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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Clinical significance of alanine aminotransferase levels and the effect of ursodeoxycholic acid in hemodialysis patients with chronic hepatitis C

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Abstract
Background The natural history of hepatitis C virus (HCV) carriers and the effect of ursodeoxycholic acid (UDCA) have not been fully elucidated among hemodialysis (HD) patients.
Methods Eighty-four anti-HCV antibody- and HCV RNA-positive and 154 anti-HCV antibody-negative HD patients who were retrospectively observed for at least 3 years were analyzed. We investigated the factors associated with thrombocytopenia ($< 1.3 \times 10^5/\mu L$) and decreased platelet count (PLT) (more than 20% decrease during the follow-up period), which were considered to be indicators of hepatic fibrosis. In addition, another 16 HD patients with HCV who received 300 mg/day UDCA orally for at least 6 months were investigated. Changes in alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT) and PLT were assessed.
Results After the 60.3-months mean follow-up period, HCV infection was independently associated with both thrombocytopenia [odds ratio (OR) 2.589] and decreased PLT (OR 2.339) in 238 HD patients. In 84 HD patients with HCV, the average ALT levels ($\geq$ 15 IU/L) during the follow-up period was associated with thrombocytopenia (OR 3.882) and decreased PLT (OR 4.470). In addition, ALT, AST and GGT significantly decreased at 6 months after starting UDCA, but PLT did not change in 16 HD patients with HCV.
Conclusions These results indicate that HCV infection is a risk for thrombocytopenia which should be associated with hepatic fibrosis in HD patients. In addition, the clinical course of ALT levels predicts the progression of thrombocytopenia, and UDCA may effectively lower ALT levels in HD patients with HCV.

Keywords Hemodialysis · HCV · Thrombocytopenia · ALT · Ursodeoxycholic acid

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Introduction

Chronic kidney disease (CKD) patients who are on hemodialysis (HD) continue to have a higher prevalence of hepatitis C virus (HCV) infection than the general population [1–4]. The prevalence of anti-HCV seropositivity among patients undergoing regular dialysis in developed countries ranges between 7 and 40% [5–8].

HCV infection in HD patients is usually recognized as asymptomatic and cirrhosis is infrequent in this population [9]. One of the reasons for these findings is that the clinical course of chronic hepatitis C extends over decades and dialysis patients generally have higher morbidity and mortality rates than the general population, making the long-term consequences of HCV infection with HD difficult to establish [6]. However, more recently, the prognosis of HD patients has been improving, so addressing HCV infection in these patients is becoming more important [10].

The strong association between serum alanine aminotransferase (ALT) levels and the fibrosis progression rate or occurrence of hepatocellular carcinoma has been well documented in HCV carriers without HD [11–13]. HD

patients with persistent HCV infection also have higher ALT levels than those patients without HCV, and ALT values may predict the outcome of HCV infection in patients with HD [14]. In contrast, ALT values are still typically within the normal range in HCV carriers with HD and ALT values are lower in HCV carriers with HD than those without HD. Recently, the risk of liver disease-related deaths is higher in chronic hepatitis C patients with ALT levels closer to the upper limit of the normal range (ULN) (20–29 IU/L) compared to patients with lower ALT levels (< 20 IU/L) [15, 16]. In addition, it has been proposed that the cut-off for serum ALT levels should be reduced by half to screen for hepatic damage in HCV carriers with HD [17]. However, the association between serum ALT levels in those patients with HCV and fibrosis progression has not been fully elucidated.

Platelet count (PLT) is a simple biomarker of hepatic fibrosis in HCV carriers [18]. PLT is also lower in HCV RNA-positive HD patients than in HD patients with HCV RNA-negative serum [16]. In addition, severe hepatic fibrosis is independently associated with thrombocytopenia ($< 1.3 \times 10^5/\mu L$) in HCV carriers with end-stage renal disease [19]. This study evaluated the association of ALT status over a long period and changes in PLT, which was considered an indicator of hepatic fibrosis, in HD patients.

Several trials have examined the efficacy of interferon monotherapy or interferon plus ribavirin combination therapy in HD patients with HCV, and some of these patients obtained a sustained virological response [20]. However, the virological response was limited and side effects may occur more frequently in patients with HD than in those without HD [21, 22]. Therefore, other therapies should be considered for these patients. For chronic hepatitis C patients with or without HD, ursodeoxycholic acid (UDCA) has already been used up to 150 mg/day as routine care in Japan. In addition, the effect of UDCA up to 900 mg/day in HCV carriers who are not undergoing HD was investigated [23], and the use of UDCA up to 900 mg/day was approved for use by chronic hepatitis C patients after April 2007 in Japan. However, the effect of UDCA was not fully elucidated in HCV carriers with HD. Therefore, in this retrospective study we investigated the clinical significance of biochemical markers in the natural course of disease with particular emphasis on PLT and assessed the effect of oral UDCA on serum biomarkers in those patients with HCV.

Materials and methods

Study population

The patients in this study were retrospectively recruited. This study was approved by the Kagoshima University Graduate School of Medical and Dental Sciences. The study population consisted of patients who were on HD in August 2008 and whose data were obtained at least 3 years before August 2008 at 17 HD facilities in Kagoshima, Japan. Their alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), total cholesterol (TC) and PLT were monitored once or twice each month. In 2539 patients, 243 patients were positive for anti-HCV, 143 patients were excluded because they were positive for hepatitis B virus surface (HBs) antigen, they were positive for anti-HCV antibody (anti-HCV) but were not examined for HCV RNA, they had received antiviral treatment or they had hepatocellular carcinoma (HCC). The final population enrolled in this study consisted of 100 patients. Among this cohort of 100 HD patients who were both anti-HCV- and HCV RNA-positive, 84 subjects had not received UDCA and were enrolled in study 1 (HD + HCV Group) and 16 subjects had already received 300 mg/day UDCA for at least 3 months after April 2007 when UDCA up to 900 mg/day was approved for use by chronic hepatitis C patients in Japan and were enrolled in study 2 (UDCA Group). The control subjects in study 1 were 154 HD patients who were anti-HCV-negative (HD Group), and the controls in study 2 were the 84 HD patients among the study 1 population who were both anti-HCV- and HCV RNA-positive but had not received previous treatments including UDCA and were observed until August 2008 (non-UDCA Group). Of the 84 HD patients who were controls in study 2, 2 patients died before November 2008. Blood samples were obtained before routine HD procedures and then were used to assay for ALT, AST, GGT, TC and PLT. The relationship of these markers to PLT and the percent change in PLT were examined, and the percent change in PLT was calculated according to the formula: $\Delta\%PLT = [PLT$ (at the end of study) − PLT (at enrollment)]/PLT (at enrollment) × 100.

Serum HCV markers

Serum anti-HCV and HBsAg were determined using a commercially available third-generation enzyme-linked immunosorbent assay and anti-HBs assay, respectively. For anti-HCV antibody-positive patients, HCV RNA was quantified using the COBAS TaqMan HCV kit (COBAS AmpliPrep/COBAS TaqMan HCV assay, Roche Diagnostics, Tokyo, Japan) during the follow-up period. The serologically defined HCV genotype (HCV serotype) was also determined with a serological genotyping assay kit (Immunocheck F-HCV Grouping, International Reagents Co., Tokyo, Japan). In some patients, the HCV genotype was examined (HCV Core Genotype, SRL, Tokyo, Japan). HCV genotype 1b was included with serotype I, and genotypes 2a and 2b with serotype II. No other HCV genotype was detected in this study population.

Study 1

The HD + HCV Group, which contained 84 HD patients with HCV, was compared to the HD Group, which contained 154 HD anti-HCV-negative patients. We compared the basal characteristics at enrollment and the changes in PLT during the follow-up period between the two groups. In addition, we divided the HD + HCV patients into the following four groups according to the average ALT level of all available ALT levels during the follow-up period: Group A, ALT < 15; Group B, $15 \leq$ ALT < 20; Group C, $20 \leq$ ALT < 30; and Group D, $30 \leq$ ALT. Clinical characteristics at baseline or average ALT levels and change in PLT during the follow-up period were compared between these four groups.

Study 2

Sixteen patients with HD and HCV had been treated with 300 mg/day UDCA orally for at least 3 months after April 2007, when UDCA up to 900 mg/day was approved for chronic hepatitis C patients, until August 2008 (UDCA Group). These patients were observed every month for at least 6 months before the administration of UDCA and then monitored for the efficacy of UDCA for more than 3 months until August 2008. Then, these patients were observed for a total of at least 6 months until November 2008. We compared the basal characteristics between the UDCA Group just before UDCA treatment and the non-UDCA Group in May 2008. In addition, the changes in ALT, AST, GGT and PLT during the follow-up period were compared between the two groups. For example, the percent of ALT was calculated according to the formula: %ALT = [ALT (−6, 0, 1, 2, 3 or 6 M]/ALT[0 M] $\times$ 100).

Statistical analysis

When appropriate, χ^2 test, Fisher's exact test, Student's *t* test and Mann–Whitney *U* test were used to compare the frequencies or means. Logistic regression models were used for calculating the odds ratios (ORs), 95% confidential intervals (CIs) and *P* values. Statistical analyses were performed using STATVIEW (version 5.0; Abacus Concepts, Berkeley, CA), or SPSS (SPSS Inc., Chicago, IL) software programs. A *P* value less than 0.05 was considered statistically significant.

Results

Demographic characteristics of study 1 subjects

As shown in Table 1, 84 HD patients among the anti-HCV-positive patients were HCV carriers (positive for HCV RNA). One hundred fifty-four HD patients were anti-HCV-negative. On average, the frequency of males, levels of AST, ALT and GGT were higher and TC and PLT were lower at baseline in patients with HCV than those in patients without HCV. The follow-up period was also shorter in patients with HCV than those in patients without HCV. In contrast, there were no significant differences between the two groups with respect to age and duration of dialysis.

Table 1 Baseline characteristics of hemodialysis patients

	HCV (+)[a]	HCV (−)[b]	*P* value
Number	84	154	
Age (year)	64.4 ± 10.3	62.2 ± 12.5	0.165
Sex (male/female)	54/30	77/77	0.034
Duration of HD (years)	13.5 ± 9.6	11.8 ± 7.4	0.669
Follow-up period (months)	56.8 ± 15.8	62.2 ± 7.9	0.039
HCV RNA (Log IU/mL)[c]	4.9 ± 1.4	–	
Serotype (I/II/undetermined)[c]	59/21/4	–	
AST (IU/L)	19.7 ± 8.5	14.9 ± 6.7	<0.001
ALT (IU/L)	18.5 ± 9.3	13.2 ± 7.1	<0.001
GGT (IU/L)	41.5 ± 43.0	30.1 ± 42.1	0.002
TC (mg/dl)	153.7 ± 41.0	167.1 ± 35.0	0.003
PLT ($\times 10^5$/μl)	1.59 ± 0.53	1.93 ± 0.73	<0.001

Unless otherwise indicated, data are given as the mean ± SD or number of patients

HD hemodialysis, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *GGT* gamma-glutamyl transpeptidase, *TC* total cholesterol, *PLT* platelet count

[a] HCV (+), both anti-HCV antibody and HCV RNA positive

[b] HCV (−); anti-HCV antibody negative

[c] HCV RNA and serotype were examined during follow-up period

Predictors of thrombocytopenia in HD patients

Table 2 summarizes the results of a univariate analysis of factors associated with thrombocytopenia (PLT $< 1.3 \times 10^5$/μl) at the end of study 1 (August 2008) using 9 baseline characteristics in all HD patients with or without HCV. Older age, HCV viremia, elevated AST, ALT, and GGT levels were significantly associated with thrombocytopenia. In addition, a multivariate analysis revealed that HCV viremia was independently associated with thrombocytopenia (Table 2). Furthermore, after the 60.3-month mean follow-up period (mean of HD + HCV Group, 56.7 months; HD Group, 62.2 months), PLT in the HD + HCV Group had decreased (from 1.59×10^5/μL to 1.22×10^5/μL) significantly compared to that in the HD Group (from 1.93×10^5/μL to 1.77×10^5/μL) (average Δ%PLT in each patient: −22.4 vs. −5.3%, $P < 0.001$). Variables that were statistically significant by a univariate analysis were further analyzed to identify variables that

Table 2 Univariate and multivariate analyses of variables associated with thrombocytopenia (< 1.3 × 10^5/μl) in HD patients

Variables	Odds ratio	95% CI	*P* value
Univariate analysis			
Age (years)			
<60	1.0		
≥60	1.994	1.141–3.484	0.015
Sex			
Female	1.0		
Male	1.494	0.868–2.571	0.147
Duration of dialysis (years)			
<10	1.0		
≥10	1.065	0.624–1.818	0.816
Follow-up period (months)			
<55	1.0		
≥55	0.727	0.4–1.321	0.296
HCV			
(−)	1.0		
(+)	4.533	2.555–8.043	<0.0001
AST (IU/L)			
<30	1.0		
≥30	7.741	2.095–28.603	0.002
ALT (IU/L)			
<20	1.0		
≥20	3.793	2.017–7.133	<0.0001
GGT (IU/L)			
<50	1.0		
≥50	2.836	1.396–5.758	0.004
TC (mg/dl)			
<150	1.0		
≥150	0.58	0.296–1.135	0.112
Multivariate analysis			
Age (years)			
<60	1.0		
≥60	1.783	0.937–3.394	0.078
HCV			
(−)	1.0		
(+)	2.589	1.317–5.091	0.006
AST (IU/L)			
<30	1.0		
≥30	5.123	0.996–26.339	0.050
ALT (IU/L)			
<20	1.0		
≥20	1.75	0.786–3.896	0.171
GGT (IU/L)			
<50	1.0		
≥50	1.743	0.783–3.88	0.174

Abbreviations as in Table 1

were independently associated with a more than 20% decrease in PLT. As a result, male sex (OR 2.375; 95% CI, 1.319–4.278; $P = 0.004$) and HCV viremia (OR 2.339; 95% CI, 1.295–4.224; $P = 0.005$) were factors that were independently associated with more than a 20% decrease in PLT.

Predictors of thrombocytopenia in HD patients with HCV

Table 3 summarizes the results of a univariate analysis of factors associated with thrombocytopenia (PLT < 1.3 × 10^5/μL) at the end of study 1 (August 2008) using 10 baseline characteristics in HD patients with HCV. The patients with HCV and thrombocytopenia had significantly higher frequencies of elevated ALT and GGT levels at baseline. However, age, sex, duration of HD, follow-up period, history of diabetes mellitus (DM), and elevated AST and TC levels were not significantly different between patients with and without thrombocytopenia. In addition, elevated ALT and GGT levels at baseline were not significantly associated with thrombocytopenia in patients with HCV by a multivariate analysis.

On the other hand, a univariate analysis that compared a decrease in PLT of more than 20% with a decrease less than 20% revealed that male sex and elevated ALT levels at baseline were associated with decreased PLT in patients with HCV. A multivariate analysis of two variables that were statistically significant by a univariate analysis also revealed that high ALT levels (ALT ≥ 20 IU/L) at baseline were independently associated with decreased PLT in patients with HCV (OR 3.318; 95% CI, 1.256-8.764; $P = 0.016$).

Furthermore, we divided patients with HCV into four groups according to average ALT levels during the follow-up period. As Table 4 shows, 30, 19, 18 and 17 patients were in Groups A, B, C and D, respectively. Age, duration of dialysis, follow-up period, HCV RNA levels, distribution of HCV serotype, frequency of diabetes mellitus, TC levels and PLT were not significantly different between the four groups. However, serum AST levels and ALT levels at baseline were significantly different, and these levels gradually increased from Group A to D. The distribution of sex was also significantly different and the frequency of males was higher in Groups B, C and D than in Group A. The decreasing rate of change in PLT was significantly higher in Groups B, C, and D compared to Group A (Fig. 1). In addition, the average ALT levels (≥ 15 IU/L) during the follow-up period were independently associated with thrombocytopenia (OR 3.882; 95% CI, 1.257–11.987;

Table 3 Univariate and multivariate analyses of variables associated with thrombocytopenia (PLT < 1.3 × 10^5/ul) in HD + HCV patients

Variables	Odds ratio	95% CI	*P* value
Univariate analysis			
Age (years)			
<60	1.0		
≥60	0.616	0.247–1.534	0.298
Sex			
Female	1.0		
Male	1.273	0.518–3.129	0.599
Duration of HD (years)			
<10	1.0		
≥10	1.321	0.555–3.141	0.529
Follow-up period (months)			
<55	1.0		
≥55	1.057	0.445–2.515	0.899
History of diabetes mellitus			
−	1.0		
+	1.426	0.557–3.646	0.459
Serotype			
I	1.0		
II	1.051	0.384–2.871	0.923
AST (IU/L)			
<30	1.0		
≥30	3.4	0.676–17.103	0.138
ALT (IU/L)			
<20	1.0		
≥20	2.686	1.083–6.662	0.033
GGT (IU/L)			
<50	1.0		
≥50	4.333	1.235–15.206	0.022
TC (mg/dl)			
<150	1.0		
≥150	0.727	0.27–1.958	0.528
Multivariate analysis			
ALT (IU/L)			
<20	1.0		
≥20	1.972	0.665–5.847	0.221
GGT (IU/L)			
<50	1.0		
≥50	3.305	0.876–12.467	0.078

Abbreviations as in Table 1

$P = 0.018$) by multivariate analysis using two variables including average ALT levels and GGT at baseline. The average ALT levels were also associated with decreased PLT (OR 4.470; 95% CI, 1.571–12.719; $P = 0.005$) by multivariate analysis using average ALT levels and sex. These results indicate that the clinical course of ALT levels is associated with thrombocytopenia and a decrease in PLT in patients with HCV.

Demographics of HD patients with HCV who were treated with UDCA

We enrolled 16 HD patients with HCV who were treated with 300 mg/day UDCA orally for more than 3 months in August 2008, and compared these patients (UDCA group) to 84 HD patients with HCV who were not treated with UDCA (non-UDCA group). The UDCA group and non-UDCA group showed similar demographics in regard to age, sex, HCV RNA levels, distribution of HCV serotype, GGT and PLT. The UDCA group, however, had a shorter duration of dialysis and higher AST and ALT levels just before UDCA administration compared to those in the non-UDCA group in May 2008 (Table 5).

Efficacy of UDCA in HD patients with HCV

After administering UDCA, percent of ALT and AST significantly decreased after one month and remained constant up to 6 months compared to the non-UDCA group (Fig. 2). Percent of GGT also significantly decreased after 2 months of UDCA treatment compared to the non-UDCA group. In addition, ALT, AST and GGT levels significantly decreased after UDCA treatment compared to levels before treatment, but PLT did not change during the 6 months of UDCA treatment (Fig. 2). In contrast, serum AST, ALT, GGT and PLT in the non-UDCA group did not change during the 6-month period from May 2008 to November 2008.

Discussion

Our study indicated that HD patients persistently infected with HCV are at risk for thrombocytopenia (less than 1.3 × 10^5/μL) and a decrease in PLT (more than 20%), although the exact dates of HCV infection were not clear in our study population. In addition, the basal or clinical course of ALT levels appears to predict decreased PLT or thrombocytopenia in patients with HCV. In this study population, the prevalence [243 anti-HCV positive among 2539 HD patients (9.6%)] and age distribution (average age was 63 years old) of anti-HCV antibody-positive subjects and the frequency of the HCV serotype I (74%) were similar to previous reports on HD patients with HCV in Japan [24–26], suggesting that the clinical course of anti-HCV-positive subjects in this study reflects those in Japan as a whole.

It is known that patients on HD often have thrombocytopenia [27], and there is a negative correlation between the dialysis period and PLTs [27, 28]. It was also reported that megakaryocytes are produced at lower levels in the bone marrow [28], platelets are destroyed due to the

Table 4 Baseline characteristics of four groups of HD patients with HCV according to the clinical course of average ALT levels

Average ALT	A; ALT < 15	B; 15 ≤ ALT < 20	C; 20 ≤ ALT < 30	D; 30 < ALT	*P* value
Number	30	19	18	17	
Age (years)	67.8 ± 10.8	60.8 ± 10.6	64.0 ± 9.7	63.1 ± 8.7	0.105
Sex male/female	11/19	15/4	14/4	14/3	0.001
Duration of dialysis (years)	14.4 ± 10.7	14.2 ± 9.2	12.8 ± 8.8	11.7 ± 9.1	0.945
Follow-up period (months)	53.2 ± 14.3	55.4 ± 16.4	64.2 ± 16.0	57.5 ± 16.3	0.290
HCV-RNA (Log IU/mL)	4.9 ± 1.6	4.8 ± 1.3	5.2 ± 1.2	4.8 ± 1.4	0.774
HCV Serotype (I/II/undetermined)	21/7/2	13/6/0	13/4/1	12/4/1	0.949
History of diabetes mellitus (-)/(+)	23/7	12/7	12/6	10/7	0.592
AST (IU/L)	15.0 ± 4.7	19.8 ± 8.6	22.8 ± 9.8	24.9 ± 8.0	<0.001
ALT (IU/L)	10.4 ± 4.1	19.3 ± 6.8	22.3 ± 8.0	27.8 ± 7.9	<0.001
GGT (IU/L)	21.3 ± 15.2	34.8 ± 22.1	81.2 ± 71.2	48.5 ± 35.2	<0.001
TC (mg/dl)	149.7 ± 31.4	152.3 ± 46.1	154.9 ± 37.0	161.2 ± 57.2	0.970
PLT ($\times 10^5/\mu l$)	1.62 ± 0.55	1.62 ± 0.61	1.46 ± 0.42	1.64 ± 0.51	0.764

Abbreviations as in Table 1

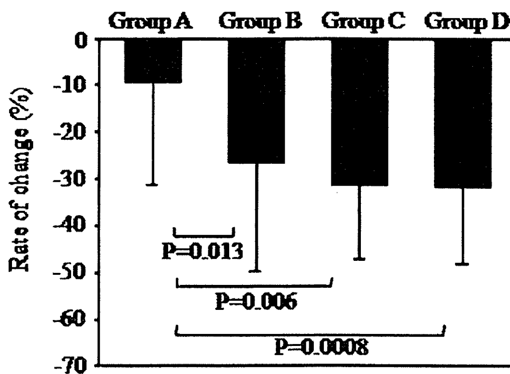

Fig. 1 Comparison of the rate of change in platelet counts by average alanine aminotransferase (ALT) levels during the follow-up period. Group A, average ALT < 15; Group B, 15 ≤ average ALT < 20; Group C, 20 ≤ average ALT < 30; Group D, 30 ≤ average ALT

Table 5 Demographics of HD patients with HCV who were treated with UDCA

	UDCA[a]	Non-UDCA[b]	*P* value
Number	16	84	
Age (years)	66.4 ± 8.6	69.2 ± 10.2	0.261
Sex male/female	9/7	54/30	0.743
Duration of dialysis (years)	6.5 ± 6.6	18.2 ± 9.9	<0.001
HCV-RNA	4.1 ± 2.6	4.9 ± 1.4	0.918
Serotype (I/II/undetermined)	12/4/0	59/21/4	0.669
AST (IU/L)	30.2 ± 24.2	19.2 ± 10.2	0.008
ALT (IU/L)	25.3 ± 16.9	17.1 ± 9.9	0.004
GGT (IU/L)	32.3 ± 23.4	41.4 ± 39.1	0.793
PLT ($\times 10^5/\mu l$)	1.55 ± 0.56	1.39 ± 0.56	0.577

Abbreviations as in Table 1

[a] Data was obtained at just before the treatment period

[b] Data was obtained in May 2008

appearance of the anti-platelet antibodies [28, 29] and uremic materials reduce the effects of hemopoietic cells [30]. In our study, PLT in HD patients without HCV was significantly decreased after 62.2 months (−5.3%). However, PLT decreased even more dramatically in HD patients with HCV after 56.7 months (−22.4%) compared to patients without HCV. In addition, persistent HCV infection was independently associated with thrombocytopenia and a decrease in PLT in HD patients by a multivariate analysis, but dialysis period was not associated with those. Although the data regarding liver histology and serum markers of hepatic fibrosis were lacking in our study, it has also been reported that severe hepatic fibrosis is associated with thrombocytopenia in HCV carriers with end-stage renal disease [19]. These results suggest that thrombocytopenia is more associated with HCV viremia than with the HD procedure or dialysis period in HD patients.

Hepatocellular carcinoma (HCC) and hepatic failure are critical complications in HCV patients, even in those undergoing HD [10, 31]. These complications occur more frequently in patients with advanced hepatic fibrosis [32, 33]. It has been reported that hepatic fibrosis can be predicted by thrombocytopenia in chronic hepatitis C patients with or without HD [19, 34]. In addition, hepatitis is usually assessed by ALT levels, and changes in ALT levels have been shown to be the most important factor that affects hepatic fibrosis in chronic hepatitis C patients without HD [11, 12]. In this study, we showed that basal ALT levels are associated with thrombocytopenia by a univariate analysis and with decreased PLT by a multivariate analysis. The clinical

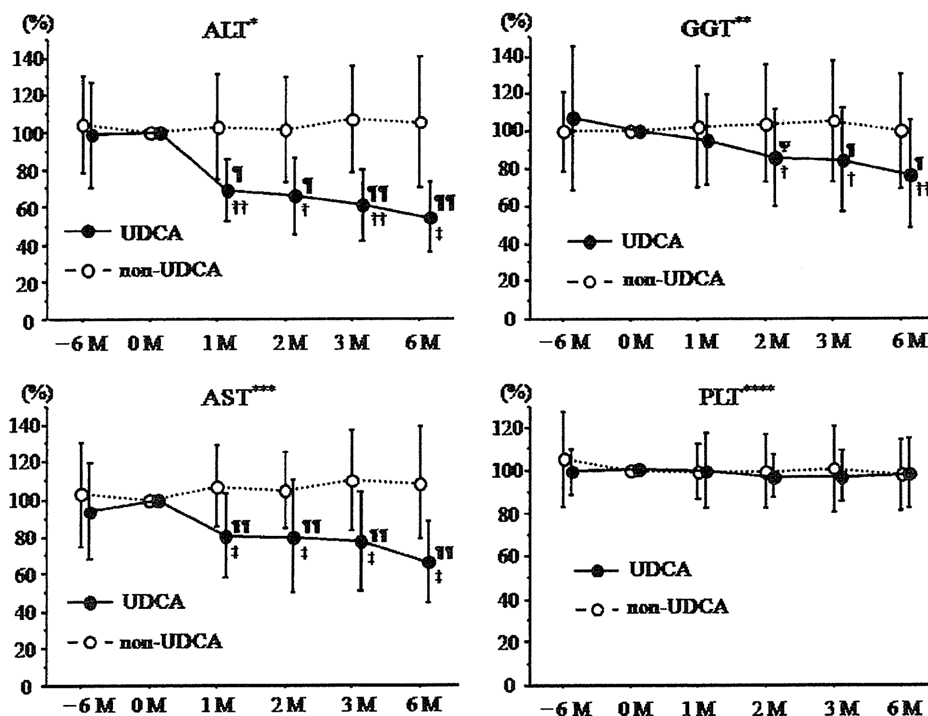

Fig. 2 Efficacy of ursodeoxycholic acid (UDCA) in hemodialysis (HD) patients with hepatitis C virus. Percent of ALT, AST, GGT and PLT in the UDCA group ($n = 16$) 6 months (−6 M) or just (0 M) before and during the treatment period [1, 2, 3 or 6 months (M)] compared to patients in the non-UDCA group ($n = 84$ excluding 6 M) in December 2007 (−6 M), May 2008 (0 M), June (1 M), July (2 M), August (3 M) or November 2008 (6 M, $n = 82$; two patients died before November 2008). Data are expressed as mean ± standard deviation. Closed (*black*) and open circles indicate the UDCA group and non-UDCA group, respectively. The percent of ALT was calculated according to the formula: %ALT = (ALT[−6 M, 0 M, 1 M, 2 M, 3 M or 6 M]/ALT[0 M]) × 100. *ALT* alanine aminotransferase, *AST* asparate aminotransferase, *GGT* gamma-glutamyl transpeptidase, *PLT* platelet count. $^{\Psi}P < 0.05$ (UDCA vs. non-UDCA). $^{¶}P < 0.01$ (UDCA vs. non-UDCA), $^{¶¶}P < 0.001$ (UDCA vs. non-UDCA), $^{\dagger}P < 0.05$ (vs. 0 M), $^{\dagger\dagger}P < 0.01$ (vs. 0 M), $^{\ddagger}P < 0.001$ (vs. 0 M)

course of ALT is also associated with these clinical changes. These results indicate that ALT is an important predictor of thrombocytopenia which should be associated with hepatic fibrosis in HD patients with HCV. In contrast, serum ALT levels are significantly lower in chronic hepatitis C patients on HD than in chronic hepatitis C patients with normal renal function [19]. It was reported that a vitamin B6 deficiency [35], uremic toxins [36], or ultraviolet-absorbing materials [37] are associated with low ALT levels in HD patients. Furthermore, ALT levels have been reported to predict liver disease-related deaths in HD patients, even when ALT levels are in the normal range [38, 39]. Our study also revealed that both patients with abnormal ALT levels (Group D) and normal ALT levels close to the ULN (Groups B and C) had a significant decrease in PLT compared to patients with low ALT levels (Group A). These findings suggest that ALT levels can be used to assess liver damage in HD patients with HCV, although the normal range of ALT should be determined in those patients with HCV in a large cohort study or by liver biopsy.

HCV carriers with persistently normal ALT (PNALT) are more often females than chronic hepatitis C patients with abnormal ALT [40]. This distinction is likely due to lifestyle differences such as alcohol consumption [40], hormonal factors [41] or lower serum iron levels [42]. Although the normal range of ALT in HD patients with HCV may be different compared to the range in HCV carriers with normal renal function, our study demonstrated that females are more likely to have lower ALT levels, even in HD patients (Table 4). This difference in sex may also affect the decrease in PLT. In contrast, the frequency of serotype II, which is reportedly higher in PNALT patients than in chronic hepatitis C patients with abnormal ALT [43], was not different between the four groups in this study (Table 4). A further analysis of the factors associated with elevated ALT levels in those patients with HCV is required.

Interferon therapy has been shown to improve hepatic fibrosis [44] and to reduce the occurrence of HCC in chronic hepatitis C patients with normal renal function. Compared to untreated patients, the risk of HCC after interferon treatment in patients who did not achieve a virological response was shown to be 0.20, 0.36 and 0.91 in chronic hepatitis C patients whose ALT levels were

normal, moderately elevated (less than twice the upper normal limit) and highly elevated, respectively [45]. These results indicate that ALT might predict the mortality of patients with liver-related diseases who have or have not received interferon treatment. Although lower serum ALT levels decreased the risk of HCC, biochemical and virological responses were limited [20, 46] and HD was one of the factors associated with patients who did not respond to interferon and ribavirin treatment [21, 22]. Other therapies that lower serum ALT levels but do not involve interferon-based treatment need to be investigated. Recently, it has been established that UDCA up to 900 mg/day dose-dependently improves biochemical indices such as serum ALT, GGT and bilirubin [23]. Although UDCA seems to lower serum ALT levels, the risk of liver fibrosis, and possibly the incidence of hepatocellular carcinoma, liver histology, serum hepatic fibrosis markers and prognosis (including the incidence of HCC) should also be evaluated over a long time period in HCV carriers with or without HD.

Our study had several limitations; a small number of patients was simply treated with UDCA as routine care, selection of patients depended on each physician and then the data collected retrospectively after a specified duration of therapy. However, this study showed that UDCA effectively had reduced serum ALT, AST and GGT levels in HD patients with HCV. Interestingly, UDCA decreased ALT levels even in patients with normal ALT levels less than 30 IU/L (data not shown). Therefore, HCV patients with normal ALT levels should also be considered for the indication of treatment.

Although the patients in this study were treated with 300 mg/day UDCA, it has also been reported that a 600 mg/day dose of UDCA more effectively decreases ALT and AST levels than a 150 mg/day dose in chronic hepatitis C patients with normal renal function [23]. In addition, PLT did not change during UDCA treatment. Future studies need to investigate the dose-dependent effects of UDCA on ALT levels and prospective double-blind UDCA treatment over a long period in HD patients with HCV.

In conclusion, HCV viremia and ALT levels at basal conditions and during the clinical course of disease were associated with thrombocytopenia and decreased PLT in HD patients. We recommend that HCV carriers on HD who have ALT levels greater than 15 IU/mL be considered for treatment. In addition, UDCA should be considered for HD patients who have chronic hepatitis due to HCV infection but cannot receive interferon-based therapy.

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NOTE

Presence of multiple copies of capsulation loci in invasive *Haemophilus influenzae* type b (Hib) strains in Japan before introduction of the Hib conjugate vaccine

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ABSTRACT

Despite the effectiveness of the Hib vaccine, multiple amplification of the *cap*b locus contributes to vaccine failure. However, there has been no report on the effect of Hib locus amplification in Japan. We examined 24 Hib strains from Japanese children with invasive diseases due to Hib. Although all strains showed the same *cap*b sequence, Southern blot analysis showed that four strains (16.7%) harbored multiple copies (more than two) of the *cap*b locus. Careful analysis of the locus in circulating Hib strains is necessary now that the Hib vaccine has been introduced into Japan.

Key words capsular polysaccharide, *Haemophilus influenzae* type b, Hib conjugate vaccine.

Hib occasionally causes invasive bacterial diseases such as meningitis, epiglottitis and sepsis, especially among young children. Hib conjugate vaccines, which consist of capsule polysaccharide conjugated with carrier protein, are very effective and safe. Since the Hib conjugate vaccine was introduced in Europe and America in the 1990s, the incidence of invasive Hib disease has decreased dramatically in many countries (1). However, despite the efficacy of the Hib vaccine, an increased number of cases of the rare invasive Hib diseases (i.e. cases of true vaccine failure) have now been reported in Europe in fully vaccinated children (2–5). Although possibly contributory host factors such as lower avidity of the anti-Hib antibody are known to occur (6, 7), amplification of the capsulation locus may also have contributed to vaccine failure (8, 9).

Type b polysaccharide capsules, polymers of PRP, are cell-surface components that serve as major virulence factors against host defense mechanisms. The genes involved in Hib capsule expression are found within the *cap*b locus, an 18-kb DNA segment of the chromosome (10). Most invasive Hib strains contain a partial duplication of the *cap*b locus which consists of one intact copy of the locus, and a second copy with a 1.2-kb deletion region containing the *bex*A gene and an IS1016 insertion element that flanks the locus (10). Polysaccharide capsule production relates to the number of copies of the locus (11). Recently, Cerquetti *et al.* reported that amplification of the *cap*b locus to as many as three to five copies is associated with vaccine failure (8, 9). In addition, Schouls *et al.* found two variants of the capsular gene cluster, designated type I and type II, which were assessed by considerable sequence divergence in the *hcs*A and *hcs*B genes of the *capb* locus. They found that type I strains carry approximately twice as much capsular polysaccharide on the cell surface as type II strains (12).

In Japan, the Hib conjugate vaccine was licensed in January 2007, and introduced in December 2008; however, the vaccination plan has not yet been fully implemented. Although 55% of bacterial meningitis cases in children in Japan were caused by Hib (13), there has been no national

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List of Abbreviations: capsulation b, *capb*; CSF, cerebrospinal fluid; DIG, digoxigenin; Hib, *Haemophilus influenzae* type b; PFGE, pulsed-field gel electrophoresis; PRP, polymers of ribose ribitol phosphate.

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Table 1. Sequence type and number of copies of the *cap*b locus of the 21 *Haemophilus influenzae* strains examined in this study

No. of cases	No. of strains	Detected date (Year/month)	Age (months)	specimen	disease	Ampicillin susceptibility	PFGE pattern	the *cap*b locus		
								Sequence type	Size of band	No. of copies
1	C1650	2004/11	14	blood	bacteremia	R[†]	H	I	45 kb	2
2	K4646	2005/7	9	blood	meningitis	R	G	I	81 kb	4
3	K5003	2005/11	53	blood	meningitis	S[‡]	A1	I	45 kb	2
4	K5154	2006/1	17	CSF	meningitis	S	D	I	45 kb	2
5	K5221	2006/1	5	CSF	meningitis	S	B	I	45 kb	2
6	K5331	2006/2	24	CSF	meningitis	S	E	I	45 kb	2
7	K5545	2006/4	12	blood	cellulitis	-	A1	I	45 kb	2
8	K5625	2006/5	31	CSF	meningitis	R	F	I	45 kb	2
9	K5905	2006/9	19	CSF	meningitis	S	A1	I	45 kb	2
10	K6066	2006/11	7	CSF	meningitis	S	B	I	63 kb	3
11	K6168	2006/12	56	CSF	meningitis	R	B	I	45 kb	2
12	K6519	2007/8	20	CSF	meningitis	S	A1	I	45 kb	2
13	K6803	2007/10	29	blood	epiglottitis	S	A1	I	45 kb	2
14	K6886	2007/12	21	CSF	meningitis	S	A1	I	45 kb	2
15	K6892	2007/12	9	CSF	meningitis	R	A1	I	45 kb	2
16	K6930	2008/1	63	blood	bacteremia	R	A1	I	45 kb	2
17	K6934	2008/1	2	CSF	meningitis	R	A1	I	45 kb	2
18	K7112	2008/3	15	blood	meningitis	S	A1	I	45 kb	2
19	K7448	2008/7	8	CSF	meningitis	S	C	I	45 kb	2
20	K7450	2008/7	7	CSF	meningitis	S	A1	I	45 kb	2
21	K7522	2008/9	14	CSF	meningitis	S	A1	I	45 kb	2
22	K7639	2009/4	4	blood	meningitis	S	A2	I	81 kb	4
23	K7641	2009/4	12	CSF	meningitis	S	A1	I	45 kb	2
24	K7721	2009/5	4	blood	bacteremia	S	I	I	63 kb	3

[†]resistant, [‡]susceptible.

survey of strains isolated from patients with invasive Hib diseases including meningitis. Furthermore, there are no reports on the amplification or sequence divergence of the *cap*b locus. The principle aim of this study was to analyze the number of *cap*b copies, and to assess sequence divergence in the *hcs*A and *hcs*B genes of Hib strains isolated from children with Hib diseases in our district before the introduction of the Hib conjugate vaccine.

A total of 24 Hib strains isolated between November 2004 and May 2009 from 24 children with invasive Hib diseases who had not received Hib conjugate vaccine in Kagoshima Prefecture, Japan, were collected and examined. Of these strains, 15 were isolated from CSF and 9 from blood. The strains were epidemiologically unrelated and individually stored at −80°C. All isolates were identified as serotype b by PCR capsular genotyping (14). PFGE was performed using a CHEF-DR 3 apparatus (Nippon Bio-Rad Laboratories, Tokyo, Japan) according to previously reported methodology (15). Briefly, DNA was digested by *Sma*I and separated on 1% agarose gels by PFGE under the following conditions: current range, 100 to 130 mA at 14°C for 16 hr; initial switch time, 5.3 s, linearly increasing to a final switch time of 49.9 s; angle, 120°; field strength, 6 volts/cm. The gels were stained with ethidium bromide and photographed. A lambda with a size range of 48.5 kb to 1 Mb (BME, Rockland, ME, USA) was used as a size marker. For interpretation of banding patterns separated by PFGE, we referred to the criteria of Tenover *et al.* (16).

Two variants of the *cap*b locus DNA sequence, type I and type II, were determined by PCR using two primer sets targeting the *hcs*A gene which could discriminate between the two capsular genotypes as described in a previous report (12). The DNA sequences of the PCR products were determined by an ABI Prism 310 sequencer (Applied Biosystems Japan, Tokyo, Japan).

The number of *cap*b locus copies was detected by Southern blotting analysis according to previously reported methods (8). Because *Kpn*I and *Sma*I restriction sites flank the *cap*b locus, extracted DNA in an agarose plug was digested with these enzymes, separated by PFGE, and transferred to a nylon membrane. A Hib capsule-specific 480-bp probe was constructed by PCR (14) and labeled with DIG using a DIG high prime DNA labeling kit (Roche Diagnostics, Mannheim, Germany). The membrane was hybridized with the probe and visualized by

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chemiluminescent detection using a DIG detection kit (Roche Diagnostics). The *Kpn* I/*Sma* I fragment of a two copy strain was expected to be 45-kb, because it includes two repeats of the locus (18 + 17 kb) plus additional segments (~10 kb) upstream and downstream of the *cap* region (17). Three-, four-, and five-copy fragments showed increased size in 18-kb increments for each additional copy (63, 81, and 99-kb, respectively) (8).

A summary of results is shown in Table 1. The type I-associated *hcs*A gene was found in all of the strains examined. The DNA sequences of all the PCR products were completely identical. PFGE analysis showed nine distinctive restriction patterns (A to I) among the 24 isolates. Fourteen strains with the A pattern were divided into A1 subtype (13 strains) and the closely-related A2 subtype (one strain). Southern blotting analysis demonstrated that 20 strains showed a two-copy arrangement of the *cap*b locus (45-kb), two strains showed three copies (63-kb), and the other two showed four copies (81-kb) (Fig. 1). The incidence of multiple-copy strains (>two copies) among examined strains was 16.7% (4/24). All of the strains with the dominant PFGE pattern (A1) possessed two copies, while one with the closely-related A2 subtype harbored four copies. The other three strains with multiple copies showed minor PFGE patterns (B, G or I). All the patients infected by strains with multiple copies were treated successfully without neurological or physical sequelae.

Amplified *cap*b sequences were detected more frequently among strains from children with true vaccine failure than among those from unvaccinated children (24% vs. 10%) in the United Kingdom (8). Furthermore, the proportion of strains with multiple copies of the *cap*b locus increased over time in Italy (9). Amplification of the *cap*b locus is associated with decreased susceptibility to complement-mediated lysis and decreased complement-mediated opsonization (11). Thus, amplification of the *cap*b locus may result in the overcoming of host defenses and contribute to vaccine failure. We have found that Hib strains with multiple (three or four) copies of the *cap*b locus were present in Japan before the introduction of the Hib conjugate vaccine. The incidence of 16.7% (4/24) of multiple-copy strains found in our study is slightly higher than that found in the UK between 1991 and 1992 before routine immunization was introduced (10.1%, 9/89) (8). In our study, most of the multiple-copy strains showed rare PFGE patterns. Thus these strains might be selected and involved in vaccine failure after the introduction of Hib conjugate vaccination in Japan.

Sequence typing of the *capb* locus is based on the considerable sequence divergence in the *hcs*A and *hcs*B genes, which are involved in the transport of capsular polysaccharides across the outer membrane (18). Schouls *et al.* have reported that type II strains display less expression of

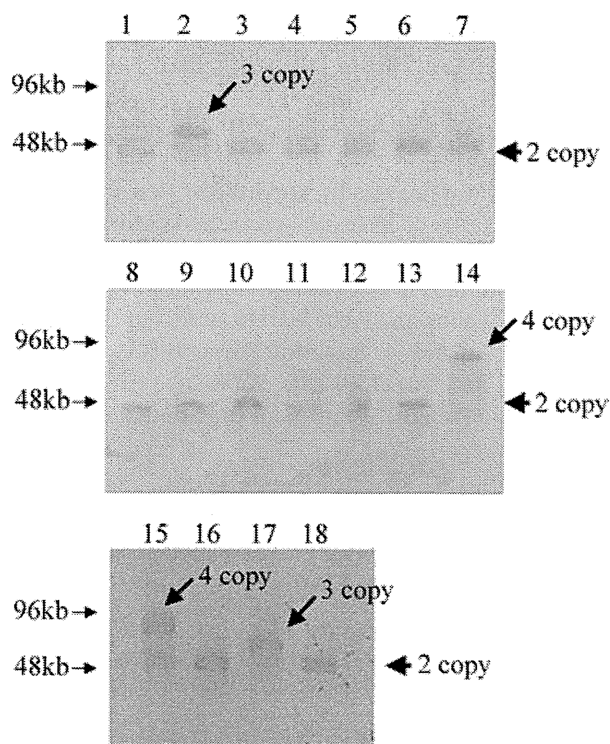

Fig. 1. Examples of Southern blot analysis of DNA from *Haemophilus influenzae* type b strains digested with KpnI/SmaI, separated by PFGE, and hybridized with the 480-bp DIG-labeled *cap*b probe. Strain K6066 in lane 2 and strain K7721 in lane 17 showed three-copy arrangement of the *cap*b locus (ca. 63-kb). Strain K4646 in lane 14 and K7639 in lane 15 had four-locus copies (ca. 81-kb). Other strains had two copies (ca. 45-kb).

capsular polysaccharide than do type I, and were isolated only during the pre-vaccination era in the Netherlands (12). The greater polysaccharide expression may have provided a selective advantage for type I strains, resulting in the rapid elimination of type II. In addition, there have been remarkable differences in the geographic distribution of type I and type II; with a higher incidence in the United States (73%) than the Netherlands (5%) of type II among Hib strains isolated from patients (12). While we did not find type II strains in this study, more Hib strains should be evaluated to clarify the exact incidence.

To our knowledge, this is the first study to investigate *cap*b locus copy number in invasive Hib strains isolated in Japan. We found that multiple-copy strains were in existence in Japan before the introduction of Hib conjugate vaccine. Molecular epidemiological surveillance of invasive Hib strains after the introduction of vaccines will allow prompt detection of any changes in bacterial properties. In addition, because higher antibody concentrations may be required to protect against Hib disease caused by strains with multiple copies of the *cap*b locus, we strongly

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recommend the complete implementation of Hib vaccination in young children in Japan.

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Role of Human Herpes Virus 6 in Corneal Inflammation Alone or With Human Herpesviruses

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Purpose: The purpose of this study was to determine the association of human herpes virus 6 (HHV-6) and/or other human herpesviruses in corneal inflammation using polymerase chain reaction (PCR).

Methods: We collected tear films, conjunctival smears, and a corneal button of inflamed cornea, and the presence of HHV-6 and other herpesviruses in these samples were assessed by a nested PCR.

Results: In tear films collected from 3 of 9 patients with dendritic keratitis, HHV-6 DNA was positive twice, together with herpes simplex virus (HSV) or varicella zoster virus DNA most often, during the acute phase of the disease. Two other patients in this group were either positive for HSV-1 and varicella zoster virus or for HSV-1 and Epstein–Barr virus DNA but negative for HHV-6. When another 12 patients' smear samples from corneal ulcer or keratouveitis were examined, 9 were positive for HHV-6 DNA. Of these, 4 were positive for HSV-1 simultaneously, whereas the remaining 5 patients were negative for HSV-1. One patient's smear was positive for HSV-1 but not for HHV-6. In the corneal button, both HSV and HHV-6 DNAs were positive by nested PCR. HHV-6 was also positive by nested PCR in the conjunctival swab obtained from the contralateral inflamed eye of the patient.

Conclusions: In 22 patients with corneal inflammation, HHV-6 was positive in 14 of 22 patients and HSV-1 was found in 9 of those patients. These data indicated that the association of HHV-6 with disease was more frequent than with other herpesviruses and that HHV-6 may be another sole causative agent for corneal inflammation.

Key Words: human herpesvirus 6, herpes simplex virus, varicella zoster virus, cornea, inflammation, polymerase chain reaction

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Human herpesvirus 6 (HHV-6) was originally isolated from patients' peripheral blood mononuclear cells (PBMCs) with lymphoproliferative disorders and/or patients infected with human immunodeficiency virus in 1986.[1] HHV-6 is classified into 2 groups, variant A (HHV-6A) and variant B (HHV-6B) according to their antigenic and biological characteristics.[2] HHV-6B causes exanthem subitum (ES),[3] whereas HHV-6A–related disease is currently still unknown.

Herpetic keratitis caused by herpes simplex virus type 1 (HSV-1) is a vision-threatening disease. HSV-1 harbored in the trigeminal ganglion frequently reactivates and causes recurrent corneal herpes, which eventually leads to herpetic stromal keratitis (HSK) with dense stromal haze in some patients. Association of HHV-6 in ocular diseases has been amply documented in the literature in which presence of HHV-6 DNA in corneal tissues was described.[4,5]

In the present study, we collected tears, smears of conjunctival and corneal epithelium, and a corneal button. Presence of HSV-1, varicella zoster virus (VZV), and HHV-6 DNAs in these samples was assessed by nested polymerase chain reaction (PCR) to see if HHV-6 associates with HSV-1 or VZV.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

The ethics committee of Hyogo College of Medicine and the National Cancer Institute and local institutional review boards approved the study. Tears were collected from 47 normal eyes, 10 eyes of patients with ES, and 9 eyes of patients with dendritic keratitis by the Schirmer method or by an aspiration of 50-μL saline instilled in the lower conjunctival sac. Conjunctival and corneal epithelial cells were scraped off from 12 patients with corneal ulcers or keratouveitis using a cotton swab. These samples were treated with buffered proteinase K at 60°C for 3 hours, then heated at 95°C to inactivate the enzymatic activity. A herpetic corneal button obtained during the corneal transplant performed in the Ophthalmology Clinic at the National Eye Institute (NEI) was cut in 8-μm thick specimens, which were boiled in sterile distilled water for 15 minutes. Supernatants of these treated samples were used as templates for a single PCR or a nested PCR amplification.

Amplification of Viral DNA

Nested PCR for HHV-6 and dot blot hybridization of PCR products were performed as described previously.[6]

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Briefly, 5 μL of each sample in a 45-μL reaction mixture containing outer primers and Taq polymerase was amplified for 30 cycles, composed of a denaturing step at 90°C for 1 minute, annealing at 62°C, and extension at 72°C for 3 minutes. Five microliters of the PCR product was then further amplified in the second round of PCR using nested primers. HHV-6A DNA, U1102 strain, and HHV-6B DNA, HST strain, served as positive controls. Electrophoresis of these PCR products was performed, and the obtained bands were size identified with ethidium bromide staining. To confirm the results further, transferred bands were hybridized with a corresponding alkaline phosphatase–conjugated oligonucleotide probe. Nested PCRs for HSV-1 were performed as described previously.[7] Briefly, 5 μL of DNAs purified from clinical samples was added to the reaction mixture containing Taq polymerase and outer primers, then amplified 40 cycles at 94°C for 1 minute, 55°C for 2 minutes, and 72°C for 1.5 minutes. Second round PCR was performed using nested primers at the same thermal cycling conditions. The corneal button and smear samples, obtained in the NEI Clinic, were subjected to nested PCR using the protocol by Mitchell et al.[7] Ten microliters of nested PCR product was run on a 4% agarose gel, transblotted to a nylon membrane, and hybridized with a specific internal HSV-1 or HHV-6 probe labeled with digoxigenin.

RESULTS

In the tear films collected from 3 of 9 patients with herpetic dendritic keratitis, HHV-6 DNA was positive for 5 of 6 times together with HSV or VZV DNA during the acute phase of the disease (Table 1). HHV-6 and HSV-1 DNAs were detected on the first day of examination in patient 1. After treating her with a topical acyclovir, HSV-1 DNA disappeared on the following day from the tear film but HHV-6 remained positive. In patients 2 and 3 who had been diagnosed with pseudodendrite, tears were positive for VZV and HHV-6 DNAs. Patient 3 had facial eruptions of zoster. Both were treated with a topical acyclovir, and in patient 2, both viral DNAs disappeared after the initiation of the topical acyclovir applications. However, both viral DNAs remained positive in patient 3 even after treatment with topical acyclovir. Another patient was positive for HHV-6 DNA twice but negative for other herpesviruses. Two patients in this group were either positive for HSV-1 and VZV or for HSV-1 and Epstein–Barr virus DNAs but negative for HHV-6. Although the remaining 3 patients with other forms of keratitis were examined, they were negative for all herpesviruses examined. As a control, tears collected from 47 normal eyes and 10 patients with ES (57) were examined. None of them showed positive results for HHV-6.

We next examined 12 patients' smear samples, of which 8 were obtained from patients with corneal ulcer and 4 from patients with keratouveitis (Table 2). Among them, 9 PCR products were positive for HHV-6 (Fig. 1A) and were further identified as HHV-6B by dot blot hybridization (Fig. 1B). Of these 9 patients, 4 patients were positive for HSV-1 simultaneously. The remaining 5 patients were positive for HHV-6 but negative for HSV-1. One patient with corneal ulcer had a smear that was positive for HSV-1 but not for HHV-6. One patient with uveitis and another patient with corneal ulcer were negative for both viruses. In the corneal button obtained from a patient in the NEI Clinic, who was living in the United States, both HSV and HHV-6 DNAs were positive by nested PCR (Fig. 2). HHV-6 was also positive by nested PCR in the conjunctival swab obtained from the contralateral inflamed eye of the patient (Fig. 2). During the follow-up study, HHV-6 was not detected in the patient's tears and conjunctival swabs at 2 weeks after the corneal allograft transplantation but it was positive in saliva at 4 weeks after the corneal graft operation (data not shown).

TABLE 1. Detection of Herpesvirus DNA in Tear Films

	Time (D)	Symptom	Treatments	Positive PCR
Patient 1	0	DK	ACV	HHV-6, HSV-1
(37 yr, female)	1	DK, herpes labialis	ACV	HHV-6
Patient 2	0	DK	—	HHV-6, VZV
(78 yr, female)	3	DK	ACV	HHV-6, VZV
	9	Cure	IDU	(−)
	41	—	IDU	(−)
Patient 3	0	Zoster, keratitis	ACV	HHV-6, VZV
(77 yr, male)	3	DK	ACV	HHV-6, VZV

ACV, acyclovir; DK, dendritic keratitis; IDU, idoxuridine.

DISCUSSION

We evaluated 22 patients with corneal inflammation. Among them, HHV-6 was positive in 14 of 22 patients and HSV-1 was found in 9 of those patients. Only 5 patients were negative for herpesvirus DNA. These results indicated that the majority of the corneal inflammation was correlated with herpesviruses and that the association of HHV-6 with disease was more frequent than with other herpesviruses. In contrast, HHV-6 DNA was not detected in 47 tear samples obtained from normal individuals and in the 10 tear samples obtained

TABLE 2. Detection of Herpesvirus DNA in Patients With Corneal Ulcer and Keratouveitis

Patient	HHV-6	HSV-1	Others	Diagnosis
1	+	+	−	UC
2	+	+	−	UC
3	+	+	−	UC
4	+	+	−	KU
5	+	−	−	UC
6	+	−	−	UC
7	+	−	−	UC
8	+	−	−	KU
9	+	−	−	KU
10	−	+	−	UC
11	−	−	−	UC
12	−	−	−	KU

+, PCR positive; −, PCR negative; KU, keratouveitis; UC, ulcer of the cornea.

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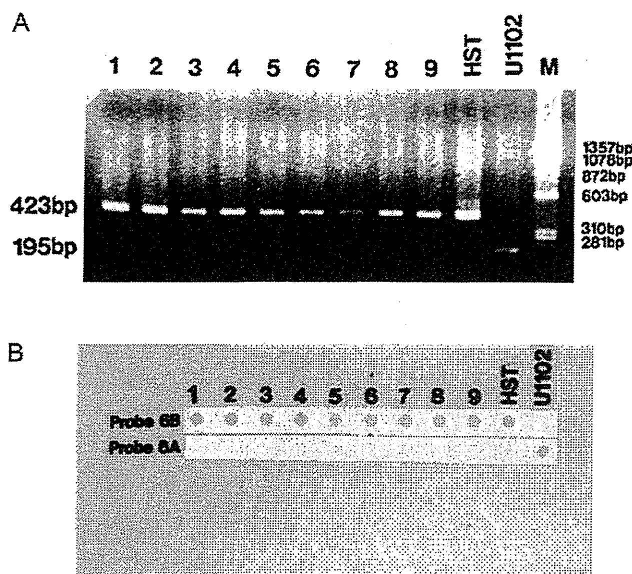

FIGURE 1. A, Agarose gel electrophoresis of 9 PCR-positive samples. U1102 and HST are representative strains of HHV-6A and HHV-6B, respectively. M, molecular size markers. B, Dot blot hybridization of 9 PCR-positive samples with variant A–specific (probe 6A) and variant B–specific (probe 6B) probes. All 9 samples amplified by nested PCR were variant B by size and hybridization with probe 6B.

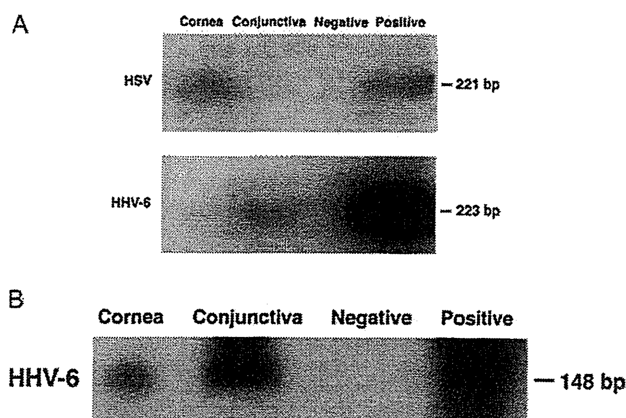

FIGURE 2. A, Detection of HSV and HHV-6 in a patient's sample by PCR. Samples were subjected to PCR followed by gel electrophoresis. Bands were transblotted onto nylon membranes and confirmed by hybridization with specific internal HSV and HHV-6 probes. B, Detection of HHV-6 in a patient's sample by nested PCR. Samples were subjected to nested PCR followed by gel electrophoresis. Transblotted bands were hybridized with a specific HHV-6 probe. Negative, negative control; Positive, positive control.

from patients with ES. Because patients with ES usually present without ocular symptoms and HHV-6 DNA was not detected in patients' secretions such as saliva shortly after onset of the disease as indicated by Suga et al,[8] these results were not unexpected. Two possibilities can be drawn from the results of these PCR analyses. First, HHV-6 is a causal agent of keratitis because in 1 tear and 5 swab samples from 9 patients with dendritic keratitis and 12 patients with corneal ulcer, respectively, HHV-6 DNA was singly detected. Second, HHV-6 reactivates and is associated with the lesions caused by HSV or other agents.

In total, 8 of 15 samples where HHV-6 DNAs were found, other herpesvirus DNAs were also found. Qavi et al[9] reported the possible worsening and long-lasting role of HHV-6 in herpetic keratitis in rabbit. They also found HHV-6 antigens, transcripts, and DNA sequences in the corneal buttons collected from patients infected with human immunodeficiency virus 1 at autopsy.[4,5] In addition, they showed HHV-6 in a few samples of a CCRF-HSB2 T cell line. This cell line is known to support only HHV-6A growth. Most of the HHV-6A strains were detected or isolated from patients with AIDS, whereas all the HHV-6 detected in other immunocompromized patients were variant B.

Robert et al[10] also detected both HHV-6 and HSV-1 DNAs in the tear film from a patient with dendritic keratitis. Recent evidence suggests that HHV-6 infection creates an immunosuppressive milieu. In the fatal immune suppression associated with disseminated HHV-6 infection, patients lack proliferation of antigen specific T lymphocytes.[11,12] Although the mechanisms of HHV-6–induced immune suppression are still largely unknown, several types of evidence have been reported, including the inhibition of interleukin (IL)-12 p70 production by macrophages,[13] a defective antigen presentation, nonmaturation of dendritic cells,[14] and aberrant cytokine production.[11,15–17] We recently found that in HHV-6 infection, $CD4^{+}CD25^{high}$ regulatory T cells suppressed conventional T cells via enhanced IL-10 production.[18] Arena et al[15] reported that PBMCs infected with HHV-6 upregulate IL-10 and downregulate interferon γ production upon lipopolysaccharide stimulation. HHV-6 also inhibits IL-2 release from PBMCs and $CD3^{+}$ and $CD4^{+}$ T cells. Wang et al[19] suggested that IL-10 produced by HHV-6-stimulated $CD4^{+}$ T cells obtained from HHV-6–uninfected individuals suppressed naive $CD4^{+}$ and $CD8^{+}$ T-cell activity. Thus, HHV-6 may enhance disease severity because of its immunosuppressive effect by facilitating HSV-1 replication. In contrast, it may be just a bystander because when HSV-1 causes corneal inflammation, infiltrating T cells or macrophages may carry HHV-6. To answer this question we have to get more clinical samples with clear clinical findings. However, we also observed HHV-6 DNA but not HSV-1 DNA results in the smear samples from 5 of 12 patients (Table 2). These data indicate that HHV-6 may be another causative agent by itself for corneal inflammation. In addition, it is not clear how the resolution of the disease changes the HHV-6 status. Because we did not get the sample after resolution of the disease, further observation and laboratory tests are necessary in the future.

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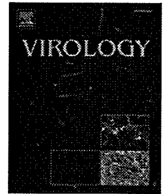

O-sulfate groups of heparin are critical for inhibition of ecotropic murine leukemia virus infection by heparin

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ABSTRACT

There is increasing evidence that soluble glycosaminoglycans such as heparin can interfere with the infectivity of various viruses, including ecotropic murine leukemia viruses (MLVs). The ecotropic MLV, Friend MLV (F-MLV) and the neuropathogenic variants A8 MLV and PVC-211 MLV, were susceptible to heparin-mediated inhibition of infection of NIH 3T3 cells. To investigate the interaction between the envelope glycoprotein (Env) of MLV and heparin, we prepared vesicular stomatitis virus-based pseudotyped viruses carrying the Env of F-, A8, or PVC-211 MLVs. Surface plasmon resonance analyses indicated that the Env of A8 and PVC-211 MLVs had a higher binding activity to heparin than that of F-MLV. We examined the influence of N- or O-sulfation of heparin on binding activity to Env and on the inhibition of the infectivity of MLV and pseudotyped viruses carrying Env. This analysis indicated that the O-sulfate groups of heparin play a major role in determining Env-dependent inhibitory effects.

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Introduction

Previous studies have shown that soluble glycosaminoglycans (GAGs), such as heparin, inhibit the infectivity of ecotropic murine leukemia virus (MLV) (Batra et al., 1997; Guibinga et al., 2002; Jinno-Oue et al., 2001; Le Doux et al., 1996, 1999; Masuda et al., 1997; Walker et al., 2002). Jinno-Oue et al. (2001) found that heparin influences Env-dependent attachment of the virus to the cell surface. By contrast, Walker et al. (2002) and Guibinga et al. (2002) reported that heparin inhibits Env-independent interaction of MLV with target molecules on the cell surface. Infection by ecotropic MLV is mediated by the binding of the viral Env protein to the rodent ortholog of cationic amino acid transporter 1 (CAT-1), which serves as the specific cellular receptor (Albritton et al., 1989; Kim et al., 1991; Wang et al., 1991). Although the Env-receptor interaction appears to be required for membrane fusion and entry of the viral capsid, it has been reported that initial attachment of a retroviral particle to the cell surface can take place in a receptor-independent manner (Guibinga et al., 2002; Pizzato et al., 1999; Walker et al., 2002). The fact that soluble GAGs, such as heparin, inhibit MLV infectivity suggests that cell surface GAGs, such as heparan sulfate, might be involved in the initial attachment (Batra et al., 1997; Guibinga et al., 2002; Jinno-Oue et al., 2001; Le Doux et al., 1996, 1999; Masuda et al., 1997; Walker et al., 2002). Similarly, it has been shown that infection by other enveloped viruses, such as herpes viruses (Neyts et al., 1992; Secchiero et al., 1997; WuDunn and Spear, 1989), respiratory syncytial virus (Krusat and Streckert, 1997) and human immunodeficiency virus (HIV) (Mondor et al., 1998; Patel et al., 1993), are inhibited by heparin, and that cell surface attachment of these viruses involves cell surface GAGs. Thus, the interaction of viral particles with soluble and cell surface GAGs is an important issue for virology in general.

In the present study, we initially investigated the influence of heparin on infection of ecotropic MLVs including Friend MLV (F-MLV) clone 57 (Oliff et al., 1980) and its neuropathogenic variants, A8 MLV (Takase-Yoden and Watanabe, 1997; Watanabe and Takase-Yoden, 1995) and PVC-211 MLV (Kai and Furuta, 1984; Masuda et al., 1992). The infection of neonatal rats with A8 or PVC-211 MLV induces spongiform neurodegeneration without inflammatory infiltrates. The primary determinant for neuropathogenicity of these viruses has been identified as Env. Comparison of the amino acid sequences of A8-Env and PVC-211-Env showed that only 3 of the 676 amino acids differ. F-MLV is non-neuropathogenic and F-Env shows differences at 26 of the 676 amino acids compared to A8-Env. Here, we performed surface plasmon resonance (SPR) analyses to compare the binding activities to heparin of pseudotyped viral particles carrying Env of ecotropic MLVs. To our knowledge, this is the first time that SPR technology has been applied in a quantitative analysis of

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the interaction between viral particles carrying MLV Env and heparin. Heparin is a linear polysaccharide composed of α1-4 linked disaccharide repeating units. The most common unit contains 2-*O*-sulfated iduronic acid and 6-*O*-sulfated, *N*-sulfated glucosamine. The negatively-charged sulfate groups of heparan sulfate (which is structurally related to heparin) are thought to play an important role in its biological activity, such as fibroblast growth factor (FGF) signaling, and to act as an entry receptor for herpes simplex virus type 1 (Capila and Linhardt, 2002; Copeland et al., 2008; Shukla et al., 1999; Xia et al., 2002; Ye et al., 2001). We also examined the relative importance of *N*- or *O*-sulfation of heparin for its inhibitory effects on infection by ecotropic MLVs and on the binding activities to viral particles using chemically modified heparins: *N*-acetylheparin (NA-H), which has diminished *N*-sulfation; de-*N*-sulfated heparin (dNS-H), which completely lacks *N*-sulfation; and *N*-acetyl-de-*O*-sulfated heparin (NAdOS-H), which has markedly diminished *N*-sulfation and completely lacks *O*-sulfation. Taken together, the present investigations have characterized the inhibitory activities of heparin against ecotropic MLV infection of NIH 3T3 cells, while the SPR analysis showed that the binding activity of ecotropic MLV with heparin may be determined by Env amino acid sequences. We also demonstrate that the *O*-sulfate groups of heparin play a major role in inhibiting the infectivity of ecotropic MLV on cells in an Env-dependent manner. The possible mechanisms of the inhibition of heparin against viral infection and the implications of heparan sulfate on the cell surface are discussed.

Results

Inhibition of ecotropic MLV infection by heparin and its derivatives

We compared the effects of heparin and its derivatives on the infectivity of F-, A8, and PVC-211 MLVs using viruses that had been pre-incubated with various concentrations of heparin, or one of the derivatives, in the absence of polybrene. After this pre-incubation step, the virus-heparin or virus-derivative mixture was inoculated onto NIH 3T3 cells at a multiplicity of infection (MOI) of 1 in the presence of 10 μg/ml polybrene. We used polybrene for viral infection, because MLV infections are usually carried out in the presence of polybrene to enhance infection. After 72 h, viral production was evaluated by measuring virion-associated reverse transcriptase (RT) activities in the culture supernatants.

Pre-incubation of the viruses with heparin at concentrations greater than 1 μg/ml resulted in a dose-dependent decrease in viral production (Fig. 1A). The 50% inhibitory dose (ID_{50}) values of heparin for F-, A8, and PVC-211 MLVs were 12.7 ± 4.5, 8.5 ± 0.9, and 13.0 ± 3.3 μg/ml, respectively; these values were not significantly different (Table 1). The structure of the most common disaccharide unit of heparin is composed of 2-*O*-sulfated iduronic acid and 6-*O*-sulfated, *N*-sulfated glucosamine (Fig. 1E). To investigate whether differences in the sulfation patterns of heparin affect the ability to inhibit ecotropic MLV infection, we pre-incubated viruses with a heparin derivative: NAdOS-H, which has markedly diminished *N*-sulfation and no *O*-sulfation; NA-H, which has diminished *N*-sulfation; or dNS-H, which completely lacks *N*-sulfation. NAdOS-H did not show inhibitory effects on infection of NIH 3T3 cells with F-, A8, or PVC-211 MLV even at a concentration of 1000 μg/ml (Fig. 1B and Table 1). In contrast, NA-H inhibited production of F-, A8, and PVC-211 MLVs in infected NIH 3T3 cells, with ID_{50} values of 54.5 ± 4.1, 62.1 ± 2.5, and 53.7 ± 2.8 μg/ml, respectively (Fig. 1C and Table 1); these values were significantly higher than for heparin ($P<0.001$). dNS-H also inhibited infection of NIH 3T3 cells with F-, A8, and PVC-211 MLVs, with ID_{50} values of 76.8 ± 3.9, 65.4 ± 2.3, and 72.4 ± 4.2 μg/ml, respectively (Fig. 1D and Table 1). The ID_{50} values for dNS-H were significantly higher than those of heparin ($P<0.001$).

Preparation of vesicular stomatitis virus (VSV)-based pseudotyped viruses carrying ecotropic MLV Env

Previous studies suggested that heparin affects ecotropic MLV infection during the early steps of viral replication. We used pseudotyped viruses bearing Env to analyze the effects of heparin on the viral replication process from the attachment to gene expression steps. We also sought to clarify the contribution of Env of ecotropic MLV to the inhibitory effects of heparin against viral infectivity by examining this response in the absence of other retroviral proteins, such as Gag and Pol, that might influence heparin-mediated reduction in infectivity. To this end, we prepared VSV based-pseudotyped viruses carrying the Env of F-, A8, or PVC-211 MLV (VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env, respectively) using 293T cells.

Initially, we performed a Western blot analysis using an anti-Env antibody to examine whether Env protein is normally expressed in the 293T cells transfected with F-, A8-, and PVC-211-Env expression vectors, and compared the expression levels of Env in these cells. In the transfected 293T cells, the primary product observed was gp70, although gpr85 was also detectable (lanes 1–3 in Fig. 2). The expression levels of Env protein were similar in cells transfected with F-, A8-, and PVC-211-Env expression vectors. The Env-expressing cells were infected with recombinant VSV, which has the green fluorescence protein (GFP) gene in place of the viral G protein gene, and VSV-pseudotyped viruses carring Env were obtained. As a control, VSV viruses-like particles lacking Env (VSV/ΔEnv) were also obtained as described in the Materials and methods section. Next, we compared the amount of Env protein packaged in VSV-pseudotyped viruses among VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env. In total, 2×10^5 infectious units of VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env were spun down and the precipitates were used in a Western blot analysis. With regard to viral particles, gp70 was observed, and the amount of Env protein showed no appreciable differences among the virions (lanes 5–7 in Fig. 2).

Inhibitory effects of heparin and its derivatives on the infectivity of VSV-based pseudotyped viruses carrying ecotropic MLV Env

We examined the inhibitory effects of heparin and its derivatives on the infectivity of VSV-based pseudotyped viruses carrying ecotropic MLV Env. VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env were pre-incubated with various concentrations of heparin, or a derivative, in the absence of polybrene, and then inoculated onto NIH 3T3 cells at an MOI of 1 in the presence of 10 μg/ml polybrene. After 16 h, GFP-positive cells were counted by fluorescence-activated cell sorting (FACS) in order to evaluate viral infectivity. Heparin was found to inhibit infection of NIH 3T3 cells with VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env (Fig. 3A) with ID_{50} values of 7.0 ± 0.3, 8.0 ± 0.3, and 7.4 ± 0.4 μg/ml, respectively (Table 2). Although NAdOS-H did not inhibit infectivity of VSV/F-Env, VSV/A8-Env, or VSV/PVC-211-Env on NIH 3T3 cells (Fig. 3B and Table 2), high concentrations of NA-H or dNS-H did show inhibition of infection of NIH 3T3 cells (Figs. 3C and D). The ID_{50} values of NA-H (50.1 ± 3.9, 53.4 ± 2.6, and 50.8 ± 3.7 μg/ml for VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env, respectively) and of dNS-H (67.8 ± 1.5, 64.8 ± 2.0, and 71.5 ± 1.8 μg/ml for VSV/F-Env, VSV/A8-Env, and VSV/PVC-211-Env, respectively) were significantly higher than those of heparin ($P<0.001$) (Table 2).

We also sought to compare the effects of heparin and its derivatives on the infectivity of ΔEnv-VSV-pseudotyped viruses. However, the efficiency of GFP gene transduction of the cells with the ΔEnv-VSV-pseudotyped viruses was too low to allow a reliable comparison to be carried out.

SPR analysis of the direct interactions of ecotropic MLV Env with heparin or its derivatives

In order to analyze direct interactions between Env and heparin, we measured the binding activities of VSV/F-Env, VSV/A8-Env, and