Functional analysis of mutations conferring lamivudine resistance on hepatitis B virus

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Two patterns of mutation are commonly observed in the polymerase gene of lamivudine [(−)-2'-deoxy-3'-thiacytidine]-resistant hepatitis B virus (HBV). The M539I substitution in the conserved YMDD motif occurs independently of other changes, whereas the M539V substitution is associated with an additional upstream change (L515M). These mutations were introduced into a common background and their effects on HBV DNA replication and lamivudine resistance studied. The L515M and M539V mutations provided only partial resistance while the M539I mutation conferred a high degree of lamivudine resistance. The combination of the L515M and M539V mutations gave an intermediate level of replication competence, compared with either mutation alone, and increased resistance to lamivudine. This probably accounts for these two mutations always being observed together. The M539I mutation reduced replication competence.

Persistent infection with hepatitis B virus (HBV) may lead to chronic active hepatitis, cirrhosis and primary liver cancer. Therapy with interferon has been the treatment of choice until recently, despite being effective only in a minority of cases. Many centres ceased to offer orthotopic liver transplantation (OLT) to patients with HBV-associated, end-stage liver disease because of almost universal infection of the graft, commonly followed by rapid disease progression. (Muller et al., 1994).

HBV replicates via reverse transcription of an RNA intermediate but, in contrast to human immunodeficiency virus (HIV), nucleoside analogues such as AZT and ddi have not proved useful for therapy of chronic hepatitis B. Recently, lamivudine [(−)-2'-deoxy-3'-thiacytidine] (Dienstag et al., 1995; Lai et al., 1997; Nevens et al., 1997) and famciclovir (Schalm et al., 1995) have been found to reduce virus load in chronically infected patients and lamivudine has been used to reduce the risk of graft infection following OLT (Bain et al., 1996; Bartholomew et al., 1997; Ben-Ari et al., 1997a, b; Grellier et al., 1996; Ling et al., 1996; Markowitz et al., 1998; Tipples et al., 1996).

For HIV, resistance to lamivudine in cell culture and in vivo (Tisdale et al., 1993; Wainberg et al., 1995) correlates with changes in the methionine residue of the YMDD motif. Two aspartate residues in this motif, which is conserved amongst hepadnavirus and retrovirus reverse transcriptases, constitute two of three in a catalytic triad (Kohlstaedt et al., 1992; Larder et al., 1989; Toh et al., 1983). Similar mutations have been reported in resistant HBV from patients receiving lamivudine in combination with OLT (Bartholomew et al., 1997; Ling et al., 1996; Tipples et al., 1996) and in chronically infected patients (Allen et al., 1998; Buti et al., 1998; Chayama et al., 1998; Honkoop et al., 1997; Niesters et al., 1998). The methionine of the YMDD motif is substituted by either isoleucine or valine in these mutants (M539I or M539V, with the numbering based on the polymerase protein sequence from 3182 bp HBV strains). A substitution of an upstream leucine residue by methionine (L515M) invariably is associated with M539V, whereas no other mutation consistently is associated with M539I. A change at position 501 previously reported by us (Ling et al., 1996) proved on further sequence analysis to be erroneous.

This report describes the effects of mutations at positions 515 and 539, in a common sequence background, on DNA replication competence and its resistance to lamivudine. The mutations were introduced into a greater than full-length HBV clone (Ling & Harrison, 1997) and the constructs transfected into HepG2 (hepatoblastoma) and HuH7 (hepatocellular carcinoma) cells. The levels of relaxed circular (RC) HBV DNA product were measured by Southern blotting. Poor replication efficiency and only partial resistance of viral DNA encoding a polymerase with the M539V change alone accounted for the requirement for the additional L515M mutation, which alone conferred only partial lamivudine resistance. The M539I mutation also resulted in poor replication efficiency but may require a different background for efficient replication since it was tested in a sequence background different from that in the corresponding patient.

A greater than full-length HBV clone generated from the...
LSH strain of HBV [EMBL accession no. D00220 (Vaudin et al., 1988)] was used to express HBV DNA replicative intermediates (Ling & Harrison, 1997). The inability of the construct to produce the surface proteins reduces the risks involved in the experiments and eliminates any effect of changes in the surface protein coding sequence. The region of the polymerase gene containing the mutations was derived from the XbaI–HpaI HBV fragment [247–961 using the numbering of Ono et al. (1983), genotype D] from the pre-treatment serum of a patient whose virus subsequently acquired the double mutation (L515M and M539V). The presence of a SpeI site between the two mutations (Fig. 1a) enabled fragments from a post-
A BstXI–SpeI fragment containing the L515M mutation was introduced to create a clone with this mutation alone (Fig. 1b). The clones containing the M539 mutation or the double mutant were slightly more difficult to construct due to downstream differences in the post-transplant clones (probably due to Taq polymerase errors during their production). The pre-treatment derived clone was digested with SpeI and HpaI and an AccI–HpaI fragment from the same clone was introduced, along with a SpeI–AccI fragment containing the M539V mutation, to create a clone with this mutation alone (Fig. 1c). The double mutant was created in a similar way using the pre-treatment clone digested with BstXI and HpaI, along with the AccI–HpaI fragment from the same clone and a BstXI–AccI fragment containing both mutations from a post-treatment clone (Fig. 1d). Clones containing both, either or neither mutation were digested with Xbal and HpaI and the fragments inserted into the greater than full-length clone in pUC19.

The YIDD mutant was generated using a PCR-based method and starting from the same pre-treatment DNA (Fig. 1e). Two fragments were generated, one from an oligonucleotide containing the SpeI site (CTCAGTCTACTAGT-GCCATT) to an antisense oligonucleotide with the desired mutation (TACACATCATCATGATATAACTGAAAG) and a second from a sense oligonucleotide with the desired mutation (CTTCAATATATCGATATGGTA) to an antisense oligonucleotide with the HpaI site (ATAAGCCTGTAAAC-AGGAAGT). These two fragments were joined together by PCR using the outer primers, digested with SpeI and HpaI and introduced into the greater than full-length clone in pUC19 digested with the same enzymes. This HBV construct was sequenced over the altered region. Finally, all the constructs were subcloned into the episomal expression vector pMEP4 (Invitrogen), using the KpnI and HindIII sites.

The extraction procedure for obtaining DNA from transiently transfected cells was designed to remove input plasmid DNA. The extraction was performed as described previously (Ling & Harrison, 1997). Comparison of the relative amounts of RC HBV DNA extracted from HepG2 or HuH7 cells transiently transfected with constructs containing the various mutations or the wild-type suggests that all the mutations suppress replication efficiency (Fig. 2). The M539V and M539I mutations have a particularly marked effect. In the case of the M539V mutation this appears to be compensated for partially in the presence of the L515M mutation. The observed differences did not correlate with variation of the SEAP (secreted alkaline phosphatase) activity expressed from a cotransfected plasmid. The data are pooled from three (HepG2) or two (HuH7) experiments with triplicate transfections in each experiment. Relative levels of SEAP activity secreted from cotransfected HepG2 cells were obtained for one experiment in each case (error bars omitted for clarity). ☺, SEAP activity secreted from cotransfected HepG2 cells. ♦, SEAP activity secreted from cotransfected HuH7 cells.

![Fig. 2. Levels of HBV RC DNA in transiently transfected HepG2 (open columns) or HuH7 (shaded columns) relative to the wild-type construct in each case.](Image 324x484 to 553x719)
phosphatase) activity suggesting that the differences observed (except possibly those for the L515M mutant) were not due to variable transfection efficiencies. A reduction in replication efficiency of YVDD mutants, combined with increased fidelity of reverse transcription, may contribute to the clinical benefit of lamivudine in HIV patients (Wainberg et al., 1996).

The levels of RC DNA in transfected cell lines were measured by Southern blotting and the levels of transcripts containing sequences around the core promoter were measured using the ribonuclease protection assay (RPAII Ambion Inc., Texas, USA). RNA samples were obtained from the same nucleic acid samples used for the Southern blot by digestion with RNase-free DNase after boiling and quenching on ice to separate DNA–RNA duplexes. The RNA was extracted with phenol–chloroform and stored in 0·3 M sodium acetate, 75 % ethanol. The levels of RC DNA extracted from the cell lines following treatment with different lamivudine concentrations are shown in Fig. 3 (a) and the RNA levels at low and high lamivudine concentrations are shown in Fig. 3 (b). The M539I mutation alone confers significant lamivudine resistance.
whereas the L515M and M539V mutations confer a similar level of resistance only when both are present. RNA levels were measured to assess whether the differing levels of DNA in the cell lines were primarily due to differences in replication competence or the level of viral RNA in the cells. The relatively low expression level of the M539I construct may have been related to the low level of transcription rather than reduced reverse transcription (Fig. 3b). This suggests that the plasmid may have been present at a lower copy number in this cell line. A comparison of the DNA to pregenomic RNA ratios for the different mutants at the low lamivudine concentration showed that there were not significant differences between the wild-type and L515M constructs or the L515M + M539V and M539I constructs. All other pairwise comparisons showed differences that were significant (P < 0.05) or (in the case of the comparisons with the wild-type which showed highly variable RNA levels) almost significant (P < 0.065). These data are consistent with that for the transient transfections except for the M539I mutation which always showed lower replication efficiency than the L515M + M539V double mutant in transient transfections. The lamivudine concentration did not influence RNA levels, indicating that the differences in DNA levels reflected differences in reverse transcription rather than template concentration.

These studies confirm the involvement of mutations identified in virus from lamivudine-treated patients in whom virus replication had resumed, with drug resistance in HBV. Several other groups have recently reported similar results (Allen et al., 1998; Fu & Cheng, 1998; Ladner et al., 1998; Melegari et al., 1998). It has been shown that the replication competence of the M539I and L515M double mutant (not observed in patients) is also greater than that of the M539I single mutant (Melegari et al., 1998). This suggests that the additional L515M mutation usually observed in combination with the M539V mutation is selected primarily due to the improved lamivudine resistance of the double mutant (compared to the M539V only mutant) rather than its improved replication competence. The M539I mutant replicates poorly in the backgrounds used by us and others but this may not be the case with different background or as yet unidentified additional mutations. An alanine substitution for proline at position 572 was observed with the M539I mutant in the patient that we studied but this has not been observed in other cases and a rare L515M/M539V double mutant from this patient had threonine instead of proline at this position.

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References


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