Mutational Pattern of Hepatitis B Virus on Sequential Therapy With Famciclovir and Lamivudine in Patients With Hepatitis B Virus Reinfection Occurring Under HBIg Immunoglobulin After Liver Transplantation

HANS L. TILLMANN,[1] CHRISTIAN TRAUTWEIN,[1] THOMAS BOCK,[1] KLAUS H. W. BÖKER,[1] ELMAR JÄCKEL,[1] MEDEA GLOWIENKA,[1] KARL OLDHAFER,[2] INSA BRUNS,[3] JOSEE GAUTHIER,[3] LYNN D. CONDREAY,[3] HANS-RUDOLF RAAB,[2] AND MICHAEL P. MANNS[1]

Famciclovir (FCV) and lamivudine (LAM) reduce viral replication in patients with recurrent hepatitis B virus (HBV) infection after orthotopic liver transplantation (OLT). Eighteen of 20 patients with insufficient response to FCV were treated with 100 mg LAM daily after OLT. These patients had shown nonresponse (n = 5), partial response (n = 7), or breakthrough (n = 6) during FCV therapy. Despite passive immunoprophylaxis with hepatitis B immunoglobulin after liver transplantation, HBV reinfection had occurred in 14 of 15 transplanted patients. HBV-DNA levels and the regions A to E of the HBV-DNA polymerase gene were analyzed before and after treatment failure to either therapy. Within 4 weeks on LAM, all but 1 patient showed a 95% average reduction of the HBV-DNA level. As with FCV, we did not observe any severe side-effects attributable to LAM. However, 7 patients developed a breakthrough within 12, 29 (n = 2), 32, 37, 54, and 145 weeks under treatment with LAM associated with the methionine-to-valine signature mutation (M552V) in the YMDD motif in all. With FCV, no unique, but a dominant, resistance pattern with the L528M mutation was identified for patients with breakthrough under FCV. In contrast, nonresponders or patients with partial response to FCV did not exhibit such mutations. Our results indicate that the L528M mutation is a risk factor for LAM breakthrough, because breakthrough during LAM occurred earlier in patients with this mutation (50 ± 10 weeks vs. 120 ± 21 weeks). Because breakthrough on either treatment is frequent for this specific group of patients, the use of combination therapy should be explored. (HEPATOLOGY 1999;30:244-256.)

Hepatitis B virus (HBV) infection affects more than 300 million people worldwide. The infection frequently leads to cirrhosis and hepatocellular carcinoma. Orthotopic liver transplantation (OLT) is a therapeutic option for end-stage liver disease. Results of liver transplantation for HBV-related end-stage liver disease are worse compared with other benign indications for liver transplantation,[1] because HBV reinfection often runs a severe course in liver graft recipients,[2,3] with an estimated 3-year survival of only 54%.[4] Treatment with passive immunoprophylaxis with hyperimmunoglobulin against hepatitis B surface antigen (HBIg) has improved the outcome of these patients.[5] In some patients, in particular if pretransplantation replication levels were high, this regime fails to prevent recurrence of HBV. Thus, additional treatment options are needed for these patients.[6] Two new oral nucleoside analogues, lamivudine (LAM) and famciclovir (FCV), have been reported as potent inhibitors of hepadnaviral replication *in vitro*[7,8] and *in vivo*.[9-12] HBV replicates via an intermediate RNA step through the process of reverse transcription. As a consequence, the mutation rate in the HBV genome is increased compared with other DNA viruses as a result of the lack of proofreading activity in the viral polymerase. Nonresponse or resistance to nucleoside analogues can be related to particular mutations conferring resistance.[13] The sequence of HBV in patients with breakthrough on LAM was found to map to the YMDD motif of the HBV-DNA polymerase.[14-19] It is unclear whether there is a specific mutation conferring resistance against FCV. Only 3 reports[20-22] described the selection of mutations in the HBV-polymerase gene on FCV treatment. Furthermore, the clinical impact of these mutations on response and development of resistance to subsequent LAM therapy remains unclear.

In our center, patients were treated with FCV according to different study protocols (SB 42810/110 and SB 42810/129[23]). In 1 study (SB 42810/110), patients positive for HBV DNA pre-OLT were treated with FCV before and FCV plus HBIg after OLT (n = 12) to prevent HBV reinfection. In the other study (SB 42810/129), patients were treated with FCV for HBV reinfection that had occurred while under or after HBIg immunoprophylaxis (n = 22). Eighteen patients of

Abbreviations: HBV, hepatitis B virus; OLT, orthotopic liver transplantation; HBIg, hepatitis B hyperimmune globulin; LAM, lamivudine; FCV, famciclovir; HBsAg, hepatitis B surface antigen; anti-HBs, hepatitis B surface antigen antibody; HCV, hepatitis C virus; ALT, alanine transaminase; CHE, cholinesterase; PCR, polymerase chain reaction; HBeAg, hepatitis B e antigen; aa, amino acid; PCV, penciclovir;

From the Departments of [1]Gastroenterology and Hepatology, and [2]Abdominal and Transplant Surgery, Medizinische Hochschule Hannover, Hannover, Germany; and [3]Glaxo Wellcome Inc., Research Triangle Park, NC.

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Address reprint requests to: Michael P. Manns, M.D., Professor and Chairman, Department of Gastroenterology and Hepatology, Medizinische Hochschule Hannover, Carl Neuberg Str. 1, 30623, Hannover, Germany. E-mail: manns.michael@mh-hannover.de; fax:49-511-532-4896.

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TABLE 1. Patient Characteristics for Those With HBV Reinfection After OLT and a Complete Response to FCV

Patients	Age at OLT	OLT	Indication for OLT	Lowest Anti-HBs Titer Before Reinfection	Anti-HBs Titer at Reinfection	Reinfection (wk after OLT)†	Start FCV (wk after OLT)§	BK FCV Weeks on FCV	Interval Between FCV and LAM (wk)	Start LAM (wk after OLT)	Weeks on LAM until BK or (by end October 98)
R01	55	29.12.95	HBV-Ci	122 U/L	37 U/L*	26	−12	38	0	41	54
R02	45	03.02.94	HBV/HDV-Ci	206 U/L	195 U/L*	5	−4	106	0	126	37
R03	56	20.06.97	HBV-Ci	‡	‡	0	−49	66	0	28	(40)
R04	57	26.06.94	HBV-Ci, HCC	741 U/L	36 U/L	4	20	34	12	74	145
R05	60	13.06.91	HBV-Ci	29 U/L	<10 U/L	17	195	140	0	350	29
R06	34	22.10.90	HBV-Ci	>150 U/L	>150 U/L	107	178	150	0	342	(76)

Abbreviations: BK, breakthrough; HBV-Ci, hepatitis B virus–induced cirrhosis; HBV/HDV-Ci, cirrhosis caused by HBV and hepatitis D virus infection; HCC, hepatocellular carcinoma; HCV, hepatitis C virus superinfection; NR, nonresponse; PR, partial response; R, response.

*Patients with a mutation within the "a-determinant" of the surface protein, previously associated with HBIg treatment.

†Reinfection was determined by positive test for HBsAg.

‡This patient had never lost his HBsAg from the circulation.

§The duration of FCV therapy is the start of LAM minus the interval between the 2 treatments and the start of FCV.

both studies, either developing breakthrough or not responding sufficiently to FCV, were subsequently treated with LAM.

To study the mutations occurring during treatment with nucleoside analogues, the HBV-DNA polymerase gene was sequenced before therapy and when nonresponse, breakthrough, or insufficient response to firstly FCV (n = 20) and then LAM (n = 7) therapy had emerged. The aims of this study were to analyze: 1) mutational selection in the domains A-E of the HBV-DNA polymerase during FCV therapy; 2) response to LAM in patients with a different response pattern to FCV (nonresponse, partial response, or breakthrough to FCV); 3) whether certain mutations can be identified for nonresponse or partial response to FCV; 4) if FCV-selected mutations might change after cessation of FCV therapy; 5) the response to LAM in relation to mutations selected on FCV; and 6) mutations selected in patients with subsequent breakthrough on LAM.

PATIENTS AND METHODS

Patients. Nineteen male and 1 female patients (PR14) (Tables 1, 2, and 3, and Fig. 1) grouped according to their response to FCV treatment were included in the study. Three patterns were distinguished: 1) patients with initial response to FCV (HBV DNA became negative by hybridization assay); 2) patients with partial response to FCV (>50% reduction of HBV DNA during FCV therapy); and 3) nonresponse to treatment (no significant reduction in HBV DNA on FCV). These 3 patient groups were identified as R (responder), PR (partial responder), or NR (nonresponder).

Of the 8 patients (R01, R02, R03, PR16, PR17 PR18, PR19, NR26) treated with FCV before liver transplantation, 4 (PR16, PR17 PR18, NR26) were switched to LAM before potential OLT. Two of these (PR16, PR17) died as a result of advanced liver disease before a suitable graft became available. A third patient (PR18) died after OLT as a result of the inability to be withdrawn from mechanical ventilation and subsequent pneumonia. Another patient (NR26) was well 12 weeks after OLT without evidence of reinfection. One (PR19) of the 8 patients remained viremic despite FCV treatment. His overall clinical condition had improved (currently taken off the waiting list), and FCV treatment was continued. The other 3 patients (R01, R02, R03) were transplanted successfully on FCV, but did experience HBV recurrence.

Twelve patients had become hepatitis B surface antigen (HBsAg)- and HBV-DNA–positive after liver transplantation when treatment with FCV was started. One (PR14) of these patients was not switched to LAM, because liver function parameters were stable.

All patients who underwent OLT had received 10.000 units of HBIg during the anhepatic phase, and thereafter HBIg was given and adjusted to maintain serum hepatitis B surface antigen antibody (anti-HBs) levels above 100 U/L. However, in some patients, it was impossible to maintain anti-HBs levels constantly above 100 U/L because of the lack of compliance or immediately before evident HBV recurrence.

Fifteen patients experienced a viral recurrence. Of the 2 patients (R03, NR24) who never became HBsAg-negative, 1 (R03) had been

TABLE 2. Patient Characteristics for Those With HBV Reinfection After OLT and a Partial or Nonresponse to FCV

Patients	Age at OLT	OLT	Indication for OLT	Lowest Anti-HBs Titer Before Reinfection	Anti-HBs Titer at Reinfection	Reinfection (wk after OLT)†	Start FCV (wk after OLT)¶	BK FCV (wk on FCV)	Interval Between FCV and LAM (wk)	Start LAM (wk after OLT)	Weeks on LAM until BK or (by end October 98)
PR11	39	08. and 10.08.91	HBV/HDV-Ci, HCV	55 U/L	41 U/L*	60	129	PR, 59‖	8	227	32
PR12	48	09.01.95	HBV/HDV-Ci, HCC	41 U/L	14 U/L	33	45	PR	3	86	(died in week 3)
PR13	63	05.06.95	HBV-Ci	86 U/L	75 U/L	7	25	PR	0	80	(98)
PR14§	53	30.07.91	HBV-Ci	31 U/L	0 U/L	80	214	PR	—	§	§
PR15	44	01.10.93	HBV-Ci	105 U/L	38 U/L	87	99	PR	0	222	(43)
NR21	38	21.08.91	HBV-Ci, HCC	40 U/L	5 U/L	110	178	NR	0	212	12
NR22	33	28.07.93	HBV-Ci	141 U/L	707 U/L*	26	54	NR	34	113	(160)
NR23	59	09.04.96	HBV-Ci	252 U/L	277 U/L	15	49	NR	0	69	(died in week 36)
NR24	59	30.01.97	HBV-Ci, HCC	‡	‡	0	2	NR	0	15	29

Abbreviations: BK, breakthrough; HBV-Ci, hepatitis B virus–induced cirrhosis; HBV/HDV-Ci, cirrhosis caused by HBV and hepatitis D virus infection; HCC, hepatocellular carcinoma; HCV, hepatitis C virus superinfection; NR, nonresponse; PR, partial response; R, response.

*Patients with a mutation within the "a-determinant" of the surface protein, previously associated with HBIg treatment.

†Reinfection was determined by positive test for HBsAg.

‡This patient had never lost his HBsAg from the circulation.

§This patient was not treated with Lamivudine after Famciclovir was withdrawn, because of only minimal changes in histology.

‖This patient developed a breakthrough on FCV after a partial response to FCV.

¶The duration of FCV therapy is the start of LAM minus the interval between the 2 treatments and the start of FCV.

TABLE 3. Patients With Insufficient Response to FCV Before Potential OLT

Patients	Age at Start FCV	Start Date FCV	Indication for OLT	Weeks on FCV	BK FCV (wk on FCV)	Interval Between FCV and LAM (wk)	Start LAM (wk after FCV)	Weeks on LAM Until Death or By End October 98	Outcome by October 1998
PR16	51	05.05.95	HBV-Ci	31	PR	0	8.12.95	9	Died before OLT
PR17	59	04.06.96	HBV-Ci	23	PR	0	15.11.96	14	Died before OLT
PR18	57	4.96	HBV-Ci	65	PR	2	27.6.97	29	Died 10 weeks after OLT
PR19	33	04.09.97	HBV-Ci.	69	NR	—	Not yet started	—	Clinical well despite BK on FCV
NR26	46	6.5.97	HBV-Ci	41	NR	0	19.2.98	36	OLT, without reinfection

Abbreviations: BK, breakthrough; HBV-Ci, hepatitis B virus–induced cirrhosis; NR, nonresponse; PR, partial response.

treated with FCV before OLT. Eleven of the other 13 patients developed HBV reinfection within 4 to 107 weeks post-OLT, despite ongoing HBIg treatment (Tables 1-3). Another 2 patients (PR14, NR21), whose anti-HBs prophylaxis was stopped 1.5 years and 2 years after OLT, respectively, became positive 80 and 110 weeks after OLT. All patients were HBsAg-positive when either FCV or LAM was started. Three patients (R02, PR11, and PR12) were coinfected with hepatitis D, and 1 (PR11) was also infected with the hepatitis C virus (HCV). In the HCV-RNA–positive patient, HCV RNA became negative 160 weeks after OLT, and his anti-HCV response disappeared 208 weeks after OLT.

Medication. Patients were given FCV adjusted to creatinine clearance: cretonne clearance >60 mL/min, 500 mg FCV, 3 times per day; creatinine clearance 30 to 60 mL/min, 500 mg FCV, twice daily; creatinine clearance <30 mL/min, 500 mg FCV, daily. LAM was started when patients failed to respond to FCV, or showed insufficient response or breakthrough. LAM dosage was 100 mg daily, except for patient WW, who received 200 mg LAM daily for the last 2 weeks of LAM treatment. Both treatment protocols were approved by the ethics committee of the Medizinische Hochschule Hannover. All patients had given written informed consent before inclusion in the studies. Response was defined as complete when HBV DNA became undetectable by hybridization assay, as partial when HBV DNA decreased but remained positive by hybridization assay, and as failed when HBV DNA remained unchanged. Breakthrough was defined as a constant increase of HBV DNA after initial reduction of HBV DNA to <50% of initial HBV-DNA level.

Alanine Transaminase, Prothrombin Time, and Cholinesterase. Alanine transaminase (ALT), prothrombin time, and cholinesterase (CHE) were determined by standard methods.

Hepatitis Markers. HBV-DNA levels were determined with the liquid hybridization assay (Genostics, Abbott Laboratories, Chicago, IL) with a cut-off level of approximately 1 pg/mL. If HBV-DNA levels were undetectable by hybridization assay, a polymerase chain reaction (PCR) was performed as previously described.[24] Serological markers for HBsAg, hepatitis B e antigen (HBeAg), anti-HBs, antibodies to hepatitis B e antigen, hepatitis B core, hepatitis D virus, and anti-HCV were determined by commercial tests (EIA, Abbott Laboratories). HCV RNA was detected as previously described.[25]

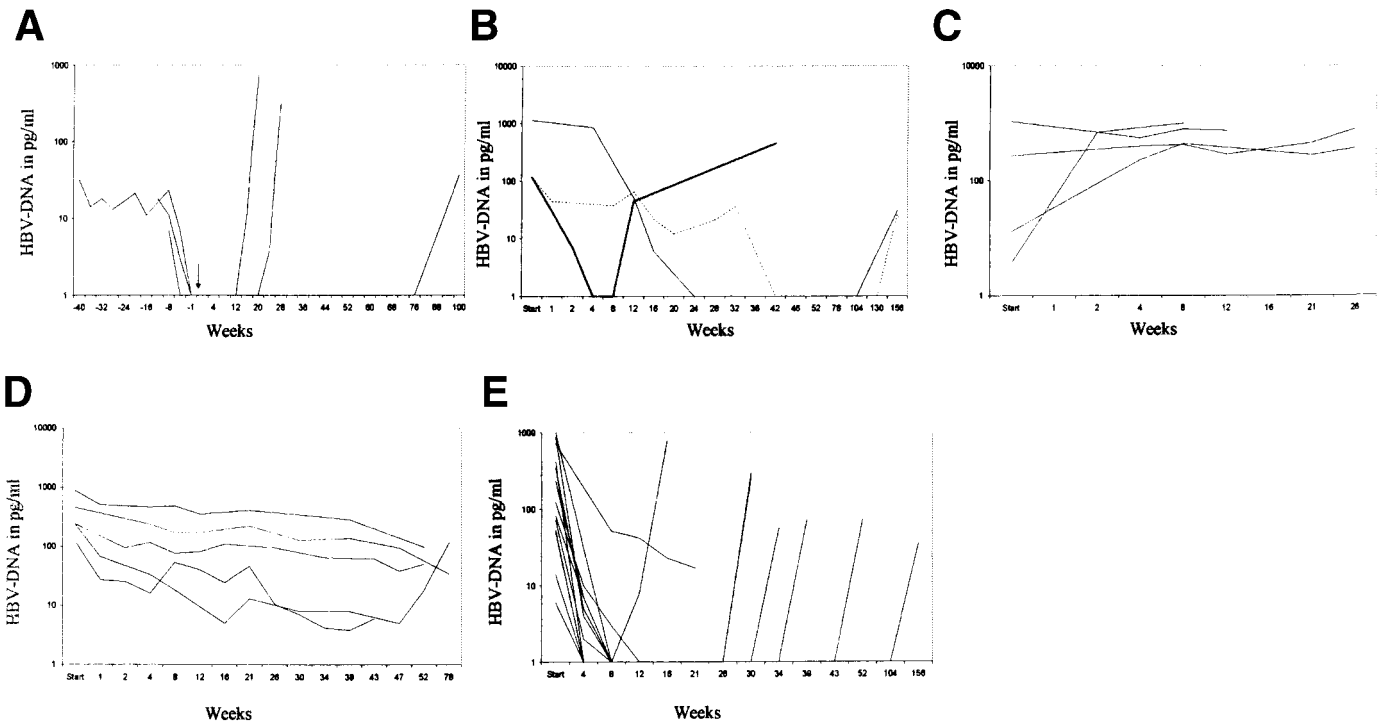

FIG. 1. HBV-DNA response on treatment. (A) HBV DNA in FCV responders started on FCV before OLT (n=3). (B) HBV DNA in FCV responders started on FCV after OLT (n=3). (C) HBV DNA in FCV nonresponders started on FCV after OLT (n=4). (D) HBV DNA in FCV partial responders started on FCV after OLT (n=5). (E) HBV DNA after switch to LAM (n=18). The HBV-DNA level is given in a logarithmic scale (pg/mL) on the *y*-axis, while the *x*-axis indicates the weeks in relation to start of either treatment (B to E) or in relation to OLT.

TABLE 4. Primers Used for PCR and Sequencing

Primers used to amplify and sequence 377/840 fragment:	
primer 377:	5′-GGA TGT GTC TGC GGC GTT T-3′
primer 840:	5′-ACC CCA TCT TTT TGT TTT GTT AGG-3′
primer 5RC:	5′-CAA AAG AAA ATT GGT AAC AGC GGT A-3′
Primers used to amplify and sequence 12F/5RC fragment:	
primer 12F:	5′-AGA CTC GTG GTG GAC TTC TCT 3′
primer 5RC:	5′-CAA AAG AAA ATT GGT AAC AGC GGT A-3′
primer 377:	5′-GGA TGT GTC TGC GGC GTT T-3′
Primers used to amplify and sequence 12F/6R fragment:	
primer 12F:	5′-AGA CTC GTG GTG GAC TTC TCT 3′
primer 840:	5′-ACC CCA TCT TTT TGT TTT GTT AGG-3′
primer 5RC:	5′-CAA AAG AAA ATT GGT AAC AGC GGT A-3′
primer 377:	5′-GGA TGT GTC TGC GGC GTT T-3′
primer 6R:*	5′-ATG AGC TTT GCT CCA GAC C-3′

*Primer 6R was not used for sequencing.

HBV-DNA Amplification Before Sequencing. HBV DNA was derived from serum samples, after lysis in sodium dodecyl sulfate buffer and proteinase K digestion with subsequent phenol/chloroform extraction. The extracted DNA was resuspended in 10 mmol/L Tris-HCl (pH 8.0) and amplified by PCR using the following protocol: the PCR was started at 94°C, followed by 30 or 40 cycles each comprising 30 seconds at 94°C, 15 seconds at 55°C, and 1 minute at 72°C; the final extension at 72°C was for 5 minutes. HBV DNA was amplified using 3 different sets of primers. One set amplified a fragment from nucleotides 377 to 840 with primer 377 (Table 4) and primer 840 (Table 4). The second fragment was amplified from nucleotides 252 to 793 with the primers 12F (Table 4) and 5RC (Table 4). The third fragment containing the nucleotides 252 to 1308 was amplified with the primers 12F (Table 4) and 6R (Table 4).

HBV-DNA Sequencing. Before sequencing, the PCR products were purified using the QIAquick PCR purification kit (Qiagen Inc., Chatsworth, CA) and semiquantified by agarose gel electrophoresis. Samples were sequenced using an automated fluorescent DNA sequencer with the primers indicated in Table 4.

Approximately 50 to 100 ng of DNA was used per sequencing reaction. DNA was dissolved in 8 µL ddH_20 and mixed with 4 µL primer (1,6 µmol/L) and 1 µL dimethylsulfoxide. HBV-DNA sequences were analyzed from about nucleotides 270 to 950 determined by direct sequencing of amplified PCR products corresponding to amino acids (aa) 396 to 622 of the polymerase gene including the domains A to E, of which regions B and C have been shown to contain mutations on FCV or LAM therapy.[26] Because of the nature of the overlapping reading frames of the HBV genome, this region also corresponds to aa 40 up to the stop codon of HBsAg.[27] The nucleotide numbering starts with the *Eco*R1 site in the HBV genome (*Eco*R1 = 1), and the sequences were compared with the appropriate wild-type sequence of ayw[27] or adw2.[28]

TABLE 5. HBeAg in Patients With Complete Response to FCV and Subsequent "Breakthrough" on FCV

	HBeAg Before FCV	Lowest HBeAg on FCV	HBeAg at BK on FCV	HBeAg Before LAM	Lowest HBeAg on LAM	HBeAg at BK on LAM
R01	+	−n.Tx.	+	+	+	+
R02	+	−n.Tx.	+	+	+	+
R03	+	−n.Tx.	+			
R04	−	−	−	−	−	−
R05	+	+	+	+	+	
R06	+	−	+	+	—	

NOTE. Three patients constantly HBeAg-negative were from Southern and Eastern Europe in accordance with previous data from Germany.[24]
Abbreviation: BK, breakthrough.

TABLE 6. HBeAg in Patients With Partial or Nonresponse to FCV After OLT

	HBeAg Before FCV	Lowest HBeAg on FCV	HBeAg at BK on FCV	HBeAg Before LAM	Lowest HBeAg on LAM	HBeAg at BK on LAM
PR11	+	+	+	+	+	+
PR12	+	+	+	+	+	
PR13	−	−	−	−	−	
PR14	+	+	+			
PR15	+	+	+	+	+	
NR21	+	+	+	+	+	+
NR22	+	+	+	−	−	
NR23	+	+	+	+	+	
NR24	+	+	+	+	+	+

NOTE. Three patients constantly HBeAg-negative were from Southern and Eastern Europe in accordance with previous data from Germany.[24]
Abbreviation: BK, breakthrough.

RESULTS

Response of HBV-DNA Level to Treatment

The HBV-DNA levels were measured at designated time points from all 20 patients receiving FCV and from 18 patients who subsequently received LAM therapy.

In 3 (R01, R02, R03) of the 8 patients receiving FCV pre-OLT, HBV-DNA levels were reduced below the level of detection of the HBV-DNA hybridization assay within 3, 12, and 38 weeks (Fig. 1A). One patient (R03) never became HBsAg-negative post-OLT and HBV DNA started to rise 4 months after OLT. Two of these patients (R01, R02) remained HBV-DNA–negative on FCV and HBIg until reinfection occurred at 5 and 26 weeks post-OLT. While HBV DNA rose immediately after reinfection in 1 patient (R02), HBV DNA remained below the detection level of the hybridization assay for another 97 weeks in patient R02 despite occurrence of HBsAg (Table 1). From the remaining 5 patients (PR16, PR17, PR18, PR19, NR26) who received FCV pre-OLT and did not sufficiently respond to therapy, 4 were switched to LAM, which resulted in negative HBV-DNA levels.

Twelve patients (R04, R05, R06, PR11, PR12, PR13, PR14, PR15, NR21, NR22, NR23, NR24) received FCV because of HBV recurrence after OLT. Three of 12 patients (R04, R05, R06) (Fig. 1B) had a complete response within 3, 24, and 40 weeks of treatment; 4 of 12 patients (NR21, NR22, NR23, NR24 (Fig. 1C) did not respond to FCV; and 5 of 12 patients

TABLE 7. HBeAg in Patients With Insufficient Response to FCV Before Potential OLT

	HBeAg Before FCV	Lowest HBeAg on FCV	HBeAg at BK on FCV	HBeAg Before LAM	Lowest HBeAg on LAM
PR16	+	+	+	+	+
PR17	+	+	+	+	+
PR18	−	−	−	−	−
PR19	+	+	+		
NR26	−	−	−	−	−

NOTE. Three patients constantly HBeAg-negative were from Southern and Eastern Europe in accordance with previous data from Germany.[24]
Abbreviation: BK, breakthrough.

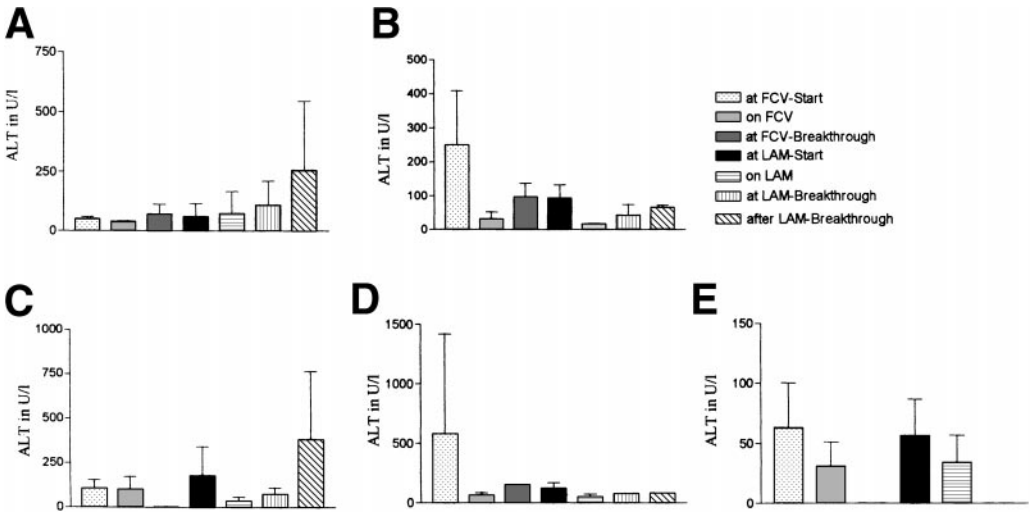

FIG. 2. ALT response on treatment. (A) ALT in FCV responders started on FCV before OLT (n=3). (B) ALT in FCV responders started on FCV after OLT (n=3). (C) ALT in FCV nonresponders started on FCV after OLT (n=4). (D) ALT in FCV partial responders started on FCV after OLT (n=5). (E) ALT in patients with insufficient response to FCV before potential OLT (n=5). ALT is given in U/L. The legend is shown at the right side for (A to E).

(PR11, PR12, PR13, PR14, PR15 (Fig. 1D) showed a partial response with a 80% to 90% reduction within 16 to 55 weeks; 1 of these patients (PR11) developed breakthrough with significant increase of HBV-DNA levels.

In summary, 7 patients (R01, R02, R03, R04, R05, R06, PR11) were switched to LAM for breakthrough under FCV, 1 of whom (PR11) had only shown a partial response, 5 patients (NR21, NR22, NR23, NR24, NR26) as a result of nonresponse, and 6 (PR12, PR13, PR15, PR16, PR17, PR18) because of partial response to FCV and deteriorating liver function. Two patients (PR14, PR19) with partial response only to FCV were not switched to LAM because of stable liver function.

All patients receiving LAM for insufficient FCV response, nonresponse, or breakthrough showed decreasing HBV-DNA levels during treatment. Within 4 weeks, HBV-DNA levels were reduced by 95%, and HBV DNA became undetectable within 4 to 12 weeks on LAM by the hybridization assay (Fig. 1E) in all patients but R03. This patient showed a slow reduction of HBV DNA without becoming HBV-DNA–negative by hybridization assay until week 24. Seven patients (R01, R02, R04, R05, PR11, NR21, NR24) developed a breakthrough on LAM within 12, 29, 29, 32, 37, 54, and 145 weeks on LAM, respectively. The 1-year resistance rate was calculated to be approximately 50% in patients treated with LAM after HBV reinfection and insufficient response to FCV (5 [R02, R05, PR11, NR21, NR24] of 10 [R02, R05, PR11, NR21, NR24, and R01, R04, R06, PR13, NR22] who completed 1-year therapy on LAM). The resistance developed faster in patients with breakthrough on FCV than in those with nonresponse or partial response without breakthrough (87 ± 26 vs. 102 ± 25; $P = .6$; not significant). Likewise, resistance developed faster in those patients in whom the HBV-DNA polymerase contained a mutation at position aa528 than in those without this mutation (50 ± 10 weeks vs. 120 ± 23 weeks; $P > .4$; not significant).

HBeAg Status on Treatment

Three patients from Eastern and Southern Europe were HBeAg-negative (R04, PR13, PR18). All the others were HBeAg-positive. The HBeAg status remained unchanged on either treatment with the exception of 6 patients (Tables 5, 6, and 7). Only 1 patient (R06) became HBeAg-negative on FCV and on LAM after breakthrough to FCV. One (NR22) lost his HBeAg after FCV was stopped before starting LAM. Four patients (R01, R02, R03, and NR26) who underwent OLT on nucleoside analogue therapy became HBeAg-negative after surgery. Recurrence of HBeAg was found in 3 (R01, R02, and R03) of the latter soon after HBsAg recurrence. Thus, only 1

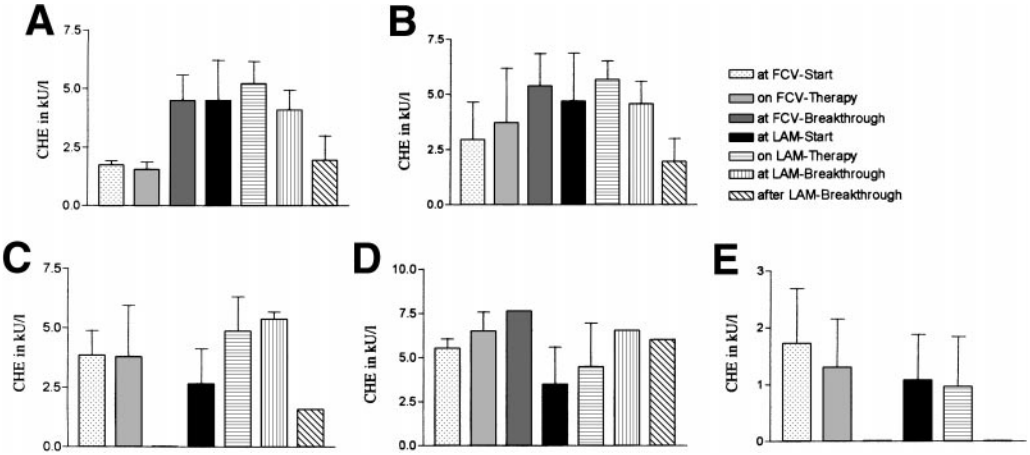

FIG. 3. CHE response on treatment. (A) CHE in FCV responders started on FCV before OLT (n=3). (B) CHE in FCV responders started on FCV after OLT (n=3). (C) CHE in FCV nonresponders started on FCV after OLT (n=4). (D) CHE in FCV partial responders started on FCV after OLT (n=5). (E) CHE in patients with insufficient response to FCV before potential OLT(n=5). CHE is given in kU/L. The legend is shown at the right side for A to E.

TABLE 8. Amino Acid Changes in the HBV-DNA Polymerase During the Course of Treatment in Patients With Complete Response to FCV and Subsequent "Breakthrough" on FCV

Corresponding aa of the HBsAg	Sample Date (wk)†	45	116-119	120	140	142	144/145	164	171	172	176	195	198/199	207	210	...
AA of Pol		402	473-475	476	496	499	501	521	528	529	532	552	555	563	567	614
Wild-type adw2		S	Q-Y-G	T	Y	Y	W	V	L	A	A	M	V	Q	S	V
Wild-type ayw		Y	Q-H-G	T	Y	F	R	V	L	A	A	M	V	Q	S	I
R01																
pre-OLT	−5, −26		del*	N*			W/E*		L						S/A	
Adw2																
Reinfection	26		del*	N*			E*		L						A	
FCV-BK	34		del*	N*			E*		L						A	
Lam-BK	41		del*	N*			E*		M*			V*			A	
R02																
pre-OLT	−7	T			F*	L			L			M				
Ayw																
Reinfection	5	T			F*	L			L			M				
FCV-BK	102, 115	T			F*	L			L/M*			M				
LAM-BK	163	T			Y	L			M*		S*	V				
R03																
pre-OLT	−49					Y*		V	L							
Ayw																
Reinfection	3					Y*		V	L							
FCV-BK	17, 44					Y*		L*	M*							
R04																
Reinfection	7								L	A		M		S/Stop*		
Ayw																
Start FCV	20								L	A		M		S/Stop*		
FCV-BK	54, 74								L	T*		M		S/Stop*		
LAM-BK	219								M*	A		V*		S		
R05																
Start FCV	195								L			M	V		A	
Adw2																
FCV-BK	350								L/M*			M	V		A	
LAM-BK	379								M*			V*	I		A	
R06																
Start FCV	178								L						A	
Adw2																
FCV-BK	328, 394								M*						A	

NOTE. "AA of Pol" indicates the position of the amino acid change within the polymerase protein. The amino acid of the wild-type adw2 and ayw are shown. The corresponding position within the HBsAg is indicated by "corresponding aa of the HBsAg." The amino acid changes or amino acids not known to be variants are indicated for each patient at the time of reinfection, FCV-BK (famciclovir breakthrough), FCV-NR (famciclovir nonresponse), FCV-PR (famciclovir partial response), and LAM-BK (lamivudine breakthrough). The amino acids are given in the 1-letter code; if 2 letters are written, there was a codominance of 2 strains. The *dotted line* indicates the amino acid within the domain A; a *single line* indicates the domain B associated amino acids; the *double line* indicates the domain C.

*Those amino acid changes associated with a mutation within the hepatitis HBsAg.

†Sample dates are shown in relation to OLT.

of 17 HBeAg-positive patients lost HBeAg as a result of nucleoside analogue therapy (Tables 5, 6, and 7).

Biochemical Liver Function Parameters

During FCV therapy, ALT levels decreased in all complete and partial responders (Fig. 2A, 2B, 2D, and 2E), while ALT remained unchanged in the nonresponders (Fig. 2C). When breakthrough occurred, the ALT level increased in complete responders (Fig. 2A and 2B), and in the patient (PR11) with partial response and subsequent breakthrough.

The liver function parameters, CHE (Fig. 3) and prothrombin time (data not shown), improved in responders started on FCV post-OLT and in partial responders (Fig. 3B and 3D), whereas there was no significant effect in those patients treated with FCV pre-OLT and in the nonresponders (Fig. 3A, 3C, and 3E). The subjective parameters at commencement of LAM are the result of the fact that treatment was switched when deterioration was suspected. (Figs. 2C-2E, 3C-3E). After breakthrough on either FCV or LAM, an increase of transaminases and a decrease of liver function parameters was evident (Figs. 2 and 3), except for the 3 responders (R01, R02, R03) who were started on FCV pre-OLT. They were switched to LAM before deterioration of liver function.

Sequencing Results

Analysis of the HBV-DNA Polymerase Region A to E on FCV Treatment. Sequence data were generated from sera before starting FCV and LAM treatment, after breakthrough on FCV, after breakthrough on LAM (R01, R02, R04, R05, PR11, NR21, NR24), and at the time of HBV reinfection (R01, R02, R03, R04, PR11, PR12, NR21, NR22, NR24). Amino acid

TABLE 9. Amino Acid Changes in the HBV-DNA Polymerase During the Course of Treatment in Patients With Partial or Nonresponse to FCV After OLT

Corresponding aa of the HBsAg	Sample Date (wk)†	45	68	101	104	106	109	109	118	120	144/145	164	171	195	206	207	210	—
AA of Pol		402	424	458	461	463	466	468	474	476	501	521	528	552	562	563	567	614
Wild-type adw2		S	N	R	A	L	N	R	Y	T	W	V	L	M	V	Q	S	V
Wild-type ayw		Y	N	R	A	L	N	R	H	T	R	V	L	M	V	Q	S	I
PR11																		
Reinfection	52									N*		V	L	M				
Ayw																		
Start FCV	129									N*		V	L	M				
FCV-BK	223, 226, 227									N*		V/L*	L/M*					
LAM-BK	253									N*		L*	M*	V*				
PR12																		
Reinfection	38	T																
Adw2																		
Start FCV	45	T																
Start LAM	86	T																
PR13																		
Start FCV	25							G							E*	S*		
Ayw																		
Start LAM	80							G							E*	S*		
PR14																		
Start FCV	214																A	
Adw2																		
Control	300																A	
PR15																		
Start FCV	99				T												A	
Adw2																		
Start LAM	222, 232				T												A	
NR21																		
Reinfection	113		N	R/K			N			T			L	M			S	
adw2																		
Start FCV	178		N	R/K			N			T			L	M			S	
Start LAM	191		K*	R			D			N*			L	M			A	
LAM-BK	234, 236		K*	R			D			N*			M	V*			A	
NR22																		
Reinfection	31	T				M					E*							
Adw2																		
Start FCV	54, 75, 85	T				M					W/E*							
Start LAM	113	T				M					W/E*							
NR23																		
Start FCV	49												L	M			A	
adw2																		
Start LAM	69, 100												L	M			A	
NR24																		
pre-OLT	−5									T/N*		V	L	M			A	
Adw2																		
Reinfection	2									N*		V	L	M			A	
Start LAM	15									T		V	L	M			A	
LAM-BK	44									T		L*	M*	V*			A	

NOTE. "AA of Pol" indicates the position of the amino acid change within the polymerase protein. The amino acid of the wild-type adw2 and ayw are shown. The corresponding position within the HBsAg is indicated by "corresponding aa of the HBsAg." The amino acid changes or amino acids not known to be variants are indicated for each patient at the time of reinfection, FCV-BK (famciclovir breakthrough), FCV-NR (famciclovir nonresponse), FCV-PR (famciclovir partial response), and LAM-BK (lamivudine breakthrough). The amino acids are given in the 1-letter code; if 2 letters are written, there was a codominance of 2 strains. The *dotted line* indicates the amino acid within the domain A; a *single line* indicates the domain B associated amino acids; the *double line* indicates the domain C.

*Those amino acid changes associated with a mutation within the hepatitis HBsAg.

†Sample dates are shown in relation to OLT.

changes occurring during therapy or amino acids not described as known variations of the HBV-DNA polymerase[26] are shown in Tables 8, 9, and 10. None of the patients with a partial response, other than 1 (PR11) who also showed an increase of HBV DNA on FCV after initial partial response to FCV (8 of 9), showed any changes of the HBV-DNA polymerase protein while on treatment with FCV (Tables 9 and 10). Likewise, none of the patients except NR21 with nonresponse on FCV exhibited any changes of the HBV-DNA polymerase protein while on treatment with FCV (Table 9). The mutations selected in the nonresponder (NR21) contained sequence changes outside the domain B of the HBV-

TABLE 10. Amino Acid Changes in the HBV-DNA Polymerase During the Course of Treatment in Patients With Insufficient Response to FCV Before Potential OLT

Corresponding aa of the HBsAg	Sample Date (wk)†	101	104	106	118	120	198/199	210
AA of Pol		458	461	463	474	476	555	567
Wild type adw2		R	A	L	Y	T	V	S
Wild type ayw		R	A	L	H	T	V	S
PR16								
Start FCV	0	G				T/I*		
Ayw								
Start LAM	31, 38	G				T/I*		
PR17								
Start FCV	0							
Adw2								
Start LAM	23							
PR18								
Start FCV	0			V	R			
Ayw								
Start LAM	65			V	R			
PR19								
Start FCV	0					N*	I*	A
Adw2								
Control on FCV	44					N*	I*	A
NR26								
Start FCV	0		T					A
Adw2								
Start LAM	41		T					A

NOTE. "AA of Pol" indicates the position of the amino acid change within the polymerase protein. The amino acid of the wild-type adw2 and ayw are shown. The corresponding position within the HBsAg is indicated by "corresponding aa of the HBsAg." The amino acids are given in the 1-letter code; if 2 letters are written, there was a codominance of 2 strains.

*Those amino acid changes associated with a mutation within the hepatitis HBsAg.

†Sample dates for patients with insufficient response to FCV before potential OLT; sample dates are given in relation to start of FCV.

DNA polymerase (N424K, R458K, N466D, S567A), and only 1 mutation (N424K) was located in the domain A as shown in Table 9. Another nonresponder (NR24) demonstrated a wild-type sequence at aa 476 of the HBV-DNA polymerase at FCV breakthrough, which had also been present before OLT. Thus, no common mutation pattern was observed in patients with partial or nonresponse to FCV (Tables 9 and 10).

In contrast, all patients showing a breakthrough on FCV after initial complete response selected mutations or at least a certain pre-existing viral strain (R01) (Table 8).

In 5 (R02, R03, R05, R06, PR11) of the 7 patients with breakthrough (all responders and PR11), a leucine-to-methionine mutation at aa528 (L528M) of the HBV-DNA polymerase was determined (Tables 8 and 9). Two (R03, PR11) of these patients additionally showed a valine-to-leucine mutation at aa 521 (V521L) (Tables 8 and 9). The sixth patient (R04) had an alanine-to-threonine mutation at aa529 (A529T), and the seventh (R01) selected a serine-to-alanine mutation at aa567 (S567A) and a tryptophan–to–glutamic acid at aa501 (W501E) already previously present as a codominant strain (Table 8). In summary, no uniform mutation was found at breakthrough on FCV, but a dominance of the L528M mutation was evident. The A529T mutation has not been described before, and is also located in the domain B of the HBV-DNA polymerase.

Analysis of the HBV-DNA Polymerase Region A to E on LAM Treatment. The HBV-DNA polymerase from 7 patients (R01, R02, R04, R05, PR11, NR21, NR24), 5 of whom had developed a breakthrough on FCV before start of LAM (R01, R02, R04, R05, PR11), who subsequently developed resistance to LAM, were analyzed. All isolates analyzed revealed the methionine-to-valine mutation in the YMDD motif (M552V) (Tables 8 and 9). Those isolates (R02, R05, PR11) with a pre-existing L528M mutation remained conserved during LAM. The L528M mutation was selected in the HBV-DNA polymerase from the others (R01, R04, NR21, NR24). R04 had selected a A529T mutation on FCV, which was lost during LAM breakthrough. R02 additionally showed an alanine-to-serine mutation at aa532 (A532S) previously described to be associated with either FCV or LAM breakthrough.[15,20] This patient (R02) also exhibited a reversion back to wild type at aa496 (F496Y) already existing before FCV (Table 8). NR24 selected the V521L mutation in addition to the L528M and M552V mutations on LAM. In conclusion, the mutations at aa528 and aa552 (M552V and L528M) were common to all 7 patients, while only 1 mutation selected on FCV (A529T in R04) reverted on LAM.

Analysis of the Hepatitis B S-Gene. The open reading frame coding for the surface protein overlaps with the polymerase gene. Therefore, a mutation is likely to lead to an amino acid change in both proteins. From previous studies, we know that certain amino acid changes are associated with failure of HBIg immunoprophylaxis.[29-33] Before the start of nucleoside analogue therapy, 8 patients (PR11, NR22, NR24, R01, R02, PR16, PR19, NR26) (Tables 11, 12, and 13) also showed such mutations within the S-gene. These mutations were a G145R mutation associated with a D144E mutation of the HBsAg corresponding to a W501E mutant of the HBV-DNA polymerase in 2 patients (R01, NR22), and a P120T mutation corresponding to a T476N mutation of the HBV-DNA polymerase in 6 (R01, PR11, NR24, PR 16, PR19, NR26), of whom 1 (R01) also had the G145R and D144E mutations. The eighth patient (R02) had a T140S mutation corresponding to a Y496F mutation of the HBV-DNA polymerase. Further mutations of the HBsAg are shown in Tables 11-13.

During FCV treatment, 5 patients (R02, R03, R04, PR11, NR21) selected mutations associated with amino acid changes in the HBsAg. One patient (NR21) selected a I68N and P120T corresponding to mutations N424K and T476N within the HBV-DNA polymerase, 2 patients (R03, PR11) selected a E164D corresponding to a V521L mutation within the HBV-DNA polymerase, 1 (R02) selected a Q120P mutation on FCV, and another 1 (R04) selected a W172Stop mutation of the HBsAg corresponding to a A529T mutation within the HBV-DNA polymerase. Only 1 patient (NR21) selected a mutation (P120T) on FCV, which has been associated with failure of HBIg prophylaxis.[29-33] However, 1 nonresponder (NR24) reverted this mutation P120T on FCV.

All patients with breakthrough on LAM (Tables 11 and 12) selected mutations associated with amino acid changes in both the HBsAg and the HBV-DNA polymerase. In 6 patients (R01, R04, R05, PR11, NR21, NR24), the mutations within the YMDD motif of the HBV-DNA polymerase led to changes in the HBsAg at aa195 (I195M), and NR24 coselected the E164D corresponding to a V521L mutation within the HBV-DNA polymerase. The seventh patient (R02) showed no

TABLE 11. Amino Acid Changes in the HBsAg During the Course of Treatment in Patients With Complete Response to FCV but Subsequent Breakthrough on FCV

Corresponding aa of the Polymerase	Sample Date (wk)†	396	401	417	424	465	473-475	476	489	496	499	501	501	518	521	529	532	552	562	563	567	580
AA of HBsAg		40	45	61	68	109	116-119	120	133	140	143	144	145	162	164	172	176	195	206	207	210	225
Wild-type adw2		N	S	S	I	L	T-S-T-G	P	M	T	T	D	G	L	E	W	L	I	Y	S	S	V
Wild-type ayw		N	T	S	T	L	T-S-T-G	P	M	T	S	D	G	L	E	W	L	I	Y	S	S	V
R01																						
pre-OLT	−5, −26						T-del*	T*	M/T			D/E*	G/R*					I			S/R	
Adw2																						
Reinfection	26						T-del*	T*	T			E*	R*					I			R	
FCV-BK	34						T-del*	T*	T			E*	R*					I			R	
LAM-BK	41						T-del*	T*	T			E*	R*					M			R	
R02																						
pre-OLT	−7		L	L	T			P		S*							L	T				
Ayw																						
Reinfection	5		S	L	I			P		S*							L	T				
FCV-BK	102, 115		L	L	T			Q		S*							L	T				
LAM-BK	163		L	L	T			P		I*							V*	T				
R03																						
pre-OLT	−49										M*				E							
Ayw																						
Reinfection	3										M*				E							
FCV-BK	17, 44										M*				D*							
R04																						
Reinfection	7					L/V										W		I	Y/C	S/R*		V/A
R04																						
Start FCV	20					L/V										W		I	Y/C	S/R*		V/A
Ayw																						
FCV-BK	54, 74					L										Stop*		I	Y/C	S/R*		V
LAM-BK	219					L										W		M*	C	R*		V
R05																						
Reinfection	195																	I			R*	
Adw2																						
FCV-BK	350																	I			R*	
LAM-BK	379																	M			R*	
R06																						
Reinfection	178	S																				
Adw2																						
FCV-BK	328, 394	S																				

NOTE. "AA of HBsAg" indicates the position of the amino acid change within the HBsAg. The amino acid of the wild-type adw2 and ayw are shown. The corresponding position within the HBV-DNA polymerase protein is indicated by "corresponding aa of the polymerase." The amino acid changes are given for each patient at the start of treatment, at the time of reinfection, FCV-BK (famciclovir breakthrough), and LAM-BK (lamivudine breakthrough). The amino acids are demonstrated in the 1-letter code; if 2 letters are given, there was a codominance of 2 strains. The *dark shaded* areas indicate amino acid changes previously associated with HBIg treatment, while the *underlined* amino acid changes indicate previously described potential epitopes within the HBsAg.[53]

*Those amino acid changes associated with a mutation within the HBV-DNA polymerase.

†Sample dates are shown in relation to OLT.

new mutation at position aa195, but selected mutations outside the YMDD motif, which led to amino acid changes at aa140 and aa176 (S140I and L176V) of the S-protein (Tables 8 and 11).

DISCUSSION

HBV reinfection after OLT is associated with a poor prognosis,[2,3] especially when fibrosing cholestatic hepatitis develops.[34,35] The successful use of FCV to control HBV replication after OLT was first described in a patient with severe HBV reinfection.[10] This patient is still on FCV for 6 years now without limitations on his everyday activities. He has no HBV DNA, but has persistent HBsAg (data not shown). More recently, other patients have been treated with FCV for HBV reinfection,[36,37] but the drug has failed to control HBV replication in a proportion of patients.[36,37]

Here, we describe patients with insufficient response or breakthrough on FCV therapy and their subsequent response to LAM. Seven patients with virological breakthrough on FCV selected mutations in the HBV polymerase gene. No unique mutation pattern was found in these patients. However, a strong predominance at aa528 of the polymerase gene was evident (Tables 8-10 and 14). In addition to the V521L mutation, the L528M mutation, and the T532S mutation, we found a further mutation in close proximity to these amino acids (aa529) (Tables 8-10 and 14). Therefore, we suggest that this region in domain B of the HBV-DNA polymerase is involved in conferring resistance to FCV therapy, because 8 of 11 patients (this and earlier studies [Table 14]) selected mutations within this region under FCV therapy. In contrast, no mutation was found in this region in nonresponders or patients with partial response without

TABLE 12. Amino Acid Changes in the HBsAg During the Course of Treatment in Patients With Partial or Nonresponse to FCV

Corresponding aa of the Polymerase	Sample Date (wk)†	396	424	458	476	490	501	501	517	518	521	533	549	552	560	562	563	567
AA of HBsAg		40	68	102	120	134	144	145	161	162	164	177	193	195	204	206	207	210
Wild-type adw2		N	I	G	P	F	D	G	Y	L	E	V	S	I	S	Y	S	S
Wild-type ayw		N	T	G	P	Y	D	G	F	L	E	V	S	I	S	Y	S	S
PR11																		
Reinfection	52				T*						E			I				
PR11																		
Start FCV	129				T*						E			I				
Ayw																		
FCV-BK	223, 226, 227				T*						E/D*			I				
LAM-BK	253				T*						D*			M				
PR12																		
Start FCV	38, 45	S				L												
Adw2																		
Start LAM	86	S				L												
PR13																		
Start FCV	25											A				S*	R*	
Ayw																		
Start LAM	80											A				S*	R*	
PR14																		
Start FCV	214																	R*
adw2																		
Control	300																	R*
PR15																		
Start FCV	99									Q							N	R
adw2																		
Start LAM	222, 232									Q							N	R
NR21																		
Start FCV	113		I	G/S	P									I	S			S
adw2																		
Start LAM	178		N*	G	T*									I	N			R
LAM-BK	1914		N*	G	T*									M*	N			R
Reinfection	234, 236		N*	G	T*									M*	N			R
NR22																		
Reinfection	31	S					E*	R*	Y									R*^{V}
adw2																		
Start FCV	54, 75, 85	S					D/E*	G/R*	Y/F									R*^{V}
Start LAM	113	S					D/E*	G/R*	Y/F									R*^{V}
NR23																		
Start FCV	49													I				
adw2																		
Start LAM	69, 100													I				
NR24																		
pre-OLT	−5				P/T*						E			I				R*
adw2																		
Reinfection	2				T						E			I				R*
FCV-BK	15				P						E			I				R*
LAM-BK	44				P						D*			M*				R*

NOTE. "AA of HBsAg" indicates the position of the amino acid change within the HBsAg. The amino acid of the wild-type adw2 and ayw are shown. The corresponding position within the HBV-DNA polymerase protein is indicated by "corresponding aa of the polymerase." The amino acid changes are given for each patient at the start of treatment, at the time of reinfection, FCV-BK (famciclovir breakthrough), and LAM-BK (lamivudine breakthrough). The amino acids are demonstrated in the 1-letter code; if 2 letters are given, there was a codominance of 2 strains. The *dark shaded* areas indicate amino acid changes previously associated with HBIg treatment, while the *underlined* amino acid changes indicate previously described potential epitopes within the HBsAg.[53]

*Those amino acid changes associated with a mutation within the HBV-DNA polymerase.

†Sample dates are shown in relation to OLT.

breakthrough, which correlates with data reported recently by a French group.[38]

Because some patients did not respond or responded slowly to FCV treatment despite lack of significant mutations within the enzymatically active domains of the HBV-DNA polymerase,[36,39] additional mechanisms seem important to confer nonresponse or resistance to FCV treatment.

The active form of FCV is penciclovir (PCV). Phosphorylation of PCV is essential for activation of the prodrug. In herpes virus–infected cells, phosphorylation of PCV can be performed by the herpes virus–encoded thymidine-kinase.[40] The genome of HBV and other hepadnaviruses do not code a related enzyme. Therefore, it is most likely that host enzymes are involved in the phosphorylation of PCV in HBV-infected

TABLE 13. Amino Acid Changes in the HBsAg During the Course of Treatment in Patients With Insufficient Response to FCV Before Potential OLT

Corresponding aa of the Polymerase	Sample Date (wk)†	428	474	476	533	549	555	555	560	563	564	567
AA of HBsAg		80	118	120	177	193	198	199	204	207	208	210
Wild-type adw2		G	T	P	V	S	M	W	S	S	I	S
Wild-type ayw		G	T	P	V	S	M	W	S	S	I	S
PR16												
Start FCV	0			S*								
Ayw												
Start LAM	31, 38			S*								
PR17												
Start FCV	0								R*			
Adw2												
Start LAM	23								R*			
PR18												
Start FCV	0		A		A*	L					T	
ayw												
Start LAM	65		A		A*	L					T	
PR19												
Start FCV	0			T*			I*	L*				R*
Adw2												
control on FCV	44			T*			I*	L*				R*
NR26												
Start FCV	0	A		T						N		K
Adw2												
Start LAM	44	A		T						N		K

NOTE. "AA of HBsAg" indicates the position of the amino acid change within the HBsAg. The amino acid of the wild-type adw2 and ayw are shown. The corresponding position within the HBV-DNA polymerase protein is indicated by "corresponding aa of the polymerase." The amino acid changes are given for each patient at the start of treatment, at the time of reinfection, FCV-BK (famciclovir breakthrough), and LAM-BK (lamivudine breakthrough). The amino acids are demonstrated in the 1-letter code; if 2 letters are given, there was a codominance of 2 strains. The *dark shaded* areas indicate amino acid changes previously associated with HBIg treatment, while the *underlined* amino acid changes indicate previously described potential epitopes within the HBsAg.[53]

*Those amino acid changes associated with a mutation within the HBV-DNA polymerase.

†Sample dates for patients with insufficient response to FCV before potential OLT; sample dates are given in relation to start of FCV.

patients. Inosine monophosphate-guanosine monophosphate 5′nucleotidase (IMP-GMP-5′nucleotidase) is a main candidate for this step.[41] Therefore, one hypothesis could be a possible polymorphism for IMP-GMP 5′nucleotidase, which might account for nonresponsiveness in some patients who lack mutations in the HBV-DNA polymerase. Interestingly, 1 patient (RO1) developed the breakthrough on FCV after OLT, despite absence of mutations within the polymerase domain B. Thus, in view of missing mutations, a modified liver metabolism of the donor organ for FCV might account for this event.

TABLE 14. Mutations Occurring on FCV Treatment, Previously Described and in This Article

424	458	466	521	525	528	529	532	552	567	Wild-type
N	R	N	V	P	L	A	T	M	S	
			L	L*	M					Aye et al.[20]
					M					Aye et al.[21]
					V		S			Naoumov NY et al.[22]
									A	R01 this work
					L/M					R02 this work
			L		M					R03 this work
						T				R04 this work
					L/M					R05 this work
					M					R06 this work
			V/L		L/M					PR11 this work
K	K	D							A	NR21 this work

NOTE. Shaded areas indicate the region dominantly mutated on either treatment. The numbers in the first line indicate the position of the amino acid, and the letters in the second line indicate the amino acid of the wild-type sequence.

*Only in about 10% of clones.

All patients previously not responding to FCV or developing breakthrough on FCV subsequently responded to LAM. Thus, primary nonresponse to LAM did not occur in our patients. The 1-year breakthrough rate was estimated to be 14% in immunocompetent patients.[42] We found a breakthrough rate of approximately 50% in patients receiving immunosuppressive therapy and previous treatment with FCV within 1 year. This appears higher than the frequency of resistance without prior FCV therapy.[43]

Two different mutational patterns have been described within the YMDD motif. However, the M552V mutation was found more frequently than the M552I mutation.[44] All patients in this study developing a breakthrough during LAM therapy selected a M552V mutation in the YMDD motif associated with a second mutation at aa 528 of the HBV-DNA polymerase.

Initially, it was suspected that the aa528 mutation is not essential for resistance to LAM.[45] Recent work revealed that the 552 methionine-to-valine and the aa528 mutation each showed a low level of resistance. However, the combination of both mutations confers a similar level of resistance against LAM as the M552I mutation.[44] In our study, all patients received FCV before LAM, and the L528M mutation was already selected in some patients when LAM therapy started. Still, they responded to LAM for up to 18 months (R06). From these data, it appears that the mutation at aa528 itself only confers low levels of resistance to LAM. However, our results indicate that the pre-existing L528M mutation predis-

poses for breakthrough on LAM associated with the M552V mutation.

In vitro data indicated impaired viral replication of LAM-resistant strains,[46,47] However, our *in vivo* data show high HBV replication after the selection of LAM-resistant strains.

None of the patients with HBV recurrence despite immunoprophylaxis showed mutations associated with resistance to either FCV or LAM at the time of reinfection (Table 4). One patient (NR21) selected a mutation at aa120 of the HBsAg on FCV, which had been associated with failure of immunoprophylaxis. However, because another patient (NR24) reverted this mutation on FCV, it is unlikely that this mutation is dominantly selected on FCV. Another patient (NR26), transplanted after being switched to LAM, is still without evidence of reinfection 12 weeks after OLT, despite a P120T mutation of the HBsAg previously associated with HBIg failure.

Knowing which mutations and mechanisms confer resistance against either FCV or LAM after liver transplantation is important to design more effective therapeutic strategies to prevent HBV reinfection and avoid selection of resistant strains. FCV and LAM have an additive to synergistic effect to reduce HBV replication *in vitro*.[48,49] Thus, it seems likely that combination therapy with different antivirals may lower the risk of HBV reinfection, based on the rationale that a more sufficiently suppressed viral replication will delay the emergence of drug-resistant mutant viral strains.[50] Therefore, our results in patients with consecutive resistance to FCV and LAM support the need for controlled trials to prevent and treat HBV reinfection with combination treatment using different antivirals pre– and post–liver transplantation with or without HBIg. This approach is particularly intriguing because similar observations have been made in treating human immunodeficiency virus infection.[51] In human immunodeficiency virus, it was clearly demonstrated that simultaneous combination therapy is more effective than sequential therapy.[52] Therefore, with the evolving number of different nucleoside analogues available, combination therapy should be encouraged in HBV infection to delay resistance against nucleoside analogues.

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