briefly, HBsAg in sera was captured in wells of a microtiter plate coated with mAbs 3207 and 5124A, both directed to the common determinant epitope a, and tested for binding with genotype-specific monoclonal antibodies labeled with horseradish peroxidase (mAb 5520, epitope b; T2741, m; K0610A, k; 4408, s; 3465, u; 5142A, f; and 5156, g) (Figure 1). From the amino acid sequence found by reaction with mAbs, genotypes A to F were determined: b, s, and u for genotype A; b and m for B; b, k, and s for C; b, k, s, and u for D, b, k, s, u, f, and g for E; and b, k, and f for F. Genotype G was determined by the combination of the above preS2-based ELISA genotype kits for genotype D and HBsAg subtype adw.[24]

Statistical analysis. Statistical analysis was done with BMDP statistical software for the IBM (Yorktown Heights, NY) 3090 system computer (BMDP Statistical Software, Inc., Saugus, MA). Continuous data were expressed as the mean ± SD. An unpaired *t*-test and the Mann-Whitney U test were used to compare the means of samples between the two groups. The chi-square test or Fisher's exact test was used for categorical variables for comparisons between the two groups. Because there were significant differences in age between the subjects of the studied areas, we used the Mantel-Haenszel test for differences in the HBeAg prevalences of the HBV carriers from these different areas. A P value < 0.05 was considered statistically significant.

RESULTS

Age-specific HBeAg prevalences by geographic region. The age-specific prevalence of HBeAg of all 403 studied individuals is shown in Figure 2. The HBeAg prevalences by age in the Solomon Islands subjects were 10–19 years old, 45.2% (19 of 42); 20–29 years old, 38.4% (38 of 99); 30–39 years old, 23.2% (13 of 56); and 40–49 years old, 22.2% (2 of 9). The Okinawa prevalences were 10–19 years old, 66.7% (4 of 6); 20–29 years old, 23.5% (3 of 12); 30–39 years old, 13.0% (3 of 23); 40–49 years old, 9.1% (2 of 22); 50–59 years old, 5.0% (1 of 20); and ≥ 60 years old, 0% (0 of 8). The Fukuoka prevalences were 20–29 years old, 62.5% (10 of 16); 30–39 years old, 58.3% (14 of 24); 40–49 years old, 32.1% (9 of 28); 50–59 years old, 20.7% (6 of 29); and ≥ 60 years old, 0% (0 of 9). The HBeAg prevalences decreased with age in each region. Because the mean ± SD ages were different in the Solomon Islands (26.0 ± 6.7 years), Fukuoka (39.5 ± 13.2 years), and Okinawa (32.5 ± 15.5 years), we used the Mantel-Haenszel test to analyze the differences of the total HBeAg

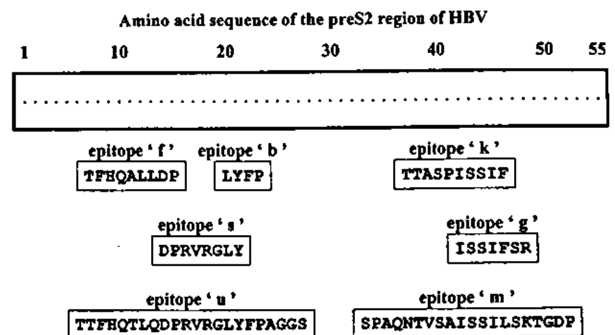

FIGURE 1. Relationship between genotype-determined epitopes and the amino acid sequence of the hepatitis B virus (HBV) preS2 region product.

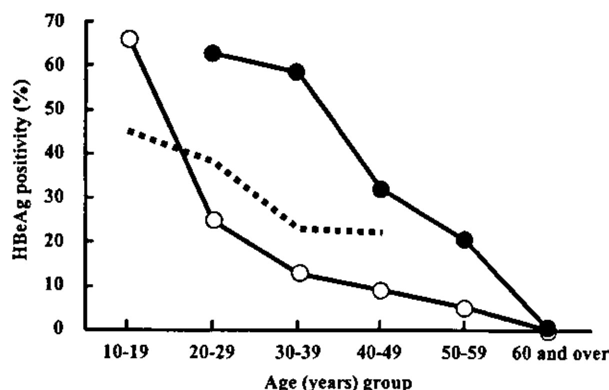

FIGURE 2. Age-specific prevalence of hepatitis B e antigen (HBeAg) in carriers of hepatitis B virus (HBV) classified by region in the Solomon Islands and Japan (Fukuoka and Okinawa). Dashed line = HBV carriers in the Solomon Islands; solid line with closed circles = HBV carriers in Fukuoka; solid line with open circles = HBV carriers in Okinawa.

prevalence of these areas. The total HBeAg prevalence of Fukuoka subjects (39 of 106, 36.8%) was significantly higher than that of Okinawa subjects (13 of 91, 14.3%) ($P < 0.0001$) and the Solomon Islands subjects (72 of 206, 35.0%) ($P = 0.0014$). There was no statistically significant difference in prevalence between Okinawa and the Solomon Islands ($P = 0.3925$). No significant difference was found in sex-specific prevalence of HBeAg in each region.

Genotype distribution of HBV. The distribution of HBV genotype in the Solomon Islands and Japan is shown in Table 2. The HBV genotypes of 206 Melanesian subjects from the Solomon Islands were 114 with genotype C (55.3%) and 92 with genotype D (44.7%). No other genotypes were found in our Melanesian patients. The HBV genotypes of 197 Japanese subjects were 74 with genotype B (37.6%) and 123 with genotype C (62.4%): 5 with genotype B (4.7%) and 101 with genotype C (95.3%) in 106 Fukuoka subjects and 69 with genotype B (75.8%) and 22 with genotype C (24.2%) in 91 Okinawa subjects. The vast majority were genotype B in Okinawa and genotype C in Fukuoka. No other genotypes were found in our Japanese subjects. The genotype C prevalences were significantly different, ranging from 24.2% in Okinawa, to 54.4% in the Solomon Islands, to 95.3% in Fukuoka (Okinawa versus Solomon Islands, Solomon Islands versus Fukuoka, Okinawa versus Fukuoka; all $P < 0.0001$, by chi-square test), which reflected the HBeAg prevalence differences by region.

Differences between Japanese patients in Okinawa and Fukuoka by HBV genotype. Among 91 Okinawa subjects, genotype B subjects had a significantly lower rate of HBeAg

TABLE 2
HBV genotype distribution of HBV carriers classified by region*

	Okinawa Japanese (n = 91)	Solomon Islands Melanesian (n = 206)	Fukuoka Japanese (n = 106)
Genotype, no. (%)			
B	69 (75.8)	0	5 (4.7)
C	22 (24.2)†‡	114 (55.3)†§	101 (95.3)‡§
D	0	92 (44.7)	0

* HBV = hepatatis B virus.
†‡§ $P < 0.0001$, by chi-square test.

positivity (7 of 69, 10.1%) than genotype C subjects (7 of 22, 31.7%) ($P = 0.0359$). The 106 Fukuoka subjects had similar characteristics to those of Okinawa; HBeAg positivity was 0% (0 of 5) in those with genotype B versus 37.6% (38 of 101) in those with genotype C. No significant difference was observed in male-to-female ratio or mean age between the genotype-classified groups between Okinawa and Fukuoka. These findings showed similar clinical outcome for subjects with the same HBV genotype in Okinawa and Fukuoka.

Prevalence of HBeAg classified by HBV genotype. The characteristics of all 403 subjects with HBV infection are shown in Table 3. As reported earlier in this study, the clinical state in Japanese subjects with chronic HBV infection depended mainly on HBV genotype, not on any geographic difference. Therefore, we pooled the two Japanese populations (Okinawa and Fukuoka) in Table 3. No significant difference was observed in male-to-female ratio or mean age between the genotype-classified groups of Japanese or in Melanesians. The major HBsAg subtype was adw (63.5%) in the Japanese genotype B group, adr (80.5% and 81.3%) in the Japanese and Melanesian genotype C groups, and ayw (91.5%) in Melanesian genotype D group. Several HBsAg subtypes and an undetermined subtype were found in each genotype group in both the Japanese and Melanesians individuals. The prevalence of HBeAg positivity was significantly higher in Melanesian genotype C subjects (42.0%) than in Melanesian genotype D subjects (26.6%) ($P = 0.0310$, by chi-square test). Similarly, the prevalence of HBeAg positivity was significantly higher in Japanese genotype C subjects (36.6%) than in Japanese genotype B subjects (9.5%) ($P < 0.0001$, by chi-square test).

Differences in the preS2 epitope reaction in the HBV genotype C between subjects in the Solomon Islands and Japan. As mentioned in the Materials and Methods, the HBV genotype is determined from the pattern of reaction with mAb (epitope) in the preS2 region. The pattern of preS2 epitope reaction in genotype C subjects in the Solomon Islands and Japan is shown in Table 4. The distribution of preS2 epitope pattern was 13 bks (11.6%), 25 bks-fg (22.3%), 73 bks-f (65.2%), and 1 bks-g (0.9%) in the Solomon Islanders with genotype C, and 5 bks (4.1%), 109 bks-fg (88.6%), 8 bks-f (6.5%), and 1 bks-g (0.9%) in Japanese with genotype C. The epitope pattern differed between Melanesians and Japanese infected with genotype C. The epitope reaction for bks-f was found most frequently in the Solomon Islands subjects, whereas the bks-fg epitope combination was found most frequently in Japanese subjects. When classified by the combination of preS2 epitope, the prevalences of HBeAg were 7.7% (1 of 13) in bks, 56.0% (14 of 25) in bks-fg, 42.5% (31 of 73) in bks-f, and 0% (0 of 1) in bks-g among the Solomon Islands subjects, and 20.0% (1 of 5) in bks, 38.6% (42 of 109) in bks-fg, 37.5% (3 of 8) in bks-f, and 0% (0 of 1) in bks-g among the Japanese subjects. There was no significant difference in HBeAg prevalence by preS2 epitope reaction between the Solomon Islands and Japan.

DISCUSSION

We previously reported that HBV infection was highly endemic in the Solomon Islands population, the major HBsAg subtype was adr, and that HBeAg positivity rate was markedly high, suggesting a correlation between HBV endemicity

TABLE 3
Characteristics of 206 Melanesians and 197 Japanese infected with HBV and classified by HBV genotype*

Characteristics	Melanesian genotype		Japanese genotype	
	C (n = 112)	D (n = 94)	B (n = 74)	C (n = 123)
Male, no. (%)	64 (57.1)	45 (47.9)	42 (56.8)	73 (59.5)
Age (mean ± SD years)	27.7 ± 7.1	24.9 ± 6.2	33.4 ± 14.7	35.9 ± 13.5
HBsAg subtype, no. (%)				
adr	91 (81.3)	0	0	99 (80.5)
adw	0	0	47 (63.5)	0
adwr	3 (2.7)	0	0	8 (6.5)
adyr	3 (2.7)	0	0	1 (0.8)
aydw	0	1 (1.1)	0	0
ayr	5 (4.5)	0	0	0
ayrw	1 (0.9)	0	0	0
ayw	0	86 (91.5)	0	0
aywr	0	4 (4.3)	0	0
Undetermined	9 (8.0)	3 (3.2)	27 (36.5)	15 (12.5)
HBeAg positivity no. (%)	47 (42.0)†	25 (26.6)†	7 (9.5)‡	45 (36.8)‡

* HBV = hepatitis B virus; HBsAg = hepatitis B surface antigen; HBeAg = hepatitis B e antigen.
† $P = 0.0310$, by chi-square test.
‡ $P < 0.0001$, by chi-square test.

and HBsAg subtype.[4] Although HBsAg subtypes adw and adr are generally associated with HBV genotypes B and C, respectively, the subtypes do not necessarily parallel the genotypes.[16] In addition, some cases of undetermined subtype found in HBV infection.[16] In Asian patients, including Japanese, genotype C is more likely to result in HBeAg positivity and severe liver damage than genotype B.[14–16] Genotypes C and D were found in the Solomon Islands. No racial differences in the pathogenesis of genotype C were found in a comparison of the Melanesian and Japanese subjects in the present study.

Due to the geographic distribution pattern, HBV genotypes B and C are commonly observed in south Asia and the Far East.[25,26] These two genotypes are found in Japanese HBV-infected patients, with genotype D found infrequently in Japan.[16] The distribution of HBV genotypes in the southwestern Pacific Ocean countries is unknown. The present study showed that genotypes C and D were observed in the Solomon Islanders, although this was expected. Data of our previous report showed the major HBsAg subtypes to be adr and ayw.[4] The population of the Solomon Islands is a mixture of ethnic groups: Melanesian, Polynesian, and Micronesian. Our recent survey showed that a majority (92.9%) of the population was Melanesian, and that a majority (94.9%) of subjects infected with HBV were also Melanesians.[4] Therefore, we believe that the data acquired in the present study, in which the subjects studied were Melanesians only, is representative of HBV infection state in the Solomon Islands.

In the present study, the Solomon Islanders and Japanese with HBV genotype C had a higher prevalence of HBeAg than the Solomon Islanders with genotype D and Japanese with genotype B, respectively. Although several reports have shown a higher HBeAg prevalence in genotype C than in genotype B,[14–16] no reports were found comparing the prevalences of genotypes C and D. The present study also showed that the HBeAg prevalence of Japanese subjects in Fukuoka (36.8%) was significantly higher than of Okinawa Japanese subjects (14.3%) and the Solomon Islands Melanesian subjects (35.0%). This clearly shows that the geographic differences found in previous studies were based on the different prevalences of HBV genotype C in HBV infection: Fukuoka (95.3%), Okinawa (24.2%), and Solomon Islands (54.4%). Our data indicate that the differences were only geographic, not racial. Because the present study was cross-sectional and our studied samples were convenience samples of the Solomon Islanders and Japanese, we can not thoroughly address the validity of conclusions that can be drawn from such samples. However, to the best of our knowledge, there have been no reports, such as ours, regarding the investigation of the relationship between HBV genotype and HBeAg prevalences comparing such different areas and races.

TABLE 4
Differences in the preS2 epitope combination in HBV genotype C between subjects in the Solomon Islands and Japan*

PreS2 epitope combination	Solomon Islands Genotype C (n = 112)	Japanese Genotype C (n = 123)
bks	13 (11.6)	5 (4.1)
bks-fg	25 (22.3)	109 (88.6)
bks-f	73 (65.2)	8 (6.5)
bks-g	1 (0.9)	1 (0.9)

* HBV = hepatitis B virus.

Recently, data has been accumulating regarding the characterization of specific viral variants and their clinical significance. For example, Sugauchi and others[27,28] reported two subgroups of the same HBV genotype B, one of which possessed the recombination with genotype C over the precore region plus core gene and was more prevalent in HBeAg, and the other that did not. The former was found in non-Japanese Asians, and the latter only in Japanese individuals. In the present study, HBV genotyping was done with a serologic ELISA with mAbs against seven genotypic epitopes in the preS2 gene product of HBsAg. Genotypes deduced from serotypes, determined by the assay, are consistent with genotypes defined by nucleotide sequences.[25,26] We found differences of preS2 epitope reaction in genotype C between subjects in the Solomon Islands and Japan, suggesting the presence of nucleotide sequence differences. Parts of the preS region contribute the most variable part of the HBV genome.[29] This region is important for virus attachment and cell entry.[29] To determine the correlations between race and genome differences, further study is needed to investigate dif-

ferences in clinical features based on large numbers of patients infected with some subgroups of genotype C.

The most important findings of the present study were that HBV was of genotypes C and D in the Solomon Islands, and that the pathogenesis of HBV-infected patients is related to HBV genotype rather than race.

Received June 4, 2003. Accepted for publication October 12, 2003.

Acknowledgments: We thank Sadakazu Usuda and Shunji Mishiro (Department of Medical Sciences, Toshiba General Hospital, Tokyo, Japan) and Ricky Eddie (Pathology Division, Central Hospital, Honiara, Solomon Islands) for their constant interest in and support of this work. We also thank Naoko Kinukawa for providing advice on the statistical assessments.

Authors' addresses: Norihiro Furusyo and Jun Hayashi, Department of General Medicine, Kyushu University Hospital, Higashi-Ku, Fukuoka, 812-8582, Japan. Norihiko Kubo, Hisashi Nakashima, and, Kenichiro Kashiwagi, Department of Environmental Medicine and Infectious Diseases, Faculty of Medical Sciences, Kyushu University, Higashi-Ku, Fukuoka, 812-8582, Japan.

Reprint requests: Norihiro Furusyo, Department of General Medicine, Kyushu University Hospital, Higashi-Ku, Fukuoka, 812-8582, Japan, Telephone: 81-92-642-5909, Fax: 81-92-642-5916, E-mail: furusyo@genmedpr.med.kyushu-u.ac.jp.

REFERENCES

1. Maynard JE, 1990. Hepatitis B: global importance and need for control. *Vaccine 8 (suppl):* S18–S20.
2. Lai CL, 1997. Chronic hepatitis B in Hong Kong: immunization strategies for the control of hepatitis B virus infection. Zuckerman AJ, ed. *Hepatitis B in the Asian-Pacific Region. Screening, Diagnosis and Control.* Volume 1. London: Royal College of Physicians of London, 79–87.
3. Gust ID, 1996. Epidemiology of hepatitis B infection in the western Pacific and south east Asia. *Gut 38 (suppl 2):* S18–S23.
4. Furusyo N, Hayashi J, Kakuda K, Sawayama Y, Ariyama I, Eddie R, Kashiwagi S, 1999. Markedly high seroprevalence of hepatitis B virus infection in comparison to hepatitis C virus and human T lymphotropic virus type-1 infections in selected Solomon Islands populations. *Am J Trop Med Hyg 61:* 85–91.
5. Le Bouvier GL, McCollum RW, Hierholzer WJ Jr, Irwin GR, Krugman S, Giles JP, 1972. Subtypes of Australia antigen and hepatitis-B virus. *JAMA 222:* 928–930.
6. Shiina S, Fujino H, Kawabe T, Tagawa K, Unuma T, Yoneyama M, Ohmori T, Suzuki S, Kurita M, Ohashi Y, 1991. Relationship of HBsAg subtypes with HBeAg/anti-HBe status and chronic liver disease. Part II: Evaluation of epidemiological factors and suspected risk factors of liver dysfunction. *Am J Gastroenterol 86:* 872–875.
7. Noguchi A, Hayashi J, Nakashima K, Hirata M, Ikematsu H, Kashiwagi S, 1994. HBsAg subtypes among HBsAg carriers in Okinawa, Japan. Evidence of an important relationship in seroconversion from HBeAg to anti-HBe. *J Infect 28:* 141–150.
8. Furusyo N, Hayashi J, Sawayama Y, Kishihara Y, Kashiwagi S, 1999. Hepatitis B surface antigen disappearance and hepatitis B surface antigen subtype: a prospective, long-term, follow-up study of Japanese residents of Okinawa, Japan with chronic hepatitis B virus infection. *Am J Trop Med Hyg 60:* 616–622.
9. Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, Mayumi M, 1988. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol 69:* 2575–2583.
10. Norder H, Courouce AM, Magnius LO, 1994. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology 198:* 489–503.
11. Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R, 2000. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol 81:* 67–74.
12. Mayerat C, Mantegani A, Frei PC, 1999. Does hepatitis B virus (HBV) genotype influence the clinical outcome of HBV infection? *J Viral Hepat 6:* 299–304.
13. Telenta PF, Poggio GP, Lopez JL, Gonzalez J, Lemberg A, Campos RH, 1997. Increased prevalence of genotype F hepatitis B virus isolates in Buenos Aires, Argentina. *J Clin Microbiol 35:* 1873–1875.
14. Kao JH, Chen PJ, Lai MY, Chen DS, 2000. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. *Gastroenterology 118:* 554–559.
15. Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, Okanoue T, Yotsuyanagi H, Iino S, 2001. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. *Hepatology 33:* 218–223.
16. Furusyo N, Nakashima H, Kashiwagi K, Kubo N, Hayashida K, Usuda S, Mishiro S, Kashiwagi S, Hayashi J, 2002. Clinical outcomes of hepatitis B virus (HBV) genotypes B and C in Japanese patients with chronic HBV infection. *Am J Trop Med Hyg 67:* 151–157.
17. Grandjacques C, Pradat P, Stuyver L, Chevallier M, Chevallier P, Pichoud C, Maisonnas M, Trepo C, Zoulim F, 2000. Rapid detection of genotypes and mutations in the pre-core promoter and the pre-core region of hepatitis B virus genome: correlation with viral persistence and disease severity. *J Hepatol 33:* 430–439.
18. Sanchez-Tapias JM, Costa J, Mas A, Bruguera M, Rodes J, 2002. Influence of hepatitis B virus genotype on the long-term outcome of chronic hepatitis B in western patients. *Gastroenterology 123:* 1848–1856.
19. Nakashima H, Furusyo N, Kubo N, Kashiwagi K, Etoh Y, Kashiwagi S, Hayashi J, 2003. A double point mutation in the core promoter region of hepatitis B virus (HBV) genotype C may be related to liver deterioration in patients with chronic HBV infection. *J Gastroenerol Hepatol* (in press).
20. Williams H, Mazzur S, 1976. Hepatitis B antigen subtypes in asymptomatic carriers in the Solomon Islands. *Aust N Z J Med 6:* 210–213.
21. Furusyo N, Hayashi J, Sawayama Y, Kawakami Y, Kishihara Y, Kashiwagi S, 1998. The elimination of hepatitis B virus infection: changing seroepidemiology of hepatitis A and B virus infection in Okinawa, Japan over a 26-year period. *Am J Trop Med Hyg 59:* 693–698.
22. Usuda S, Okamoto H, Iwanari H, Baba K, Tsuda F, Miyakawa Y, Mayumi M, 1999. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. *J Virol Methods 80:* 97–112.
23. Usuda S, Okamoto H, Tanaka T, Kidd-Ljunggren K, Holland PV, Miyakawa Y, Mayumi M, 2000. Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. *J Virol Methods 87:* 81–89.
24. Kato H, Orito E, Sugauchi F, Ueda R, Gish RG, Usuda S, Miyakawa Y, Mizokami M, 2001. Determination of hepatitis B virus genotype G by polymerase chain reaction with hemi-nested primers. *J Virol Methods 98:* 153–159.
25. Norder H, Hammas B, Lee SD, Bile K, Courouce AM, Mushahwar IK, Magnius LO, 1993. Genetic relatedness of hepatitis B viral strains of diverse geographical origin and natural variations in the primary structure of the surface antigen. *J Gen Virol 74:* 1341–1348.
26. Magnius LO, Norder H, 1995. Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. *Intervirology 38:* 24–34.
27. Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, Ishida T, Chutaputti A, Lai CL, Ueda R, Miyakawa Y, Mizokami M, 2002. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. *J Virol 76:* 5985–5992.
28. Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, Ishida T, Chutaputti A, Lai CL, Gish RG, Ueda R, Miyakawa Y, Mizokami M, 2003. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. *Gastroenterology 124:* 925–932.
29. Kidd-Ljunggren K, Miyakawa Y, Kidd AH, 2002. Genetic variability in hepatitis B viruses. *J Gen Virol 83:* 1267–1280.

Journal of Medical Virology 73:362–367 (2004)

Downregulation of CXCR5 in CD27^{-} B Cells of HIV-1 Infected Patients

Yong Chong,[1]* Shigeki Nabeshima,[1] Norihiro Furusyo,[1] Masayuki Murata,[1] Kouzaburo Yamaji,[1] and Jun Hayashi[1,2]

[1]*Department of General Medicine, Kyushu University Hospital, Fukuoka, Japan*
[2]*Department of Environmental Medicine and Infectious Diseases, Faculty of Medical Sciences Kyushu University, Fukuoka, Japan*

The CD27^{-} (naive) B cells of HIV-1 infected patients have been shown to be increased in frequency and to be activated, as indicated by high CD38 expression on the cell surface. CXCR5, a B cell chemokine receptor, is expressed on circulating CD27^{-} (naive) B cells and plays a pivotal role in peripheral B cell development. To investigate the effect of HIV-1 infection on the expression of this chemokine receptor on naive B cells, the expression level of CXCR5 on CD27^{-} B cells was examined in 19 drug-naive HIV-1 infected patients, 27 HAART-treated patients, and 20 controls. CXCR5 expression on CD27^{-} B cells was significantly lower in drug-naive patients than in HAART-treated patients and controls ($P < 0.01$). CD27^{-} B cells with high CD38 expression exhibited low CXCR5 expression. The CXCR5 expression level on CD27^{-} B cells recovered to within the normal range after effective antiretroviral therapy. These findings suggested that HIV-1 infection induces a remarkable phenotypic alteration of naive B cells and that the activated naive B cells found in HIV-1 infection downregulate CXCR5 on their surface. Impaired homing of naive B cells may contribute to HIV-1 induced immunological deficiencies. ***J. Med. Virol. 73:362–367, 2004.*** © 2004 Wiley-Liss, Inc.

KEY WORDS: HIV; B cells; chemokine receptor

INTRODUCTION

HIV-1 related B cell disorders are characterised by hypergammaglobulinaemia [Pahwa et al., 1984; Nicholson et al., 1985; Miedema et al., 1988] and a high prevalence of B cell lymphoma [Davi et al., 1998; Ng and McGrath, 1998]. Chronic B cell activation during HIV-1 infection is known to be related to pathogenesis [Lane et al., 1983; Shirai et al., 1992]. It was demonstrated, at a molecular level, that the peripheral B cells of HIV-1 infected patients are expanded clonally throughout the infection [Chong et al., 2001]. Aberrant development of circulating B cells from HIV-1 infected patients has been demonstrated recently by the identification of a memory B cell marker, CD27 antigen. Reductions in the frequency and absolute number of peripheral memory (CD27 positive) B cells of HIV-1 infected patients have been reported [de Milito et al., 2001; Nagase et al., 2001]. Peripheral naive (CD27 negative) B cells, the number of which was not altered, have been shown to be highly activated and to exhibit an increased susceptibility to apoptosis (manuscript submitted).

Chemokines and their corresponding receptors play an important role in the host defence system [Moser and Loetscher, 2001]. Chemokine receptors for lymphocytes maintain the development of peripheral lymphocytes by inducing homing to second lymphoid organs [Ansel and Cyster, 2001]. CXCR-5 has been identified as a chemokine receptor expressed mainly by B cells and has been shown to play a critical role in directing B cell migration into lymphoid organs [Förster et al., 1996]. CXCR-5 deficient mice have shown abnormal lymphoid organs morphologically and functionally [Förster et al., 1996].

It was found that aberrant naive B cells are the main population of circulating B cells during the progression of HIV-1 infection. In this study, the expression level of CXCR-5 on naive B cells from drug-naive and highly active antiretroviral therapy (HAART)-treated HIV-1 infected patients was examined to investigate B cell dysfunction caused by HIV-1 infection. The relationship of CXCR-5 expression with other surface markers on B cells was also examined.

MATERIALS AND METHODS

Patients

Forty-six HIV-1 infected patients and 20 HIV-1 negative, healthy individuals were registered in this

*Correspondence to: Yong Chong, MD, Department of General Medicine, Kyushu University Hospital, Higashiku Fukuoka, 812-8582, Japan. E-mail: chong@genmedpr.med.kyushu-u.ac.jp

Accepted 3 March 2004

DOI 10.1002/jmv.20099

Published online in Wiley InterScience (www.interscience.wiley.com)

© 2004 WILEY-LISS, INC.

study with informed consent. Among the infected patients, 19 were drug-naive and 27 had received HAART. Nucleoside reverse transcriptase inhibitors (NRTIs) were taken by all HAART-treated patients, 10 of whom had regimens including zidovudine (AZT) and 14 of whom had regimens including stavudine (d4T). Nonnucleoside reverse transcriptase inhibitors (NNRTIs) were given to seven patients, all of whom received efavirenz (EFV). Protease inhibitors (PIs) were given to 22 patients, 15 of whom took nelfinavir (NFV). Drug-naive patients were divided into two categories according to the Centers for Disease Control and Prevention (CDC, Atlanta, GA) criteria: 14 patients were at stage A and five were at stage C. Blood sampling was done from November, 2000 to May, 2003. Blood samples of three patients were obtained before and after antiviral therapy. These samples were used as the data of drug-naive patients and were not included with the data of HAART-treated patients. CXCR5 levels were not obtained for one drug-naive and one HAART-treated patient, due to the lack of testing material. The mean HIV-1 RNA load of drug-naive patients was 103,000 copies/ml. Stage A and C patients had mean viral loads of 85,000 and 250,000 copies/ml, respectively. The HIV-1 RNA loads of all HAART-treated patients were less than 400 copies/ml at the time of blood sampling. Twenty-four of the 27 HAART-treated patients had less than 50 HIV-1 RNA copies/ml at the time of blood sampling.

Measurement of Serum Igs and HIV RNA Load

Serum γ-globulin, IgG, IgA, and IgM levels were determined by turbidimetric immunoassay or fluorescence-enzyme immunoassay at the Mitsubishi Kagaku Bio-Clinical Laboratories (Tokyo, Japan). Plasma HIV-1 RNA loads were determined by reverse-transcriptase polymerase chain reaction using Amplicor HIV-1 Monitor assay (Roche Diagnostic Systems, Branchburg, NJ). The threshold detection level was 50 HIV-1 RNA copies/ml.

Flow Cytometry

For phenotypic analysis of peripheral CD 19 positive ($CD19^+$) B cells, two or three-color flow cytometry was done using the following fluorescein isothiocyanate (FITC), phycoerythrin (PE), or phycoerythrin-cyanin 5.1 (PC5) conjugated mouse anti-human monoclonal antibodies (mAbs): CD3-PC5, CD19-PC5, CD38-PC5, CD38-FITC, CD4-PE (Immunotech, Marseille Cedex, France), CD8-FITC (Ortho Diagnostic Systems, Raritan, NJ), CD27-FITC, CD27-PE (Becton Dickinson, NJ), CXCR5-PE (Dako Japan, Kyoto, Japan), and IgD-FITC (Southern Biotechnology Associates, Birmingham, AL). Ig isotype-matched FITC, PE, or PC5-conjugated mouse antibodies were used as negative controls for non-specific staining. Whole blood of 50 μl in volume was incubated with mAbs for 30 min at 4°C. The incubated blood samples were lysed in 1 ml of lysing reagent (Ortho Diagnostic Systems) for 10 min at room temperature and washed twice with Dulbecco's phosphate-buffered saline (PBS) (Nisssui Chemical Industries, Tokyo, Japan) with 0.5% bovine serum albumin (BSA) (Sigma, St. Louis, MO) plus 0.1% sodium azide (Wako Pure Chemical Industries, Osaka, Japan). This analysis was done with the following combinations of conjugated mAbs: CD3-PC5, CD4-PE, and CD8-FITC; CD19-PC5 and CD27-PE; CD19-PC5, CD27-PE, and CD38-FITC; CD19-PC5, CD27-FITC, and CXCR5-PE.

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised venous blood by density centrifugation over LSM solution (Organon Teknika-Cappel, Durham, NC). Freshly isolated PBMCs were stained with CD19-PC5, CD27-PE, and IgD-FITC for 30 min at 4°C, washed twice with PBS/BSA, and prepared with PBS with 1% formaldehyde.

B cells were isolated by positive selection using anti-CD19 mAb coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacture's instructions. The purity of the B cell suspensions was greater than 95%. The isolated B cells were stained with CD27-FITC, CD38-PC5, and CXCR5-PE.

Stained cells were analysed by the flow cytometer, CYTORON ABSOLUTE (Ortho Diagnostic Systems) and ImmunoCount 2 software (Ortho Diagnostic Systems). Live-gating of lymphocytes was performed, and up to 30,000 events were acquired for each analysis.

Statistical Analysis

The Mann–Whitney *U*-test was performed to compare differences in the analysed data among the three groups. The association between two related variables was analysed by the Spearman's rank correlation test.

RESULTS

Immunological Characteristics of HIV-1 Infected Patients

The immunological characteristics of the individuals examined in this study are summarised in Table I. The $CD4^+$ cell count of drug-naive and HAART-treated patients was significantly lower than that of controls. The $CD4^+$ cell count of HAART-treated patients was higher than that of drug-naive patients, but the difference was not statistically significant. The serum γ-globulin level of drug-naive patients was significantly higher than that of controls. The serum γ-globulin level of HAART-treated patients was significantly lower than that of drug-naive patients, but was still higher than controls, although the difference was not significant. Results of serum IgG and IgA levels were similar to that of the serum γ-globulin level. The absolute number of circulating total B cells was comparable among the three groups. The absolute number of circulating $CD27^+$ B cells was significantly lower in both drug-naive and HAART-treated patients than in controls (both $P<0.01$). In contrast, the absolute number of circulating $CD27^-$ B cells was comparable among the three groups.

TABLE I. Immunological Characteristics in HIV-1 Infected Patients and Controls

	HIV patients		
	Drug-naive	HAART-treated	Controls
Number of patients	19	27	20
Age (years, mean)	37.2	38.4	32.1
$CD4^+$ T cell count (cells/μl, mean)	303[a]	409[b]	664[a,b]
γ-globulin (mg/dl, mean)	1,881.7[c,d]	1,293.5[d]	1,143.6[c]
IgG (mg/dl, mean)	2,041.2[e,f]	1,491.6[f]	1,255.2[e]
IgA (mg/dl, mean)	458.7[g,h]	279.8[h]	219.5[g]
IgM (mg/dl, mean)	162.6[i]	102.0[i]	146.6
B cell count (cells/μl, mean)	193	223	218
$CD27^-$ B cell count (cells/μl, mean)	169	189	151
$CD27^+$ B cell count (cells/μl, mean)	24[j]	34[k]	67[j,k]

[a–k] $P < 0.01$.

Circulating $CD27^-$ B Cells of HIV-1 Infected Patients

Representative FACS analysis of PBMCs stained by anti-CD19 and CD27 antibodies is shown in Figure 1a. $CD27^-$ B cells were distinct from $CD27^+$ B cells. As shown in Figure 1a, the frequency of $CD27^-$ B cells appeared to increase as the stage of HIV-1 infection progressed. The mean percentage of circulating $CD27^-$ B cells was significantly higher in drug-naive (86.2%) and HAART-treated patients (83.9%) than in controls (68.6%) (both $P < 0.01$). The percentage of $CD27^-$ B cells was significantly higher in stage C patients (mean, 93.8%) than in stage A patients (mean, 83.5%) ($P < 0.05$). These results are consistent with those reported previously (manuscript submitted). $CD27^-$ B cells have been reported to be naive IgD positive B cells (IgD^+ B cells) [Klein et al., 1998]. The percentage of circulating $CD27^-$ B cells found in HIV-1 infected patients was positively correlated with the percentage of circulating IgD^+ $CD27^-$ B cells ($P < 0.01$, $r = 0.9$) (Fig. 1b), suggesting that the increased percentage of $CD27^-$ B cells found in HIV-1 infected patients is not a result of CD27 downregulation on memory B cells.

CXCR5 Expression on Circulating $CD27^-$ B Cells in Drug-Naive and HAART-Treated Patients

CXCR5 expression on $CD27^-$ B cells from drug-naive patients was significantly lower than that of controls ($P < 0.01$, Fig. 2). CXCR5 expression on $CD27^-$ B cells from HAART-treated patients was significantly higher than that of drug-naive patients and was the same as that of controls ($P < 0.01$, Fig. 2). CXCR5 expression on

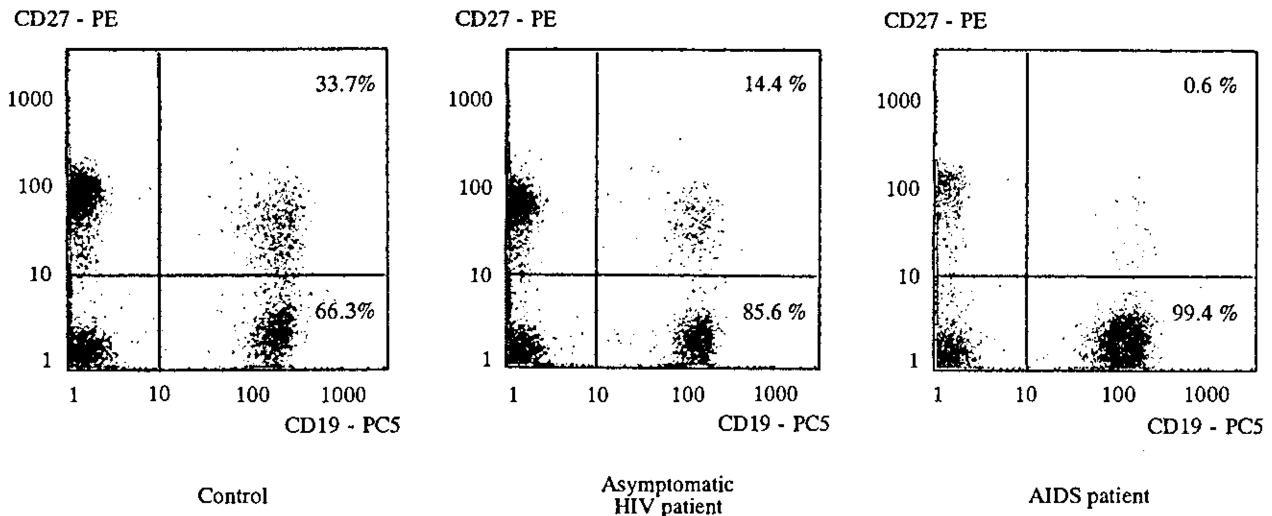

Fig. 1. a: Representative FACS analysis of anti-CD19 and CD27 stained peripheral blood mononuclear cells (PBMCs) in one asymptomatic HIV-1 infected patient, one HIV-1 infected patient with AIDS, and one control individual. The numbers in the upper and lower right quadrants show $CD27^+$ and $CD27^-$ B cells as a percentage of total peripheral B cells, respectively. b: Correlation between $CD27^-$ and IgD^+ $CD27^-$ cells as a percentage of total peripheral B cells. Data were obtained from drug-naive and HAART-treated HIV-1 infected patients.

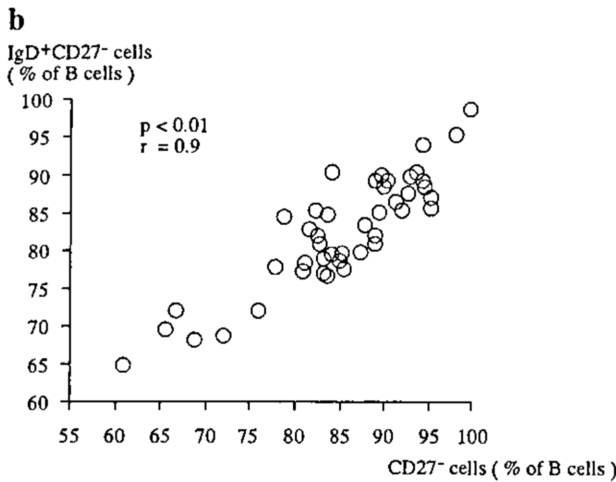

Fig. 1. (*Continued*)

CD27⁻ B cells from drug-naive patients was comparable between stage A and C patients. The expression level of CXCR5 on CD27⁻ B cells from drug-naive patients was not associated with viral load or CD4 cell count ($P = 0.2$ and $P = 0.8$, respectively).

To investigate the relationship between the activational and chemotactic states of CD27⁻ B cells, the expression levels of CD38 and CXCR5 on CD27⁻ B cells were examined. CD38 expression on CD27⁻ B cells from drug-naive patients was significantly higher than that of HAART-treated patients and controls (manuscript submitted). A certain relationship was found between CD38 and CXCR5 expression on CD27⁻ B cells from drug-naive patients. In drug-naive patients, CD27⁻ B cells with highly expressed CD38 exhibited extremely low levels of CXCR5 (Fig. 3). Few CD27⁻ B cells with high CD38 and low CXCR5 expression were observed in HAART-treated patients and controls (Fig. 3).

The expression levels of CD38 and CXCR5 on CD27⁻ B cells from three patients were examined before and after antiviral therapy (Table II). An increase in the CD4 cell count and a decrease in the IgG level were seen in each patient, along with a reduction of viral load. Also, the CD38 expression level on CD27⁻ B cells decreased after therapy and the CXCR5 expression level on CD27⁻ B cells increased to within the normal range after therapy.

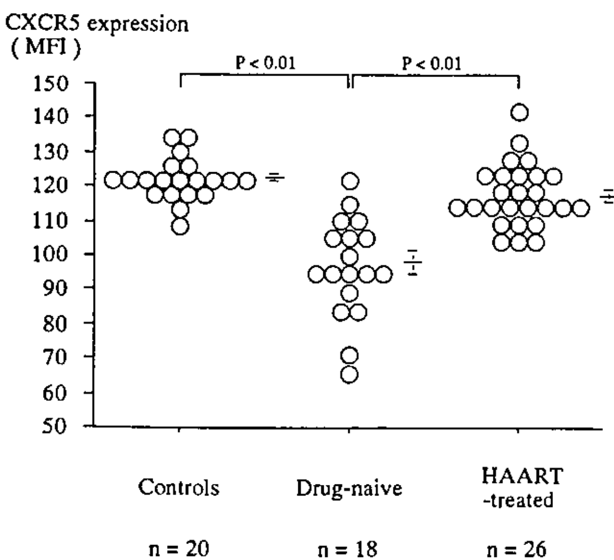

Fig. 2. Mean fluorescence intensity (MFI) of CXCR5 on peripheral CD27⁻ B cells in HIV-1 infected patients. The horizontal and vertical dotted bars represent the mean levels and SE, respectively.

DISCUSSION

In contrast with CD27⁺ (memory) B cells, the absolute number of peripheral CD27⁻ (naive) B cells was not decreased in HIV-1 infected patients. The CD27⁻ B cells represented an activated phenotype with an increased susceptibility to apoptosis (manuscript submitted). In this study, the CXCR5 expression level on CD27⁻ B cells of HIV-1 infected patients was decreased. Thus, HIV-1 infection would seem to induce a phenotypic alteration of CD27⁻ (naive) B cells.

CXCR5, a chemokine receptor, is expressed on all peripheral B cells and is involved in homing to second lymphoid tissues, which plays an important role in peripheral B cell development [Förster et al., 1996]. CD27⁻ B cells, the dominant population of the peripheral B cells of HIV-1 infected patients, were shown to be a phenotype of naive B cells (Fig. 1a,b). The CXCR5 expression level on CD27⁻ B cells was significantly lower in drug-naive patients than in HAART-treated patients and controls (Fig. 2). Although the capacity of CD27⁻ B cells to migrate to BCA-1, the ligand of CXCR5, was not examined in this study, the homing of naive B cells into lymphoid tissues may be impaired in HIV-1 infected patients. The structure of lymphoid tissues was reported to have been markedly destroyed in the AIDS stage [Pantaleo et al., 1994]. CXCR5 downregulation and the presumed dysfunction of lymphoid tissues possibly affect the homing of circulating naive B cells and inhibit the differentiation of naive B cells into memory B cells.

The expression of chemokine receptors on lymphocytes is tightly regulated by their developmental stage [Ansel and Cyster, 2001]. Peripheral blood B cell subsets, CD27⁻ (naive) B cells and CD27⁺ (memory) B cells, express CXCR5 and are recruited into lymphoid tissues. Plasma cells, which are produced mainly by a specific antigenic stimulation, downregulate CXCR5 expression on their surface and are recruited into other sites [Calame et al., 2003]. In this respect, this CXCR5 downregulation of CD27⁻ B cells from HIV-1 infected patients is an intriguing phenomenon, because these B cells remain a phenotype of naive B cells (IgD positive). The expression of chemokine receptors on lymphocytes is also influenced by the state of activation. Activated T cells detected in HIV-1 infected patients have been

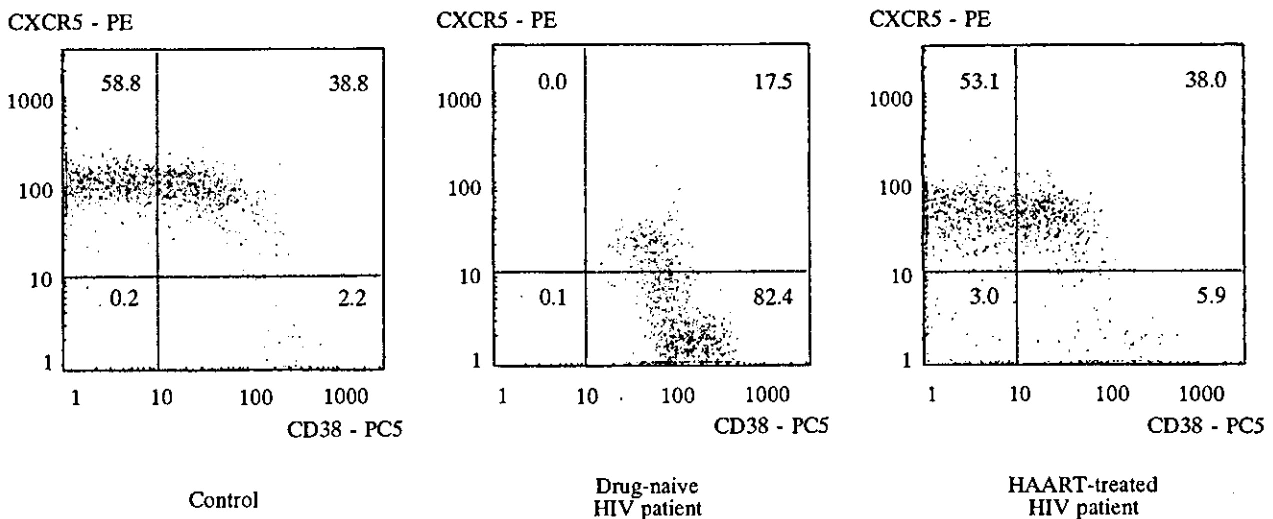

Fig. 3. Relationship between CD38 and CXCR5 expression on peripheral CD27⁻ B cells. Three-color flow cytometry was done using anti-CD27, CD38, and CXCR5 stained B cells after B cell isolation. Representative FACS analysis is shown for one drug-naive HIV-1 infected patient, one HAART-treated patient, and one control individual. Each number in the quadrant shows the percentage of total $CD27^-$ B cells.

shown to upregulate CCR5, a T cell chemokine receptor expressed on peripheral T cells [Ostrowski et al., 1998]. The activation of circulating $CD27^-$ B cells, indicated by high CD38 expression, has been observed in HIV-1 infected patients (manuscript submitted). In this study, $CD27^-$ B cells with high CD38 expression exhibited extremely low CXCR5 expression (Fig. 3). Most of these B cells were detected in drug-naive patients, but not in HAART-treated patients (Fig. 3). Thus, it is possible that the activated naive B cells observed during HIV-1 infection downregulate CXCR5 expression on their surface. The absolute number of peripheral naive B cells of HIV-1 infected patients remains unchanged (Table I), but their quality is markedly changed.

Some cytokines, such as IL-6 and TNF-α, have been reported to be related to B cell activation and to be elevated in HIV-1 infected patients [Lahdevirta et al., 1988; Breen et al., 1990; Kehrl et al., 1992; Bergamini et al., 1999]. In HIV-1 infected patients, CD40 dependent T–B interaction, which generally results from a specific antigenic stimulation, might be impaired due to the lack of $CD4^+$ T cells. If the naive B cells of HIV-1 infected patients are activated under a CD40 independent process, it would be interesting to determine how these cytokines affect the phenotypic alteration of naive B cells in HIV-1 infected patients. Persistent stimulation by nonspecific factors such as the above cytokines, not by a specific antigen, may induce CXCR5 downregulation in naive B cells, which is not seen in the peripheral naive B cells of healthy individuals.

A previous study [Morris et al., 1998] reported that the frequency of B cells secreting Ig spontaneously is significantly higher in the peripheral blood of HIV infected patients with detectable viral loads than in controls. We found that IgD^+ $CD27^-$ (naive) B cells were predominant in the peripheral blood during HIV infection. These findings seem to be paradoxical. de Milito et al. [2001] proposed a persistent differentiation of $CD27^+$ B cells into plasma cells during HIV infection. Based on this mechanism, $CD27^+$ B cells differentiate into antibody-secreting cells during HIV infection, resulting in memory B cell depletion and naive B cell predominance in peripheral blood. Further investigation will be necessary to address this issue.

TABLE II. Comparison of Clinical Parameters and Flow Cytometric Analysis Before and After Antiretroviral Therapy

Case number	Medication (before/after)	$CD4^+$ T cell count (cells/μl)	HIV RNA load (copies/ml)	IgG (mg/dl)	CD38 expression on $CD27^-$ B cell (MFI)	CXCR5 expression on $CD27^-$ B cell (MFI)
1	Before	234	251,000	1,810	113.0	91.0
	After	379	<50	1,346	105.2	118.1
2	Before	111	230,000	1,403	132.7	75.5
	After	301	<50	901	78.2	109.3
3	Before	29	2,500	2,155	124.9	102.7
	After	174	<50	1,203	89.3	130.7

MFI, mean fluorescence intensity.

In conclusion, the frequency of $CD27^-$ (naive) B cells increased as the stage of HIV-1 infection progressed. HIV-1 infection induced the downregulation of CXCR5 on $CD27^-$ (naive) B cells. This is possibly related to the activation of naive B cells. The putative homing impairment of naive B cells into lymphoid tissues may contribute to the pathogenesis of HIV-1 related immunodeficiencies.

REFERENCES

Ansel KM, Cyster JG. 2001. Chemokines in lymphopoiesis and lymphoid organ development. Curr Opin Immunol 13:172–179.

Bergamini A, Faggioli E, Bolacchi F, Gessani S, Cappannoli L, Uccella I, Demin F, Capozzi M, Cicconi R, Placido R, Vendetti S, Mancino G, Colizzi V, Rocchi G. 1999. Enhanced production of tumor necrosis factor-α and interleukin-6 due to prolonged response to lipopolysaccharide in human macrophages infected in vitro with human immunodeficiency virus type 1. J Infect Dis 179:832–842.

Breen EC, Rezai AR, Nakajima K, Beall GN, Mitsuyasu RT, Hirano T, Kishimoto T, Martinez-Maza O. 1990. Infection with HIV is associated with elevated IL-6 levels and production. J Immunol 144:480–484.

Calame KL, Lin KI, Tunyaplin C. 2003. Regulatory mechanisms that determine the development and function of plasma cells. Annu Rev Immunol 21:205–230.

Chong Y, Ikematsu H, Ariyama I, Chijiwa K, Li W, Yamaji K, Kashiwagi S, Hayashi J. 2001. Evidence of B cell clonal expansion in HIV type 1-infected patients. AIDS Res Hum Retroviruses 17:1507–1515.

Davi F, Delecluse HJ, Guiet P, Gabarre J, Fayon A, Gentilhomme O, Felman P, Bayle C, Berger F, Audouin J, Bryon PA, Diebold J, Raphaël M. 1998. Burkitt-like lymphomas in AIDS patients: Characterization within a series of 103 human immunodeficiency virus-associated non-Hodgkin's lymphomas. J Clin Oncol 16:3788–3795.

de Milito A, Mörch C, Sönnerborg A, Chiodi F. 2001. Loss of memory (CD27) B lymphocytes in HIV-1 infection. AIDS 15:957–964.

Förster R, Mattis AE, Kremmer E, Wolf E, Brem G, Lipp M. 1996. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. Cell 87:1037–1047.

Kehrl JH, Rieckmann P, Kozlow E, Fauci AS. 1992. Lymphokine production by B cells from normal and HIV-infected individuals. Ann NY Acad Sci 651:220–227.

Klein U, Goossens T, Fischer M, Kanzler H, Braeuninger A, Rajewsky K, Küppers R. 1998. Somatic hypermutation in normal and transformed human B cells. Immunol Rev 162:261–280.

Lahdevirta J, Maury CP, Teppo AM, Repo H. 1988. Elevated levels of circulating cachectin/tumor necrosis factor in patients with acquired immunodificiency syndrome. Am J Med 85:289–291.

Lane HC, Masur H, Edgar LC, Whalen G, Rook AH, Fauci AS. 1983. Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. N Engl J Med 309:453–458.

Miedema F, Petit AJC, Terpstra FG, Schattenkerk JKME, de Wolf F, Al BJM, Roos M, Lange JMA, Danner SA, Goudsmit J, Schellekens PTA. 1988. Immunological abnormalities in human immunodeficiency virus (HIV)-infected asymptomatic homosexual men. J Clin Invest 82:1908–1914.

Morris L, Binley JM, Clas BA, Bonhoeffer S, Astill TP, Kost R, Hurley A, Cao Y, Markowitz M, Ho DD, Moore JP. 1998. HIV-1 antigen-specific and -nonspecific B cell responses are sensitive to combination antiretroviral therapy. J Exp Med 188:233–245.

Moser B, Loetscher P. 2001. Lymphocyte traffic control by chemokines. Nature Immunol 2:123–128.

Nagase H, Agematsu K, Kitano K, Takamoto M, Okubo Y, Komiyama A, Sugane K. 2001. Mechanism of hypergammaglobulinemia by HIV infection: Circulating memory B-cell reduction with plasmacytosis. Clin Immunol 100:250–259.

Ng VL, McGrath MS. 1998. The immunology of AIDS-associated lymphomas. Immunol Rev 162:293–298.

Nicholson JKA, McDougal JS, Jaffe HW, Spira TJ, Kennedy MS, Jones BM, Darrow WW, Morgan M, Hubbard M. 1985. Exposure to human T-lymphotropic virus type III lymphadenopathy-associated virus and immunologic abnormalities in asymptomatic homosexual men. Ann Intern Med 103:37–42.

Ostrowski MA, Justement SJ, Catanzaro A, Hallahan CA, Ehler LA, Mizell SB, Kumar PN, Mican JA, Chun TW, Fauci AS. 1998. Expression of chemokine receptors CXCR4 and CCR5 in HIV-1-infected and uninfected individuals. J Immunol 161:3195–3201.

Pahwa SG, Quilop MTJ, Lange M, Pahwa RN, Grieco MH. 1984. Defective B-lymphocyte function in homosexual men in relation to the acquired immunodeficiency syndrome. Ann Intern Med 101: 757–763.

Pantaleo G, Graziosi C, Demarest JF, Cohen OJ, Vaccarezza M, Gantt K, Muro-Cacho C, Fauci AS. 1994. Role of lymphoid organs in the pathogenesis of human immunodeficiency virus (HIV) infection. Immunol Rev 140:105–130.

Shirai A, Cosentino M, Leitman-Klinman SF, Klinman DM. 1992. Human immunodeficiency virus infection induces both polyclonal and virus-specific B cell activation. J Clin Invest 89:561–566.

CONFIRMATION OF NOSOCOMIAL HEPATITIS C VIRUS INFECTION IN A HEMODIALYSIS UNIT

Norihiro Furusyo, MD, PhD; Norihiko Kubo, MD; Hisashi Nakashima, MD; Kenichiro Kashiwagi, MD; Yoshitaka Etoh, BS; Jun Hayashi, MD, PhD

ABSTRACT

OBJECTIVES: To investigate a hepatitis C virus (HCV) outbreak in a hemodialysis unit and determine the source of transmission.

METHODS: We have prospectively investigated the epidemiology of hemodialysis-related HCV infection in a single unit since 1989. In September 2000, acute hepatitis C (AH-C) was diagnosed in 5 patients by alanine aminotransferase elevation and HCV genotype 1b viremia without antibody to HCV. We surveyed the epidemiologic situation and performed polymerase chain reaction sequence analysis of the HCV 5'-noncoding (5'NC) region in the patients for comparison with 9 patients with chronic HCV genotype 1b viremia.

RESULTS: Sequence analysis of the 5'NC region showed the consistency in the 5 independent clones from each AH-C patient and those from each chronic HCV viremia patient and no quasispecies over time in the clones of any of 14 analyzed patients. All AH-C patients had the same sequencing of the 6 variations in the region with the only other patient. A saline ampoule, used for heparin solution during hemodialysis, had a recap function. It was difficult to determine whether the ampoule was new or had already been used. The source-patient often underwent hemodialysis before the AH-C patients and most of their hemodialysis-related medicine was prepared during the source-patient's treatment. These findings suggested a high possibility that the AH-C patients shared a single heparin–saline solution ampoule contaminated by HCV from the source-patient.

CONCLUSION: Nosocomial HCV infection occurred as a result of poor infection control practice when a patient with chronic HCV viremia received treatment prior to other hemodialysis patients (*Infect Control Hosp Epidemiol* 2004;25:584-590).

Since 1988, hepatitis C virus (HCV) infection has been documented to be a hazard for hemodialysis patients throughout the world.[1,2] In Japan, the reported prevalence of HCV is remarkably higher for hemodialysis patients (22.0% to 55.5%) than for the general population (0.7% to 3.7%).[1,3-5] HCV is acquired from parenteral exposure with two risk factors identified for transmission of HCV to hemodialysis patients: blood transfusion and long-term hemodialysis.[3] The spread of HCV in Japan was mainly due to transfusion of contaminated blood and blood products before the establishment of screening for HCV markers.[6] Although blood transfusion has historically been the most common therapy for the renal anemia of hemodialysis patients,[7] during the past decade erythropoietin treatment has gained favor.[8] Also important is that the prevalence of antibody to HCV (anti-HCV) has been shown to increase with the duration of hemodialysis for patients with no history of blood transfusion, and that the prevalence was not related to age.[3] Hemodialysis itself is a risk factor for HCV infection and occasional transmission of HCV to hemodialysis patients still occurs, despite the screening of blood products for anti-HCV.[9] Documentation of the routes of transmission of nosocomial HCV in hemodialysis units is important for developing measures to prevent HCV infection among hemodialysis patients. Nucleotide sequence analysis has been recently used extensively to characterize the route of transmission of HCV in different epidemiologic settings. No data are available concerning the transmission of nosocomial HCV based on genomic sequencing of the HCV 5'-noncoding (5'NC) region.

Since 1989, we have been conducting an epidemiologic survey of HCV infection in a hemodialysis unit in a hospital in Fukuoka, Japan.[9-13] In September 2000, newly acquired HCV infection was found in five patients at the same time. We describe this outbreak of nosocomial HCV infection and analyze the source of horizontal transmission by use of epidemiologic data and molecular evidence from polymerase chain reaction (PCR) sequencing of the HCV 5'NC region.

METHODS

Patients

Patients of this hemodialysis unit have been prospectively studied to investigate the epidemiology of hemodialysis-related HCV infection since 1989.[9-13] In May 2000, 239 individuals were regularly receiving dialysis as outpatients

Drs. Furusyo and Hayashi and Mr. Etoh are from the Department of General Medicine, Kyushu University Hospital; and Drs. Furusyo, Kubo, Nakashima, Kashiwagi, and Hayashi are from the Department of Environmental Medicine and Infectious Disease, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan.

Address reprint requests to Norihiro Furusyo, MD, PhD, Department of General Medicine, Kyushu University Hospital, Higashi-Ku, Fukuoka, 812-8582, Japan.

Reprinted with permission; SLACK Incorporated, © 2004. All rights reserved.

TABLE 1
HEPATITIS C VIRUS MARKERS OF THE FIVE PATIENTS WITH ACUTE HEPATITIS C AND 35 OTHER PATIENTS WHO UNDERWENT HEMODIALYSIS IN THE SAME ROOM ON THE SAME DAYS

Shift 1 (Morning)				Shift 2 (Afternoon)			
Bed No.*	Anti-HCV	HCV RNA	Genotype	Bed No.*	Anti-HCV	HCV RNA	Genotype
1	Negative	Negative		1	Positive	Positive	ND
2	Positive	Positive	1b	2	Negative	Negative	
3	Positive	Positive	1b	3	Negative	Negative	
4	Negative	Negative		4	Positive	Positive	1b
5	Negative	Negative		5	Negative	Negative	
6	Positive	Positive	1b	6	Negative	Negative	
7	Positive	Positive	1b	7	Negative	Negative	
8	Negative	Negative		8	Negative	Negative	
9	Negative	Negative		9	Negative	Negative	
10	Positive	Positive	ND	10	Negative	Negative	
11	Negative	Negative		11	Positive	Positive	2a
12	Negative	Negative		12	Negative	Negative	
13	Negative	Negative		13	Negative	Negative	
14	Positive	Negative		14	Negative	Negative	
15	Negative	Negative		15	Positive	Positive	1b
16	Negative	Negative		16, patient A	Negative	Positive	1b
17	Positive	Positive	1b	17, patient B	Negative	Positive	1b
18	Positive	Positive	1b	18, patient C	Negative	Positive	1b
19	Negative	Negative		19, patient D	Negative	Positive	1b
20	Positive	Positive	1b	20, patient E	Negative	Positive	1b

Anti-HCV = antibody to hepatitis C virus; ND = not determinable.
*Bed locations are shown in Figure 2.

(131 men and 108 women; mean age, 59.6 years). Sixty-seven (28.0%) were positive for anti-HCV and 59 (24.7%) were positive for HCV RNA. No patient who was negative for anti-HCV was positive for HCV RNA. Among the 59 patients with chronic HCV viremia, 42 (71.2%) had genotype 1b, 7 (11.9%) had genotype 2a, 2 (3.4%) had genotype 2b, and 8 (13.5%) had an undeterminable genotype.

All 239 patients had laboratory blood tests performed monthly and serologic viral hepatitis tests performed every 3 months after the start of hemodialysis. The HCV outbreak in this hospital was recognized on September 11, 2000. Acute hepatitis C was diagnosed in 5 patients (patients A, B, C, D, and E) by alanine aminotransferase level elevation and serum HCV RNA positivity without anti-HCV in each patient for the first time after the start of hemodialysis. A hospital institutional review board approved the study protocol, and the patients provided written informed consent.

Clinical Setting

Hemodialysis was performed in three physically separated rooms with a maximum of 20 beds. Patients received dialysis in each room during either the morning or the afternoon in the following four shifts: shift 1, Monday, Wednesday, and Friday mornings; shift 2, Monday, Wednesday, and Friday afternoons; shift 3, Tuesday, Thursday, and Saturday mornings; and shift 4, Tuesday, Thursday, and Saturday afternoons. The 5 patients with acute hepatitis C regularly received dialysis in the same room and were all on shift 2. The patient charts showed that 20 patients on shift 1 and 15 on shift 2 shared the same room and equipment with the 5 patients with acute hepatitis C after May 2000. Chronic HCV viremia had been confirmed in 8 of 20 patients on shift 1 and 4 of 15 patients on shift 2 in our previous epidemiologic study.[9] Among these 12 patients with chronic HCV viremia, 9 had genotype 1b, 1 had genotype 2a, and 2 had an undeterminable genotype (Table 1).

Routine hemodialysis techniques were used for all of the patients during treatment for 3 to 4 hours three times a week. The dialysis machines had automated ultra-filtration control. No dialyzers were reused. At least two staff members who wore aprons and masks were present at the start of each dialysis session for each patient. General hygiene precautions, including separate vials of physiologic saline, separate gloves, and sterile conditions at the start of dialysis, were taken for all patients. Testing of all hospital staff members working on shifts 1 and 2 revealed no anti-HCV.

Assay Methods

Starting in 1989, serum samples were drawn approximately every 3 months,[9-13] stored at -20°C, and

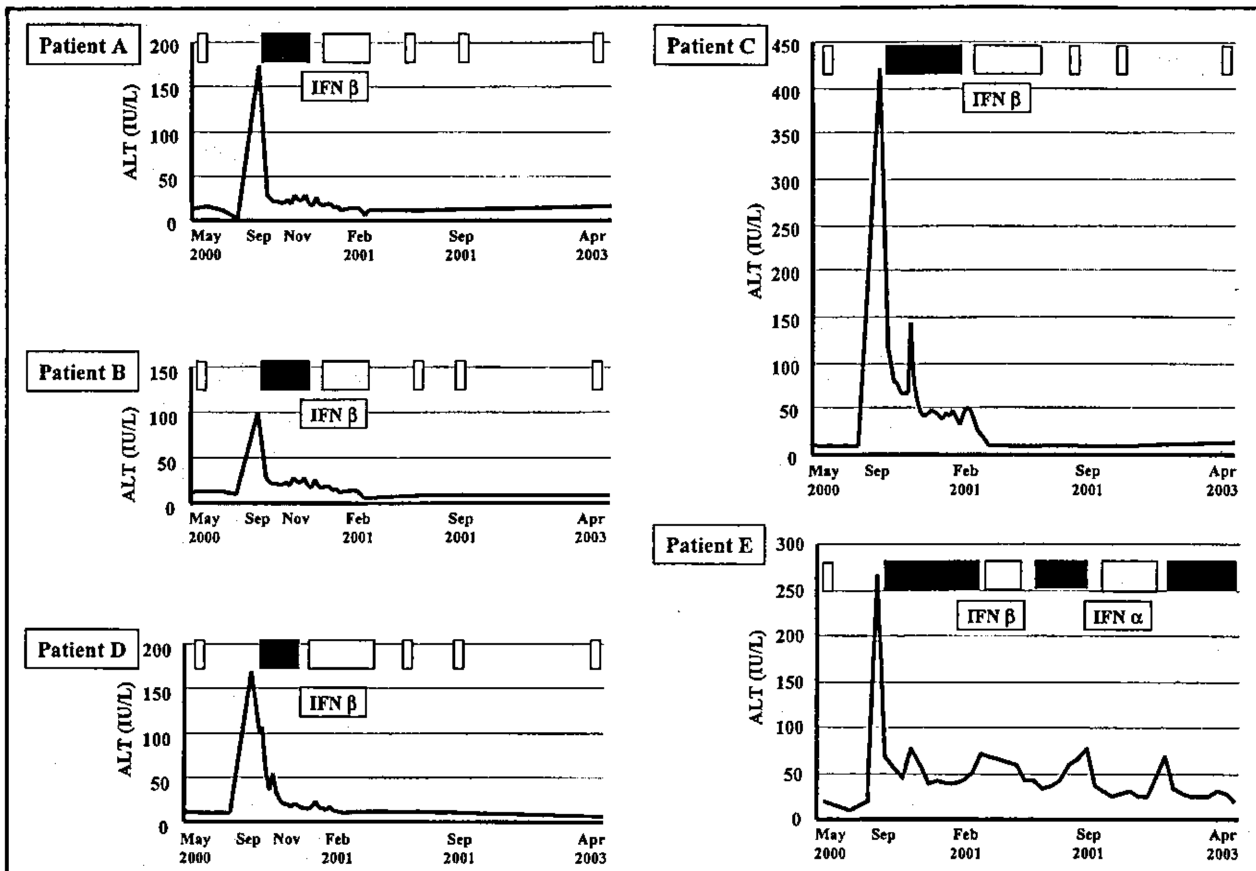

FIGURE 1. Biochemical and virologic course of the 5 patients involved in the outbreak of hepatitis C virus (HCV). The white bars indicate negativity for HCV RNA and the dark bars indicate positivity. Administration of intravenous natural interferon-beta (IFN β) (3 megaunits thrice weekly for 6 months) is also shown. Only patient E received natural IFN-alpha (α) (3 megaunits thrice weekly for 6 months) after IFN β. ALT = alanine aminotransferase.

frozen and thawed only once before testing for anti-HCV, HCV genotype, HCV RNA level, and HCV sequencing. Anti-HCV was determined by enzyme-linked immunosorbent assay (HCV EIA II, Abbott Laboratories, Abbott Park, IL). PCR was done to detect HCV RNA and to determine the HCV RNA genotype.

Determination of HCV RNA by PCR

RNA was extracted from 50 μL of serum by Sep Gene RV (Sanko Junyaku, Tokyo, Japan). Complementary DNA was synthesized by use of random primers and reverse transcriptase (Super Script II, Life Technologies, Gaithersburg, MD). HCV RNA was detected by 2-stage PCR with primers from the 5'NC region of the HCV genome[14]: 5'-CTGTGAGGAACTACTGTCTT-3' (sense) and 5'-AACACTACTCGGCTAGCAGT-3' (antisense) in the first stage and 5'-TTCACGCAGAAAGCGTCTGT-3' (sense) and 5'-GTTGATCCAAGAAAGGACCC-3' (antisense) in the second stage.

HCV RNA Genotyping

The HCV RNA genotype of each patient with HCV viremia was determined by 2-stage PCR using universal and type-specific primers from the putative core gene of the HCV genome by a modification of the method of Okamoto et al.[15] and our previous report.[16] The genotype nomenclature was based on the system proposed by Simmonds et al.[17]

Quantitative Detection of Serum HCV DNA

HCV RNA levels of patients with HCV viremia were determined by use of a second-generation branched-DNA probe assay (Quantiplex HCV RNA 2.0 assay, Chiron, Emeryville, CA). The linear relationship provided ranged from 0.2 to 60 mega-genome equivalents per milliliter for the branched-DNA probe assay.

Sequence Analysis

Using serial sequence variation analysis to determine quasispecies in the 5'NC and core regions of HCV, we analyzed the serum samples from 1 and 2 weeks (September 18 and 25) after elevation of alanine aminotransferase level in the 5 patients newly diagnosed with acute hepatitis C for comparison with those from 9 patients with chronic HCV viremia of genotype 1b who had received dialysis in the same room. The same