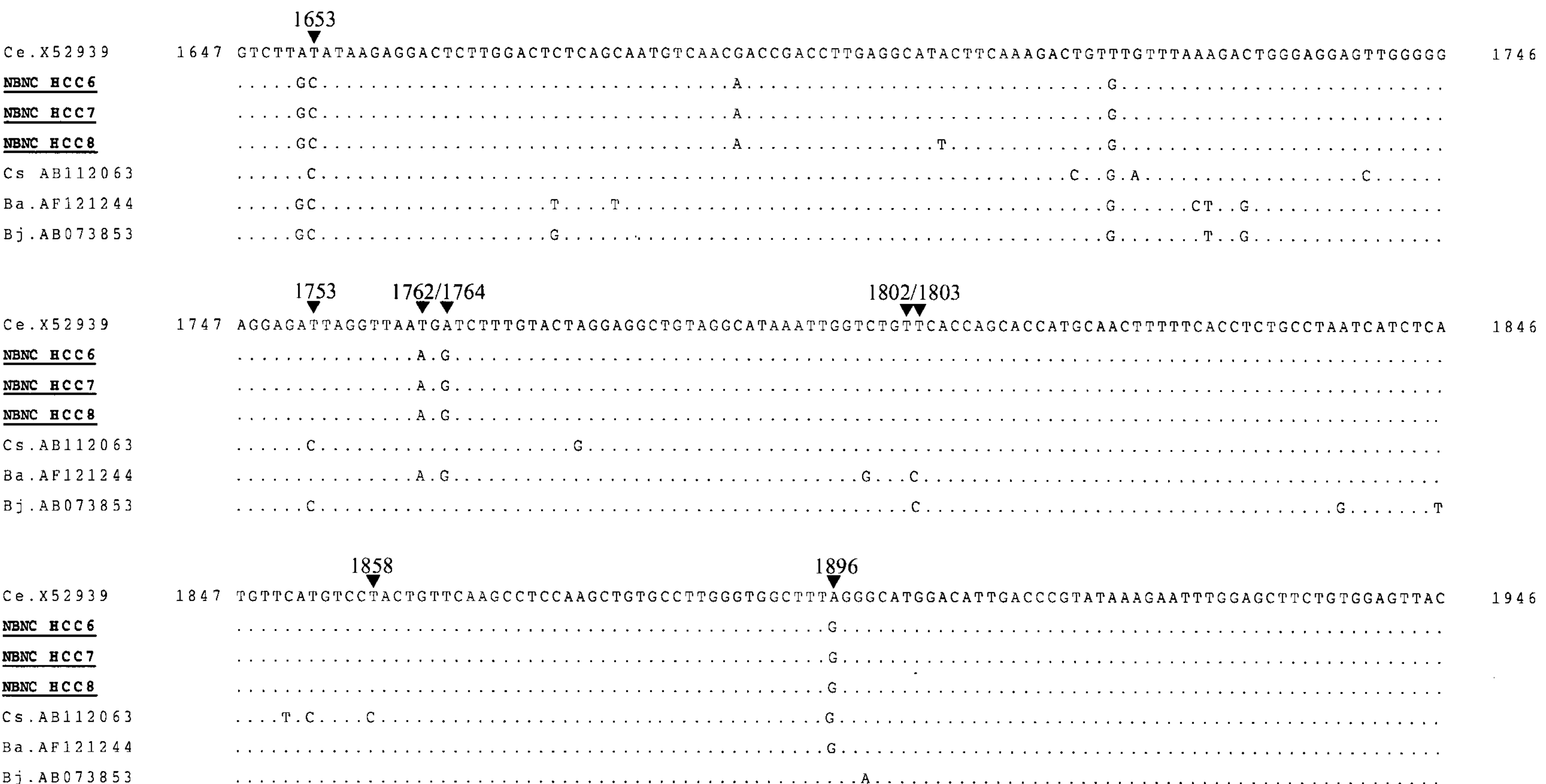

Fig. 3. Alignment of the three non-B non-C patients in regard to enhancer II/core promoter/precore/core region. No specific mutations, such as C1653T, T1753V (not T), or A1762T/G1764A, which are associated with HCC development, were found. All sequences from the database are identified with *accession numbers, followed by subtype. Arrowheads* show representative mutation points of HBV

and HCC) in further investigations, which should include prospective studies.

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Original Article

Accidental exposure to HCV antibody-positive blood in hospital and pre-emptive one-shot interferon alpha-2b treatment

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Aims: Infection with hepatitis viruses following blood exposure accidents, such as needle stick injuries, is a serious issue for medical staff. In particular, although accidental exposure to hepatitis C virus (HCV) occurs frequently, postexposure prophylactic measures have not been established yet. In this study we investigate the efficacy of recombinant α-2b interferon (IFN) as a single, 10 MU intramuscular injection for preventing transmission.

Methods: 264 incidents of accidental blood to HCV antibody-positive blood, occurring between 1993 and 2003 in the Social Insurance Chukyo Hospital, were surveyed. Accident reports, which described in detail the circumstances and the presence or absence of infectious disease in the blood, and accidental exposure to HCV antibody-positive blood was investigated.

Results: Pre-emptive IFN treatment was given in 115 out of 157 cases occurring between 1993 and 1998. One case developed acute HCV. Phylogenetic analysis provided evidence that the accident caused the infection and the patient was cured by immediate IFN therapy. Between 1999 and 2003, the exposed were in principle followed-up without IFN treatment; IFN treatment was only given when requested. As a result, IFN was given in 14 of 107 cases. During this period, no transmission was observed.

Conclusion: Taken together, the benefits of pre-emptive IFN treatment were considered unremarkable and a follow-up without treatment, or immediate IFN therapy after confirmation of the onset, was recommended.

Key words: accidental blood exposure, HCV, medical staff, phylogenetic analysis, pre-emptive interferon

INTRODUCTION

HEPATITIS VIRUS INFECTION following a blood exposure accident, such as a needle stick injury, is a critical issue for medical staff. For the prevention of infection with hepatitis B virus (HBV), regimens of inoculation with a hepatitis B vaccine and the injection of hepatitis B immune globulin have been established.[1,2] However, despite a large number of accidents, measures have not been established for the prevention of hepatitis C virus (HCV) infection. In this study, surveying exposure accidents to blood positive for HCV antibody between 1993 and 2003, we analyzed retrospectively the efficacy of recombinant α-2b interferon (IFN) as a single, 10 MU intramuscular injection for preventing the transmission of HCV.

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METHODS

BLOOD EXPOSURE ACCIDENTS during the 11 years between 1993 and 2003 in the Social Insurance Chukyo Hospital were surveyed. When accidents occurred, accident reports, which described in detail the circumstances and the presence or absence of infectious disease in the blood of the patient source and the exposed individuals were submitted and accidental

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exposure to HCV antibody-positive blood was investigated.

After the accident, circumstances and test results were taken into account and informed consent was obtained; a single 10 MU intramuscular injection of recombinant α-2b IFN was given in principle to prevent transmission of HCV during the six years between 1993 and 1998. During the five years between 1999 and 2003, pre-emptive IFN injection was not given in principle, except when requested by the exposed individual. In all cases, liver function tests and HCV antibody were examined once a month for one year following the accident.

RNA was extracted from the serum of the exposed patient who developed acute hepatitis and the source patient positive for HCV antibody. Nucleotide sequences were determined after amplification of the E1 region by polymerase chain reaction (PCR) and a phylogenetic tree was constructed for analysis.

RESULTS

TABLE 1 SHOWS the number of blood exposure accidents reported, the results of HCV antibody and HCV-RNA testing in the source patients, and the number of cases treated pre-emptively with IFN. One case developed acute hepatitis C during the 11 years between 1993 and 2003. There were 696 accidents in total, 264 and 333 accidents involved blood positive and negative for HCV antibody, respectively, while 99 accidents involved blood of unknown status for HCV antibody.

IFN treatment was given to prevent transmission in 115 out of 157 exposure accidents from blood positive for HCV antibody in all departments of the hospital between 1993 and 1998. The fee was paid by the hospital, but the number of IFN treated cases was only three in the fiscal year 1994, because the exposed individuals had to pay the fee, according to the policy of the hospital. HCV-RNA in the blood from the source patients was measured (by PCR) in 133 of 157 exposure accidents to blood positive for HCV antibody, and 99 and 34 cases were found to be positive and negative, respectively. However, HCV-RNA was not quantified.

Of 157 cases, pre-emptive IFN was given in 73 of 99 exposure accidents with blood positive for HCV-RNA in the source patient. Of these, one case developed acute hepatitis. In 26 cases without IFN treatment, no case developed acute hepatitis. None developed acute hepatitis in 34 cases with negative HCV-RNA or 24 with unidentified HCV-RNA status. During the follow-up period, none of the 157 cases became positive for HCV antibody except the one case who developed acute hepatitis. At this point, pre-emptive IFN treatment under the present regimen was judged to have had no preventive effect against the onset of acute hepatitis C.

Therefore between 1999 and 2003, pre-emptive IFN treatment was not given under the hospital's policy, except at the request of the individual exposed. It was decided to follow up those exposed for one year after their accident. During five years from 1999 through to 2003, there were 107 exposure accidents involving blood positive for HCV antibody and pre-emptive IFN

Table 1 Accidental blood exposure and pre-emptive IFN treatment

Year	Number	Anti-HCV positive	HCV-RNA positive	Preventive IFN	HCV transmission
1993	66	29	19	23	0
1994	66	24	20	3‡	0
1995	66	24	16	20	0
1996	66	28	16	25	0
1997	53	24	12	20	0
1998	70	28	16	24	1
1999	66	27	NT	14†	0
2000	58	23	NT	0	0
2001	63	20	NT	0	0
2002	61	20	NT	0	0
2003	61	17	NT	0	0
Total (93–03)	696	264	99	129	1

†1999 was a transition year when pre-emptive IFN treatment was stopped and only a total of 14 cases received IFN. ‡The number of IFN treated cases was limited to three in fiscal year 1994, because the exposed individuals had to pay the fee for the treatment, according to the policy of the hospital.

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Table 2 Comparing patients exposed to blood positive for HCV who received pre-emptive IFN and those who did not

Patient	Received pre-emptive IFN	Did not receive pre-emptive IFN
Age (mean ± SD)	28.9 ± 7.3	29.1 ± 7.5
Sex (male:female)	31:98	29:106
Episode		
Needle stick	114	113
Surgical knife	8	6
Splash accident	7	16
HCV-RNA		
Positive	73	26
Negative	20	14
Not tested	26	95
HCV transmission	1	0

was given in 14 cases. The fiscal year 1999 was a transition year when pre-emptive IFN treatment was stopped and a total of 14 cases received IFN. No one has received pre-emptive IFN since 2000. Regardless of pre-emptive IFN treatment, none developed acute hepatitis C or seroconverted to HCV antibody during the follow-up period.

Table 2 compares the patients who were exposed to blood positive for HCV who received pre-emptive IFN and those who did not. Age and sex was not correlated significantly with the receipt of pre-emptive IFN treatment. In the circumstances of the accidents, needle stick injuries and cut injuries by knives were not correlated significantly, but in splash accidents to eyes, mouths and skin, the number of patients who did not receive pre-emptive IFN is higher than the number of patients who did receive pre-emptive IFN. In the period in which HCV-RNA was measured in the source patients, between 1993 and 1998, the number of patients who received pre-emptive IFN was more than the number of patients who did not, and in the period when HCV-RNA was not tested, between 1999 and 2003, the number of patients who did not receive pre-emptive IFN is more than the number of the patients who did receive it.

The case that developed acute hepatitis after accidental blood exposure involved a 21-year-old female nurse (K.F.) who complained of general fatigue, anorexia and nausea. On 16 February 1998 she had accidentally pierced her finger deeply with a needle used for blood sampling from a patient positive for HCV antibody. HCV-RNA by the probe method was 1.3 Meq/mL in the blood of the source patient (M.T.). The next day, a single, pre-emptive dose of IFN (10 MU recombinant IFN α-2b) was given. Symptoms appeared at the beginning of March, blood collected on 9 March (three weeks after the needle stick injury) revealed abnormal results (AST 1180, ALT 1657 and T-Bil 3.1) and she was admitted to hospital with a diagnosis of acute hepatitis. Table 3 shows the laboratory results on admission. ALT elevation and coagulation dysfunction were observed. HCV-RNA was positive by PCR and 40 Meq/mL or higher by the DNA probe method, but HCV antibody (second generation) was negative.

Table 3 Laboratory data on admission

Hematology	
WBC	4400/μg
RBC	453×10^4/μg
Hb	13.7 g/dL
Ht	39.1%
PLT	16.9×10^4/μg
Coagulation test	
HPT	39%
PT	12.4 s
(control)	10.9
APTT	38.8 s
(control)	26.6
Biochemistry	
BUN	10 mg/dL
Cre	0.6 mg/dL
NH3	79 μg/dL
Na	137 mEq/l
K	4.4 mEq/l
Cl	104 mEq/l
AST	1185 IU
ALT	1751 IU
LDH	1672 IU
ALP	222 IU
γ-GTP	119 IU
Ch-E	183 I.U
Amy	86 I.U
CPK	52 I.U
T.Bil	3.5 mg/dL
D.Bil	2 mg/dL
ZTT	5 U
TTT	6 U
TP	6.8 g/dL
Alb	4.5 g/dL
T-Cho	106 mg/dL
TG	54 mg/dL
FBS	90 mg/dL
Serological examination	
CRP	0.3> mg/dL
HBsAg	negative
Anti-HCV (2nd)	negative
HCV-RNA (RT-PCR)	positive
HCV-RNA (DNA-probe)	40< MEQ/ML

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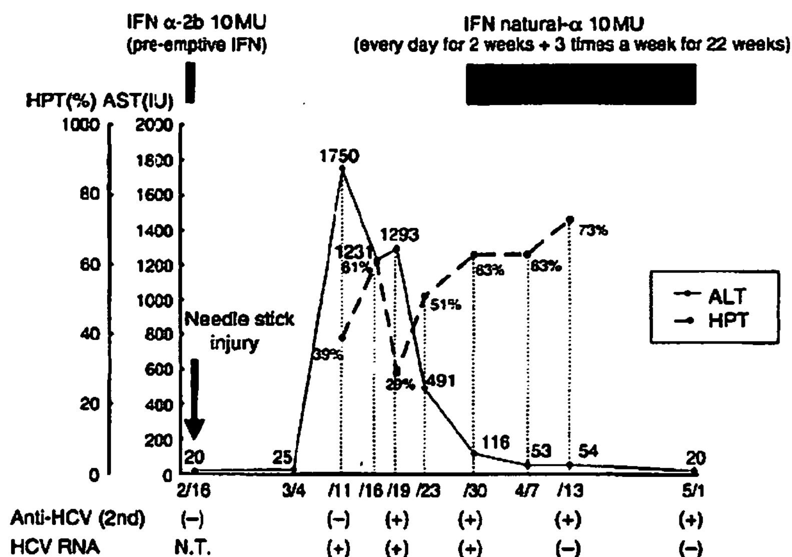

Figure 1 The clinical course of the patient who developed acute hepatitis C after exposure.

Figure 1 shows the clinical course of this case. ALT elevated to 1751 on 11 March, and dropped later. T.Bil elevated to 12.5 on 20 March, while HPT dropped to a nadir of 29%. At that point, general fatigue and nausea were severe, and drip infusion of prednisolone was started at 40 mg/day. Then the two parameters peaked and began to improve, and prednisolone was gradually decreased and stopped on 27 March when T.Bil and HPT became 2.3 and 102%, respectively. Despite the alleviation of symptoms, HCV-RNA and HCV antibody were both positive, and on 31 March she started to receive IFN (natural-α, 10 MU) treatment according to a 24-week schedule of daily injections for two weeks, followed by three times a week for 22 weeks. On 14 April (two weeks later), HCV-RNA became undetectable (by PCR) and liver functions were normalized.

HCV-RNA was examined by PCR on 1 May (four weeks later) and found to be negative. One year after the end of IFN treatment, liver functions were normal and HCV-RNA was negative by PCR. To confirm that HCV-RNA detected in the blood of the exposed individual was derived from the source patient, phylogenetic analysis was performed, targeting the HCV E1 region. Figure 2 shows the phylogenetic tree. The clone extracted from the exposed (K.F.) markedly resembled that from the source patient (M.T.), and the phylogenetic tree formed one cluster. Taken together, it was concluded that the infection was caused by the accidental exposure.

DISCUSSION

BLOOD EXPOSURE ACCIDENTS, including needle stick injuries, are a critical issue for medical staff. Recently, there have been reports of the development of acute hepatitis due to accidental exposure to blood positive for HCV antibody.[3–9] Table 4 shows the onset rates in a number of reports.[4,5,8–22] The onset rates of acute hepatitis ranged from 0% by Okamoto *et al.*[10] and Hernandez *et al.*[12] to 5.4% by Arai *et al.*[5] Based on all these reports, the average rate was estimated to be 2–3%.

Our results revealed relatively low rates of transmission of 0.4% and 1.1% when the source patient was positive for HCV antibody and HCV-RNA, respectively. These results are based on a larger number of cases than the previous reports. Mitsui *et al.*[13] followed up 68 exposure accidents to blood positive for HCV-RNA and reported that HCV-RNA became detectable in seven cases (10%). In our analysis, 81 cases were followed up for one year in 99 incidents of exposure to blood positive for HCV-RNA, among 157 incidents of exposure to blood positive for HCV antibody, and only one case became positive for HCV antibody. Although HCV-RNA in the source patients was not quantified, it was considered that infection could be established through accidental blood exposure but the onset rates were not so high.

In terms of risk factors influencing the onset of acute hepatitis, it should be noted that the case that developed

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Figure 2 Phylogenetic analysis of the HCV strains isolated from the patient with acute hepatitis C (K.F.) and the source patient (M.T.). As shown here, the strains isolated from both the patients were clustered in the same branch, indicating that the infection was caused by the accidental exposure.

acute hepatitis had a deep piercing with a contaminated needle. Yazdanpanah *et al.*[23] reported that risk factors for HCV infection due to accidental blood exposure comprised exposure to a large amount of blood and high HCV titers. In this study, unfortunately, HCV-RNA in the blood from the source patient was not quantified.

Denise *et al.*[24] investigated risk factors in medical staff who were exposed percutaneously to HIV-positive blood, and suggested several significant factors influencing risks for HIV infection. Of them, deep piercing was the greatest risk factor.

De carli G *et al.*[9] concluded that deep injury and injury with a blood-filled needle seem to be associated with occupational transmission and increased the transmission risk.

Next, we analyzed the outcomes of pre-emptive IFN treatment in exposure accidents to blood positive for HCV-RNA. Currently, IFN has been given to cases of chronic hepatitis C to eliminate HCV. Considering the effect of eliminating viruses by IFN, a beneficial effect was expected in accidental blood exposure. There are two methods of IFN treatment after blood exposure accidents: pre-emptive therapy for a short period immediately after the accident, and administration after the onset.

Although the types and amounts of IFN and treatment regimens have been controversial in pre-emptive treatment, the amount of virus introduced into the body by accidental blood exposure is far less than that by transfusion. Therefore, the treatment regimen we employed (10 MU dose of recombinant α-2b IFN) was expected to yield a beneficial effect. In this study, however, acute hepatitis developed in only one of 129 cases treated pre-emptively with IFN among 264 exposure accidents involving blood positive for HCV antibody, but not in any of the 146 cases without treatment during 11 years from 1993 through 2003, which suggested that the beneficial effect of pre-emptive IFN treatment according to the regimen in this study was not apparent and the amounts and schedules of IFN treat-

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Table 4 Reported cases of accidental exposure to HCV antibody-positive blood and the onset rate of acute hepatitis C

Reporter	Reported year	No. of accident	No. of hepatitis C transmission	Rate (%)	HCV-RNA positive in the source	No. of pre-emptive IFN
Okamoto *et al.*[10]	1990	16	0	0	NT	0
Kiyosawa *et al.*[11]	1991	110	3	2.7	NT	0
Hernandez *et al.*[12]	1992	81	0	0	NT	0
Mitui *et al.*[13]	1992	76	3	3.9	76	0
Marannconi *et al.*[14]	1992	117	3	2.6	NT	0
Sodeyama *et al.*[15]	1993	88	2	2.3	16	0
Fujise *et al.*[17]	1994	41	1	2.4	1	0
Yamamoto *et al.*[16]	1994	60	1	1.7	1	0
Bruce P *et al.*[22]	1994	176	4	2.3	NT	0
Kidouchi *et al.*[18]	1995	41	1	2.4	NT	0
Takagi *et al.*[19]	1996	251	4	1.6	NT	0
Arai *et al.*[3]	1996	56	3	5.4	NT	0
Hamid S *et al.*[20]	1999	53	2	3.8	17	0
Isozaki Y *et al.*[4]	1999	166	1	0.6	1	unknown
Ogasawara Y *et al.*[21]	2000	98	0	0	NT	0
Chung H *et al.*[8]	2003	684	2	0.3	NT	279
De Carli G *et al.*[9]	2003	4403	14	0.3	566	0
Present study	2006	264	1	0.4	99	129

ment should be modified if pre-emptive treatment is performed. Chung *et al.*[8] reported that of the 684 patients exposed to an anti-HCV-positive source, one case of HCV infection was found in each of the treated by pre-emptive IFN group (1/279; 0.4%) and non-treated group (1/405; 0.2%) and there was no significant difference in the transmission of HCV between the two groups. They concluded that short duration IFN administration to prevent HCV transmission is unnecessary.

However, previous reports on blood exposure accidents and the onset of hepatitis C revealed that HCV-RNA often became negative a few months after temporary aggravation by acute hepatitis, but some developed chronic hepatitis.[13,15] Acute hepatitis C can be cured and the development of chronic hepatitis can be prevented at high rates by immediate IFN treatment for a short period (four weeks).[25] Even in persistent cases, IFN treatment at early stages is expected to provide a beneficial effect.[6,26] This expectation is based on the fact that IFN treatment is excellent for acute hepatitis C.[27,28] Taken together, it is therefore more practical to follow up those exposed carefully and give IFN treatment as soon as the onset of hepatitis is confirmed, rather than to explore the best pre-emptive treatment.

The infected case in our study developed symptoms despite pre-emptive IFN treatment, but was cured later by a course of IFN treatment. There was a possibility that viruses were suppressed temporarily by pre-emptive IFN treatment but activated later by the rebound in this case. Therefore, we considered that pre-emptive IFN treatment under the present regimen was not necessary for all individuals exposed to blood positive for HCV antibody, and a careful follow up was sufficient in cases with a high risk of infection.

CONCLUSION

THERE WERE 696 reported cases of accidental blood exposure at the Social Insurance Chukyo Hospital from 1993 through to September 2003 and 264 cases were exposed to blood positive for HCV antibody. During the six years between 1993 and 1998, HCV-RNA in the blood from the source patients was measured in 133 of 157 exposure accidents involving blood positive for HCV antibody. HCV-RNA was positive in 99 of the 133 cases. Pre-emptive treatment was given in 73 of the 99 cases, and one case of a finger pierced deeply with a needle developed acute hepatitis C. Transmission by the needle stick injury was confirmed by phylogenetic analysis in the case with the onset. After the onset, the case was cured by IFN treatment. In 264 exposure accidents involving blood positive for HCV antibody, 129 cases were treated with pre-emptive IFN while 135 cases were not. Among the treated cases, one case developed acute hepatitis. The beneficial effect of pre-emptive IFN

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treatment according to the present regimen (10 MU intramuscular injection of recombinant α-2b) was uncertain. There was no necessity for pre-emptive IFN treatment according to the present regimen, and it was considered more practical to follow up high-risk cases carefully and commence IFN treatment as soon as possible after the onset of acute hepatitis.

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Adefovir dipivoxil monotherapy and combination therapy with lamivudine for the treatment of chronic hepatitis B in an Asian population

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Aim: To determine differences in Chinese patients treated with adefovir (ADV) monotherapy or ADV in combination with lamivudine (3TC) after development of resistance to 3TC, with respect to biochemical improvement, HBV DNA suppression and development of subsequent ADV resistance.
Methods: All hepatitis B patients with 3TC resistance treated with ADV for 3 months or more at our centre were included, and monitored 3-6 monthly for biochemical and virological response, and development of ADV resistance.
Results: A total of 56 patients were included, 50% switched to ADV monotherapy and 50% received combination 3TC/ADV therapy. Median follow-up was 15.5 months. Normalization of alanine aminotransferase (ALT) occurred in 25 (89%) patients in the ADV group compared with 24 (86%) in the 3TC/ADV group (P=0.686). Virological response (VR) was achieved in seven (35%) patients in the ADV group at 12 months compared with five (28%) in the 3TC/ADV group (P=0.637). By 24 months, seven (64%) patients in the ADV group achieved VR compared with two (40%) in the 3TC/ADV group (P=0.377). Cumulative probability of developing genotypic ADV resistance in the ADV group at 24 months was 18% compared with 7% in the 3TC/ADV group (P=0.94).
Conclusion: There was no obvious improvement in ALT normalization and virological suppression or reduction in the development of ADV-resistant mutations with 3TC/ADV therapy compared with ADV monotherapy. Further studies with longer follow-ups are required to determine whether combination 3TC/ADV therapy will reduce the emergence of ADV resistance compared with ADV monotherapy.

Introduction

Approximately 400 million people worldwide are infected with chronic hepatitis B (CHB), contributing to 1 million deaths per year [1]. Lamivudine (3TC), a nucleoside analogue, and adefovir dipivoxil (ADV), a nucleotide analogue, are commonly used for the treatment of CHB. Both suppress hepatitis B virus (HBV) DNA by inhibition of HBV polymerase through chain termination. However, treatment with these agents has been associated with development of drug resistance, which increases with duration of treatment.

3TC resistance occurs as a result of mutation at position 204 of the HBV DNA polymerase, with substitution of methionine by valine or isoleucine (M204V/I) in the tyrosine-methionine-aspartate-aspartate (YMDD) motif [2]. These YMDD variants can be detected in up to 70% of patients after 4 years of treatment with 3TC. However, they remain sensitive to ADV [3]. Resistance to ADV is less common, with an estimated rate of 28% after 5 years in treatment-naive hepatitis B e antigen (HBeAg)-negative patients [4]. Mutations conferring resistance to ADV include substitution of asparagine by threonine at position 236 (N236T) and alanine by valine or threonine at position 181 (A181V/T). These remain sensitive to 3TC [5].

Patients who develop 3TC resistance are commonly switched to ADV monotherapy. However, given the complementary resistance profile of HBV with these two drugs, there may be potential benefits in combination therapy. The benefits of combination therapy are well established in the treatment of HIV infection, and may be of importance in the treatment of CHB as more antiviral drugs become available.

In this study we aimed to determine whether there were any differences in Chinese CHB patients on 3TC treatment who were subsequently treated either with ADV monotherapy or ADV in combination with 3TC, with respect to biochemical improvement, HBV DNA suppression and development of subsequent ADV resistance.

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Patients and methods

Patients

During the period of October 2002 to June 2005, all CHB patients over the age of 15 referred to the hepatitis clinic, Queen Mary Hospital, the University of Hong Kong, who had documented 3TC resistance defined by HBV DNA >10^5 copies/ml or the presence of YMDD mutations, and who had been given ADV therapy for 3 months or more with preceding 3TC treatment were included. Those that received *de novo* ADV therapy or another antiviral agent during the same period were excluded. Patients were followed up every 3 to 6 months with determination of liver biochemistry, HBeAg and antibody to HBeAg (Anti-HBe) (enzyme-linked immunosorbent assay (ELISA); Abbott Laboratories, Chicago, IL, USA) and serum alpha-fetoprotein (AFP) levels. All samples were stored at −80°C until time of use.

Patients were divided into two groups. The first group included patients who were switched to ADV monotherapy with or without a period of overlap with 3TC therapy. The second group included patients who received combination therapy, with continuation of 3TC therapy after addition of ADV.

Liver cirrhosis was diagnosed with a combination of ultrasonography findings of a small-sized liver and enlarged spleen, and low platelet count of $<100\times10^9$/l.

HBV DNA measurement

HBV DNA was measured at the time of starting ADV therapy, and subsequently at 3, 6, 12, 18 and 24 months using the Versant HBV DNA 3.0 Assay (bDNA) (Bayer Healthcare, Berkeley, CA, USA) with a lower limit of detection of 2,000 copies/ml.

Testing for antiviral resistance and HBV genotypes

Testing for 3TC resistance at rt204 was performed prior to commencing ADV therapy using the second generation INNO-LiPA HBV DR v2 (Innogenetics NV, Ghent, Belgium). The same line probe assay also detected the presence of ADV-resistant mutations at rt181 and rt236. This assay had a high degree of concordance with direct sequencing [6].

Testing for ADV resistance was performed at the time of initiation of ADV therapy and at 3, 6, 12, 18 and 24 months, regardless of HBV DNA levels. Initial HBV DNA extraction was performed using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) as per the manufacturer's instructions. The HBV genotypes were determined by ELISA as described previously [7].

Statistical analysis

All statistical analyses were performed using SPSS 10.0 (SPSS, Inc., Chicago, IL, USA). Categorical variables were analysed using the Chi-square test and Fisher's exact test when appropriate. Continuous variables with skewed distribution were analysed using the Mann–Whitney U test. Kaplan–Meier analysis was used to determine the cumulative probability of developing ADV-resistant mutations with log-rank testing for significance. A P-value of ≤ 0.05 was considered statistically significant.

Results

A total of 56 patients were enrolled in this study of which 28 (50%) patients received combination therapy with the addition of ADV to 3TC. The remaining 28 patients (50%) switched from 3TC to ADV monotherapy with a median overlap period of 2 months (range 0–14 months). All patients were ethnically Chinese. The baseline characteristics in both groups were well-matched in terms of age, HBeAg status, presence of cirrhosis, baseline HBV DNA levels, presence of YMDD mutations, duration of 3TC therapy and HBV genotype (Table 1). The median baseline alanine aminotransferase (ALT) was higher in the ADV monotherapy group compared with the 3TC/ADV group (360 IU/l versus 106 IU/l, respectively, P=0.021). The median (range) length of follow-up was 19 (6–36) months in the ADV monotherapy group and 11 (6–31) months in the 3TC/ADV group (P=0.011).

A set of 14 patients (67%) in the ADV monotherapy group and seven (39%) patients in the 3TC/ADV group had evidence of reversion back to YMDD wild-type, although this did not reach statistical significance (P=0.083).

Normalization of ALT

There were no significant differences in rates of normalization of ALT between the ADV monotherapy and 3TC/ADV combination therapy groups. At 3 months, 13/24 (54%) patients had normalization of ALT compared with 13/25 (52%) patients in those receiving combination 3TC/ADV therapy (P=0.879). At 6 months, 19/27 (70%) patients had normal ALT in both groups. At 12 months, 20/25 (80%) patients in the ADV monotherapy group had normal ALT compared with 14/19 (74%) patients in the combination 3TC/ADV group (P=0.72).

A total of seven (13%) patients had elevated ALT at the end of the follow-up period. Of the three patients in the ADV monotherapy group who had elevated ALT at the end of the follow-up period, two had ALT less than twice the upper limit of normal (2×ULN) whereas one patient had ALT above 2×ULN. Four patients in the 3TC/ADV group had elevated ALT at the end of the follow-up period of which three had ALT less than 2×ULN and one patient had ALT above 2×ULN. None

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Table 1. Baseline demographic data, liver biochemistry, HBV DNA levels, YMDD mutations and duration of therapy

	ADV monotherapy (switch; n=28)	ADV + 3TC (combination; n=28)	P-value
Age, years	36 (19–68)	44 (24–68)	0.129
Sex, M:F	23:5	25:3	0.445
Follow-up, months	19 (6–36)	11 (5–31)	0.011
HBeAg positive, n (%)	23 (82%)	22 (79%)	0.737
Cirrhosis, n (%)	7 (25%)	6 (21%)	0.752
ALT, IU/l	360 (23–2,689)	106 (19–2,079)	0.021
Log HBV DNA, copies/ml	8.0 (3.0–9.0)	7.5 (5.2–8.0)	0.132
3TC overlap, months	2 (0–14)	-	
Duration of 3TC therapy, months	39.5 (2–117)	50 (9–134)	0.093
YMDD mutation, n (%)	24 (86%)	26 (93%)	0.388
Genotype B:C	8:20	7:21	0.763

Continuous variables are expresses in median (range). 3TC, lamivudine; ADV, adefovir; ALT, alanine aminotransferase; F, female; M, male.

of the seven patients with elevated ALT developed ADV-resistant mutations and six of the patients had HBV DNA levels of <5 $\log_{10}$ copies/ml. Three patients had underlying liver cirrhosis and two patients had evidence of fatty liver on ultrasonography, which might account for the mild elevation of ALT in these patients.

Virological response

The baseline median HBV DNA for the ADV monotherapy and combination 3TC/ADV group was 8.0 log and 7.5 log copies/ml, respectively (P=0.132). The median levels of viral HBV DNA in the ADV monotherapy group at 3, 6, 12, 18 and 24 months were 3.9 log, 4.5 log, 4.8 log, 3.7 log and 3.6 log copies/ml, respectively. The corresponding median HBV DNA levels in the 3TC/ADV group at 3, 6, 12, 18 and 24 months were 5.3 log, 5.0 log, 5.5 log, 4.7 log and 4.1 log copies/ml respectively (P=NS for all comparisons) (Figure 1).

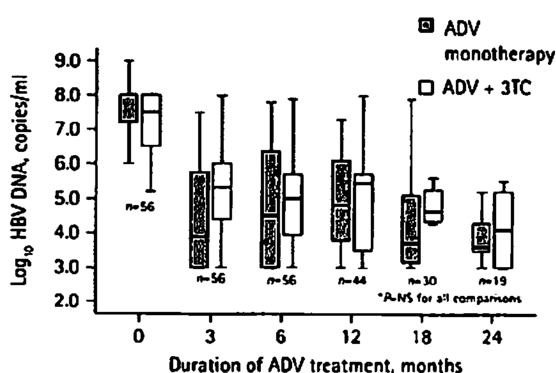
Virological response (VR), defined as suppression of HBV DNA below 4 log copies/ml, was assessed at 12 and 24 months. At 12 months, seven (35%) patients in the ADV monotherapy group achieved VR compared with five (28%) patients in the 3TC/ADV group (P=0.637). Four (20%) patients had undetectable HBV DNA in the ADV monotherapy group compared with three (27%) in the combination therapy group (P=1.0). By 24 months, seven (64%) patients in the ADV monotherapy achieved VR compared with two (40%) patients in the 3TC/ADV group (P=0.377). Of these, two (18%) patients in the ADV monotherapy group had undetectable HBV DNA compared with two patients (40%) in the combination therapy group (P=0.350).

Virological rebound defined as an increase in HBV DNA of 1 log copies/ml or greater after initial viral suppression was observed in seven (25%) patients in the ADV monotherapy group compared with four (14%) in the 3TC/ADV group (P=0.313).

Emergence of ADV resistance

During a median (range) follow-up period of 15.5 (5–36) months for the entire patient population, a total of five (9%) patients developed ADV-resistant mutations. Three patients (11%) were in the ADV monotherapy group and two (7%) were in the 3TC/ADV group (P=1.0). Of the five patients with genotypic resistance, three patients had genotype B and two patients had genotype C. All patients with genotype B who developed resistance were in the ADV monotherapy group and all patients with genotype C who developed resistance were in the combination group. ADV resistance developed in 20% of patients

Figure 1. Comparison of HBV DNA levels at 0,3,6,12,18 and 24 months between ADV monotherapy and 3TC/ADV combination therapy



Graph showing ranges, 25th and 75th percentiles and median values. ADV, adefovir; 3TC, lamivudine.

with genotype B and 5% of patients with genotype C. However, the number of patients who developed resistance was too small for any firm conclusions.

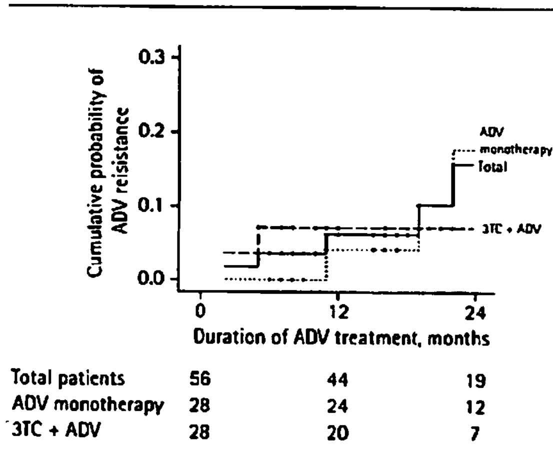
A mutation at rt181 was detected in all five patients but only one patient developed both rt181 and rt236 mutations. None of these five patients developed subsequent flares in ALT or HBV DNA rebound, and continued to be treated with ADV +/– 3TC. The cumulative probabilities of developing genotypic resistance to ADV in the ADV monotherapy group at 12 and 24 months were 4% and 18%, respectively, compared with 7% at both 12 and 24 months in the 3TC/ADV group (P=0.94) (Figure 2). The time to development of ADV-resistant mutations in the group treated with ADV alone was 11, 19 and 22 months. Within the group treated with combination 3TC/ADV, ADV-resistant mutations occurred much earlier at 2 and 5 months, both having wild-type virus confirmed at baseline. The overall cumulative probability of developing ADV-resistant mutation at 2 years was 16% (Figure 2).

Discussion

Viral kinetics studies in patients with 3TC-resistant CHB show an additional effect of 3TC on infected cell loss during combination therapy with 3TC/ADV [8].

In our study of Asian CHB patients treated with ADV after prior 3TC therapy, we found no significant difference in patients who were taken off 3TC and maintained on ADV monotherapy compared with those treated with a combination therapy of 3TC and ADV at 2 years of assessment. There were no significant differences in normalization of ALT, virological

Figure 2. Cumulative probability of developing ADV-resistant mutations in ADV monotherapy, 3TC/ADV combination therapy and overall



ADV, adefovir; 3TC, lamivudine.

suppression or virological rebound between those treated with ADV monotherapy and those treated with the combination therapy of 3TC/ADV. An earlier study also found no difference in antiviral response in those treated with ADV alone compared with those treated with additional 3TC after development of 3TC-resistant CHB [9]. This would suggest that 3TC has minimal additional antiviral benefit in patients who have developed 3TC resistance, and is consistent with previous studies showing ADV alone is active against both wild-type and 3TC-resistant HBV [9–13]. A more recent study of 30 patients treated for 48 weeks with ADV after development of 3TC resistance suggested that an overlap of 3TC for 2 months or more may lead to better virological but not biochemical outcomes [14].

Our results actually showed a poorer virological suppression with the combination group although this was not statistically significant. This raises the possibility of competing pharmacodynamics in the incorporation of the drugs into HBV polymerase, and also in intracellular phosphorylation although this is less likely as ADV and 3TC are analogues of different nucleosides/nucleotides, and since phosphorylation occurs via different intracellular kinase enzymes for 3TC and ADV [15].

Preliminary data suggest that in treatment-naive patients, combination therapy with 3TC/ADV may reduce the probability of developing YMDD mutations and subsequent 3TC resistance, without any additional benefit in antiviral effect, or biochemical or serological outcome compared with 3TC alone [16]. A randomized controlled trial of 26 patients showed better HBV DNA suppression in the combination therapy group compared with ADV monotherapy without any benefit in biochemical or histological outcome [17]. However this study included both treatment-naive patients and patients with prior 3TC therapy.

Our results showed ADV resistance increased to 18% at 2 years in the monotherapy group compared with 7% in the combination group, although the difference did not reach statistical significance. This is probably due to the small number of patients in this study. However, continuation of 3TC may be of theoretical benefit in terms of reduction of ADV resistance. Ongoing 3TC therapy may confer a selective advantage to YMDD mutants over wild-type HBV and decrease the reversion of YMDD mutant virus to YMDD wild-type HBV as shown in the present study. Since ADV is active against both wild-type and YMDD mutant virus, the clinical significance of this YMDD wild-type reversion remains to be determined. *In vitro* studies have shown ADV to be equally effective as an inhibitor of replication of 3TC-resistant HBV mutants and in wild-type HBV [18].

A total of five patients in the current study developed ADV-resistant mutations by 2 years, with an

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estimated cumulative probability of 16%. This is higher than the previously described figures in studies of patients who are naive to nucleotide/nucleoside analogues [19,20] and those with pre-existing 3TC resistance [21,22]. However, testing for ADV-resistant mutations in these studies was performed only with emergence of phenotypic resistance and therefore clinically silent genotypic resistance might remain undetected, accounting for the lower incidence. In addition, the LiPA assay used in this study is proven to be a sensitive assay which can detect the mutants when they contribute only 5% of the total viral population [23]. Our study did not show any phenotypic resistance in those patients with ADV-resistant mutations, although the duration of follow-up was short, with HBV DNA rebound and elevation of ALT usually occurring sometime after the development of genotypic resistance. More recent studies have reported higher incidence of ADV-resistant mutations in patients with pre-existing 3TC-resistant mutations [24–26]. Although the development of ADV resistance has been shown to occur later compared with the development of 3TC resistance, the earliest detected ADV-resistant mutation occurred at 2 months in our study. A recent study in patients with pre-existing 3TC resistance treated with ADV also shows emergence of ADV-resistant mutations as early as 3 months, suggesting that patients with pre-existing 3TC-resistant mutations probably develop ADV-resistant mutations more rapidly [25].

The long-term goal for the treatment of CHB is the prevention of cirrhosis and its complications including the development of hepatocellular carcinoma. This is likely to be achieved only through effective prolonged suppression of viral load. Although our results suggest no benefit in the degree of viral load suppression, larger studies with longer follow-up duration are required to determine whether combination 3TC/ADV therapy will decrease the rate of ADV resistance compared with ADV monotherapy.

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Early Dynamics of Hepatitis B Virus in Chimeric Mice Carrying Human Hepatocytes Monoinfected or Coinfected with Genotype G

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Of the 8 genotypes of HBV (genotypes A-H), genotype G is unique in that it has an insertion in the core gene and two stop codons in the precore region preventing the synthesis of hepatitis B e antigen. Most individuals with genotype G are coinfected with other genotypes, typically genotype A. Mice with severe combined immunodeficiency disease carrying human hepatocytes were infected with HBV particles propagated in Huh7 cells in culture. Mice monoinfected with genotype G did not raise detectable HBV DNA in serum, although products of the core gene emerged 4 to 8 weeks after inoculation. When they were superinfected with genotype A at week 10, however, HBV DNA of genotype A developed, which was replaced almost completely by that of genotype G within 10 weeks. Such a rapid takeover was also observed in mice initially infected with genotype A or C and superinfected with genotype G. Similar viral dynamics occurred in mice simultaneously coinfected with genotypes G and A. Takeover was markedly enhanced in mice inoculated with a serum passage containing genotype G with a trace of genotype A. Coinfection of mice with genotypes G and A induced abundant cellular steatosis along with increased fibrosis in the liver, which was not detected in mice monoinfected with genotype A or G. *Conclusion:* Genotype G can monoinfect chimeric mice at very low levels, and its replication increases maredly when coinfected with other genotypes. Coinfection with genotype G could enhance fibrosis under immunocompromised states. (HEPATOLOGY 2007;45:929-937.)

HBV infects an estimated 350 million people worldwide and causes 1 million deaths annually.[1] Eight genotypes of HBV have been classified by the sequence divergence in the entire genome exceeding 8% and have been assigned the names A through H in order of discovery.[2-6] HBV genotypes have distinct geographic distributions and can influence the severity of liver disease and response to antiviral therapies.[7-10] HBV genotypes are further divided into subgenotypes, such as A1/Aa and A2/Ae, B1/Bj and B2/Ba, and C1/Ce and C2/Cs.[11-13] These genotypes may influence clinical outcomes of HBV infection.[14,15]

HBV genotype G (HBV/G) was first described in 2000 among inhabitants of France and the state of Georgia.[5] It has an insertion of 36 base pairs in the core gene and two stop codons in the precore region.[5,16] Despite the inability in encoding hepatitis B e antigen (HBeAg), carriers of HBV/G possess it in serum.[5] They are usually coinfected with HBV of other genotypes, most frequently HBV/A, which is responsible for serum HBeAg.[17] Coinfection with HBV/C, F, and H has also been reported.[18-20] In spite of heavy dependence on other genotypes for replication, HBV/G outgrows them and eventually takes over the great majority of HBV DNA in the circulation.[16,17]

Recently, HBV/G DNA in low levels was reported in a German donor of plasmapheresis who had transmitted it to 2 recipients in look-back studies.[21] Hence, HBV/G would be able to infect recipients by itself. Furthermore,

Abbreviations: ChiM, chimeric mice; HBcrAg, HBV core-related antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; RFLP, restriction fragment length polymorphism; SCID, severe combined immunodeficiency disease.

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HBV/G has been detected in 25 of the 104 (24%) French patients coinfected with HIV-1 and HBV and was associated with a high risk of fibrosis at an odds ratio of 12.6.[22]

Mice with severe combined immunodeficiency disease (SCID) transgenic for the urokinase-type plasminogen activator gene under control of albumin promoter (uPA/SCID mice) have received human hepatocyte transplants.[23-25] These mice [hereafter referred to as chimeric (ChiM) mice] have been instrumental in experiments with hepatitis viruses *in vivo*[26,27] and offer a rare opportunity in portraying the early kinetics of HBV replication,[28] without having to resort to the ever-endangered species of chimpanzees. In this study, ChiM mice were monoinfected with HBV/G or coinfected with other genotypes, either simultaneously or in sequence, and followed for circulating HBV/G DNA. It is hoped that the emerging dynamics of HBV DNA will further characterize the dependence of HBV/G on other genotypes and unfold the pathogenicity intrinsic to this parasitic genotype.

Patients and Methods

Patients. Sera were obtained from 4 patients with chronic hepatitis B. One HBV DNA clone of subgenotype A2 and two of HBV/C2 were recovered from 3 Japanese patients in our recent study.[28] Because all HBV DNA clones of HBV/A were classified into subgenotype A2, they will be called HBV/A comprehensively in the present study. The other HBV/A and G clones were obtained from a coinfected Caucasian patient in San Francisco who represented patient 1 in our previous study.[17] All the HBV/A or C clones did not have precore or core promoter mutations affecting the expression of HBeAg. The study design conformed to the 1975 Declaration of Helsinki and was approved by the institutional ethics committees. Written informed consent was obtained from each patient.

Plasmid Constructs of HBV DNA and Sequencing. HBV DNA was extracted from 100 μL of serum using the QIAamp DNA blood kit (Qiagen, GmbH, Hilden, Germany). Four primer sets were designed for amplification of 2 fragments (A and B) covering the entire HBV/G genome. PCR with nested primers was performed with TaKaRa LA Taq polymerase (Takara Biochemicals, Kyoto, Japan) for 35 cycles (30 s at 95°C; 30 s at 60°C; 2 min at 72°C). Primer pairs and protocols for plasmid construction were described in supporting information. As reported previously,[28] these fragments were constructed into the pUC19 vector deprived of promoters (Invitrogen Corp., Carlsbad, CA) by digestion with *Hin*dIII and *Eco*RI, resulting in 1.24-fold the HBV genome—just enough to transcribe oversized pregenome and precore messenger RNA. Cloned HBV DNA sequences were confirmed with Prism BigDye (Applied Biosystems, Foster City, CA) using the ABI 3100 automated sequencer. Additionally, HBV DNA spanning the complete genome were amplified in mouse sera, cloned in the pGEM-T Easy Vector, and then sequenced.

Cell Culture and Transfection. Huh7 cells were transfected with plasmids equivalent to 5 μg of HBV DNA constructs with use of the Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN), and harvested after 3 days in culture. Transfection efficiency was monitored by 0.5 μg of coinfecting reporter plasmids expressing secreted alkaline phosphatase to estimate the latter's enzymatic activity in the culture supernatant.

Determination of HBV Markers. Hepatitis B surface antigen (HBsAg) and HBeAg were determined via chemiluminescent enzyme immunoassay using commercial assay kits (Fujirebio Inc., Tokyo, Japan). HBV core-related antigens (HBcrAg) were measured in serum using the chemiluminescent enzyme immunoassay described previously.[29,30]

Detection and Quantification of Serum HBV DNA. HBV DNA sequences spanning the S gene were determined via real-time detection PCR according to the method of Abe et al.[31] It had a sensitivity of 100 copies/ml (equivalent to 20 IU/ml) on the assay curve obtained with a calibrated World Health Organization standard serum containing HBV of genotype A (kindly provided by Dr. Hiroshi Yoshizawa of Hiroshima University) when 100 μl of the test sample was used. However, in assays for HBV DNA in mouse sera, in which only 10 μl of sample is used, the sensitivity decreased to 1,000 copies/ml (200 IU/ml). For real-time detection PCR specific for HBV/G, 10 μl of DNA sample was amplified in a 25-μl mixture containing 2 $\times$ SYBR Green PCR Master Mix (Applied Biosystems) and 2 primers specific for HBV/G: a forward primer (HBVG1620F: ACG TTA CAT GGA AAC CGC CA) and reverse primer (HBVHKR2: AGC CAA AAA GGC CAT ATG GCA) covering the 36–base pair insertion characteristic of this genotype.[5,16] Amplification and detection were performed in the ABI Prism 7700 Sequence Detection System (Applied Biosystems) with an initial activation of UNG at 50°C for 2 minutes, followed by incubation at 95°C for 10 minutes and subsequently, 40 three-step cycles (30 s at 95°C; 30 s at 60°C; 1 min at 72°C) were performed. The standard was prepared on serial dilutions of a known amount of the cloned HBV plasmid of HBV/G. The specificity of 2 primers (HBVG1620F and HBVHKR2) was confirmed in every PCR run via dissociation curve analysis (ABI Prism 7700 dissociation curve software; Applied Biosystems). The

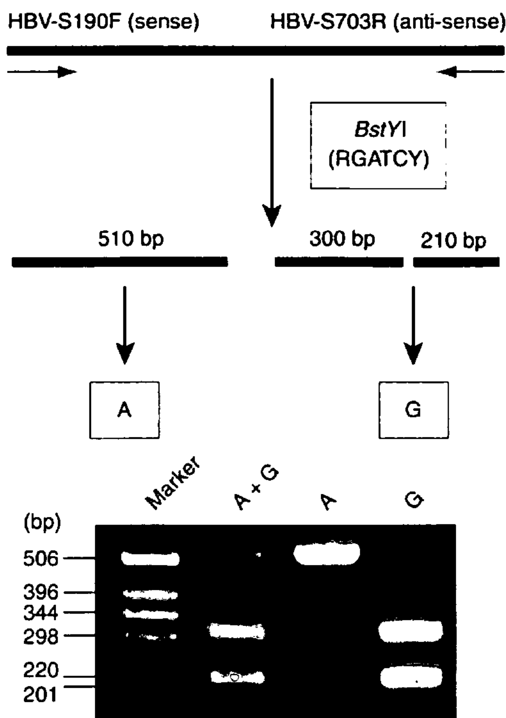

Fig. 1. PCR-RFLP for distinguishing between genotypes A and G. Products of PCR, when digested with *BstYI*, split into 2 fragments for genotype G (upper panel). Infection with genotype A or G, or coinfection with these genotypes, can be determined by analyzing the patterns of electrophoresis of digests (lower panel).

sensitivity of detecting HBV/G via real-time detection PCR was 1000 copies/ml (200 IU/ml).

PCR Restriction Fragment Length Polymorphism for Distinguishing HBV DNA of Genotype G from Others. A novel method for specific determination of HBV/G DNA in the presence of other genotypes has been developed. It involved single-cycle PCR followed by restriction fragment length polymorphism (RFLP) with an endonuclease having the restriction site specific for HBV/G (Fig. 1). PCR was performed with a forward primer (HBV-S190F: GCT CGT GTT ACA GGC GGG) and reverse primer (HBV-S703R: GAA CCA CTG AAC AAA TGG CAC TAG TA) within the S region. To distinguish HBV/G from other genotypes such as HBV/A and C, a portion (5 μl) of amplification products of 510 base pairs was digested with 5 U *BstYI* (restriction site: RGATCY) at 60°C for 2 hours. Digests were run on electrophoresis in 3.0% agarose gel, stained with ethidium bromide and examined for their sizes under the ultraviolet light. The results were supported by another method (Supplementary Fig. 1).

Inoculation of Chimeric Mice with the Liver Repopulated for Human Hepatocytes. SCID mice transgenic for the urokinase-type plasminogen activator gene with the liver repopulated for human hepatocytes (chimeric mice) were purchased from Phoenix Bio Co., Ltd. (Hiroshima, Japan). Human serum albumin was measured via ELISA using commercial assay kits (Eiken Chemical Co. Ltd, Tokyo, Japan). They were inoculated with HBV recovered from culture supernatants of Huh7 cells transfected with plasmids constructed with 1.24-fold the HBV genome of genotype HBV/A, C, or G after the method previously reported.[28]

Histopathological Examination. Liver tissues were fixed in buffered formalin, embedded in paraffin, and stained with hematoxylin-eosin or Masson's trichrome. The fibrosis stage was evaluated by an expert pathologist (S. T.) who was blinded to the nature of inocula.

Results

ChiM Mice Monoinfected with HBV/G. Two ChiM mice (ChiM92-3 and ChiM184-4) received an inoculum containing approximately 10^5 copies of HBV/G (G_US1646 strain) and were followed for 12 and 24 weeks, respectively (Fig. 2A,B). HBV DNA remained in undetectable levels ($<10^3$ copies/ml) in them both, but they developed low levels of HBcrAg (1 kU/ml) 4 and 8 weeks after inoculation, respectively. Despite absence of detectable HBV DNA in the circulation, therefore, these mice had contracted infection with HBV/G in very low levels. Intrahepatic cccDNA (covalently closed circular DNA) was detected via PCR specific for it,[32] and HBV/G DNA was detected in hepatocytes via PCR with type-specific primers[33]; they attested to infection with HBV/G in them (data not shown).

Superinfection With HBV/A on Mice Infected with HBV/G. Two chimeric mice with occult infection with HBV/G received 10^5 copies of HBV/A2 of different strains (A2_JPN to ChiM93-4 and A2_USA to ChiM172-3) 10 weeks after initial inoculation with HBV/G (Fig. 3A,B). They both developed HBV DNA in serum in titers $>10^6$ copies/ml at week 17, 7 weeks after superinfection with HBV/A, accompanied by HBcrAg and HBsAg; HBeAg appeared soon thereafter at week 22. HBV DNA and antigens increased, peaked at week 26, and then decreased in exactly the same patterns. HBV/G DNA, which was determined via PCR with type-specific primers, developed 12 weeks after the inoculation at week 22. It increased rapidly, and after the peak, took the same time course as total HBV DNA in serum—it had replaced HBV/A in the two chimeric mice.

Genotypes of HBV in ChiM93-4 were determined at the appearance of HBV DNA (week 17), at peak (week 26), and at the end of observation (week 38) via PCR-

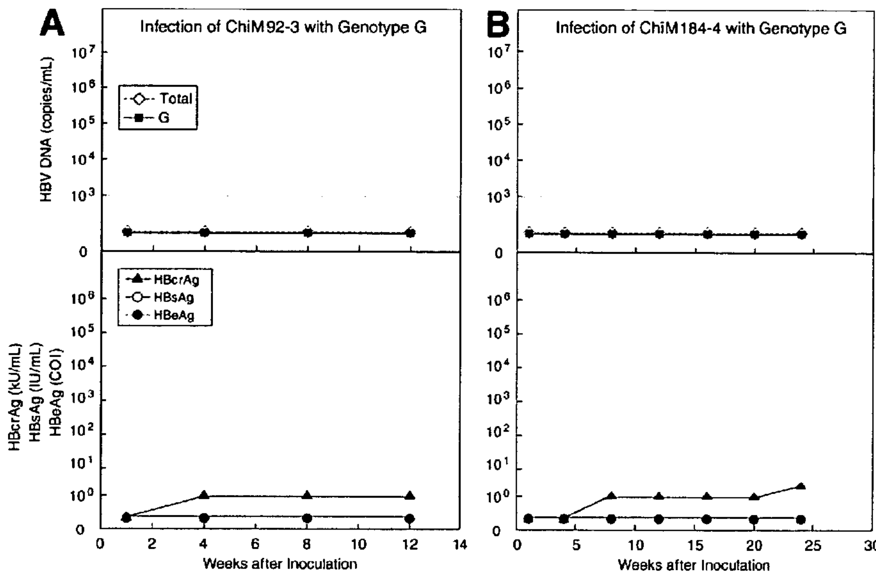

Fig. 2. (A) ChiM92-3 and (B) ChiM184-4 mice monoinfected with HBV/G. Profiles of total HBV DNA and HBV DNA of genotype G, determined via PCR with type-specific primers, are shown in the upper panels, and those of HBV antigens HBVcrAg, HBsAg, and HBeAg are shown in the lower panels. The shaded areas represent HBV DNA titers below the detection limit (<10^3 copies/ml).

RFLP (Fig. 3A). HBV/A accounted for all HBV DNA at week 17. At weeks 26 and 38, however, the vast majority of HBV DNA were of HBV/G with a trace of HBV/A. Thus, HBV/G needed coinfection with HBV/A for active replication, and took it over very swiftly.

Superinfection with HVB/G on Mice Infected with HBV/A. The chronological order of superinfection was reversed in ChiM92-9 and ChiM124-11 mice (Fig. 4A,B). The mice received 10^5 copies of HBV/A strains A2_JPN and A2_USA, respectively, and were superinfected with HBV/G (10^5 copies of G_US1646 strain) 10 weeks thereafter, when HBV/A DNA was elevated to $>5 \times 10^7$ copies/ml in both strains. Profiles of HBV DNA and antigens in these mice were quite similar but differed from those with A-on-G superinfection (Fig. 3A,B). HBV/A DNA was detected at week 1 in both groups and increased by approximately 2 logs within the next 3 weeks. HBV/G DNA developed within 3 weeks after superinfection with it, much sooner than the 12 weeks in ChiM mice superinfected with 2 genotypes in the reverse order. Three HBV antigens (HBcrAg, HBsAg, and HBeAg) waxed and waned in profiles similar to that of HBV DNA. HBV DNA levels decreased after they had peaked in ChiM92-9 as in A-on-G mice (Fig. 3A,B) by a margin close to log 2; the decrease was less prominent in ChiM124-11 by merely 1 log.

Composition of different genotypes in serum HBV DNA was followed in ChiM92-9 (Fig. 4A). Rapid replacement of HBV/A with HBV/G was obvious in G-on-A superinfection as in A-on-G superinfection (Fig. 3A). The takeover by HBV/G was not complete as in

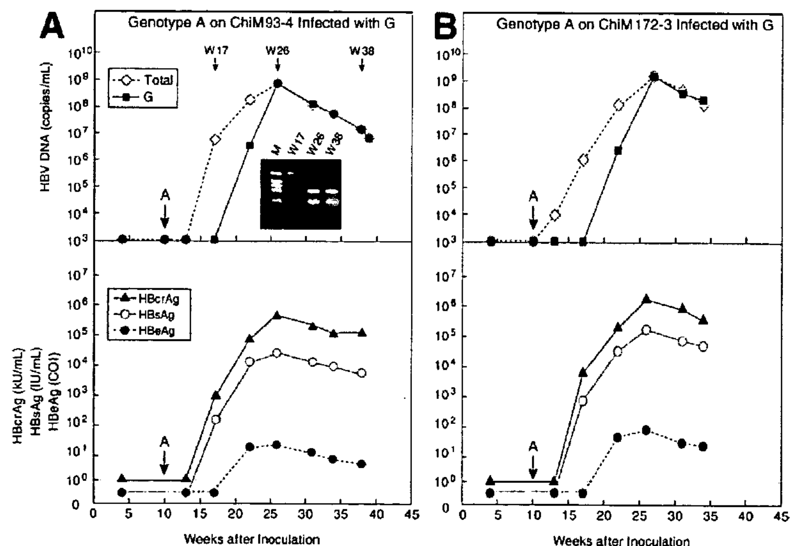

Fig. 3. Superinfection with HBV/A on (A) ChiM93-4 and (B) ChiM172-3 mice infected with HBV/G. Patterns of PCR-RFLP at different time points are shown in the insert in the upper panel of (A). Inoculation with genotype A is indicated by large arrows.

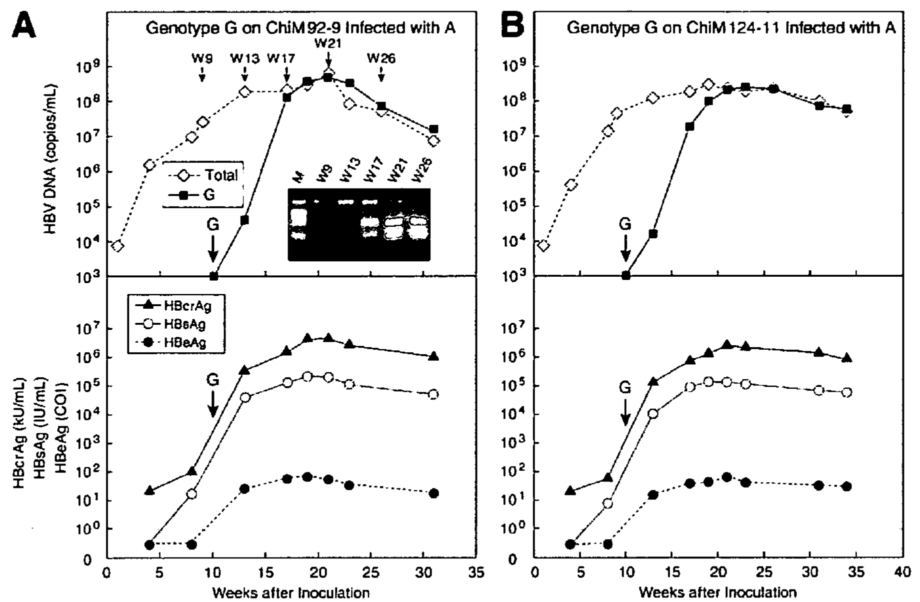

Fig. 4. Superinfection with HBV/G on (A) ChiM92-9 and (B) ChiM124-11 mice infected with HBV/A. Patterns of PCR-RFLP at different time points are shown in the insert in the upper panel of (A). Inoculation with genotype G is indicated by large arrows.

A-on-G superinfection, and HBV/A remained at very low levels throughout the weeks of observation.

Superinfection with HBV/G on Mice Infected with HBV/C. Similar superinfection with HBV/G was performed on ChiM mice that had been infected with HBV/C2 (Fig. 5A,B). Thus, C_22 and C_AT strains (10^5 copies) of HBV/C were injected intravenously into ChiM91-21 and ChiM95-11, respectively. They were superinfected with HBV/G (10^5 copies of G_US1646 strain) at week 10, when HBV DNA stabilized at approximately 10^9 copies/ml. HBV/G appeared in serum 3 weeks thereafter, at week 13 in both groups, and increased exponentially until weeks 21-23. The time required for an increase in HBV DNA level by 10-fold (log time) was 3.3 weeks in both groups, which was twice as long as the 1.6 weeks in mice with A-on-G and G-on-A superinfections (Figs. 3, 4). Likewise, the takeover of HBV/C by HBV/G in these mice was not as rapid or extensive as in superinfection with HBV/G on HBV/A (Figs. 3A, 4A, 5A). HBV antigens took time courses similar to that of HBV DNA, and they never waned after they had stabilized; however, mice were followed until 26 and 34 weeks.

Simultaneous Coinfection of Mice with HBV/A and HBV/G. Two ChiM mice (ChiM93-10 and ChiM93-

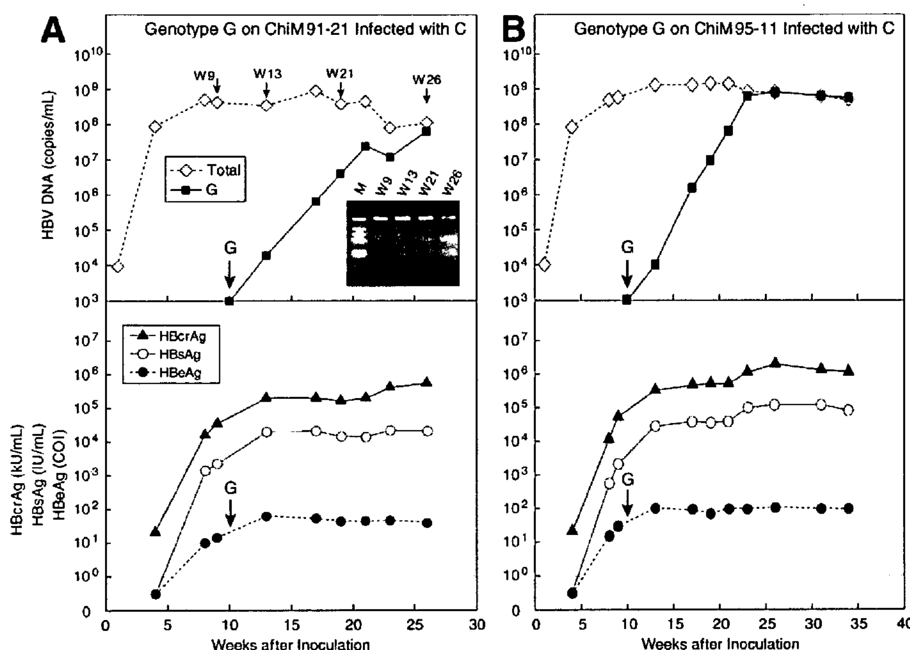

Fig. 5. Superinfection with HBV/G on (A) ChiM91-21 and (B) ChiM95-11 mice infected with HBV/C. Patterns of PCR-RFLP at different time points are shown in the insert the in upper panel of (A). Inoculation with genotype G is indicated by large arrows.