Patients after liver transplantation for HBV-related chronic liver disease who had received anti-HBs antibodies to prevent re-infection of the graft showed an "immune escape mutant".[116–118] Furthermore, "diagnosis escape mutants" have also been described because HBsAg detection assays are based on anti-HBs antibodies.[119] The emergence of these variants may contribute to occult HBsAg negative HBV infection.[120]

The HBV genome is organized in such a way that the envelope gene is overlapped by the polymerase gene; therefore, HBV with changes in the polymerase gene associated with resistance to the nucleos(t)ide analog which are described in detail in section 5 may have consequent changes in the envelope gene. A triple mutant causing LVD resistance (rtV173L + rtL180M + rtM204V), which have an enhanced replication capacity compared with rtL180M + rtM204V alone, causes two amino acid changes in the overlapping surface gene (sE164D + sI195M). This mutant reduces anti-HBs binding to levels seen only with the vaccine escape mutant sG145R.[121] Some patients treated with LVD showed seroclearance of HBsAg with detectable circulating HBV DNA. An sP120A mutation was associated with HBsAg seroconversion in these patients and this mutation produces a reduced anti-HBs binding which causes the failure to detect HBsAg.[122]

Consensus 7
Amino acid substitutions, deletions or insertions across the "a" determinant of HBsAg, such as a substitution sG145R, give rise to vaccine and immunoglobulin escape mutant. (Level 4, grade C.)

INDICATIONS FOR ANTIVIRAL TREATMENT OF CHRONIC HEPATITIS B

ONCE THE LIVER is persistently infected with HBV, it is difficult to eradicate the virus. It is reported that the natural clearance rate of HBsAg in asymptomatic HBsAg carriers is approximately 1–2% per year.[123] Therefore, the first goal in treating chronic hepatitis B is to prevent patients from progression to cirrhosis and occurrence of HCC.

When the initiation of antiviral therapy for chronic hepatitis B is considered, it is very important to estimate the fibrosis stage of each patient. If possible, a liver biopsy should be performed in order to obtain sufficient information to determine the extent of hepatic fibrosis. When the fibrosis stage of patients with chronic hepatitis B is moderate to severe, or when the patients have cirrhotic liver, the administration of antiviral therapy should be considered. When inflammatory activity is high and the fibrosis seems to be progressive, the introduction of antiviral therapy should also be considered.

In order to prevent the occurrence of hepatic fibrosis and HCC, virological factors as well as biochemical factors are important. A long-term follow-up study of untreated HBsAg positive individuals in Taiwan in which the cumulative incidence of HCC and cirrhosis were studied for 13 years revealed that high baseline HBV DNA was associated with increased risk of HCC and cirrhosis. Incidence rate of HCC in patients whose viral load of HBV DNA was less than 300 copies/mL was 1.3%, whereas in patients whose viral load was more than 1 000 000 copies/mL the incidence rate was 14.9%.[33] Moreover, incidence of cirrhosis in patients whose viral load was less than 300 copies/mL was 4.5%, whereas it was 36.2% in patients whose viral load was more than 1 000 000 copies/mL.[124] Therefore, the introduction of antiviral therapy should be considered based on biochemical and virological findings.

As mentioned above, although high viral load of HBV DNA is one of the strong risk factors in predicting poor prognosis of HBV carriers, low HBV DNA level does not rule out risk in Asian patients. Among HBeAg positive patients, HBV DNA levels of less than 10^5 copies/mL predicted better histological outcome; however, 14.3% of patients still had established fibrosis.[125] The liver biopsy is also very useful for such cases.

Recommendation 4
4-1 Introduction of antiviral therapy should be considered on the biochemical and virological findings. (Level 2a, grade B.)
4-2 Antiviral therapy should be considered for patients with low virus load but progressed hepatic fibrosis. (Level 2a, grade B.)
4-3 Liver biopsy finding (if available) should be useful to determine the introduction of antiviral therapy. (Level 2a, grade B.)

On the other hand, when patients with HBV have obscure or mild fibrosis, a close observation without any medication could be considered for them. Once antiviral therapy with a nucleos(t)ide analogue is started, it is very difficult to stop. Therefore, for patients who are in an inactive carrier state and whose fibrosis stage is relatively mild, a coarse observation without any treatment could be a useful choice to treat the patients.

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Young patients with chronic hepatitis B, especially those who are HBeAg positive, often face the flare-up of hepatitis. Because such patients are likely to achieve spontaneous HBe seroconversion and go into an inactive carrier state, unnecessary antiviral therapy should be avoided for them. A coarse observation without any medications should be considered for young patients or those with mild fibrosis.

> Recommendation 5
> Indication of antiviral therapy for chronic hepatitis B: Observation without therapy should be considered for young patients or those with mild fibrosis. (Level 3, grade B.)

NUCLEOS(T)IDE ANALOGUES FOR CHRONIC HEPATITIS B

AS STATED ABOVE, the goal of antiviral therapy in patients with chronic hepatitis B is to prevent cirrhosis and HCC. Maintaining persistent suppression of HBV replication reduces the development of cirrhosis and HCC. In the last decade, there has been a major advance in the treatment of chronic hepatitis B with nucleos(t)ide analogues such as LVD, adefovir (ADV), entecavir (ETV), telbivudine and tenofovir.[126–132] In treatment by nucleos(t)ide analogues for chronic hepatitis B in Japan, LVD, ADV and ETV are mainly used at present. Nucleos(t)ide analogues are potent inhibitors of the polymerase/reverse transcriptase and are easy to administrate p.o. to chronic hepatitis B patients because of low adverse effects and strong efficacy to suppress HBV replication. Thus, nucleotide analogue therapy could rescue liner decompensation, reduce fibrosis progression and prevent the development of HCC.[133–136] On the other hand, there are major disadvantages including requirement of prolonged or even indefinite therapy for most patients and the high incidence of antiviral resistance. Disadvantages of nucleos(t)ide analogues include the development of antiviral resistance.[137–140] Drug-resistant viruses emerge during the treatment and could be associated with flare-up of hepatitis. Due to no proof of reading activity of HBV polymerase, the spontaneous substitution rate of HBV genome is high in the natural course of the disease. Through the selection of pre-existing resistant variants and gradual accumulation of new a.a. substitutions, the mutations exhibiting the best replication capacity in the presence of the drug are selected under the circumstance of antiviral pressure. The level of intrinsic resistance and the replicative fitness determine the mutant spread and hence the annual incidence of drug resistance.

LVD

Lamivudine was the first nucleoside analogue licensed for the treatment of chronic HBV infection in Japan in 1999. LVD was given at a dose of 100 mg daily and has excellent safety and tolerability.[141–143]

Liaw *et al.* reported that continuous treatment with LVD delays the clinical progression of chronic hepatitis B with advanced fibrosis or cirrhosis by significantly reducing the incidence of hepatic decompensation and risk of HCC (level 1b).[134] Matsumoto *et al.* also showed that LVD therapy effectively reduces the incidence of HCC in Japanese patients with chronic hepatitis B.[144] Thus, it is generally considered that control of viral load using nucleos(t)ide analogues is effective to prevent complicating HCC in patients with active chronic hepatitis B.

> Consensus 8
> The control of viral load using nucleos(t)ide analogues reduces the risk of complicating HCC in patients with chronic hepatitis B. (Level 1b, grade B.)

Lamivudine resistance is characterized by the mutation of the highly conserved tyrosine, methionine, aspartate, aspartate (YMDD) nucleotide-binding motif in the catalytic domain of the enzyme. YMDD to YIDD (rtM204I) or YVDD (rtM204V) mutations are associated with LVD resistance.[142,145,146] These resistant mutants appear to replicate less efficiently than the wild-type virus *in vitro*, however, additional mutations such as rtV173L and rtL180M can restore partially the replication capacity *in vitro*.[147,148] LVD resistance occurred in approximately 20% of patients after 1 year, which increased to approximately 70% after 5 years (Fig. 1).

A meta-analysis, which included Asian patients and North American/European patients, indicated that HBV subtype ayw (genotype D) appears to respond significantly better to LVD treatment than does HBV subtype adw (genotype A). Insufficient suppression of the adw subtype during the early phase of treatment may lead to the high incidence of LVD resistance in HBV subtype adw.[149] In a study comparing the virological outcome among infections with HBV genotypes A, B and C, patients infected with genotype A had the lowest rate of HBV DNA clearance than those with genotype B or C, and had the highest incidence of resistant mutations.[150]

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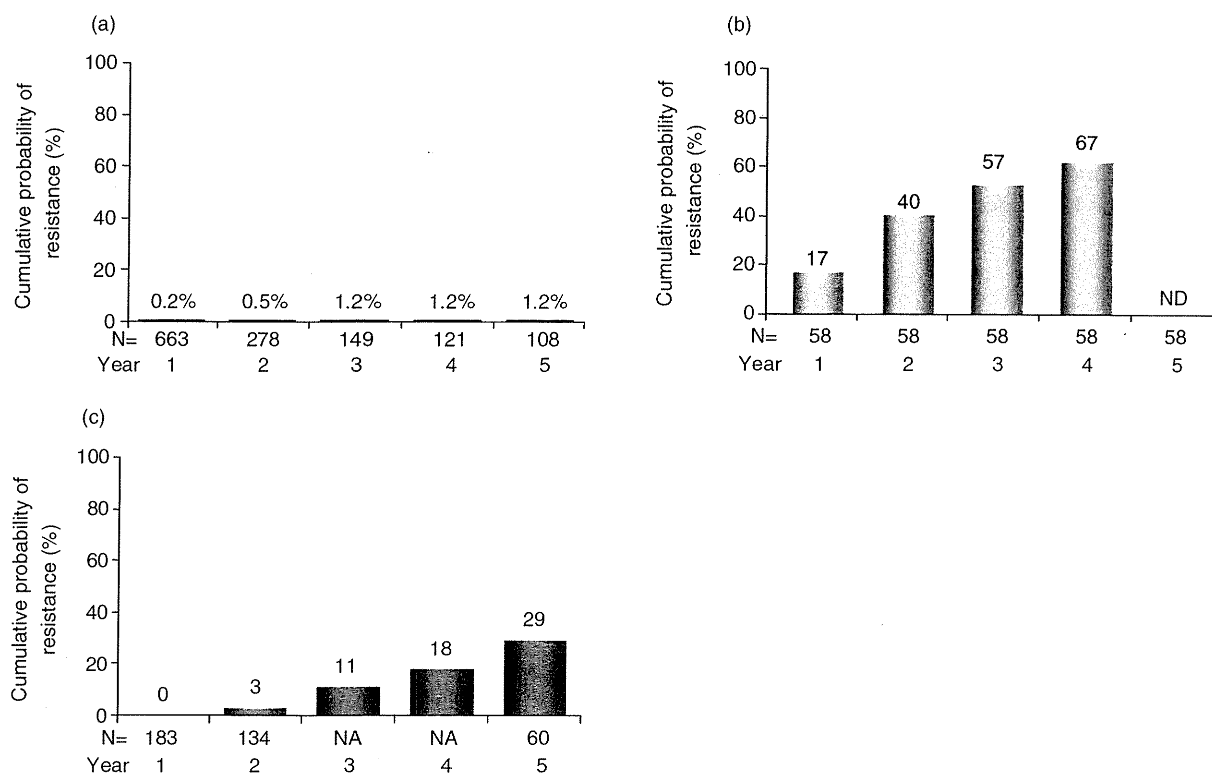

Figure 1 Cumulative probability of resistance after the initiation of entecavir (ETV), lamivudine (LVD) and adefovir (ADV) for patients with hepatitis B e-antigen. (a) Cumulative probability of resistance after the initiation of ETV.[159] (b) Cumulative probability of resistance after the initiation of LVD.[138] (c) Cumulative probability of resistance after the initiation of ADV.[153]

Lamivudine or hepatitis B immunoglobulin (HBIg) treatment induced vaccine/HBIg-escape mutations sP120T and sG145R in combination with LVD-resistance mutations. These mutations are associated with rtT128N and rtW153Q in the polymerase protein and have been found to partially restore the *in vitro* replicative capacity of LVD-resistant HBV.[121]

Another LVD resistant mutation, rtA181T, concomitantly generates a stop codon in the surface antigen (sW172stop), resulting in impaired secretion of HBsAg.[151] Neither the adefovir associated resistance mutation rtN236T nor the tenofovir associated resistance mutation rtA194T causes changes in the envelop protein.

ADV

Adefovir dipivoxil is a prodrug of ADV and has structural similarity to the natural substrate, dATP. Several studies have also been conducted using ADV.[128,152–154] In HBeAg positive patients, treatment with ADV for 1 year resulted in HBeAg seroconversion in 12%, serum HBV DNA in less than 10^3 copies/mL in 21% and normalization of ALT in approximately 48% of patients.[127] The rate of HBeAg seroconversion increased to 29% after 2 years and 43% after 3 years of treatment. In HBeAg negative patients, serum HBV DNA of less than 10^3 copies/mL and normalization of ALT were observed in 51% and 72%, respectively, after 1 year of ADV.[154] After 5 years of therapy, the serum HBV DNA were less than 10^3 copies/mL in 67% of patients, and ALT level normalized in 69%. The reported incidence of ADV resistance is 0% after 1 year, 3% after 2 years and 29% after 5 years of antiviral therapy (Fig. 1).[154] The primary mutations associated with ADV resistance are rtN236T and rtI233V in the D domain and rtA181V in the B domain of HBV polymerase. In comparison with more than 100-fold decrease in sensitivity to LVD associated with the two primary mutations, the rtN236T mutation confers only a 5–10-fold decrease in sensitivity to ADV *in vitro*,[155] which may explain the delayed emergence of this mutant.

In LVD-resistant patients treated with ADV monotherapy, the rate of antiviral resistance was 6–18% after

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1 year and 21–38% after 2 years.[156,157] Switching therapy from LVD to ADV may enhance the acquisition of another mutation and induce replication of HBV DNA.[158–160] On the other hand, combination therapy of LVD and ADV effectively suppressed viral replication and maintained high efficacy in LVD-resistant patients with chronic HBV infection.

ETV

Entecavir is a guanine analogue and Chang *et al.* have reported that ETV is effective in reducing the serum level of HBV DNA compared with LVD in HBeAg positive patients (Table 2).[159] The cumulative proportion of patients with undetectable HBV DNA (<300 copies/mL) increased to 81% after 1 year of therapy and 93% after 5 years of therapy.[160] After 1 year of treatment with ETV, the serum ALT level was normalized in approximately 70% of patients, and increased to 90% of patients after 5 years. Lai *et al.* have reported that ETV is more efficacious in HBeAg negative patients compared with LVD (Table 2).[161] ETV is the most potent of the currently available anti-HBV drugs because it affects multiple functions of the polymerase, including priming, reverse transcription and DNA elongation.[162]

Entecavir was licensed for the treatment of chronic hepatitis B in Japan in 2006. In nucleos(t)ide-naive patients, ETV is given at dose of 0.5 mg/day.

The rate of ETV resistance was extremely low in nucleoside-naive patients.[160,163,164] The incidence of ETV resistance in nucleos(t)ide analogue-naive patients was reported to be 1.2% at 3 years (Fig. 1).[160,163,164] HBeAg loss was observed in 8% of these patients. The response to ETV was lower in LVD-resistant patients than in nucleos(t)ide analogue-naive patients. In LVD-resistant patients, 20% of patients had undetectable HBV DNA levels after 48 weeks of ETV therapy, and the resistance rate to ETV was 26% at 3 years. Patients with HBeAg at the initiation of ETV had a resistance rate to ETV of 36% at 3 years. On the other hand, patients without HBeAg at the initiation of ETV did not have resistance to ETV at 3 years (Fig. 2).[160,165] In LVD-resistant patients, the risk of the development of resistance to ETV is much higher than those without LVD resistance.[160,165]

The resistance to ETV is principally associated with the mutations rtM250V, rtI169T or rtS202I in addition to the primary LVD resistance mutations rtM204V + rtL180M. The need for multiple mutations to induce ETV resistance suggests a higher genetic barrier to resistance and explains the low rate of resistance to ETV in nucleos(t)ide analogue-naive patients.

Table 2 Efficacy of nucleoside analogues for chronic hepatitis B

	Subject: HBeAg positive patients[159]								
	n	Change of HBV DNA (log copies/mL)		Negativity of HBV DNA of <300 copies/mL		Normalization of ALT		SC	
ETV 0.5 mg	354	−6.9	$P < 0.001$	67%	$P < 0.001$	68%	$P < 0.05$	21%	$P = 0.33$
LVD 100 mg	355	−5.4		36%		60%		18%	
	Subject: HBeAg negative patients[161]								
	n	Change of HBV DNA (log copies/mL)		Negativity of HBV DNA of <300 copies/mL		Normalization of ALT			
ETV 0.5 mg	325	−5.0	$P < 0.001$	90%	$P = 0.001$	78%	$P < 0.05$		
LVD 100 mg	323	−4.5		72%		71%			

ALT, alanine aminotransferase; ETV, entecavir; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus; LVD, lamivudine; SC, seroconversion; VR, virological response.

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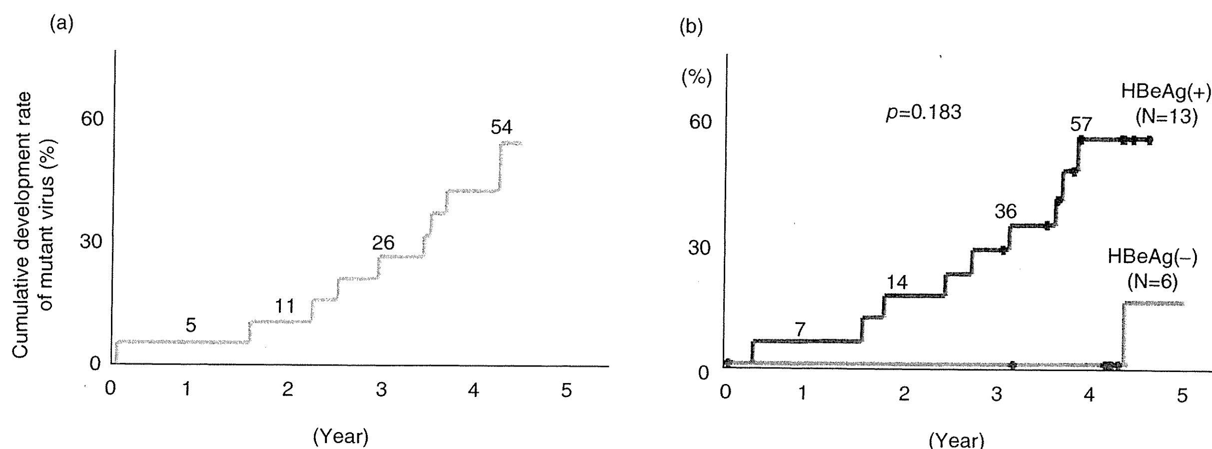

Figure 2 Cumulative development rate of mutant virus after the initiation of entecavir monotherapy in hepatitis B patients with resistance after the administration of lamivudine monotherapy.[164] (a) Cumulative development rate of mutant virus in all patients. (b) Cumulative development rate of mutant virus based on the difference of hepatitis B patients with positive hepatitis B e-antigen (HBeAg) and hepatitis B patients with negative HBeAg.

Consensus 9
Drug-resistant virus with specific mutations in the polymerase/reverse transcriptase gene emerges during nucleos(t)ide analogue therapy in chronic hepatitis B patients. The rtM204V/I and rtL180M mutations are associated with LVD resistance, the rtN236T and rtI233V or rtA181V with ADV resistance, and the rtM250V or rtT184G or rtS202I combined with rtM204V + rtL180M with ETV resistance. (Level 4, grade C.)

Recommendation 6
When patients with chronic hepatitis B are treated with nucleos(t)ide analogues, ETV should be given as the first-line drug because of its high efficacy and low emergence of viral resistant mutant. (Level 1b, grade A.)

Recommendation 7
The combination therapy of LVD and ADV is an effective treatment for LVD-resistant patients. (Level 1b, grade B.)

INTERFERON THERAPY FOR CHRONIC HEPATITIS B

INTERFERON (IFN) WAS the first antiviral treatment approved for chronic HBV infection. IFN-α and -β have a predominantly antiviral effect but also have an immunomodulatory effect and antiproliferative effect which is in contrast to direct antiviral agents such as nucleos(t)ide analogues. The duration of treatment is defined (usually 24–48 weeks) in IFN therapy. This finite duration of therapy is an advantage over direct antiviral agents which are usually given indefinitely. The long-term outcome of therapy is more precisely described in IFN compared to LVD due to its longer history of clinical usage.

Selection of patients

Factors associated with favorable response to IFN therapy are vigorously studied (Table 3). For HBeAg positive patients, high pretreatment ALT levels,[166] high grade of necroinflammation on liver histology and low serum HBV DNA level have consistently been shown to be predictive of favorable response.[167] Other predictive factors include female sex,[166] younger age,[168,169] and HBV genotype A versus D or B versus C.[169,170] Patients fulfilling these predictors are the best candidates for IFN treatment. For HBeAg negative patients, there is no consistent predictor of response. Adverse events such as severe infection or exacerbations of liver disease were common when IFN was given for decompensated cirrhosis. Thus, patients with decompensated cirrhosis should not be treated with IFN due to a risk of precipitating hepatic failure and fatal complications.[171,172]

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Table 3 Predictive factors for response to interferon therapy

Predictive factors	HBeAg positive	HBeAg negative
Race	No correlation	No correlation
Age	No correlation or Younger	No correlation or Younger
Sex	No correlation or Female	No correlation or Female
ALT	Higher level	No correlation or Higher level
Activity	Higher grade	No correlation
Fibrosis	Conflicting	No correlation
HBV DNA titer	Lower titer	No correlation or lower titer
Genotype	A > D, B > C	A > D, B > C
Precore	Conflicting	No correlation
Core promoter	mutant	

ALT, alanine aminotransferase; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus.

Recommendation 8
Younger age, high ALT levels, low HBV load, genotype A or B and high inflammatory activity in liver biopsy are predictive of good response to IFN. IFN therapy should be considered in patients fulfilling these predictors. (Level 2a, 2b, grade B.)

Recommendation 9
Interferon should be avoided for patients with decompensated cirrhosis. (Level 4, grade D.)

Standard IFN therapy in HBeAg positive chronic hepatitis B

A meta-analysis of 16 randomized controlled studies have shown that treatment with IFN-α for 16–24 weeks versus an untreated control is associated with higher rate of HBeAg loss (33% vs 12%), HBeAg seroconversion (difference of 18%), undetectable HBV DNA by hybridization or branched chain assay (37% vs 17%), HBsAg loss (7.8% vs 1.8%) and ALT normalization (difference of 23%) (Table 4).[173] A controlled trial has shown that extending therapy for up to 32 weeks in patients who remained HBeAg positive at the end of 16 weeks of therapy improved the rate of HBeAg seroconversion.[174] The durability of HBeAg seroconversion is more than 80%, and even delayed seroconversion could occur in 10–15% of patients 1–2 years after completion of therapy.[175–177] The loss of HBsAg is reported to occur in 12–65% of patients who cleared HBeAg.[175,178] However, this is a rare event in Asian patients.[176,177]

Consensus statement 10
10-1 In HBeAg positive patients, treatment with IFN versus untreated control is associated with higher rate of HBeAg loss, HBeAg seroconversion, undetectable HBV DNA, HBsAg loss and ALT normalization. Extension of therapy improves the rate of HBeAg seroconversion. (Level 1a,1b.)
10-2 Durability of HBeAg seroconversion is more than 80%. The loss of HBsAg is rare in Asian patients. (Level 1b.)

Standard IFN therapy in HBeAg negative chronic hepatitis B

Although the rate of response at the end of therapy is 60–90%, the durability of long-term response is less

Table 4 Standard interferon therapy for HBeAg positive chronic hepatitis B. Meta-analysis of 16 randomized controlled trials

	Interferon		Control	*P*-value
Loss of HBV DNA	37%		17%	0.0001
Loss of HBeAg	33%		12%	0.0001
Loss of HBsAg	7.8%		1.8%	0.001
Seroconversion		Difference of 18%		0.002
ALT normalization		Difference of 23%		0.0001

ALT, alanine aminotransferase; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus.

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than 50%.[179,180] Longer duration of therapy is associated with improved durability of response: 10–15% with 4–6 months of therapy, 22–30% with 6–12 months of therapy and 30% with 24 months of therapy.[181–184]

Consensus statement 11

11-1 Durability of response is less than 50% in HBeAg negative patients. (Level 1b.)

11-2 Longer duration of therapy (>48 weeks) is associated with improved durability of response. (Level 2b.)

Pegylated IFN (PEG IFN)

Twenty four weeks of PEG IFN-α-2a monotherapy had higher rate of combined response (loss of HBeAg, suppression of HBV DNA <500 000 copies/mL and ALT normalization) compared to standard IFN-α-2a.[185] Another study with 24 weeks of PEG IFN-α-2b monotherapy also showed a higher rate of HBeAg loss and HBV DNA suppression compared to standard IFN-α-2b.[169]

Controlled studies comparing the 48 weeks of PEG IFN-α-2a and LVD in HBeAg positive and negative patients revealed that PEG IFN had a higher rate of sustained response.[170,171] Seroconversion of HBeAg (32% vs 19%), ALT normalization (41% vs 28% in HBeAg positives and 59% vs 44% in HBeAg negatives), HBV DNA suppression (HBV DNA <10 000 copies/mL, 32% vs 22% in HBeAg positives; HBV DNA <20 000 copies/mL, 43% vs 29% in HBeAg negatives) and negative HBV DNA (14% vs 5% in HBeAg positives and 19% vs 7% in HBeAg negatives) were more frequent in PEG IFN treated patients.

Differences were reported in outcome of the antiviral treatment of patients infected with different genotypes; genotype B is associated with a higher rate of antiviral response to IFN treatment than HBV genotype C among Asian patients with HBeAg positive chronic hepatitis B.[169,186,187] In multicenter trials comparing combination therapy of PEG IFN-α-2b and LVD versus PEG IFN-α-2b alone, it was shown that treatment with PEG IFN-α-2b is the best therapy to achieve HBsAg clearance in patients with genotype A compared with D.[188,189]

Combination or sequential therapy

Combination of two antiviral agents with different mechanisms of action seems a logical approach to improve efficacy. In fact, simultaneous combination of LVD and PEG IFN has a higher rate of HBV suppression, ALT normalization and less frequent emergence of LVD-resistant mutant virus compared to LVD alone. However, there is no difference in treatment response between the simultaneous combination of LVD and IFN or PEG IFN compared to IFN or PEG IFN alone (Table 5).[132,133,170]

There are several clinical trials of sequential therapy with LVD followed by IFN.[190–194] Common to all studies is that the sequential therapy had no advantage over IFN alone. Some studies have shown the suggestive evidence that sequential therapy had a higher rate of HBV suppression, ALT normalization and less frequent emergence of LVD-resistant mutant virus compared to LVD alone (Table 5).[190–194] However, because the study protocols and their results are variable, a conclusive result could not be drawn.

Table 5 Sequential therapy of lamivudine and interferon

		BR	SC	VR	LVD-R
Manesis *et al.* 2006	Sequential	39%	NA	28%	
(*n* = 36)[190]	IFN	22%	NA	19%	
Shi *et al.* 2006	Sequential	53%	NA	14%	0%
(*n* = 162)[191]	LVD	36%	NA	18%	23%
Yurdaydin *et al.* 2005	Sequential	51%	NA	54%	24%
(*n* = 78)[193]	LVD	41%	NA	59%	53%
Sarin *et al.* 2005	Sequential	40%	40%	40%	15%
(*n* = 75)[194]	LVD	14%	11%	16%	8%
Schalm *et al.* 2000	Sequential	50%	36%	55%	0%
(*n* = 226)[192]	IFN	50%	22%	49%	0%
	LVD	63%	19%	63%	31%

BR, biochemical response; IFN, interferon; LVD, lamivudine; LVD-R, lamivudine resistant mutation; NA, not applicable because hepatitis B e-antigen patients are studied; SC, seroconversion; VR, virological response.

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Long-term outcome

The end-point of antiviral therapy is to prevent liver cirrhosis and HCC. Meta-analysis of five studies including 935 patients revealed that IFN treatment significantly decreased the incidence of cirrhosis with the combined risk ratio of 0.65 (95% confidence interval [CI] = 0.47–0.91).[195] Meta-analysis of 11 studies including 2082 patients revealed that IFN treatment significantly decreased the incidence of HCC with the combined risk ratio of 0.59 (95% CI = 0.43–0.81).[195] These results suggest that IFN prevents progression of liver disease to liver cirrhosis or delays the development of HCC, as long as it is within 4–7 years of follow up which is the length of follow up in these studies. Sustained response to IFN therapy was associated with increased survival.[175,181,196,197] To further elucidate the impact of IFN on the natural course of chronic hepatitis B, studies with larger populations followed for longer periods may be needed.

Consensus statement 12

12-1 IFN therapy prevents progression to cirrhosis or the development of HCC. (Level 1a.)

12-2 IFN therapy is associated with improved survival. (Level 1b.)

Adverse effects

The most frequent adverse effects are flu-like symptoms, fatigue, myelosuppression and dermal reaction at the injection site. Others include alopecia, depression and thyroid dysfunction. Less frequent but severe adverse events include interstitial pneumonitis, exacerbation of underlying autoimmune disorders, cerebral vascular events and flare of hepatitis.

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MAJOR ARTICLE

Transmissibility of Hepatitis B Virus (HBV) Infection through Blood Transfusion from Blood Donors with Occult HBV Infection

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Background. Studies of the transmissibility of hepatitis B virus (HBV) in occult hepatitis B (OHB) through blood transfusion are scarce. We aimed to determine the transmissibility of HBV in blood donors with OHB through transfusion in animal and human studies.

Methods. Among 217,595 blood donors, 67 donors with OHB were identified. Four chimeric mice populated with human hepatocytes were inoculated with 2 donor serum samples. Serial serum and liver HBV DNA levels were measured. Forty-nine recipients of blood transfusions traced from 10 donors with OHB (9 of whom were positive for antibody to hepatitis B surface antigen [anti-HBs]) were tested for HBV infection. Homology and phylogenetic analyses between the HBV genomic sequences of donors and recipients were performed.

Results. Serum HBV DNA was detectable (10^4 copies/mL) in 1 mouse at weeks 5 and 7 after inoculation. Total HBV DNA and HBV replication template (covalently closed circular DNA) and hepatitis B core antigen were detected in the mouse liver. After transfusion, 45 recipients (91.8%) had no HBV infection (ie, they tested negative for hepatitis B surface antigen and HBV DNA). Four tested positive for HBV DNA. In 3 recipients, 83%–86% homology and distant phylogenetic relatedness with their donor HBV excluded transmission through transfusion. The remaining recipient HBV had 95% sequence homology with her donor HBV, compatible with acquisition of HBV infection from the transfusion. High anti-HBs levels in 7 other recipients suggested recent transfusion-related HBV immune response.

Conclusions. OHB donor blood infectivity was shown in our animal and human studies. However, the risk of HBV transmission in humans was low, especially from blood products obtained from donors with OHB who were anti-HBs positive.

Occult hepatitis B (OHB) virus infection is defined as having detectable hepatitis B virus (HBV) DNA in the serum or livers of subjects who test negative for the hepatitis B surface antigen (HBsAg). Owing to the extremely low serum HBV DNA level (usually <100 IU/mL) [1], which requires highly sensitive assays to confirm the diagnosis, accurate data on the prevalence and transmissibility of OHB are very limited. The latter issue is of particular importance in improving transfusion safety. Posttransfusion acute hepatitis B has been reported to occur at a rate of 0.9% [2], even after the implementation of serological screening for HBV.

Current information on the transmissibility of OHB through transfusion mainly relies on a few case series in which the possible link has not been clearly established by strict viral sequence comparisons between the HBV strains in the donors and the recipients. OHB is not

Received 6 September 2010; accepted 17 December 2010.

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Clinical Infectious Diseases 2011;52(5):624–632

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1058-4838/2011/525-0001$37.00
DOI: 10.1093/cid/ciq247

a common entity in most countries, with a typical prevalence of <1% [3–6]. Identification of a blood donor with OHB requires considerable time for performance of nucleic acid testing (NAT) followed by extremely sensitive polymerase chain reaction (PCR) assays. These also require a high expenditure on resources. Once the donated blood is identified as OHB positive, it is discarded for obvious ethical reasons. Retrospective tracing studies are difficult to conduct because of the time lag between identification of the OHB products and contact of recipients. In addition, in areas where HBV is highly endemic, a large proportion of the population would have been naturally infected with or vaccinated against HBV.

We conducted the present study to determine the transmissibility of HBV from donors with OHB in 2 parts: (1) a chimeric mice study, to determine whether serum obtained from donors with OHB was infectious; and (2) a recipient look-back study to determine whether OHB-containing blood components were transfusion-transmissible in human recipients.

SUBJECTS AND METHODS

Subjects

The present study recruited all healthy blood donors found to have OHB during the period from 1 April 2007 through 31 January 2009 in the Hong Kong Red Cross Blood Transfusion Service. These subjects were identified by testing their blood samples for HBsAg (Prism HBsAg; Abbott Laboratories) and NAT for HBV DNA (Procleix Trigis system; Chiron; 95% detection limit, 12.2 IU/mL). All HBsAg-negative and HBV DNA–positive samples had HBV DNA levels measured by real-time PCR in the Mx4000 Multiplex Quantitative PCR System (Stratagene), using primers and 6-carbosyfluorescein (FAM)–labeled TaqMan probe targeting to conserved regions in the HBV surface gene, as described previously [7].

Of 217,595 donors, 67 subjects with OHB (0.031%) were identified. They were all negative for antibodies to human immunodeficiency virus and hepatitis C virus. Of these 67 subjects with OHB, 44 consented to participate in this study for the following additional tests: determination of the HBV DNA level, with the Artus HBV RG assay (Qiagen), which has a linear range of detection of 1.1 IU/mL to $>4 \times 10^9$ IU/mL; assessment for antibodies to HBsAg (anti-HBs) and hepatitis B core antigen (anti-HBc; Roche Diagnostics); the chimeric mice study; and the recipient look-back study with HBV genome sequencing.

Chimeric Mice Study

Two donor samples indicating OHB—1 with undetectable anti-HBs and 1 with anti-HBs titer ≥10 IU/L (conventionally taken as "protective" against HBV infection)—were used to inoculate 4 chimeric mice (2 mice for each sample). These were severe combined immunodeficiency (SCID) mice transgenic for the urokinase-type plasminogen activator gene (uPA+/+/SCID+/+ mice), with the livers replaced by human hepatocytes [8] (Phoenix Bio). Human serum albumin produced by human hepatocytes was measured by enzyme-linked immunosorbent assay (Bethyl Laboratories). One hundred microliters of the donor serum, after 10-fold concentration, were intravenously injected into the mice. Serum samples were taken weekly from the mice from week 4– to 9 after inoculation. At week 9, the mice were killed for liver immunostaining and intrahepatic HBV DNA and covalently closed circular DNA (cccDNA) measurements. HBV DNA levels were quantified by real-time PCR targeting a small S region [9], with the sensitivity of 1000 copies/mL (200 IU/mL). The cccDNA, replicative template of HBV was quantified by real-time detection–PCR using a set of conserved primers [10]. Animal protocols were performed in accordance with guidelines for animal experimentation.

Liver sections were blocked with antibody diluent (Dako), incubated with rabbit anti-HBc (Dako,) at room temperature for 1 h, washed in phosphate-buffered saline, and then incubated with goat anti-rabbit immunoglobulin G conjugated with Cy3 (Chemicon international) or goat anti-human albumin labeled with fluorescein isothiocyanate (Bethyl Laboratories). Sections were washed with phosphate-buffered saline and observed in a fluorescent microscope (Eclipse E800M; Nikon).

Look-Back Study

Data tracing of all recipients of blood components from the 44 donors with OHB were retrieved from the database of the Hong Kong Red Cross Blood Transfusion Service. Data obtained included sex, age, the date and type of blood products transfused, indication for transfusion, the nature of the illness, and HBsAg status prior to the targeted (look-back) transfusion.

Two genomic regions (pre-S and core) were sequenced from the serum of the 44 donors with OHB. In brief, HBV DNA was extracted from their serum samples using the QIAamp MinElute Virus Vacuum Kit (Qiagen). The pre-S and core regions were amplified by nested PCR, and the 2 resulting amplicons, covering the pre-S and core regions at nt 2814–237 and 1607–2385, respectively, were sequenced bi-directionally. These 2 most variable HBV regions [1, 11] were used for homology and phylogenetic comparisons between paired donors and recipients strains. Thirty-one of the 44 donors with OHB had successful viral sequencing results at either the pre-S or core regions. Recipients of transfusions from these 31 donors with OHB were contacted. HBsAg, anti-HBs, anti-HBc, and HBV DNA levels (determined as described above) were checked in recipients who consented to take part in the study. For recipients with detectable HBV DNA, the pre-S and core regions were also sequenced by PCR. Viral sequence homology of the donor and recipient

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strains was performed using the CLUSTAL X program [12]. Genetic distances were estimated by the maximum composite likelihood method, and phylogenetic trees were constructed by using the neighbor-joining method, using the Molecular Evolutionary Genetics Analysis (MEGA) software, version 4 [13]. Bootstrap resampling tests were performed 1000 times to confirm the reliability of the phylogenetic tree, and values >70% were considered to be significant [14, 15]. The presence of the common surface gene mutants (G145R) were determined by PCR sequencing using primers spanning the HBV surface gene at nucleotides 426–600 (amino acid residues, 134–148).

The study was approved by the Institutional Review Board of the University of Hong Kong and the Hospital Authority Hong Kong West Cluster. Written, informed consent was obtained from both the donors and recipients. For those recipients aged <18 years, consent was obtained from parents.

Statistical Analysis

The present study was descriptive in nature. Continuous variables were expressed as mean values with standard deviations and ranges.

RESULTS

Forty-four of the 67 identified OHB donors consented to have additional blood samples obtained for further studies. Thirty-three donors (75%) had anti-HBs levels ≥10 IU/L. The donors' demographic characteristics, serologic parameters, and HBV DNA levels are listed in Table 1.

Table 1. Demographic Characteristics, Serologic Parameters, and Hepatitis B Virus (HBV) DNA Levels for 44 Donors with Occult Hepatitis B Infection

Characteristic	Value
Ratio of male to female subjects	32:12
Age, years	
Mean ± SD	46.9 ± 11.9
Range	16.8–61.2
HBV DNA level	
No. (%) of donors with an HBV DNA level <1.1 IU/mL (%)	26 (59.1)
No. (%) of donors with an HBV DNA level ≥1.1 IU/mL, (%)	18 (40.9)
Mean	15.8
SD	45.5
Range	1.1–196.6
G145R mutations (the vaccine escape mutants), no. (%) of donors	0 (0)
Anti-HBs level	
No. (%) of donors with anti-HBs ≥10 IU/mL	33 (75%)
Titer	
Mean ± SD	217.4 ± 309.9
Range	11.1 to >1000
Anti-HBc	
No. of donors with positive anti-HBc	42 (95.5%)

NOTE. All samples positive for antigen to hepatitis B core antigen (anti-HBc) samples were of immunoglobulin G subtype. Anti-HBs, antibody to hepatitis B surface antigen.

Chimeric Mice Study

Serum samples from 2 randomly selected donors (Rx5 and Rx7) with HBV DNA levels of 9.1 and 11.0 IU/mL, respectively, and anti-HBs levels of 34 IU/L and below the detection limit, respectively, were inoculated into 4 mice (2 mice for each serum sample at the DNA amount of 52.8 and 63.8 copies). Serum HBV DNA was detectable in 1 mouse (PXB 8–22), which was inoculated with serum with undetectable anti-HBs from donor Rx7 at weeks 5 and 7 (2.4×10^4 and 2.0×10^4 copies/mL, respectively). The other 3 mice had no detectable serum HBV DNA up to 9 weeks. Total DNA and cccDNA (0.0458 and 0.0103 copies/cells, respectively) were detected in the liver of the PXB 8-22 mouse, whereas HBV DNA and cccDNA were not detectable in the livers of the other 3 mice. The immunofluorescent microcopy for hepatitis B core antigen (HBcAg) and human albumin in the liver tissues showed that the human hepatocytes expressed HBcAg (Figure 1).

Look-Back Study

Thirty-one donors with OHB had successful viral sequence generation. Two hundred seventy-two recipients who had received blood products within 3 years from these 31 donors with OHB were identified. Forty-nine recipients from 10 donors were successfully traced and consented to participate in the study. The remaining 223 recipients were not being able to undergo further investigations because they could not be contacted ($n = 46$), refused to participate in the study ($n = 30$), or died ($n = 147$). The causes of death for these 147 subjects were traced successfully. Only 16 deaths (10.9%) were liver related (3 recipients died of hepatitis C virus–related cirrhosis and/or liver cancer, and 13 recipients were known to have chronic HBV infection before transfusion and died of HBV related cirrhosis and/or liver cancer). None died because of fulminant HBV infection.

Detailed demographic characteristics and clinical, serological, and virological parameters of the 10 donors with OHB and the 49 recipients (ratio of male to female subjects, 24:25) are listed in Table 2. All 10 donors were repeated donors. The sample with which OHB was first diagnosed by NAT is defined as the "index sample"; the sample from the donation received by the 49 recipients is the "look-back sample" (HBV DNA levels were available in some cases); the sample taken when they were recalled for further study is the "sample at the time of further investigations." The median age of the recipients was 49.9 years

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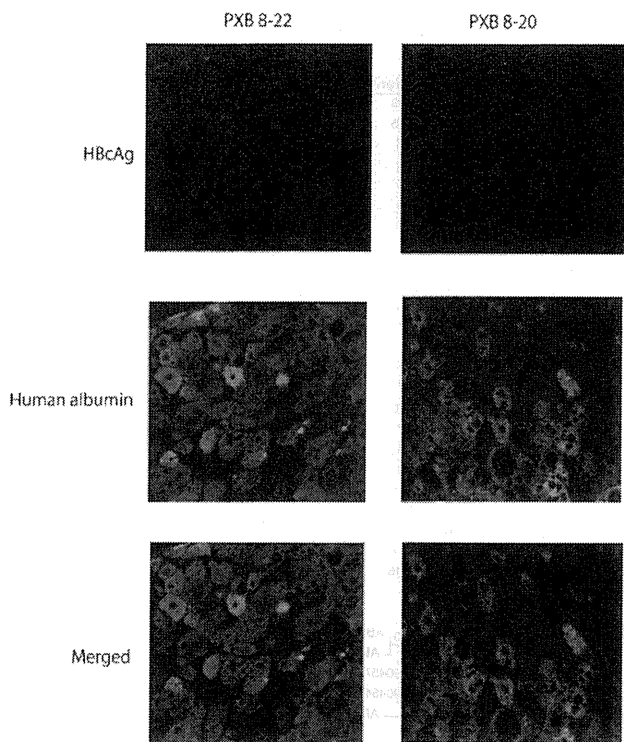

Figure 1. Staining for hepatitis B core antigen (HBcAg). In the PXB 8-22 mouse, staining for HBcAg was confined to areas where mouse liver had been replaced for human hepatocytes (the areas stained for human albumin). Co-localization of HBcAg and human hepatocytes (~40% of total human hepatocytes at this area) was demonstrated by double-staining for HBcAg and human albumin, whereas no merged stain was found in the PXB 8-20 mouse.

(range, 3.1–87.4 years). Fifteen recipients were immunocompromised because they had undergone organ transplantation with immunosuppressive therapy, had a hematologic malignancy, or had end-staged renal failure.

Twenty recipients (40.8%) were positive for immunoglobulin G anti-HBc (15 with anti-HBs) (Table 2). Thirteen (26.5%) had detectable anti-HBs levels but no anti-HBc, suggesting HBV vaccination. Nine of these 13 recipients were aged <35 years. Both anti-HBc and anti-HBs were undetectable in 16 recipients (32.7%), who were considered to be susceptible to HBV infection but noninfected. Only the 15 recipients carrying both anti-HBc and anti-HBs were considered possibly HBV infected by transfusion, and among these, 2 (Rx69 and Rx96) were found to be HBV DNA positive (HBV DNA level, 9.3 and 14 IU/mL, respectively), indicating an OHB carrier state. The viral sequence homology between the recipients and donors HBV was 83% and 95%, respectively, with values confirmed by the phylogenetic analyses (Figure 2). Another 2 recipients (Rx42 and Rx70) tested positive for HBsAg with detectable HBV DNA, indicating chronic HBV carriage (one was immunocompromised [viral load, 7.3×10^7 IU/mL]; the other had a viral load of 33 IU/mL). The viral sequence homology for these 2 recipients' and donors' HBV was only 88% and 86%, respectively, with values confirmed by the phylogenetic analyses (Figure 2). The liver biochemistries of these 4 recipients were all normal.

Of the remaining 45 recipients, 16 had undetectable anti-HBc and anti-HBs; 3 were positive for anti-HBc, but anti-HBs was undetectable; 13 had undetectable anti-HBc but were positive for anti-HBs; and 13 were positive for both anti-HBc and anti-HBs (Table 2). Of the 26 recipients with positive anti-HBs, 8 had moderately high anti-HBs titers (≥200 IU/mL). Two (Rx39 and Rx66) of these 8 recipients were negative for anti-HBc, and 6 (Rx40, Rx47, Rx67, Rx71, Rx95, and Rx97) were positive for anti-HBc. One 12-year-old recipient (Rx66) had an anti-HBs titer of 412 IU/L tested 26 months after transfusion. The other 7 recipients (age, 47–75 years) had anti-HBs levels between 299 and >1000 IU/L tested 20–52 months after transfusion. These 7 recipients received blood components carrying HBV DNA in all tested donations (5–27 /mL) from 4 donors (Rx36, Rx34, Rx58, and Rx15). Only 6 of the 27 recipients of transfusions from these 4 donors remained susceptible and noninfected (ie, negative for both anti-HBc and anti-HBs).

There were 223 recipients for whom further investigations were not performed. Twenty-four were HBsAg positive and 199 had unknown HBsAg status before transfusions. Of these 199 recipients, 24 underwent HBsAg testing after transfusions. Twenty-three of these 24 recipients were negative for HBsAg. The remaining recipient (who died of lung cancer 4 months after the transfusion) was positive for HBsAg.

DISCUSSION

In the present study, we demonstrated in the chimeric mice that serum obtained from donors with OHB with undetectable anti-HBs could be infectious. Serum HBV DNA was detected in 1 infected mouse, and more importantly, intrahepatic HBV DNA, cccDNA (the viral template in hepatocyte nucleus for viral transcription), and HBcAg (the viral protein) were found in the human hepatocytes of the mouse liver. On the contrary, the sample with positive anti-HBs titer did not infect the mice. Additional animal studies using chimeric mice model should be performed with more samples from donors with OHB, with and without anti-HBs, to confirm our preliminary finding.

It has been shown that anti-HBc–positive blood products can cause acute HBV infection in recipients [16–19]. The infectivity of anti-HBs–positive blood products is still not well defined in humans [20]. Of the 49 recipient follow-up samples, 4 were positive for HBV DNA. Two were HBsAg positive, indicating chronic hepatitis B carriage; 2 were HBsAg negative, indicating OHB. The viral sequence homology of only 83%–86%, confirmed by the distant phylogenetic relatedness between donor and recipient strains, excluded a transfusion origin in 3 of these

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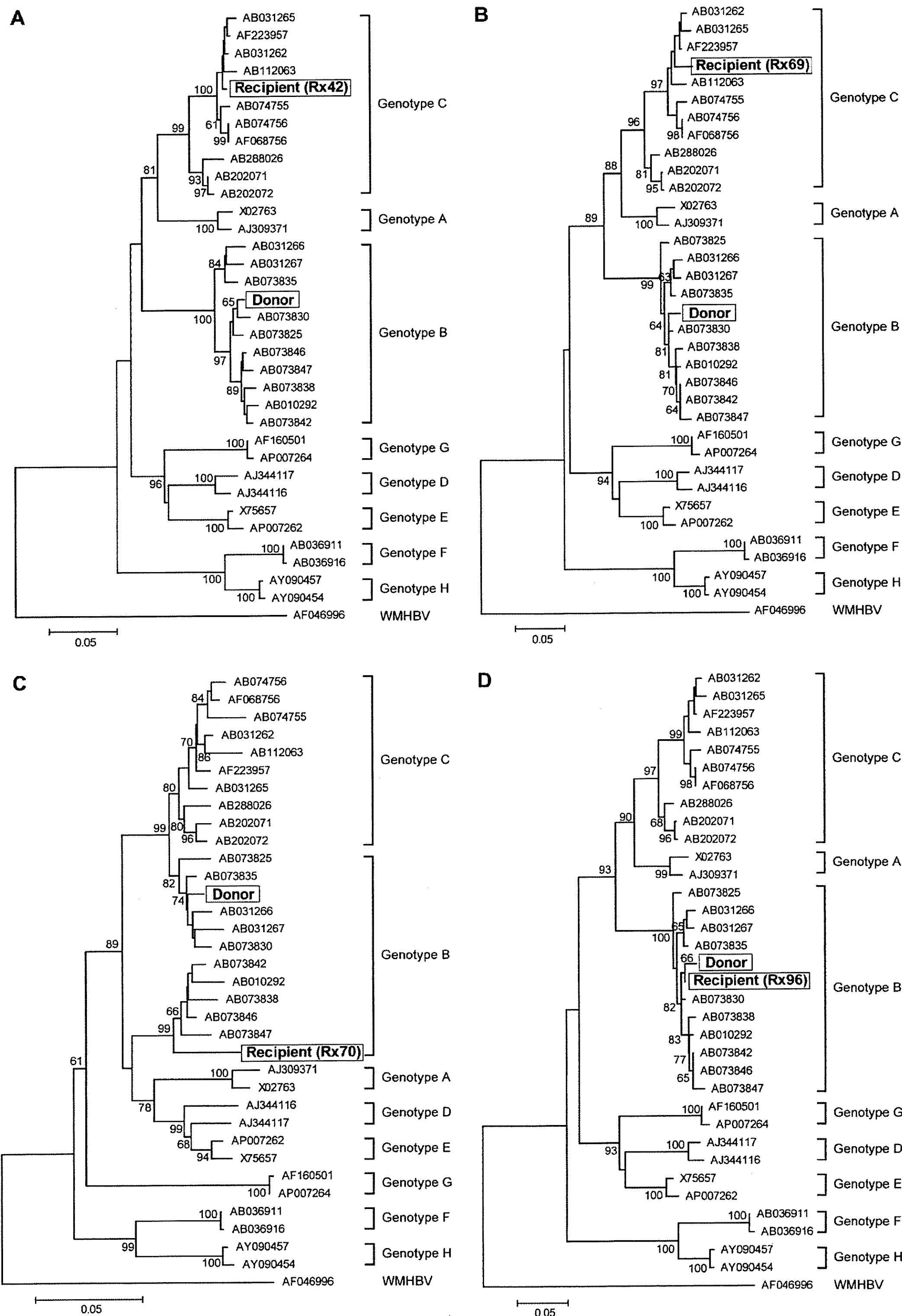

Figure 2. Phylogenetic analysis of the pre-S or core sequences of the 4 recipients with detectable HBV DNA (*A,* Rx42; *B,* Rx69; *C,* Rx70; and *D,* Rx96) and their corresponding donors. Sequences from the recipients and donors (boxed and in bold-face fonts) were subjected to phylogenetic analysis, along with the same pre-S or core regions from GenBank reference HBV sequences of various HBV genotypes (10 each of genotypes B and C, the 2 most common genotypes in Hong Kong, and 2 each of genotypes A, D, E, F, G, and H) and a reference woolly monkey hepatitis B virus (WMHBV) sequence. Sequences retrieved from the GenBank are designated by their accession numbers. The figures on the nodes indicate the percentage of occurrences by bootstrap analysis of 1000 replicates. Only bootstrap values >70% are shown. The length of the horizontal bars indicates the number of nucleotide substitutions per site.

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