Transient Selection of a Hepatitis B Virus Polymerase Gene Mutant Associated With a Decreased Replication Capacity and Famciclovir Resistance

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Prolonged therapy for chronic hepatitis B (HBV) with nucleoside analogs may result in the emergence of HBV mutants resistant to antivirals. Here, we describe the transient selection of an HBV polymerase gene mutant that was associated with viral persistence in an immune competent patient treated with famciclovir. Viral polymerase gene sequence was analyzed directly on polymerase chain reaction (PCR) products and also after cloning. The results showed the transient selection of a V542I mutant in the C domain of the viral polymerase. This mutation was associated with a stop codon at amino acid position 199 in the overlapping S gene. The mutated sequence was subcloned in a vector expressing the entire HBV pregenome to study its replication capacity after transient transfection in cultured hepatoma cells. The results showed that the V542I mutant has a decreased replication capacity compared with wild type virus and does not produce HBsAg. The sensitivity of the V542I mutant to penciclovir, the active metabolite of famciclovir, was further studied in tissue culture. This mutant was shown to be resistant to penciclovir, but remained sensitive to lamivudine, as was subsequently observed *in vivo*. These findings indicate that a prolonged administration of famciclovir may allow for the selection of HBV polymerase gene mutants in immune competent patients. The impaired replication capacity of this V542I mutant may have contributed to the absence of outgrowth of this viral strain *in vivo*. The study of the *in vitro* sensitivity of HBV polymerase mutants to nucleoside analogs will be important to design new anti-HBV strategies. (HEPATOLOGY 1999;29:230-237.)

Recently, the antiviral efficacy of new nucleoside analogs, famciclovir and lamivudine, has been assessed in chronically hepatitis B virus (HBV)-infected patients.[1,2] Famciclovir is the oral prodrug of penciclovir (9-[4-hydroxy-3 hydroxymethyl-but-1-yl] guanine; BRL 39123)-triphosphate, which was shown to inhibit hepadnavirus replication *in vitro* in tissue culture and *in vivo* in animal models of HBV infection.[3,4] It was further shown that penciclovir triphosphate not only inhibits the DNA dependent DNA polymerase activity of the HBV polymerase,[5] but also the RNA-dependent activity of this enzyme, i.e., reverse transcription, by inhibiting the synthesis of the short DNA primer for reverse transcription.[6] Preliminary evidence suggested that famciclovir may have clinical utility in the treatment of chronic hepatitis B patients[7] and in orthotopic liver transplant patients.[8] A first placebo-controlled pilot study showed a fall of HBV DNA levels by more than 90% in 6 out of 11 evaluable patients.[7] Kruger et al. have reported, in 11 patients with HBV recurrence after orthotopic liver transplantation, that famciclovir treatment could induce a significant decrease of serum viral DNA levels in 90% of the patients and a clearance of HBV DNA detected by PCR in 36%, without significant side effect.[8] A large multicenter placebo controlled trial including 333 patients showed the clinical efficacy of a 16-week course of famciclovir with a dose-dependent antiviral effect accompanied by a decrease of serum ALT levels and a significant increase of anti-HBe antibody seroconversion compared with the placebo group.[9]

By using the results of clinical trials for the evaluation of another promising nucleoside analog, lamivudine (3TC or [(−)-β-L-2′, 3′-Dideoxy-3′thiacytidine]), Nowak et al. have determined the kinetics of viral clearance during antiviral therapy of chronic HBV patients.[10] When the viral kinetics and the inhibition of viral replication by lamivudine were all taken into account, it was calculated that, in most patients, a prolonged therapy for more than 12 months is required to clear viral infection,[10] explaining why a short-term therapy with a nucleoside analog is insufficient to clear viral infection.[9,11,12] These findings underlined the need for prolonged antiviral therapy to eradicate chronic HBV infection. One problem that may be frequently encountered during prolonged administration of nucleoside analogs is the selection of HBV resistant strains with mutation in the viral polymerase as already described with antiviral therapy of HIV infection.[1,13] Sequence alignment and comparison with the crystal structure of the HIV reverse transcriptase have shown that most of the mutations associated with resistance to famciclovir or lamivudine reside in domains B and C of the HBV polymerase.[14-16] Resistant viruses, with mutations in the catalytic site (domain C) of the viral polymerase (M539V and M539I), similar to that observed in HIV resistant strains to

Abbreviations: HBV, hepatitis B virus; PCR, polymerase chain reaction; ALT, alanine aminotranferase; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; tid, thrice daily.

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3TC, may be selected after 12 months of lamivudine treatment in approximately 15% of chronic HBV patients and up to 25% of the patients in the liver transplant setting.[1,17-22] With long-term administration of famciclovir, viral resistance associated with mutation in the viral polymerase gene has been described only in immunocompromised patients.[1,16] One case of resistance to famciclovir has been fully described in one patient with severe recurrent HBV infection following liver transplantation who had been treated with famciclovir for 370 days. Two mutations, V508L and L515M, were found in domain B of the viral polymerase at the time of the viral breakthrough without any sequence change in the YMDD motif.[23] Other reports have described the occurrence of other HBV polymerase mutations (P512L, T519S, I522L) in patients with liver allograft who had been treated for chronic HBV infection with famciclovir for more than 6 months.[24,25] Figure 1 depicts the main mutations in domains B and C of the viral polymerase that have been associated with resistance to famciclovir or lamivudine (for more details see Zoulim and Trépo[1]).

In this article, we describe the case of an immune competent patient with chronic HBV undergoing long-term famciclovir therapy, in whom a novel HBV polymerase mutant was associated with viral resistance. Results of *in vitro* experiments indicate that this mutant has a decreased replication capacity and is indeed resistant to penciclovir. These data indicate that famciclovir resistant strains of HBV may also be selected in immune competent patients and underline the need for a careful monitoring of antiviral therapy and for the design of new antiviral strategies to prevent the occurrence of viral resistance.

PATIENTS AND METHODS

Patients. We describe here the case report of patient C.G., a 25-year-old man, who was referred to our center in 1992 for the management of a chronic HBV. HBV infection was acquired during childhood via intrafamilial contact. The patient was positive for HBsAg and HBeAg in serum detected with standard procedures. Serum HBV DNA was positive at low levels, and ALT elevation was mild (1.5-fold the upper limit of normal). Analysis of liver biopsy performed in April 1992 showed an active cirrhosis (Knodell score: 3, 1, 3, 4). In May 1992, the patient was treated with recombinant interferon-α 2b at a dose of 8 MU thrice weekly followed by a progressive dose tapering down to 1 MU thrice weekly until April 1995. Because HBeAg and HBV DNA were still positive in serum, interferon-α was stopped and the patient received famciclovir on a compassionate use program from May 1995. Oral administration of famciclovir at a dose of 500 mg thrice daily (tid) was well tolerated, and treatment was continued until April 1997. Because of the reappearence of markers of HBV replication, famciclovir was switched to Lamivudine. Viral markers and HBV DNA polymerase gene sequence were analyzed throughout the course of therapy.

The HBV polymerase gene sequence was also analyzed in a control group of 10 patients with chronic HBV who were enrolled in a dose-ranging placebo-controlled trial of famciclovir therapy.[9] All patients were positive for HBsAg, HBeAg and HBV DNA in serum. All patients had raised serum ALT and liver biopsy proven chronic hepatitis before therapy. Five had received famciclovir at a dose of 500 mg tid, 1 patient received a dose of 250 mg tid, 3 patients received a dose of 125 mg tid and 1 patient received placebo for a period of 16 weeks. Patients were selected for viral DNA sequence analysis on the absence of HBV DNA clearance from serum during therapy, without knowing the code of study medication.

Viral Markers in Serum and Assessment of Response to Therapy. Viral markers including HBsAg, HBeAg and antibody were determined routinely in the serum of the patients by standard procedure (Monolisa, Pasteur, France and EBK, Sorin, Italy, respectively). Quantitative determination of viral DNA in serum was performed by the branched DNA assay (Quantiplex, Chiron, Emeryville, CA) and by liquid hybridization assay (Genostic Abbott, Abbot Park, IL). Serum HBV DNA levels were determined every 2 months during therapy and serum ALT activity was monitored with the same timing. Viral genotypes were determined by fragment length polymorphism of the pre-S and core region amplified by PCR, as described previously.[26]

Determination of Viral Polymerase Gene Sequence. The DNA sequence of the polymerase gene from selected patients was determined either directly from PCR products or from PCR clones. Two conserved regions among all known reverse transcriptases[14,15,27] were studied: region I from amino acid 309 to 413 (nucleotide position 54 to 366) and region II from amino acid 490 to 631 (nucleotide position 596 to 1016), which includes domains B and C of the viral reverse transcriptase. The first AUG codon used for the initiation of the translation of the viral polymerase polypeptide is used for the numbering of amino acid changes. Figure 1 indicates the position of these conserved domains as well as that of the tyrosine-methionine-aspartic acid-aspartic acid (YMDD) motif, which is part of the catalytic domain (domain C).[1] Viral DNA was extracted from serum by using standard procedures. One tenth of extracted DNA was amplified in a 50 μL reaction containing 50 mmol/L KCl; 10 mmol/L Tris-HCl; 1.5 mmol/L $MgCl_2$; 0.2 mmol/L of each deoxynucleotide tiphosphate (dNTP); 1 unit of Taq polymerase (Promega, Madison, WI); and 1 pmol/L of each primer (pPOL1: 5′-CCTGCTGGTG-

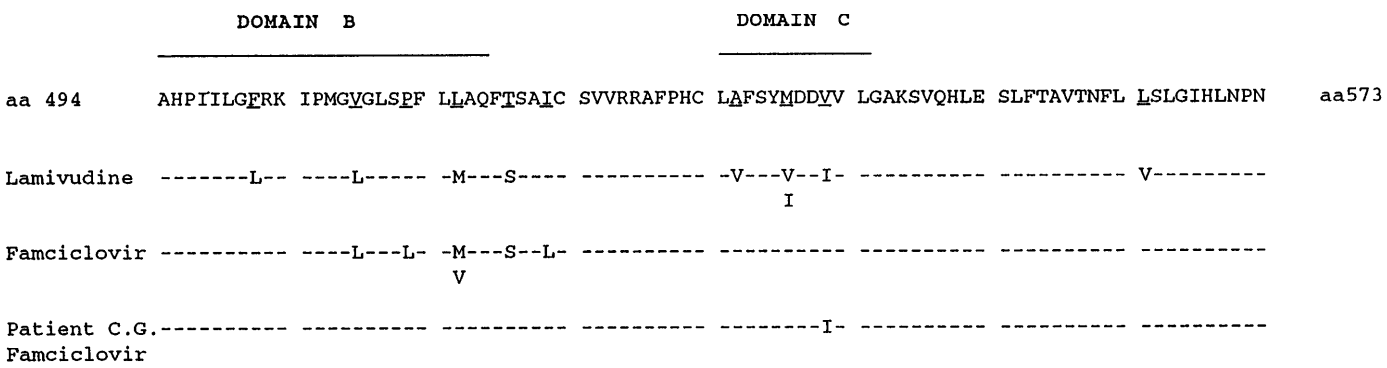

FIG. 1. HBV polymerase gene mutations associated with resistance to nucleoside analogs. The figure shows the amino acid changes in domains B and C of the viral polymerase that have been associated with resistance to famciclovir or Lamivudine (see reference 1 for more detail). These HBV mutants were shown to be selected during antiviral therapy. Mutations associated with famciclovir resistance are mainly located in the B domain of the viral polymerase. Mutations associated with Lamivudine resistance are located within the C domain and may be accompanied by amino acid variations in the B domain. The amino acid change in the viral polymerase described in the present report (patient C.G.) is indicated.

GCTCCAGTTC-3′ at nucleotide position 55-76, and primer pPOR1: 5′-CCAGGACAAGTTGGAGGACA -3′ at position 367-347 were used for amplification of region I; primers pPOL2: 5′-ACCTGTATTC-CCATCCCATC-3′ at position 597-615 and pPOR2: 5′-AAAC-CCAAAAGACCCACAAT-3′ at position 1017-997 were used for amplification of region II, or primers pPOL1 and pPOR2 for amplification of a larger fragment containing both regions). Thirty cycles of amplification were performed with denaturation at 95°C for 1 minute, annealing at 50°C for 1 minute, and extension at 72°C for 2 minutes. Viral DNA sequence analysis was performed directly on amplification products using the USB (Cleveland, OH) PCR product sequence kit. To obtain PCR clones, amplified products were electrophoresed, purified, and extracted from the gel by using a Geneclean II kit (BIO 101 Inc, Vista, CA). The DNA fragment was then cloned into a pGEM-T vector (pGEM-T vector system, Promega). DNA sequencing of both strands was performed with primer pairs pPOL2 and pPOR2, and pPOL1 and pPOR1, by using the Pharmacia T7 sequencing kit (Amersham Biotechnologies, Uppsala, Sweden). Sequencing reactions were run on 6% polyacrylamide/urea gels and autoradiographed with Hyperfilm Beta Max (Amersham Biotechnologies, Uppsala, Sweden).

Cloning, Transfection of Hepatitis B Virus Polymerase Gene Mutants and Analysis of Viral Replication in Cultured Hepatoma Cells. HBV polymerase gene sequences within *Xho*I (nucleotide position 129) and *Acc*I (nucleotide position 826) restriction sites, including the desired mutated sequence, were then subcloned in a pBR322 vector allowing the expression of the HBV pregenome under the control of the cytomegalovirus immediate early promoter, kindly provided by Dr. Christoph Seeger (Philadelphia, PA).[28] The presence of the mutations were confirmed after subcloning by DNA sequence analysis. Eight micrograms of the different plasmids were then transfected according to the calcium-phosphate precipitation method as previously described.[29] Huh7 cells were plated at a density of 1.3×10^6 cells per 60 mm diameter petri dish. The medium was changed 1 day after transfection and cells were harvested at day 7 post-transfection. Transfection was performed at least 5 times for each construct. Cotransfection with 2 μg of plasmid pSEAP (Clontech laboratories, Palo Alto, CA) encoding for alkaline phosphatase was performed to control the efficiency of transfection. Alkaline phosphatase activity was assessed on 25 μL of cell culture supernatant following manufacturer's recommendation. When indicated penciclovir was added to the culture medium at different concentrations. Penciclovir was dissolved in dimethylsulfoxide and cultures were incubated with the same final concentration of 1% dimethylsulfoxide with or without antivirals. Lamivudine was administered with the same schedule at the indicated concentrations. HBsAg and HBeAg were assayed in the culture supernatant by using commercially available kits (AUSRIA II, Abbott Laboratories, Abbot Park, IL, and EBK, Sorin Diagnostics, Saluggia, Italy, respectively).

HBV DNA was purified from intracellular core particles as described in detail by Horwich et al.[30] Briefly, after addition of lysis buffer, the lysed cells were transferred to Eppendorf tubes, vortexed and subjected to centrifugation. The supernatant was adjusted to 10 mmol/L $MgCl_2$ and treated DNAseI and RNAse (Boehringer, Mannheim, Germany) for 30 minutes at 37°C. The reaction was stopped by the addition of EDTA. Core particles were precipitated in 5% polyethyleneglycol and then subjected to proteinase K digestion. Nucleic acid was purified by phenol/chloroform extraction and ethanol precipitation. DNAs isolated from cytoplasmic core particles were separated on 1.5% agarose gels, blotted onto Hybond C membranes (Amersham), hybridized with a 32P-labelled full-length HBV fragment, followed by autoradiography of the blots.[29]

Total cellular RNAs were extracted with trizol (Gibco-BRL), treated with RNAse-free DNAseI. Total RNAs were glyoxalated and separated on 1% agarose gels, by using as running buffer sodium phosphate, pH6.5, 10mmol/L. After capillary transfer to Hybond C membranes (Amersham), RNA was hybridized with a specific 32P-labelled full-length HBV fragment as described elsewhere.[31] Quantitation of viral nucleic acids after Northern or Southern blot analysis was performed by laser densitometry analysis after scanning the autoradiograms.

RESULTS

Occurrence of Viral Breakthrough During Prolonged Famciclovir Therapy of Chronic Hepatitis B in an Immune Competent Patient. Virological markers were studied in patient C.G., suffering from chronic HBV, who was treated with famciclovir on a compassionate use. Analysis of fragment length polymorphism of the core and pre-S regions showed that the patient was infected with HBV genotype A (data not shown). Analysis of HBV DNA levels in serum by the bDNA assay is shown in Fig. 2 and demonstrated a viral breakthrough at month 9 (February 1996), 13 (June 1996), and 23 (April 1997) while the patient was still under famciclovir therapy. These episodes of breakthrough were transient and confirmed by analysis of viral DNA after amplification of the whole genome by PCR by using a method described by Günther et al.[32] (data not shown). Interestingly, analysis of serum HBV DNA by the liquid hybridization assay (Genostic, Abbott) was found negative during the entire follow up except in June 1996 after 13 months of therapy where this assay showed the presence of low level HBV DNA at 4 pg/mL (data not shown). At month 23 (April 1997), famciclovir therapy was stopped, followed by lamivudine administration (100 mg/day, per os), which was associated with a rapid and sustained decline of HBV DNA titer in serum until the last visit (month 31, December, 1997) (Fig. 2). HBeAg remained positive in serum during the entire follow-up.

Analysis of Hepatitis B Virus Polymerase Gene Sequence Showed the Appearance of a Polymerase Gene Mutant During the Episode of Viral Breakthrough. PCR amplification of the polymerase gene sequence with primers pPOL1 and pPOR2, followed by direct sequencing of the PCR products, was performed before the beginning of therapy and during therapy in all available samples. Analysis of viral DNA sequence during famciclovir therapy showed the emergence of a mutant with two nucleotide changes (GTG to ATA at codon 542) leading to a Valine to Isoleucine change at position 542 (V542I) in domain C of the viral polymerase. This V542I mutant was always found as

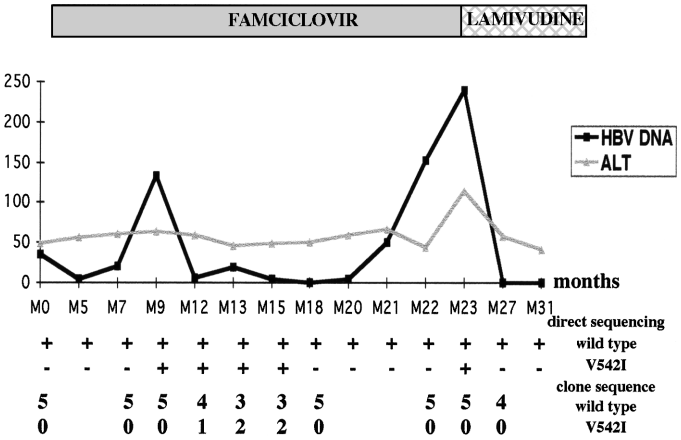

FIG. 2. Analysis of biochemical and virological markers in patient C.G. showed the occurrence of viral breakthrough during famciclovir therapy. The figure shows the serum ALT levels (IU/L) and the results of serum HBV DNA quantitation by the branched DNA assay (mega genome eq./ mL). The results of HBV polymerase gene sequencing on PCR products and after cloning are also indicated at the different time points.

a mixture with wild type sequence (Fig. 2). This mutation leads to a stop codon in the overlapping S gene at amino acid position 199. The PCR fragments obtained at different time points of therapy were further cloned in a PCR vector (see Fig. 2). Five clones of each isolate were analyzed to confirm the DNA sequence data (Fig. 3). Interestingly, the V542I mutant could never dominate the wild type strain (2 mutated clones out of 5). The mutant was then replaced by the wild type strain when HBV DNA decreased again while famciclovir therapy was continued (Fig. 2). At month 23, a mixture of wild type and V542I mutant sequence was again observed at the time of another viral breakthrough. In two samples obtained at months 12 and 23, the mutations could be detected as weak bands by direct sequencing but not in the five PCR clones analyzed, suggesting that this mutant circulated as a minor viral population. Lamivudine therapy was started and the wild type sequence became dominant as the viral titers drop dramatically (Fig. 2).

Comparison With Patients Treated With Famciclovir for 16 Weeks Showed the Absence of Selection of Hepatitis B Virus Polymerase Gene Mutants. As a control experiment of viral DNA sequence determination, we have analyzed, in parallel, a group of 10 patients who had been included in a placebo-controlled trial of famciclovir therapy. These patients were selected for the study because of the absence of viral clearance from serum during therapy. Analysis of DNA sequence of regions I (a.a. 309 to 413) and II (490 to 631) of the viral polymerase gene and that of viral genotypes was performed blindly without knowledge of the code, before therapy and at the end of the 16-week therapy, in 10 patients who had received famciclovir at a dose of 500 mg tid (five patients), 250 mg tid (one patient) and 125 mg tid (three patients) or placebo (one patient). The distribution of viral genotypes was similar to what is observed in the population of HBeAg positive carriers of HBV in our liver unit (unpublished results). In region I of the viral polymerase, no variation of nucleotide sequence was observed in all patients in both pre- and post-treatment samples. Analysis of region II sequence showed no variation in all five patients treated with famciclovir at a dose of 500 mg tid, in one patient who received famciclovir at a dose of 125 mg tid and in one placebo-treated patient. Single nucleotide changes associated with amino acid changes in the viral polymerase were observed in one patient who received famciclovir at a dose of 250 mg tid and in two who received a dose of 125 mg tid (Table 1). These amino acid changes were already observed before therapy and remained stable at the end of the 16 weeks therapy, suggesting that these variations were linked to the spontaneous heterogeneity of the viral genome.[33] All three patients had a partial response to famciclovir therapy with a decrease of viral DNA titers greater than 50%. These findings indicate that the absence of viral DNA clearance from serum in these patients was not associated with a particular viral genotype nor with the selection of polymerase gene mutants.

Functional Study of the V542I Polymerase Mutant Showed a Decreased Replication Capacity. The polymerase gene mutant was then subcloned in a pCMV vector for the expression of the viral pregenome to allow for the initiation viral DNA synthesis. Transfection of Huh7 cells was performed with both wild type and V542I mutant expressing vectors. Quantitative analysis of viral replication was made possible with the use of a control for transfection efficiency, i.e., cotransfection of a pSEAP plasmid followed by the analysis of alkaline phosphatase activity in each experimental condition. Southern blot analysis of intracellular viral core DNA reproducibly showed a 20-fold decrease of viral DNA synthesis in the case of the polymerase gene V542I mutant by comparison with the wild type genome (Fig. 4).

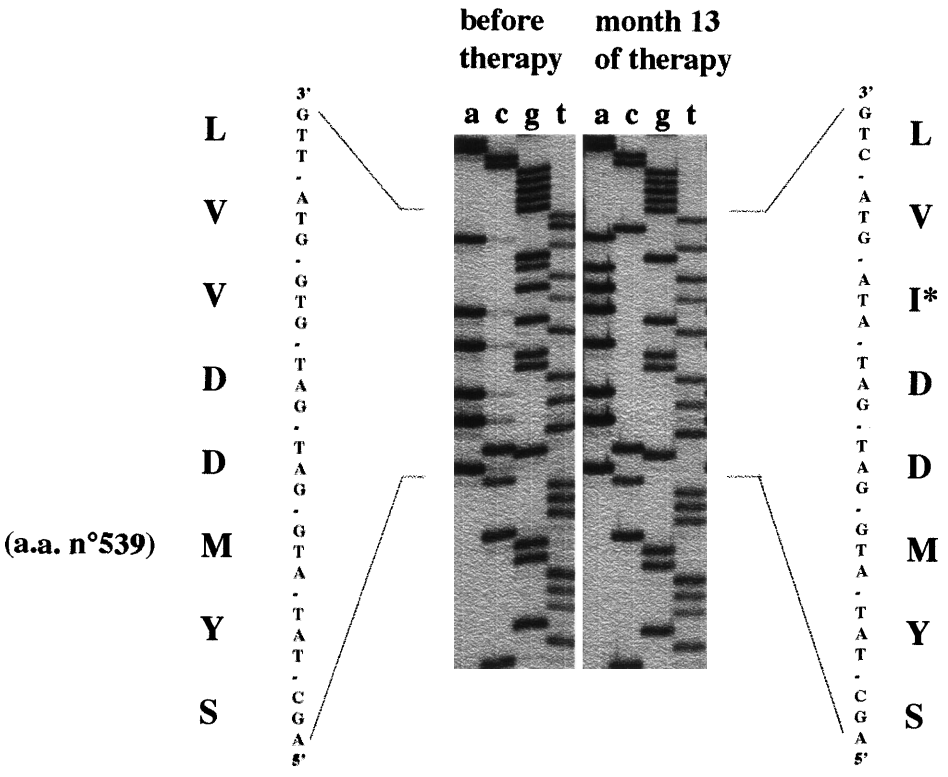

FIG. 3. HBV polymerase gene sequence in patient C.G., before therapy and during viral breakthrough under famciclovir therapy. The figure shows the results of the DNA sequence analysis of the viral polymerase gene performed after cloning of PCR products obtained before therapy (5 out of 5 wild type clones) and after 13 months of therapy, during viral breakthrough (2 mutated clones out of 5). The asterisk indicates the amino acid change.

TABLE 1. Amino Acid Changes in the Viral Polymerase Gene in 10 Patients Who Were Enrolled in a Clinical Trial of 16-Week Administration of Famciclovir and Did Not Clear Viral DNA From Serum

Patient	Famciclovir	% Decrease of HBV DNA	Amino Acid Change and Position	Viral Genotype
1.	500 mg	50%	None	A
2.	500 mg	69%	None	A
3.	500 mg	56%	None	A
4.	500 mg	0%	None	A
5.	500 mg	0%	None	A
6.	250 mg	73%	R577T	D/E
7.	125 mg	79%	V549I G579D Y580N	B/C
8.	125 mg	69%	E598D	D/E
9.	125 mg	53%	None	A
10.	placebo	61%	None	D/E

NOTE. Results of HBV polymerase sequence determination, before and at the end of therapy, in 10 patients enrolled in a placebo controlled trial of 16 week Famciclovir administration. All ten patients were selected for the study on the basis of viral persistence during famciclovir therapy. The table indicates the percentage of reduction of serum viral DNA levels at the end of therapy as compared with the pretreatment sample. Amino acid changes and their position in the viral polymerase are also indicated as well as the viral genotype. All amino acid changes observed in this group of patients were present before therapy and were stable until the end of the 16-week period of administration of the study medication.

No change in the levels of viral messenger RNA synthesis was observed between wild type and V542I mutant by Northern blot analysis of total cellular RNA (Fig. 4).

HBsAg detection in the supernatant of transfected cells showed the absence of HBsAg in the case of the V542I polymerase gene mutant, consistent with the disruption of the S gene by the mutation. HBeAg detection showed the absence of significant difference of viral antigen expression with the wild type and mutant constructs (see Fig. 2). Altogether these results show that the V542I polymerase gene mutant, which was transiently selected during famciclovir therapy, has an impaired capacity for DNA synthesis.

The V542I Polymerase Mutant Is Resistant to Penciclovir but Sensitive to Lamivudine in Tissue Culture. Then, we examined whether this mutant was sensitive to penciclovir, the active metabolite of famciclovir, *in vitro* in tissue culture after transfection of Huh7 cells. All experiments were performed after co-transfection of plasmid pSEAP as a control for transfection efficiency. Penciclovir was added into cell supernatant 2 days after transfection at concentrations of 0, 5, 20, 100 and 400 µmol/L. Medium containing the antiviral was changed every day. Southern blot analysis of viral core DNA was performed after 5 days of treatment. Results showed that replication of wild type HBV genome was inhibited by increasing concentrations of penciclovir, with a 45% and 65% inhibition of viral DNA synthesis at 100 and 400 µmol/L, respectively. Replication of the V542I mutant genome was not affected by penciclovir even at concentrations up to 400 µmol/L (Fig. 5).

In parallel, the replication capacity of this mutant was analyzed after transfection of Huh7 cells, in the presence of lamivudine at a concentration of 1, 10, and 100 nmol/L. Analysis of intracellular viral core DNA showed that lamivudine inhibited viral DNA synthesis of both wild type HBV and V542I mutant (Fig. 5). Lamivudine inhibited wild type viral DNA synthesis by 40% and 55%, at 10 and 100 nmol/L, respectively. Mutant virus replication was reproducibly inhibited by lamivudine and suppressed at 100 nmol/L.

Thus these data show that the HBV polymerase gene

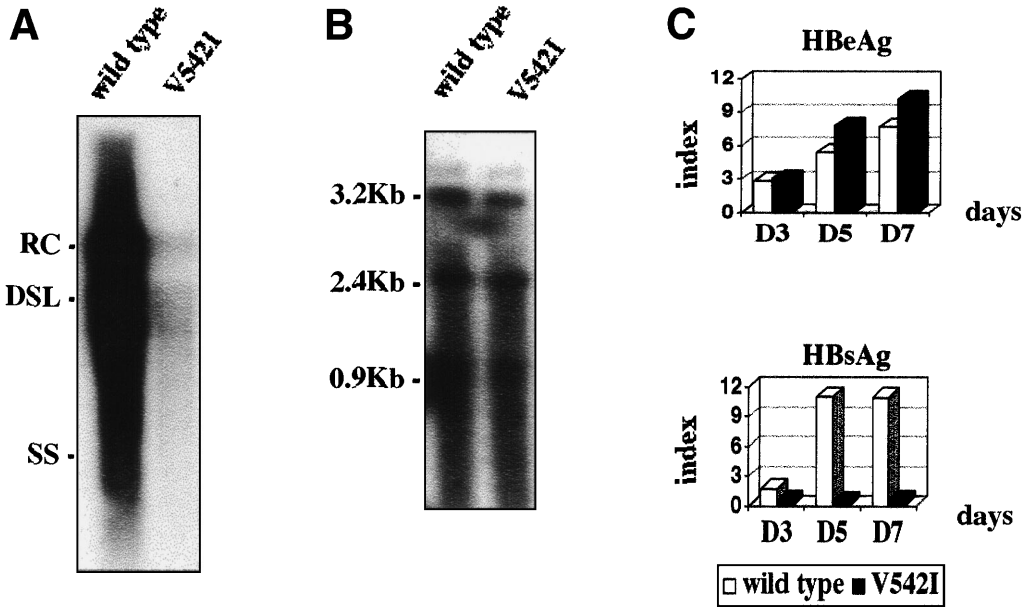

FIG. 4. Analysis of the viral replication capacity of the V542I polymerase mutant after transfection of cultured hepatoma cells. The viral polymerase mutant observed in patient C.G. was subcloned in a pCMV expression vector and transfected in Huh7 cells in parallel with a wild type HBV construct as described in Material and Methods. (A) Viral capsid DNA was extracted and subjected to agarose gel electrophoresis and Southern blot analysis. Relaxed circular (RC), double-stranded linear (DSL) and single stranded (SS) HBV DNA species are indicated. (B) Total cellular RNAs were extracted and subjected to agarose gel electrophoresis and Northern blot analysis. The arrows indicate the 3.5 Kb pregenomic, 2.4/2.1 Kb pre-S/S and 0.9 Kb × subgenomic RNAs. (C) Analysis of HBsAg and HBeAg in the supernatant of transfected cells at days 3, 5, and 7 post-transfection, by radioimmunoassay as described in Material and Methods. Results are expressed as a ratio of cpm of sample / negative control.

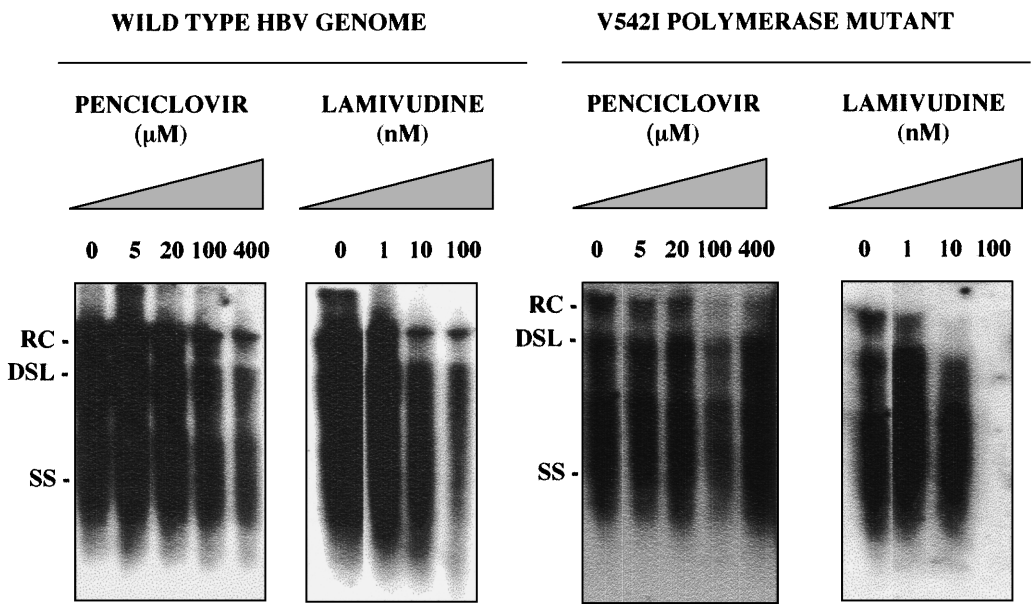

FIG. 5. Analysis of the antiviral effect of penciclovir and lamivudine on the wild type and the V542I polymerase mutant after transfection of cultured hepatoma cells. Wild type and HBV polymerase mutant V542I constructs were transfected in Huh7 cells. Penciclovir was added at a concentration of 0, 5, 20, 100 and 400 μmol/L in the culture supernatant 3 days after transfection for 5 days. Lamivudine was administered with the same schedule at concentrations of 1, 10 and 100 nmol/L. Culture medium was changed daily. Analysis of viral replication in the presence or the absence of antiviral was performed after 5 days of antiviral administration. Intracellular viral core DNA was extracted and analyzed after agarose gel electrophoresis and Southern blot as described in material and methods. Two-fold more cellular DNA was loaded on agarose gels in the case of the V542I mutant by comparison with the wild type construct. Exposure time was 12 and 48 hours for the wild type virus and the V542I mutant, respectively. Note that the V542I mutant was resistant to penciclovir but remained sensitive to Lamivudine.

mutant selected *in vivo* is indeed resistant to penciclovir but remains sensitive to lamivudine.

DISCUSSION

In this report, we describe the case of an immune competent patient who developed famciclovir resistance in association with a novel HBV polymerase mutation. This patient with chronic hepatitis B, previously nonresponding to interferon-α treatment, was treated with famciclovir for a prolonged time. Serum viral DNA analysis showed a first antiviral response that was followed by three episodes of viral breakthrough authenticated by an increase in serum viral DNA titers (see Fig. 2). Interestingly these episodes of viral breakthrough were not diagnosed with the liquid hybridization assay (Genostic, Abbott) but could be detected by the branched DNA assay (Quantiplex, Chiron), emphasizing that monitoring of the antiviral response during nucleoside analog therapy should use the most sensitive of the quantitative assays that are available. By contrast to other reports on HBV resistance, these episodes of viral breakthrough were transient. Viral DNA sequence analysis showed the occurrence of a mutation in the viral polymerase gene leading to a valine to isoleucine change in the viral polymerase at position 542 and to a stop codon at position 199 of the HBs surface protein (see Fig. 3). While several cases of resistance to famciclovir have been fully described in the liver transplant setting,[1,16,24,34] this new observation indicates that HBV polymerase gene mutants associated with resistance to famciclovir may be also selected in immune competent patients. In the present case, resistance to famciclovir was associated with a unique mutation located in the C domain of the HBV polymerase, whereas in patients with HBV recurrence on the liver graft who developed resistance to famciclovir, mutations were mainly described in the B domain of the viral polymerase (see Fig. 1).[1,16,24,34] This V542I mutation has been described in lamivudine resistant HBV strains, but was always found in association with the M539I mutation in the YMDD motif,[16,19] which contrasts with our observation. It remains to determine what is the rate of selection of this V542I mutant in patients undergoing long-term famciclovir treatment.

Interestingly, when we compared the sequence data of patient C.G. with that of patients who were enrolled in a placebo-controlled trial of 16-week administration of famciclovir and did not clear viral DNA from serum, we observed amino acid changes in the viral polymerase of 3 patients (see Table 1). By contrast with our patient C.G., these amino acid changes were already present before the beginning of therapy and were stable until the end of the 16-week trial. These amino acid changes were associated with a reduction of viral DNA levels in serum by more than 50% during famciclovir administration. In this group of patients, the results also suggest that the lack of complete antiviral response is not related to a particular viral polymerase mutant in the very conserved B and C domains. This observation has also been made in liver transplant patients receiving famciclovir for HBV recurrence.[25] This may suggest that other viral genomic determinants or the replication of HBV in nonhepatocyte cells (Dr. Locarnini, personal communication, 1997), as well as host factors such as the metabolism of the drug, may be involved in viral resistance to famciclovir. Further studies are required to gain insight in this phenomenon.

Furthermore, the replication competence of the V542I polymerase mutant observed in patient C.G. was analyzed *in vitro* after transfection of Huh7 cells. Interestingly, we found that this mutant has a decreased capacity for viral DNA synthesis (see Fig. 4). This defect in viral replication has been

also observed by Melegari et al. who have engineered by site directed mutagenesis other HBV mutant clones associated with resistance to lamivudine or to famciclovir (F501L, L515M, M539I, M539V).[35] However, the replication capacity of this mutant V542I has not been studied so far. Because we used different controls for transfection efficiency, and because there was no significant difference in pregenomic RNA transcription and HBeAg secretion in the supernatant of transfected cells, our results suggest that this polymerase mutant has an impaired capacity of viral DNA synthesis (see Fig. 4). This is consistent with the observation that mutations within the very conserved C domain of the viral polymerase were found to affect dramatically viral fitness[35-37] by a processivity defect of the mutant polymerase that was dependent on the intracellular pool of nucleotides.[35] Whether this V542I polymerase mutant has a processivity defect remains to be determined. In addition, we could confirm *in vitro* that this clinical isolate is defective in the synthesis of HBs envelope proteins (see Fig. 4). The low replication capacity and the inability of this mutant to express the viral envelope proteins may explain why this mutant could not dominate the wild type virus and required the wild type virus as a helper virus to provide the envelope proteins in *trans*. Altogether, these data may explain why this mutant did not outgrow wild type HBV during famciclovir therapy. However, it remains difficult to explain why the mutant virus emerged transiently and then appeared to wane in relative titer only to re-emerge later (see Fig. 2). One may hypothesize that a proportion of wild type genomes were packaged and replicated by the mutated polymerase provided in *trans* or that mutations at other sites in viral genomes harboring wild type sequence within the reverse transcriptase domain may also have contributed to viral persistence during famciclovir therapy. Another possible explanation could be that the pharmacokinetic of the drug may have been altered in this patient with a cirrhotic liver. This may in turn have decreased the antiviral pressure on wild type virus allowing its re-emergence and the decline of the V542I mutant. Indeed, the pharmacokinetic of famciclovir has been studied in subjects with various chronic liver diseases.[38] The results showed a decreased C_{max} and an increased T_{max} whereas the area under the curve of penciclovir was not modified, suggesting a decrease in the rate, but not in the extent, of systemic availability of penciclovir in patients with liver disease compared to healthy subjects.[38] A careful pharmacokinetic study in patients with HBV related liver cirrhosis is currently ongoing and its results are awaited to confirm this hypothesis.

The sensitivity of this HBV mutant to penciclovir, the active metabolite of famciclovir, was analyzed *in vitro* after transient transfection of cultured Huh7 cells in comparison with the wild type HBV expressing vector (see Fig. 5). This study could determine that wild type HBV replication was inhibited by penciclovir in a concentration dependent manner with an IC_{50} around 100 μmol/L as already described in a different cell culture system, i.e., the 2.2.1.5. cell line,[39] whereas another report has described a much lower IC_{50} (0.6 μmol/L) for penciclovir in the 2.2.1.5. cell line.[40] Although the replication capacity of the HBV polymerase mutant was much weaker than that of the wild type, the replication of this polymerase mutant was resistant to high concentrations of penciclovir up to 400 μmol/L. These data clearly indicate that the V542I mutation of the HBV polymerase confers resistance to famciclovir. The IC50 of lamivudine on wild type virus replication was found to be in the same range (100 nmol/L) as observed in other reports,[39,40] and the administration of the drug at 100 nmol/L almost completely suppressed mutant virus replication (see Fig. 5). Therefore, the results of our *in vitro* experiments suggest that this V542I mutant strain is not cross resistant to lamivudine. This was further shown *in vivo* by the rapid decrease of viral DNA titers that was accompanied by the disappearance of the mutant virus on DNA sequence analysis of PCR products when famciclovir therapy was replaced by lamivudine (see Fig. 2).

Our findings therefore suggest that HBV polymerase gene mutants may be selected even in immune competent patients with chronic hepatitis B treated with famciclovir. This novel V542I mutation, which was shown to be defective for viral replication, increases the spectrum of viral polymerase mutants resistant to famciclovir. Our results emphasize the need to characterize the biology and the cross resistance profile of each new mutant that is selected during antiviral therapy to further establish the best strategy for the prevention and the management of HBV resistance .

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