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Emergence of YMDD Motif Mutants of Hepatitis B Virus During Lamivudine Treatment of Immunocompetent Type B Hepatitis Patients

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Lamivudine is an effective antiviral agent for the treatment of chronic type B hepatitis. Recent studies have shown the appearance of lamivudine resistant viruses with mutations at the tyrosine-methionine-aspartate-aspartate (YMDD) motif of the viral polymerase in hepatitis B virus (HBV) infected patients who received orthotopic liver transplantation. In order to confirm the appearance of such mutant HBV in immunocompetent patients, the HBV sequences in and around the YMDD motif of HBV DNA polymerase were examined in the sera from 16 lamivudine treated and 10 untreated control patients. Approximately 200 bases including the YMDD motif of HBV DNA polymerase were amplified by polymerase chain reaction (PCR) and sequenced directly by an automated sequencer. Of the 16 patients receiving lamivudine, mutant viruses with mutations in the YMDD motif were found in 3 of 8 patients treated with lamivudine for 52 weeks. However, this mutation was not found in any of the 8 patients treated for 32 weeks or a shorter period. Mutant viruses appeared after 40 weeks of treatment and were undetectable within 12 weeks after the cessation of the treatment. Such mutant viruses were not detected in any of the 10 untreated patients. This study confirms the emergence of YMDD mutant viruses during long-term lamivudine treatment in immunocompetent type B hepatitis patients. The results from this study suggest the need for combination therapies to reduce the levels of such mutant viruses in some patients. ***J. Med. Virol. 60:8–16, 2000.***

KEY WORDS: HBV DNA polymerase; antiviral agent; PCR; direct sequencing

INTRODUCTION

Since chronic infection with hepatitis B virus (HBV) is associated with chronic hepatitis, cirrhosis of the liver, and hepatocellular carcinoma [Beasly et al.,1981; Weissberg et al., 1984; Chen, 1993], cessation of HBV replication is most important. Antiviral treatments such as interferon (IFN) alone and/or IFN in combination with corticosteroid priming have been used and shown to have limited efficacy [Lok et al., 1992; Wong et al., 1993]. However, more efficient treatment with less adverse effects are desirable.

Lamivudine [(-) enantiomer of 3′-thiacytidine] has been found to be a potent specific inhibitor of the reverse transcriptase of human immunodeficiency virus (HIV) [Soudeyns et al., 1991; Coates et al., 1992] and HBV [Doong et al., 1991; Severini et al., 1995]. The antiviral effect of lamivudine in human was clearly demonstrated in patients infected with HIV [Pluda et al., 1995; Van Leeuwen et al., 1995] and HBV [Tyrell et al., 1993; Zoulium and Trepo, 1994; Benhamou et al., 1995; Dienstag et al., 1995; Schalm et al., 1995; Benhamou et al., 1996; Lai et al., 1997]. The drug was also shown to be well tolerated and to have few adverse effects [Lau et al., 1994; Zoulium and Trepo, 1994; Dienstag et al., 1995; Schalm et al., 1995; Lai et al., 1997]. However, the recurrence of viremia after the cessation of the treatment occurs in majority of patients [Dienstag et al., 1995; Lai et al., 1997] and therefore the effect of long-term treatment with lamivudine is needed to be understood and has been investigated in the present study.

Recurrence of HIV viremia during the treatment was reported in HIV positive patients and it was found that viruses with mutation at the tyrosine-methionine-aspartate-aspartate (YMDD) motif of the HIV reverse-transcriptase were responsible for resistance [Boucher et al., 1993; Gao et al., 1993; Tisdale et al., 1993; Schuurman et al., 1995]. Recently, reactivation of HBV during lamivudine treatment in patients who received orthotopic liver transplantation (OLT) were reported [Ling et al., 1996; Tipples et al., 1996; Bartholomew et al., 1997] and it was also found that HBV found in such

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patients had mutations in the YMDD motif of HBV DNA polymerase. Since patients with HIV and those that received OLT were both immunocompromised, breakthrough of viremia during lamivudine treatment might be associated with immunosuppression.

Regarding the emergence of such mutant viruses in immunocompetent patients, little is known except for a report of Honkoop et al. [1997] who examined the incidence of such mutants in patients who had an incomplete response or viral breakthrough during lamivudine treatment. The emergence of such mutants in and around the YMDD motif of HBV DNA polymerase was studied in immunocompetent hepatitis B patients, some of whom were treated with lamivudine.

MATERIALS AND METHODS

Patients

Serum samples from 16 patients who were treated with lamivudine for various lenghts of time during April 1994 and March 1997 were examined. These patients were positive for HBsAg and negative for anti-HCV antibody and all had abnormal liver functions. They were diagnosed as chronic hepatitis B based on histological findings (Table I). Four patients received lamivudine orally for 4 weeks at the dosage of 2.5–200 mg per day. Two patients received lamivudine for 16 weeks, two patients for 32 weeks and eight patients for 52 weeks at the dosage of 100 mg per day. Data on their age, sex, histology are given in Table 1. Sera from 10 chronic hepatitis B patients who were not treated with lamivudine served as controls (Table I, Cases 17– 26).

Serum Samples

Sera from patients receiving lamivudine at dosages mentioned above for as short as 4 weeks to as long as 52 weeks were collected and examined. Sera were collected before, during, at the end, and several weeks after stopping the treatment (Fig. 1). Four patients received lamivudine for 4 weeks. Sera were collected before and at the end of 4 weeks. In addition, the sera from these patients were collected 48 weeks after cessation of lamivudine treatnent. Two patients received lamivudine for 16 weeks and sera from these patients were collected before beginning, 4 weeks after, at the end of 16 weeks, and 36 weeks after cessation. Two patients were given lamivudine for 32 weeks. The sera were collected before, beginning, after 4, 16, and 32 weeks of treatment, and 20 weeks after cessation of lamivudine treatment. Similarly, sera from eight patients who received lamivudine for 52 weeks were collected before, 4, 16, 32, and 52 weeks of treatment.

Sera from 10 patients who did not receive lamivudine (untreated) were also examined at the time of biopsy and 52 weeks afterwards (Fig. 1). In cases where mutant viruses were detected at the end of the treatment, sera obtained at 1, 2, and 3 months before the end of treatment and 1, 2, and 3 months after the end of treatment were also examined (Fig.1, Table II). Serum samples were frozen at –20°C until taken up for analysis.

Detection of HBV DNA by Polymerase Chain Reaction

Nucleic acids were extracted from 100 μl of the sera by the method of Ehata et al. [1992]. For amplifying nucleotides around YMDD motif of HBV DNA polymerase by the polymerase chain reaction (PCR), two sets of primers (P1: 5′-CTCCACCACTGGGGACCCTC-3′, PR1: 5′-GCTGCTAGGAGTTCCGCAGT-3′, P2: 5′-GGCCTCAGTCCGTTTCTCCT-3′ and PR2: 5′-TGAAGTTAAGGGAGTAGCCC-3′) were synthesized and used for nested PCR. The PCR was performed as follows. The extracted DNA was dissolved in 77 μl double distilled water and mixed with 10 μl of 10X PCR buffer [100 mM Tris-HCl (pH8.3), 500 mM KCl, 15 mM $MgCl_2$], 10 μl of 2.5 mM dNTP mixture and 1 μl (100 pmol) each of primers P1 and PR1 and 1 μl of Taq polymerase (2.5 units) (Takara Shuzo Co., Kyoto, Japan). The PCR was performed as follows: denaturing at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 70°C for 1 min for 35 cycles. Two microliters of the first round PCR products were added to a mixture of 10 μl of 10X PCR buffer, 75 μl of double distilled water, 10 μl of 2.5 mM dNTP mixture, 1 μl (100 pmol) each of primers P2 and PR2, and 1 μl of Taq polymerase (2.5 units) and then amplified with the same conditions of the PCR as in the first round.

Detection of Mutation in Active Domain of HBV DNA Polymerase

For the analysis of nucleotide sequences around YMDD motif of HBV DNA polymerase, excess primers and salts were removed from the amplified products using SUPREC-02 kit (Takara Shuzo Co., Kyoto, Japan) and cycle amplification was carried out with either primer P2 or PR2 using Ready Reaction Kit (Perkin Elmer, Foster City, CA, USA). Then the primers used for cycle amplification were excluded with Centri-Sep spin columns (Perkin Elmer). The sequences were determined bidirectionally with an automated ABI PRISM 377 DNA Sequencer (Perkin Elmer) as per the protocols given by the manufacturer.

Detection of HBV DNA Concentration

HBV DNA in sera during the treatment was measured serially by a branched chain DNA probe assay (Chiron, Emeryville, CA) and levels equal to or below 2.5 pg per milliliter have been considered undetectable by this method.

RESULTS

Detection of HBV DNA by PCR

All the 16 patients were found to have HBV DNA by the PCR before the beginning of lamivudine treatment. Although the levels of HBV DNA by the DNA probe assay were undetectable in all but 3 patients by the end of treatment, it is important to note that all of the 16

TABLE I. Detailed Data on Patients Treated or Untreated With Lamivudine

	Age	Sex	Histology	Dose	Duration of Rx	ALT (IU/L) Before Rx	ALT (IU/L) At end of Rx	HBeAg/ HBeAb	HBV-DNA (pg/ml) Before Rx	HBV-DNA (pg/ml) At end of Rx
Patient 1	24	M	CAH2a	2.5 mg/day	4 w	70	30	+/–	2.0×10^3	<2.5
Patient 2	22	F	CAH2a	25 mg/day	4 w	343	33	+/–	1.1×10^3	<2.5
Patient 3	22	M	CAH2a	100 mg/day	4 w	167	33	+/–	1.2×10^2	<2.5
Patient 4	38	M	CAH2b	200 mg/day	4 w	63	50	+/–	1.7×10^2	<2.5
Patient 5	33	M	CPH	100 mg/day	16 w	108	66	+/–	3.0×10^8	<2.5
Patient 6	20	M	CAH2b	100 mg/day	16 w	123	33	+/–	1.5×10^2	<2.5
Patient 7	31	M	CPH	100 mg/day	32 w	13	81	+/–	7.7×10^3	<2.5
Patient 8	23	F	CAH2a	100 mg/day	32 w	285	20	+/–	4.9×10^1	<2.5
Patient 9	20	M	CAH2a	100 mg/day	52 w	126	15	+/–	6.2×10^2	<2.5
Patient 10	24	M	CAH2a	100 mg/day	52 w	214	18	+/–	6.4×10^3	<2.5
Patient 11	20	M	CAH2a	100 mg/day	52 w	168	19	+/–	3.6×10^3	8.4
Patient 12	21	M	CPH	100 mg/day	52 w	43	29	–/+	10	<2.5
Patient 13	19	F	CAH2a	100 mg/day	52 w	109	12	+/–	1.0×10^2	<2.5
Patient 14	35	M	CAH2a	100 mg/day	52 w	77	20	+/–	2.5×10^3	<2.5
Patient 15	31	M	CAH2a	100 mg/day	52 w	209	248	+/–	2.1×10^4	3.3×10^3
Patient 16	33	M	CAH2b	100 mg/day	52 w	162	307	+/–	7.8×10^3	4.2×10^3
Patient 17	24	M	CAH2b	(–)	(–)	161	146	+/–	2.3×10^3	1.1×10^3
Patient 18	39	M	CAH2b	(–)	(–)	65	60	+/+	2.7×10^1	1.1×10^3
Patient 19	20	F	CAH2a	(–)	(–)	82	80	–/+	4.2×10^3	<2.5
Patient 20	28	F	CAH2a	(–)	(–)	150	54	+/–	1.2×10^3	3.1×10^3
Patient 21	45	M	CAH2a	(–)	(–)	66	20	+/–	5.0	8.1×10^2
Patient 22	38	M	CAH2a	(–)	(–)	115	123	+/–	1.5×10^3	1.2×10^3
Patient 23	45	M	CAH2b	(–)	(–)	116	94	+/–	6.7×10^2	5.3×10^2
Patient 24	41	M	CAH2b	(–)	(–)	113	189	+/–	1.6×10^2	4.9
Patient 25	41	M	CPH	(–)	(–)	50	81	+/–	1.3×10^2	6.7×10^1
Patient 26	62	M	CAH2a	(–)	(–)	50	49	+/–	9.8×10^2	7.6

Rx: Treatment; NT: not tested; CPH: chronic persistenthepatitis; CAH: chronic active hepatitis.

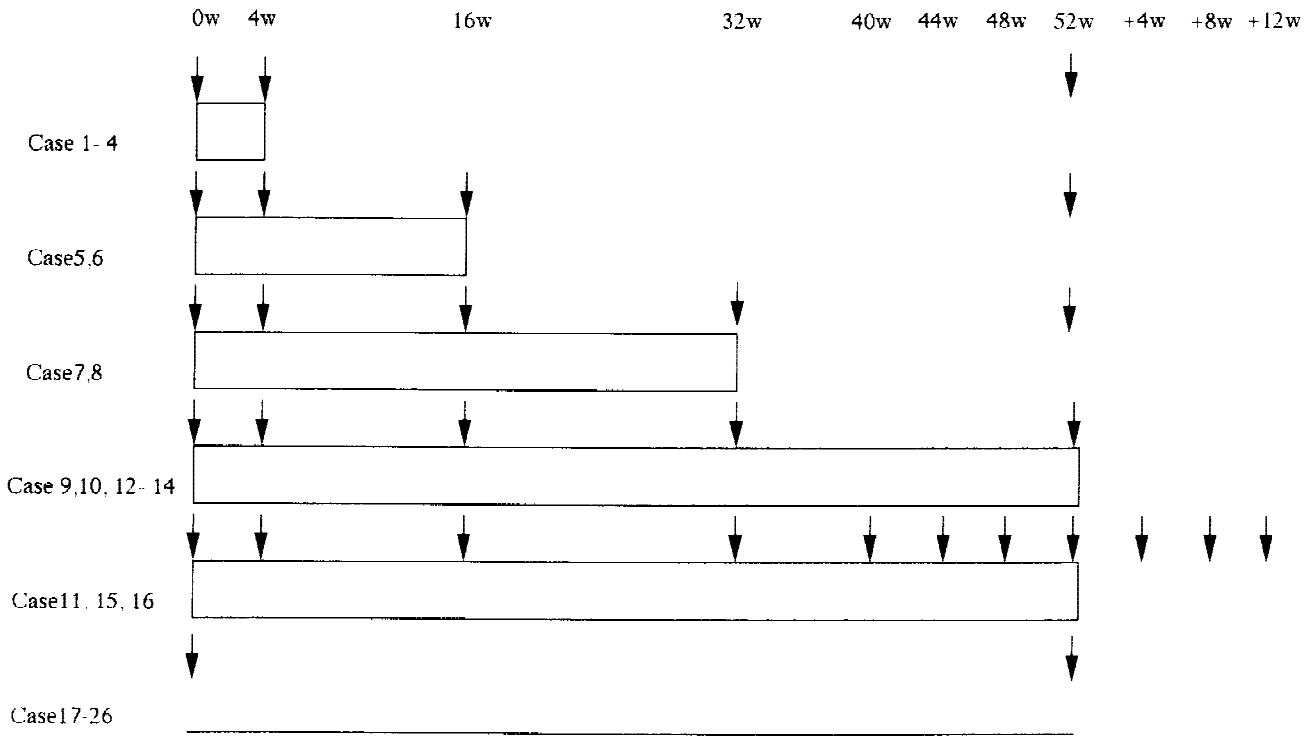

Fig. 1. Time points (arrows) for examination of mutation in YMDD motif of HBV DNA polymerase in lamivudine treated and unterated cases are shown in relation to the duration of treatment. Boxes indicate the duration of treatment.

TABLE II. Amino Acid Sequences Around YMDD Motif in 3 Patients Who Showed Mutation in YMDD Motif

	Before	4w	16w	32W	40w	44w	48w	52w	After 4W	After 8W	After 12W
Patient 9	YMDD	YMDD	YMDD	YMDD	NT	NT	NT	YMDD	NT	NT	NT
Patient 10	YMDD	YMDD	YMDD	YMDD	NT	NT	NT	YMDD	NT	NT	NT
Patient 11	YMDD	YMDD	YMDD	YMDD	YMDD	YMDD	YMDD	YIDD	YMDD	YMDD	YMDD
Patient 12	YMDD	YMDD	YMDD	YMDD	NT	NT	NT	YMDD	NT	NT	NT
Patient 13	YMDD	YMDD	YMDD	YMDD	NT	NT	NT	YMDD	NT	NT	NT
Patient 14	YMDD	YMDD	YMDD	YMDD	NT	NT	NT	YMDD	NT	NT	NT
Patient 15	YMDD	YMDD	YMDD	YMDD	YI/MDD	YI/MDD	YIDD	YIDD	YIDD	YI/MDD	YMDD
Patient 16	YMDD	YMDD	YMDD	YMDD	YV/MDD	YV/MDD	YVDD	YVDD	YV/MDD	YMDD	YMDD

NT, not tested.

patients were positive for HBV DNA by PCR at the end of treatment (Table I). Additionally, as expected, all 10 untreated patients were positive for HBV DNA by PCR at the time of liver biopsy and also at 52 weeks of follow-up (Table I).

Detection of HBV With Mutation in YMDD Motif

Before beginning lamivudine treatment, all 16 patients had the wild type YMDD motif. Only the wild type motif was observed during the treatment in the eight patients treated for less than 32 weeks. All these patients had virus with the wild type motif at 52 weeks after the beginning of the treatment.

Of the 8 cases treated for 52 weeks, viruses with wild type YMDD motif were observed in 5 cases and, with mutations in YMDD motif in 3 cases at the end of the treatment. The nucleotide sequences around the YMDD motif of HBV DNA polymerase at the end of the treatment are shown in Table 3. There are 2 types of mutants among the cases examined, one with YIDD (tyrosine, isoleucine, aspartate, aspartate) motif (Cases 11, 15), the other with YVDD (tyrosine, valine, aspartate, aspartate) motif (Case 16) (Table III). A second substitution from leucine to methionine was found at the position of amino acid 515 of HBV DNA polymerase in patient 16 who had YVDD motif. Excepting patient 16, the second mutation was not found in the current study including Cases 11 and 15 who had YIDD motif (Table III).

Serial analysis of the virus from three patients revealed that only the wild type motif was observed at 32 weeks of therapy. The mutant viruses were first observed at 40 weeks in two cases (Cases 15, 16) and at 52 weeks in one case (Case 11). All these three cases were positive for HBV DNA by branched chain DNA probe method at the end of 52 week treatment (Table I). The

TABLE III. Amino Acid Sequence Around the YMDD Motif of HBV DNA Polymerase in 16 Patients at the End of Lamivudine Treatment

PT	AA.	PFLLAQFTSAICSVVRRAFPHCLAFSYMDDVVLGAKSVQHLESLFTSITNFLLSLGIHLNPNKTKR
Patient	1	. . . . .
Patient	2	. . . . .
Patient	3	. . . . .
Patient	4	. . . . .
Patient	5	. . . . .
Patient	6	. . . . .
Patient	7	. . . . .
Patient	8	. . . . .
Patient	9	. . . . .
Patient	10	. . . . .
Patient	11	. . . . . I . . . . .
Patient	12	. . . . .
Patient	13	. . . . .
Patient	14	. . . . .
Patient	15	. . . . . I . . . . .
Patient	16	. . . M . . . . . V . . . . .

follow-up examination of these patients revealed that the mutant virus became undetectable within 3 months after the cessation of the treatment (Table 2). In patient 11, seroconversion from HBeAg to anti-HBe was observed at the end of treatment. Whereas, in patient 15 and 16, the HBeAg remained positive after the end of the treatment. Of the remaining 5 patients treated for 52 weeks, only the wild type motif was present throughout the observation period.

The HBV DNA level before treatment was higher in three patients who acquired the mutation ($1.1\text{x}10^4 \pm 0.91\times10^4$) than in five patients who did not ($2.0\times10^3 \pm 2.7\times10^3$). However, there were no statistically significant differences between these patients.

All of the 10 untreated (control) patients had YMDD motif at the beginning of the follow-up and at 52 weeks after the follow-up.

Correlation Between Mutations in YMDD Motif and Serum ALT Level

Among the 8 patients who received 52 weeks of lamivudine treatment (Patients 9–16), only two patients (patients 15, 16) had elevated levels of alanine aminotransferase (ALT) at the end of treatment (Table I), and mutations in YMDD motif were also found. The clinical courses of these patients are depicted in Figure 2a and b. In both cases, changes in YMDD motif were observed from 40 weeks after the beginning of treatment. The increase in HBV DNA level was observed after the detection of viruses with mutation in YMDD motif and then followed the elevations in ALT levels. Thus, the viruses with such mutations at YMDD motif might be selected under the presence of lamivudine, replicated selectively and resulted in the return of ALTs to initial levels. In the remaining 6 cases, the ALT levels that were elevated before treatment became normal and remained within normal limits at the end of the treatment. In patient 11, the change in YMDD motif was found only after 52 weeks of treatment and wild type motif was observed afterwards (Fig. 2c). Marked elevations of HBV DNA and ALT levels were observed from 8 weeks after the cessation of treatment in this case. This post- treatment elevation of ALT is most likely due to the re-emergence of wild type virus.

DISCUSSION

Lamivudine has been shown to be useful for reducing the levels of HBV viremia and in reducing the ALT levels in chronic hepatitis B [Dienstag et al., 1995; Lai et al., 1997]. Although recurrence of HBV replication was observed frequently after the cessation of the treatment [Dienstag et al., 1995; Lai et al., 1997], prolonged treatment with lamivudine might be useful for suppressing the recurrence of HBV for a longer duration. During the extended period of treatment of patients infected with HBV who received OLT, the occurrence of mutation in YMDD motif of HBV DNA polymerase and viral replication were reported recently [Ling et al., 1996; Tipples et al., 1996; Bartholomew et al., 1997]. This emergence of mutant viruses may be more frequent due to immunocompromized state in such patients. In contrast however, the present study confirms the appearance of such mutants in immunocompetent patients during long-term lamivudine monothrapy as observed by Honkoop et al. [1997]. It was also observed the second substitution from leucine to methionine at amino acid 515 of HBV DNA polymerase in a case with YVDD motif but not in two cases with YIDD motif as described in the previous reports [Ling et al, 1996; Bartholomew et al, 1997].

In the current study, the mutant viruses were only found in patients treated with lamivudine and not in untreated patients during the same observation period. Lamivudine inhibits the viral replication by suppressing the activity of viral reverse transcriptase and also by acting as a chain terminator [Doong et al., 1991; Zoulium et al., 1994; Schalm et al., 1995; Severini et al., 1995]. HBV DNA polymerase activity in the wild type YMDD motif was found to be inhibited in the presence of lamivudine. However, lamivudine did not inhibit mutant viruses with YIDD or YVDD motifs. Thus

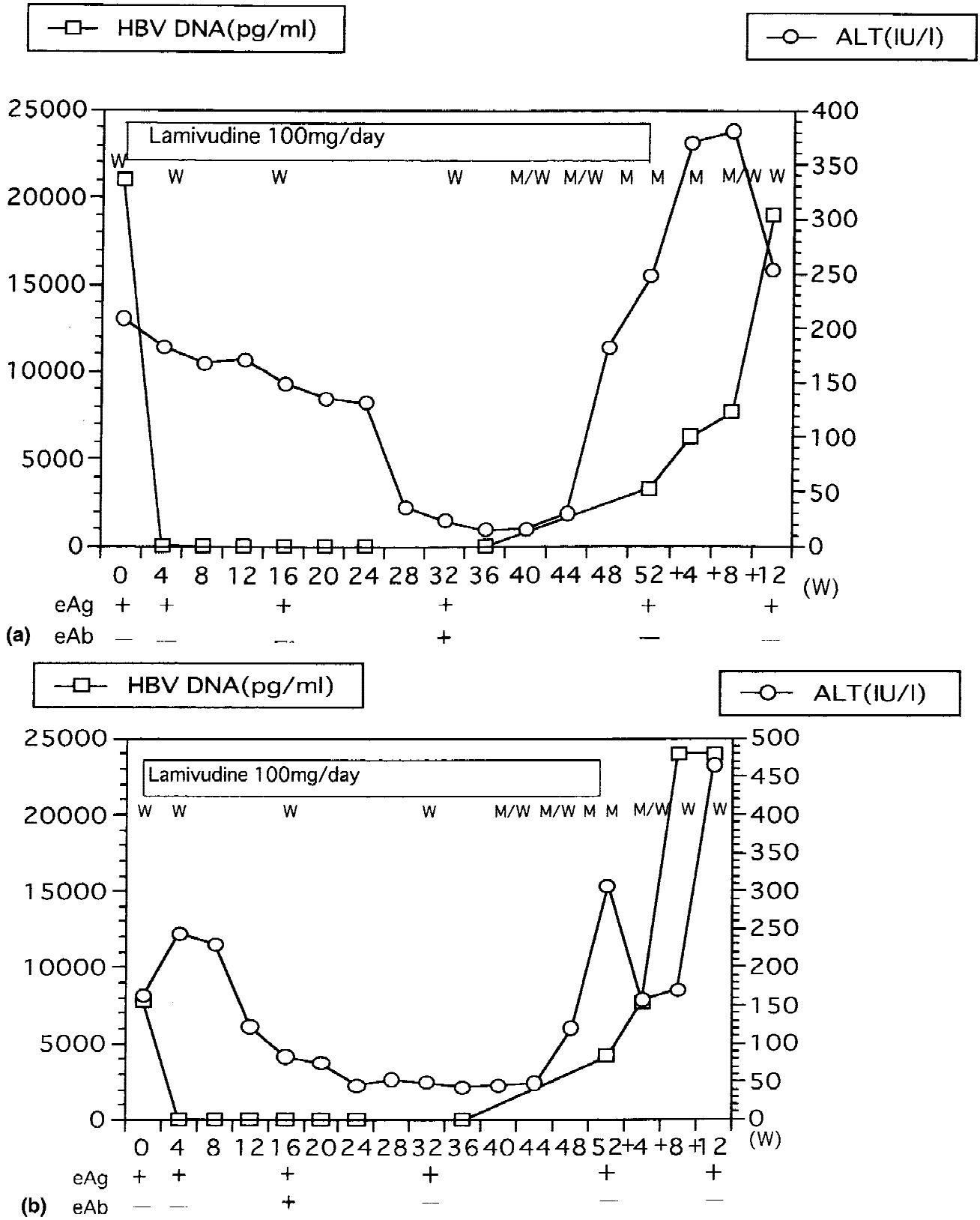

Fig. 2. Serial changes of alanine aminotransferase (ALT) levels and mutation at YMDD motif of HBV DNA polymerase and the amounts of HBV DNA as determined by branched chain DNA probe assay are shown in three cases [(**a**), Case 15, (**b**) , Case 16 and (**c**), Case 11 (see next page)] presenting with such mutations. W, wild type, and M, mutant type.

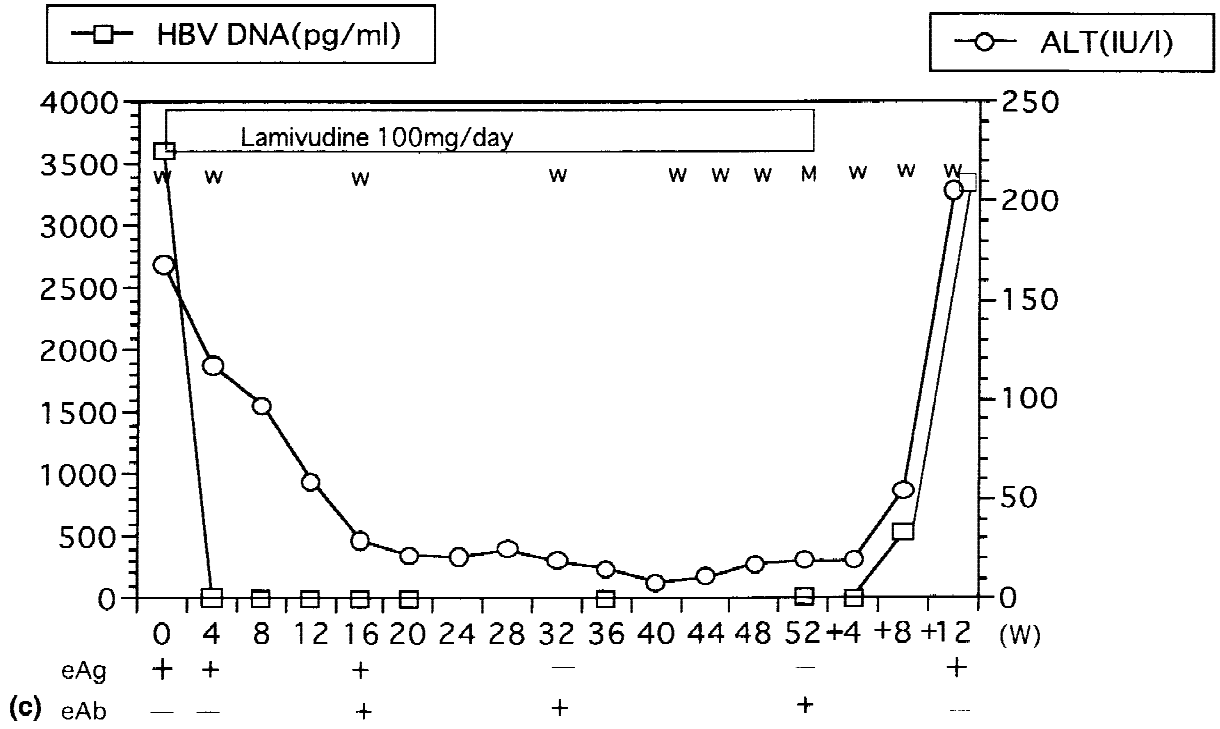

Fig. 2. Continued.

it is apparent that the mutants which can replicate in the presence of lamivudine may become selected during the treatment.

Viruses with mutations in the YMDD motif of DNA polymerase were observed in 3 of 8 (38%) immunocompetent patients receiving lamivudine for 52 weeks. The incidence of such mutations is similar to that reported by Honkoop et al. [1997] who described the appearance of mutant viruses in 4 of 14 immunocompetent patients (39% accuturial cumulative mutation rate at 52 weeks).

The YMDD motif mutants were observed in the present study only in cases treated with lamivudine for more than 40 weeks and not in any of the eight cases treated for equal to or less than 32 weeks. So far, there are no reports of such mutants emerging during therapy of less than 6 months. In patients with HIV infection, a frequent (more than 90%) and rapid appearance (within a few weeks) of mutant HIV with mutation in YMDD motif during lamivudine treatment has been reported [Boucher et al., 1993; Gao et al., 1993; Tisdale et al., 1993; Schuurman et al., 1995]. Compared to the HIV, the HBV mutant viruses seem to appear rather infrequently and relatively lately during treatment. The fidelity of HBV reverse transcriptase (DNA polymerase) was considered to be better than that of RNA viruses [Okamoto et al., 1987; Girones and Miller, 1989; Orito et al.,1989] and this might be one of the reasons for the difference in frequency and timing of appearance of such mutants.

It is not known whether such mutants exist prior to the beginning of lamivudine treatment or arise *de novo* during the treatment. However, from the relatively late appearance of HBV mutants, the mutation appears to be acquired *de novo* during lamivudine treatment.

Unexpectedly, the timing of appearance of the HBV mutants was not different from that found in the patients after OLT with immunosuppressive therapy (7–11 months after the lamivudine treatment [Ling et al., 1996; Tipples et al., 1996; Bartholomew et al., 1997]) and in immunocompetent patients [10–12 months after the treatment]. It seems the timing of appearance of such mutants was not influenced by immunosuppressive treatment.

In the current study, the YVDD motif was found 52 weeks after the beginning of the treatment in one case and YIDD in two cases after 40 weeks. In the HIV infection, YIDD motif is reported to appear first, and YVDD motif subsequently [Keulen et al., 1997]. However, this study, such change from YIDD to YVDD was not observed, indicating that the appearance of these HBV mutants was independent and the mode of appearance of these mutants is also different between the HBV and the HIV. In the present study, the mutant viruses became undetectable within 12 weeks after the cessation of the treatment. It is reported that replication ability of the mutant viruses (with YIDD or YVDD motif) of HIV is less than that of wild type virus (with YMDD motif) due to inefficient processing ability [Back et al., 1996]. Recently, the HBV with YIDD or YVDD motifs were demonstrated to have greatly impaired replication ability compared to that of wild type HBV through the defect in HBV DNA synthesis at the RNA packaging

level [Melegari et al, 1998]. This difference in replication capability between the HBV wild types and the mutants might result in reduction of the proportion of mutant viruses after the cessation of the treatment. In addition, residual wild type viruses remaining in hepatocytes during treatment start replicating once the treatment is stopped. Together, this may lead to false diagnosis of "disappearace" of mutant viruses after the cessation of treatment.

With regard to the clinical features, two of the 3 patients in whom mutant viruses were observed in this study showed the increase of the serum HBV DNA, then the elevation of ALT levels. Thus, such mutants seem to have a potential to cause hepatitis.

In conclusion, lamivudine resistant mutant HBV can emerge during long-term treatment of chronic hepatitis B even in immunocompetent patients. Thus, long-term lamivudine monotherapy may be limited due to an emergence of such HBV mutants in some patients. Combination therapies with other antiviral agents that enable the inhibition of replication of such mutants and/or with interferons which have different mechanisms of suppressing viral replication may be necessary to eliminate HBV in such patients.

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