In Vitro Characterization of Viral Fitness of Therapy-Resistant Hepatitis B Variants

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Background & Aims: **Because of the overlapping of polymerase and envelope genes in the hepatitis B virus (HBV) genome, nucleoside analog therapy can lead to the emergence of complex HBV variants that harbor mutations in both the reverse transcriptase and the envelope proteins. To understand the selection process of HBV variants during antiviral therapy, we analyzed the in vitro fitness (the ability to produce infectious progeny) of 4 mutant viral genomes isolated from one patient who developed resistance to a triple therapy (lamivudine, adefovir, and anti-HBV immunoglobulins).** ***Methods:*** **The 4 mutant and the wild-type forms of HBV were expressed from vectors in hepatoma cell lines; replication and viral particle secretion capacities then were analyzed. The impact of envelope gene mutations on infectivity was tested in HepaRG cells using the hepatitis delta virus (HDV) model as a reporter for infection.** ***Results:*** **The dominant HBV variant characterized from the therapy-resistant patient was found to have the best replicative capacity in vitro in the presence of high concentrations of lamivudine and adefovir. The expression of envelope proteins and secretion of subviral and Dane particles by this mutant was comparable with that of wild-type HBV. HDV particles enveloped by surface proteins from the selected mutant had the highest rates of infection in HepaRG cells compared with other mutants.** ***Conclusions:*** **These results illustrate the importance of viral fitness and infectivity as a major determinant of antiviral therapy resistance in patients. Understanding HBV mutant selection in vivo will help to optimize new anti-HBV therapeutic strategies.**

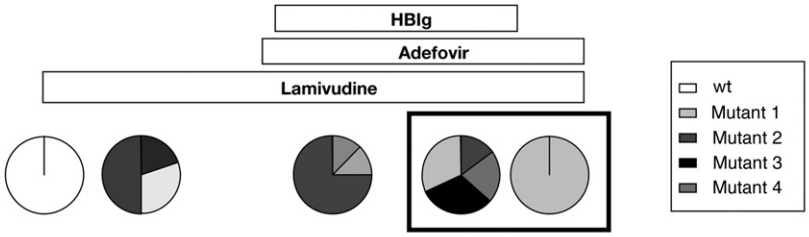
Treatment of chronic hepatitis B virus (HBV) infection with nucleos(t)ide analogs has been shown to be very effective in suppressing HBV replication.[1] In view of the long half-life of both covalently closed circular DNA and infected hepatocytes, long-term treatment is required to achieve complete HBV elimination. Unfortunately, the benefit of these treatments often is abolished by the selection of HBV drug-resistant mutants. Viral resistance to anti-HBV nucleos(t)ides occurs in 70% of patients after 4 years of lamivudine therapy and in 29% of patients after 5 years of adefovir therapy.[2] In nucleoside-naive patients, the rate of resistance to entecavir and tenofovir seems to be considerably lower. The main lamivudine resistance–associated substitution is located in the reverse transcriptase (RT) C-domain and corresponds to rtM204V/I.[3] The main variant isolated from patients with adefovir resistance has rtN236T substitution.[4] Moreover, substitution at position rt181 (rtA181V/T) may be responsible for cross-resistance to lamivudine and adefovir.[5] All these amino acid changes in the viral polymerase may affect viral replication capacity.[6–9]

The HBV genome is very compact and is organized into overlapping open reading frames, with the envelope(s) gene overlapping a part of the polymerase gene. Thus, mutations selected as a consequence of nucleoside analog therapy within the polymerase gene may result in sequence and structural changes in the 3 envelope proteins encoded by a single open reading frame.[10] Indeed, mutations in the polymerase selected during the course of antiviral nucleoside analog therapy can induce changes in the HBs antigen (HBsAg).[11] For example, lamivudine- or adefovir-resistance–associated mutations occurring in codon 173, 181, and 204 of the polymerase open reading frame also may result in substitution of amino acids in the S domain such as sE164D, sW172stop, sL173F, sI195M, or sW196L/S/stop.[12,13] These changes may alter the main functions of the HBV envelope proteins including empty subviral particles (SVP) or hepatitis delta virus (HDV) assembly, envelopment of HBV nucleocapsids in Dane particles, and binding or entry into hepatocytes.[13]

Mathematic modeling of the outcome of hepadnavirus infection, based on virologic observations made in the woodchuck model, indicated that the most important factors to consider are as follows: (1) the rate of immune killing of infected hepatocytes leading to the clearance of infected cells and to the generation of a pool of unin-

Abbreviations used in this paper: RT, reverse transcriptase; SVP, subviral particles; TP, Tri-Phosphate; wt, wild-type.

0016-5085/09/$36.00
doi:10.1053/j.gastro.2008.09.068



Polymerase clonal genetic analysis

	Polymerase gene mutations	S gene mutations
wt	none	none
Mutant 1	T128I; **V173L; L180M; A181V;** **N236T**	**F20S;** **P120S**; E164D; L173F
Mutant 2	T128I; **V173L; L180M; A181V; M204V**	**R79H;** **P120S**; E164D; L173F; I195M; **Y206F**
Mutant 3	Δ111-120; T128I; **V173L; L180M; A181V**	**F20S;** Δ102-111; **P120S;** E164D; L173F
Mutant 4	T128I; **V173L; L180M; A181V; M204V; L220I; N236T**	**P120S;** E164D; L173F; I195M

Figure 1. Evolution of HBV variants selection during anti-HBV therapy. In the first part of the figure are represented previous results of polymerase gene clonal analysis performed on HBV DNA isolated from patient sera.[8] Each diagram corresponds to HBV quasispecies isolated from one patient's serum during lamivudine monotherapy or lamivudine plus adefovir plus HB Ig tritherapy. At the different time points, the co-existence of different mutants was observed, each represented by a specific color or motif. The study was focused on the last 2 time points, illustrating the selection of mutant 1 among HBV quasispecies. In the second part of the figure are listed mutations in RT and S-associated domains for the 4 main mutants. Specific RT or S domain mutations are highlighted in *bold*. The other mutations are the consequences of mutations in the overlapping gene.

fected cells susceptible to newly selected quasispecies, and (2) the rate of replication and spread of mutant viruses in the chronically infected liver.[14] Small changes in these factors were found to have profound effect on whether treatment response is durable or subject to rapid rebound. The fitness of mutants defined by their ability to produce infectious progeny, in a defined environment, was proposed to be an important parameter in the selection process of resistant mutants.[14]

To investigate the impact of viral fitness on the selection of antiviral drug–resistant mutants, HBV variants that emerged sequentially in the serum of a patient treated by triple antiviral therapy (lamivudine, adefovir, and HB immunoglobulins [HB Igs]) were studied.[8] Polymerase and envelope sequence analyses have revealed complex HBV quasispecies composed of resistant mutants that evolved towards the selection of one specific dominant strain. To understand the mechanism of selection of this mutant within the viral population, we analyzed the fitness of 4 mutant strains co-existing in the patient's serum before the takeover by the final one. Our results indicate that mutant fitness, including infectivity, is a major determinant linked to the emergence of drug-resistant mutants during antiviral therapy.

Materials and Methods

Plasmids

Plasmids expressing 1.1 HBV genome unit length were described previously.[8] Briefly, in pTriex–wild-type (wt) HBV plasmid cloned from patient baseline serum, the RT domain was replaced by its counterpart from 4 mutants named mutants 1, 2, 3, and 4 (Figure 1). As a result, all mutants only differ by the RT and the envelope S overlapping domains. The pT7HB2.7 vector[15] was used to construct plasmids expressing L, M, and S wt or mutant envelope proteins under the control of the endogenous HBV promoter. These pHBV-env vectors were created by subcloning a *Bgl*II to *Nco*I fragment containing the wt or mutant L, M, and S envelope proteins into the blunt end-modified *Hind*III and *Nco*I sites of the pT7HB2.7 vector. The HDV recombinant plasmid pSVLD3[16] drives the replication of HDV RNA and the expression of the HDV ribonucleoprotein.

Cell Culture, Reagents, and Transfections

Huh-7 cells were grown in Dulbecco's modified Eagle medium (Eurobio, Courtaboeuf, France) supplemented with 10% fetal bovine serum. HepaRG cells were maintained in William's medium (Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal bovine serum, 5 μg/mL bovine insulin, and 5×10^{-5} mol/L hydrocortisone hemisuccinate.[17] For differentiation, HepaRG cells were maintained for 2 weeks in standard medium, then for at least 2 additional weeks in medium supplemented with 1.8% of dimethyl sulfoxide and epidermal growth factor 10 ng/mL. After virus inoculation, HepaRG cells were grown in medium supplemented with 2% dimethyl sulfoxide and epidermal growth factor 10 ng/mL. For transfection experiments, Huh-7 cells were seeded at 70% confluency and transfected with recombinant pTriex-HBV or pHBV-env clones additionally or not to pSVLD3 plasmid, using *Trans*TI-LT1 transfection reagent (Mirus bio corporation, Madison, WI) according to the manufacturer's protocol. Co-transfections with green fluorescent protein reporter plasmid were performed to normalize transfection efficiency.