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Differential Impact of Immune Escape Mutations G145R and P120T on the Replication of Lamivudine-Resistant Hepatitis B Virus e Antigen-Positive and -Negative Strains[▽]

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Immune escape variants of the hepatitis B virus (HBV) represent an emerging clinical challenge, because they can be associated with vaccine escape, HBV reactivation, and failure of diagnostic tests. Recent data suggest a preferential selection of immune escape mutants in distinct peripheral blood leukocyte compartments of infected individuals. We therefore systematically analyzed the functional impact of the most prevalent immune escape variants, the sG145R and sP120T mutants, on the viral replication efficacy and antiviral drug susceptibility of common treatment-associated mutants with resistance to lamivudine (LAM) and/or HBeAg negativity. Replication-competent HBV strains with sG145R or sP120T and LAM resistance (rtM204I or rtL180M/rtM204V) were generated on an HBeAg-positive and an HBeAg-negative background with precore (PC) and basal core promoter (BCP) mutants. The sG145R mutation strongly reduced HBsAg levels and was able to fully restore the impaired replication of LAM-resistant HBV mutants to the levels of wild-type HBV, and PC or BCP mutations further enhanced viral replication. Although the sP120T substitution also impaired HBsAg secretion, it did not enhance the replication of LAM-resistant clones. However, the concomitant occurrence of HBeAg negativity (PC/BCP), sP120T, and LAM resistance resulted in the restoration of replication to levels of wild-type HBV. In all clones with combined immune escape and LAM resistance mutations, the nucleotide analogues adefovir and tenofovir remained effective in suppressing viral replication in vitro. These findings reveal the differential impact of immune escape variants on the replication and drug susceptibility of complex HBV mutants, supporting the need of close surveillance and treatment adjustment in response to the selection of distinct mutational patterns.

Mutations within the hepatitis B virus (HBV) envelope gene can affect the antigenicity of the HBV surface antigen (HBsAg), thereby inducing an "immune escape," as protective antibodies (anti-HBs) cannot bind to the mutated epitopes of the HBsAg and neutralize the virions (34). Several conditions have been recognized in which HBV escape mutants arise in clinical practice: (i) treatment with anti-HBs immunoglobulin, e.g., after liver transplantation to avoid reinfection of the graft (23, 32); (ii) antiviral therapy by itself, as the reading frames of the envelope and polymerase genes overlap and several resistance mutations in the polymerase selected during antiviral therapy simultaneously alter the antigenicity of HBsAg (20, 21, 31); and (iii) de novo infection of vaccinated individuals with escape mutants (19). Moreover, Datta et al. recently reported that HBV variants with the sG145R immune escape mutation may very frequently "hide" in the peripheral blood leukocyte compartment of infected individuals (7).

Antibodies against HBsAg are predominantly directed toward the "a-determinant" domain of the HBs envelope protein, a highly conformational and cysteine-rich domain (34). Within this "a-determinant" domain (amino acids 124 to 147) and the major hydrophilic region 2 (amino acids 120 to 123) (23, 28), the sG145R (s denotes the amino acid position in the surface protein) and the sP120T substitutions are the most common immune escape mutations with reduced anti-HBs binding unraveled under various clinical conditions. Although the sG145R and sP120T mutations alter the sequence in the overlapping polymerase gene promoting the rtW153Q (rt denotes the amino acid position in the reverse transcriptase protein) and rtT128N exchanges, respectively, independent studies consistently reveal that these two immune escape mutations do not affect the replicative capacity of the virus per se (15, 16, 30). However, this might be very different in clinical practice, as immune escape mutations regularly arise as the consequence of (long-term) treatment modalities that by themselves carry the risk for selecting distinct HBV mutations. Thereby, immune escape variants do not usually occur alone in HBV strains of infected patients but, rather, in conjunction with additional polymerase and/or core mutations (26). In contrast to observations from HBV constructs with only one mutation, Torresi et al. reported that sG145R and sP120T envelope substitutions may enhance the replication of HBV mutants that are resistant to lamivudine (LAM) (30). Additionally, Bock et al. suggested that the susceptibility of such combination mutants to antivirals, at least to LAM, is considerably affected (4), raising the question of how to treat these compound mutants.

Our study aimed at comprehensively analyzing the effects of the most common immune escape mutations, sG145R and sP120T, in the HBV envelope protein on the replicative capacity of LAM-resistant polymerase mutants. Moreover, this issue was addressed on the background of HBeAg-positive and -negative virus strains, because precore (PC) and basal core

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promoter (BCP) mutations (conferring HBeAg negativity) by themselves alter the replication of LAM-resistant mutants (24). We also systematically addressed which nucleotide analogues remain effective in treating these complex compound HBV mutants.

MATERIALS AND METHODS

Generation of HBV vectors. The 1.28-fold replication-competent HBV plasmid (genotype A, subtype adw2) served as a wild-type (WT) vector (25), and the rtM204I and rtL180M/rtM204V LAM-resistant vectors had been generated previously (24). Notably, the rtL180M mutation will create no change in the envelope; the rtM204V will change the HBsAg to sI195M. Introduction of rtM204I can change, depending on the underlying genomic mutation, the envelope to either sW196S, sW196L, or sW196stop (20). In our constructs, the rtM204I changed the envelope protein to sW196S. The most common immune escape mutants, namely, the sG145R and sP120T mutants, had been introduced into each LAM-resistant vector by site-directed mutagenesis (4). All constructs were confirmed by digestion analysis and direct sequencing (Applied Biosystems, Foster City, CA) (24). To generate HBeAg-negative vectors, the 1.28-fold HBV replication-competent plasmids harboring G1896A/C1858T PC and A1762T/G1764A BCP mutations were used (2, 24). The fragment containing the LAM-resistant/immune escape mutant was excised by EcoRI and NcoI restriction enzymes (New England Biolabs, Ipswich, MA) and ligated into the same region within the PC and BCP plasmids. All clones were reconfirmed by digestion analysis and direct sequencing in both regions (Pol/S and BCP/C) using the BigDye terminator sequencing kit (Applied Biosystems).

Cell culture and transfection experiments. Huh-7 human hepatoma cells were seeded in 6-cm^2 petri dishes (BD Biosciences, Bedford, MA). Cells were fed by Dulbecco's modified Eagle's medium, 10% fetal bovine serum, as well as penicillin-streptomycin antibiotics (PAA, Coble, Germany). Cells were kept in an incubator with 5% CO_2 and 95% humidity. At the next day (70% confluence of cells), Huh-7 cells were transiently transfected by HBV vectors (5 μg per 6-cm^2 dishes) by using the standard calcium phosphate DNA precipitation method (24). Also, 0.5 μg of a pCMV-β-galactosidase (β-Gal) plasmid (Stratagene, La Jolla, CA) was cotransfected simultaneously with HBV vectors to monitor the transfection efficiency, using measurements of β-galactosidase activity from cell lysates (3). The transfection efficiency usually ranged between 25 and 30% of the cells. Transfected cells were harvested after 5 days. Cell lysates from three 6-cm^2 dishes were pooled for further analysis and treated as one experiment, while the cell lysate from one 6-cm^2-dish was always used for the β-Gal activity assay. All experiments were performed at least in triplicate.

Isolation of progeny HBV DNA and dot blot assay. To measure intracellular HBV replication, HBV progeny DNA was extracted and then quantified (24). In brief, 5 days after transfection, Huh-7 cells were lysed, and intracellular HBV progeny capsids were immunoprecipitated using polyclonal rabbit anti-HBc antibody (Dako, Carpinteria, CA) and protein A agarose (Roche Diagnostics, Mannheim, Germany). Input HBV plasmids were eliminated using DNase/RNase digestion (Promega, Madison, WI). Afterwards, HBV progeny capsids were digested with proteinase K-sodium dodecyl sulfate (Roche), and HBV progeny DNA was extracted by phenol-chloroform and precipitated by ethanol. HBV progeny DNA was then blotted onto a Hybond-N+ nylon membrane (Amersham Biosciences, Buckinghamshire, United Kingdom), using the Minifold I dot blot apparatus (Schleicher & Schuell, Dassel, Germany). HBV DNA was hybridized with a 3.2-kb [α^{32}P]dCTP radiolabeled HBV full-genome fragment. The signals were developed on an autoradiography film (Amersham), and data were quantified by the Scion Image software and normalized to total protein content of the cell lysate as well as the transfection efficiency.

Northern blot analysis. Five days after transfection, total RNA was extracted from transfected cells using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Concentration of RNA was measured by a spectrophotometer (Eppendorf). Northern blot analysis was performed as described before (24). Briefly, 25 μg total RNA per sample was electrophoresed on an RNA formaldehyde-denaturing agarose gel. After transferring RNAs to the Hybond-N+ nylon membrane (Amersham Biosciences), a 3.2-kb α-^{32}P-radiolabeled HBV probe was utilized for detection of all HBV transcripts. After detecting the signals via autoradiography (Amersham Hyperfilm), the density of pregenomic and surface RNA (S-RNA) was measured by the Scion Image software. Data were normalized to β-Gal transfection efficiency and also to the density of the 28S/18S rRNA as a loading control.

HBV viral load. HBV virions were harvested from the cell culture supernatant by using polyethylene glycol (PEG) precipitation (Sigma, St. Louis, MO) by an overnight incubation at 4°C under continuous rotation. Elimination of input contaminant HBV vectors was done by incubation with DNase/RNase enzymes (Promega). HBV DNA was extracted as described above. To measure HBV viral load, a real-time PCR was carried out based on the amplification of a 98-bp fragment from a highly conserved region of HBV S gene by using SYBR green quantitative PCR SuperMix (Invitrogen, Carlsbad, CA) (14). A standard curve was prepared using 10-fold serial dilutions of the HBV WT plasmid, and viral load was quantified by the ABI 7300 real-time PCR apparatus (Applied Biosystems). After quantification of HBV DNA, the viral load was normalized to the β-Gal transfection efficiency.

Monitoring contamination by the remaining HBV vectors. In order to exclude the possibility of contamination by the remaining HBV plasmids in the progeny DNA and the viral load assay, a quantitative real-time PCR approach was conducted with a primer pair targeting the pBluescript backbone of the HBV vectors. All extracted DNA from progeny assays (as well as in drug susceptibility assays) and extracted DNA from viral load assays were tested for DNA vector contamination by real-time PCR. The primer sequences were 5′-GAT GCT TTT CTG TGA CTG GTG AG-3′ for pBS-F and 5′-CGT TTT CCA ATG ATG AGC ACT T-3′ for pBS-R (2). No contamination was detected in the experiments (data not shown).

HBV proteins in supernatant. Supernatant was collected from transfected cells at day 5 after transfection. It was centrifuged for 10 min at 10,000 rpm at 4°C to remove debris and then used for quantification of HBV proteins. HBsAg and HBeAg were measured by Architect HBsAg (product number 6C36) and HBeAg (product number 6C32) kits (Architect; Abbott Laboratories, Chicago, IL) based on chemiluminescent microparticle immunoassay technology. The protein concentrations were normalized to the β-Gal transfection efficiency.

Analysis of large HBV envelope protein by Western blotting from supernatant. Supernatant was harvested 5 days after transfection. HBV virions and large envelope proteins (LHBs) were immunoprecipitated using mouse monoclonal antibody against HBV preS1 protein (clone AP1, sc-57761; Santa Cruz Biotechnology, Santa Cruz, CA) and protein A agarose (Roche Diagnostics, Mannheim, Germany) as described before (1). Laemmli sample buffer (Bio-Rad Laboratories, Inc. Hercules, CA) complemented with 5% β-mercaptoethanol was added to sedimented beads, heated for 5 min, and centrifuged briefly. Whole supernatant was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were then transferred to polyvinylidene difluoride Immobilon-P Transfer membrane (Millipore Corporation, Billerica, MA) and stained with a 1:1,000 dilution of anti-preS1 antibody (Santa Cruz Biotechnology). Subsequently, labeled horseradish peroxidase ECL anti-mouse immunoglobulin G (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom) was utilized as a secondary antibody (1:2,000 dilution). The ECL Plus Western blotting detection kit (GE Healthcare Life Sciences) was applied to develop signals on an autoradiography film (Amersham) (17). The density of LHBs proteins was measured by the Scion Image software. The protein concentrations were normalized to the total protein content of the cells and the β-Gal transfection efficiency.

Antiviral compounds and drug susceptibility analysis. LAM was purchased from Moravek Biochemicals, Brea, CA. Adefovir (ADV) and tenofovir (TDF) were kindly provided by Gilead Sciences, Foster City, CA. Stock solutions (50 mM) of the antiviral agents were prepared in sterile distilled water, aliquoted in single-use volumes, and stored frozen at −20°C. To determine the 50% effective concentration (EC_{50}) of drugs (1, 2), Huh-7 cells were transfected with the WT replication-competent HBV plasmid supplemented with different concentrations of ADV (0, 1, 5, 10, and 25 μM), LAM (0, 1, 5, 10, and 25 μM), and TDF (0, 1, 10, 50, and 200 μM). Medium containing drugs was changed every other day at day 1 and day 3 after transfection. To calculate the susceptibility of HBV to antiviral drugs, the dot blot assay of extracted HBV progeny DNA was performed as described above. The concentration of the antiviral component resulting in the inhibition of HBV replication by 50% was considered the EC_{50}. Dot blot results were normalized to the total protein content of the cell lysate and transfection efficiency based on β-Gal activity. The EC_{50} values for WT HBV were determined to be 6.1 μM for LAM, 11.5 μM for ADV, and 22.7 μM for TDF, which were in accordance with previous experiments (1, 2; data not shown). Efficient inhibitory concentrations around the EC_{50} of LAM (7 μM), ADV (15 μM), and TDF (30 μM) were then applied for antiviral susceptibility assays.

Statistical analysis. Results are reported as the mean ± standard deviation. The *t* test was used for comparisons between the groups, and a *P* value of <0.05 was considered statistically significant (SPSS, Chicago, IL).

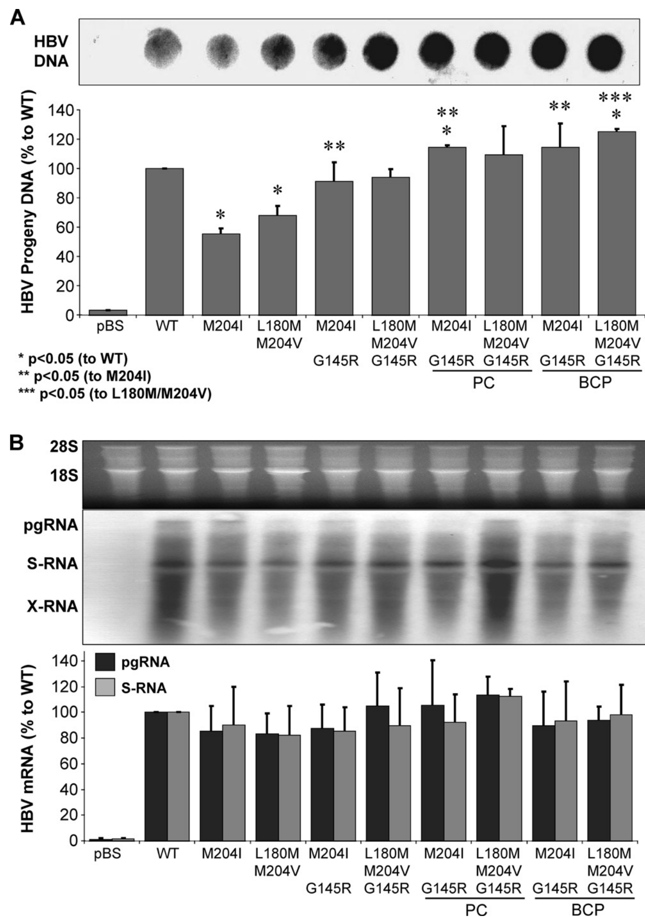

FIG. 1. Intracellular HBV replication of sG145R combination mutants. (A) Five days after transfection, intracellular viral replication efficacies were assessed by HBV progeny DNA. A representative dot blot is shown. Progeny DNA levels were quantified and normalized to the protein concentration of the cell lysates and to the β-Gal transfection efficiency. (B) Five days after transfection, intracellular HBV mRNA transcripts were analyzed by Northern blot analysis, and S-RNA and pregenomic (pg) RNA were quantified and normalized to 18S/28S RNA and transfection efficiency. Means and standard deviations are based on the results of at least three independent tests; values are given relative to those of the WT. One representative Northern blot is illustrated. Means and standard deviations are given relative to the WT, based on the results of three independent experiments. Significant differences compared to WT are marked by a single asterisk, those compared to rtM204I mutants by two asterisks, and those compared to rtL180M/rtM204V mutants by three asterisks. pBS, pBluescript control vector; X-RNA, regulatory X RNA.

RESULTS

The sG145R surface mutation increases the impaired replication of LAM-resistant clones in HBeAg-positive and -negative constructs. The most frequently detected immune escape variant of HBV is a glycine-to-arginine substitution at amino acid position 145 (sG145R) of the envelope protein (20, 23). Previous studies demonstrated that sG145R variants replicate with efficiency equal to that of WT HBV (4, 15). Because this mutation simultaneously results in a substitution in the polymerase protein (rtW153Q), it could possibly affect the already impaired replication of LAM-resistant viruses. To assess the level of intracellular viral replication, HBV progeny DNA was analyzed after immunoprecipitation of intracellular HBV core particles (Fig. 1A). As anticipated (1, 24), HBeAg-positive constructs with LAM resistance (rtM204I or rtL180M/rtM204V mutants) displayed significantly reduced replication. The additional presence of the "a-determinant" sG145R mutation increased the replication of LAM-resistant clones, resulting in replication efficacies at the level of those of WT HBV vectors (Fig. 1A).

We next examined if viral replication is altered, in case LAM resistance and sG145R mutations occur on the background of HBeAg negativity. We had previously demonstrated that the concomitant presence of PC and BCP mutants can enhance replication of LAM-resistant constructs in vitro (24). In fact, PC and BCP mutants moderately increased replication of sG145R/LAM-resistant clones, as HBV progeny DNA amounts were above the WT levels (significant differences are marked by asterisks in Fig. 1A). Northern blot analysis of cells transfected with the different HBV constructs showed that comparable amounts of viral mRNA were expressed (Fig. 1B), confirming that the polymerase activity is the main regulator of replication fitness in these mutants (24).

It has been suggested before that the sG145R mutation by itself may hamper the secretion of virions (16). We therefore assessed the number of HBV virions from the supernatant of transfected cells by precipitating virions and quantifying HBV copy numbers by the use of real-time PCR (Fig. 2A). In full agreement with the intracellular replication data, LAM-resistant clones released reduced quantities of HBV virions, whereas the combination of sG145R and LAM resistance resulted in virion numbers at the level of WT virus. In tendency, addition of PC or BCP mutations to these clones further enhanced virion release, but the differences were statistically indistinguishable from WT copy numbers, most likely due to higher variations in this assay (Fig. 2A). However, the concentration of HBsAg protein in the supernatant was significantly reduced in all clones containing the sG145R substitution (Fig. 2B), as the binding of polyclonal anti-HBs antibodies to HBs proteins with the sG145R substitution affecting the "a-determinant" epitope is impaired (13, 31). Constructs with PC mutants showed an absence of HBeAg in the supernatant, and BCP mutants displayed strongly reduced HBeAg concentrations (Fig. 2C).

The sP120T surface mutation does not influence replication of LAM-resistant mutants. The sP120T envelope mutation has been mainly observed in patients after liver transplantation during combined treatment with anti-HBs immunoglobulin and LAM (20). It simultaneously creates an rtT128N exchange in the polymerase protein. We addressed the replication of sP120T mutants in combination with LAM resistance (rtM204I or rtL180M/rtM204V). In contrast with the findings for sG145R combination mutants, the sP120T variant did not affect the replication of LAM-resistant clones (Fig. 3A; compare also to LAM resistance alone presented in Fig. 1A). The presence of PC or BCP mutations restored the replication to the level of WT HBV (Fig. 3A), as we observed it for only LAM-resistant constructs (24 and data not shown). Again, mRNA expression levels did not vary between the different mutants (Fig. 3B).

In addition to the analysis of progeny DNA for intracellular

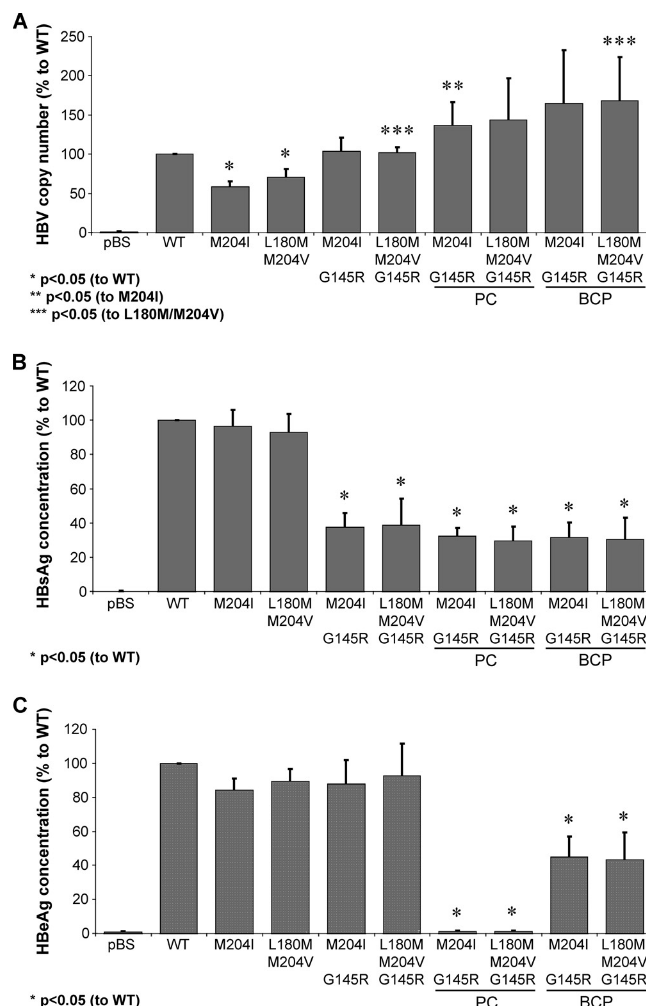

FIG. 2. Released HBV virion and proteins of sG145R combination mutants. Supernatant was analyzed 5 days after transfection. Significant differences compared to WT are marked by a single asterisk, those compared to M204I mutants by two asterisks, and those compared to L180M/M204V mutants by three asterisks. Means and standard deviations are given relative to the WT, based on results from three independent experiments. (A) HBV viral load was quantified from cell culture supernatant by real-time PCR after overnight PEG precipitation of secreted virions, normalized to the β-Gal transfection efficiency. (B) HBsAg concentrations were determined in the supernatant. (C) HBeAg concentrations were measured in the supernatant.

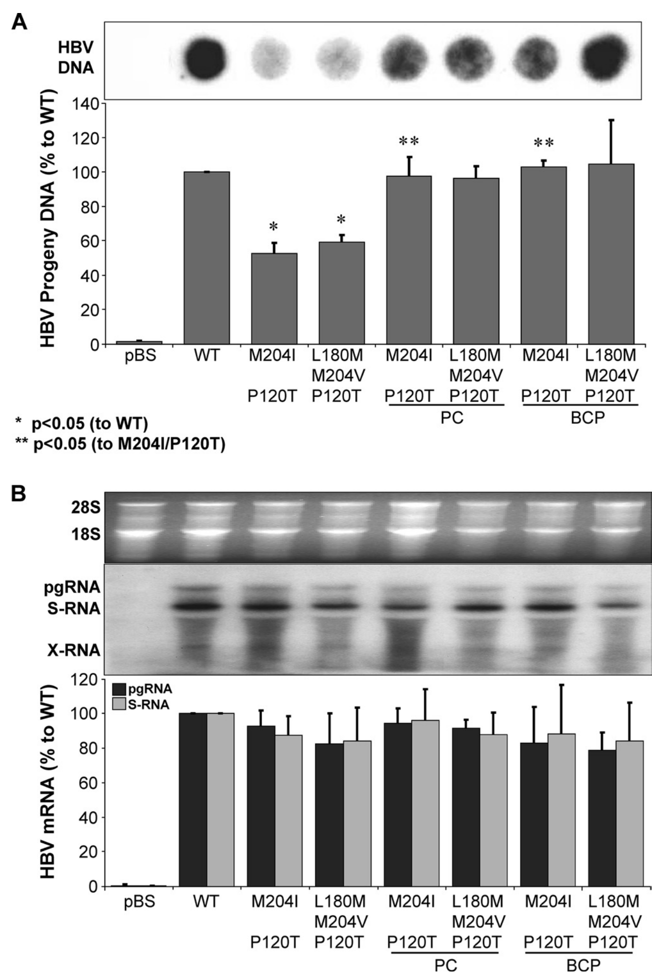

FIG. 3. Intracellular HBV replication of sP120T combination mutants. (A) Five days after transfection, intracellular viral replication efficacies were assessed by HBV progeny DNA. A representative dot blot is shown. Progeny DNA levels were quantified and normalized to the protein concentration of the cell lysates and to the β-Gal transfection efficiency. (B) Five days after transfection, intracellular HBV mRNA transcripts were analyzed by Northern blot analysis, and S-RNA and pregenomic (pg) RNA were quantified and normalized to 18S/28S RNA and transfection efficiency. One representative Northern blot is illustrated. Means and standard deviations are given relative to the WT, based on results from three independent experiments. Significant differences compared to WT are marked by a single asterisk and those compared to rtM204I/sP120T mutants by two asterisks.

replication, HBV virions were analyzed from supernatant of transfected cells. Constructs with sP120T and LAM resistance were found to have reduced HBV virions in the supernatant (Fig. 4A), at a level similar to that of constructs harboring only LAM resistance without additional envelope variants (compare to Fig. 2A). PC or BCP mutations were able to restore the virion release of constructs with sP120T/LAM resistance to the level of WT HBV (Fig. 4A).

As the sP120T mutation is known to change the antigenicity of the HBs protein (28), all constructs with sP120T mutations were found to have lower HBsAg concentrations in the supernatant (Fig. 4B), as detected by an advanced commercial test system (Abbott Architect). Moreover, clones with a combination of sP120T and the rtM204I mutation had HBsAg levels in the supernatant that were significantly lower than those of sP120T/rtL180M/rtM204V constructs, irrespective of the presence of PC or BCP mutations (Fig. 4B). HBeAg concentrations were absent in PC and reduced in BCP constructs (Fig. 4C).

Immune escape sG145R and sP120T mutations impair the release of HBV envelope proteins. Measurement of HBsAg in the supernatant of transfected cells demonstrated reduced HBsAg concentrations, using the sG145R and sP120T constructs (Fig. 2B and 4B). However, from these experiments, it remained unclear if this was solely due to a reduced detectability by the polyclonal anti-HBs antibodies applied in the HBsAg detection assay. We therefore immunopre-

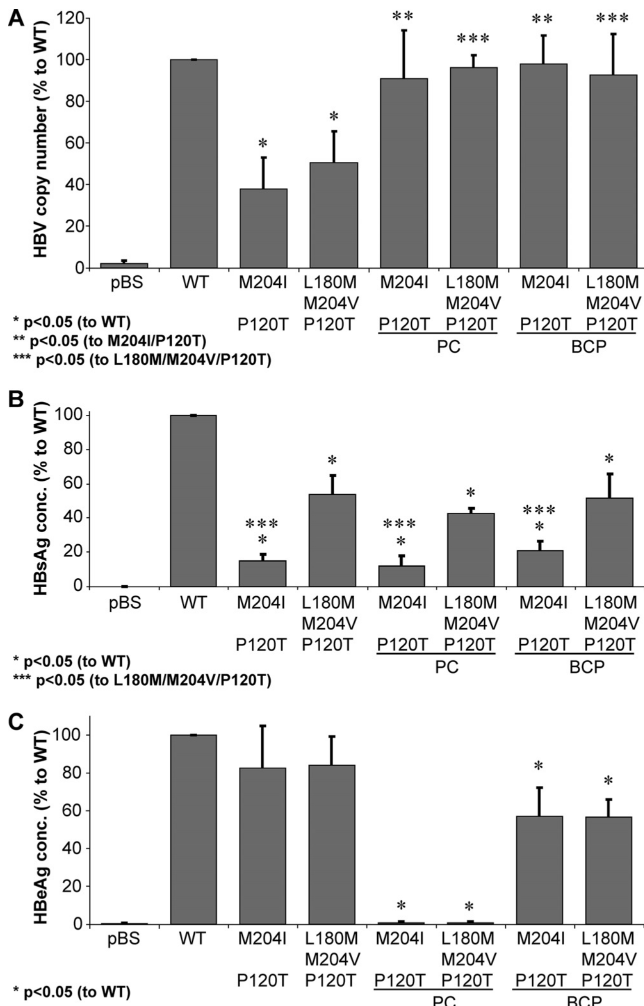

FIG. 4. Released HBV virion and proteins of sP120T combination mutants. Supernatant was analyzed 5 days after transfection. Significant differences compared to the WT are marked by a single asterisk, those compared to rtM204I/sP120T mutants by two asterisks, and those compared to rtL180M/rtM204V/sP120T mutants by three asterisks. Means and standard deviations are given relative to the WT, based on results from three independent experiments. (A) HBV viral load was quantified from cell culture supernatant by real-time PCR after overnight PEG precipitation of secreted virions, normalized to the β-Gal transfection efficiency. (B) HBsAg concentrations were determined in the supernatant. (C) HBeAg concentrations were measured in the supernatant.

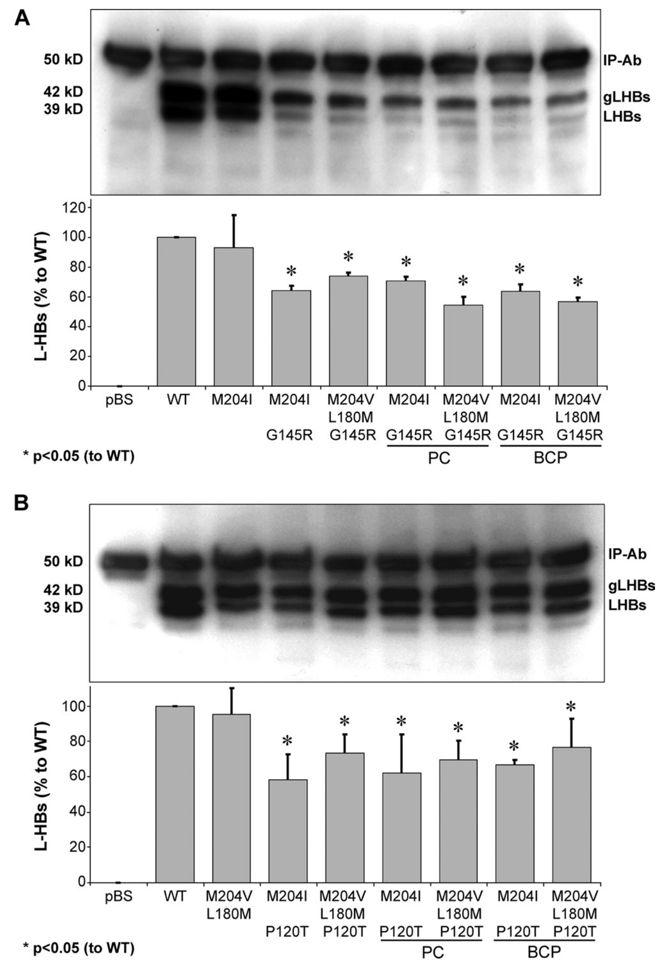

FIG. 5. Released HBV envelope proteins of sG145R and sP120T combination mutants. Supernatant was analyzed 5 days after transfection. Immunoprecipitation was performed with monoclonal pre-S1 antibody and subjected to Western blot analysis by using the pre-S1 antibody. Representative Western blots are shown for sG145R (A) and sP120T (B) combination mutants. The upper band represents the antibody used for immunoprecipitation (IP-Ab) and demonstrates equal loading. The specific bands show the glycosylated (gLHBs) and nonglycosylated (LHBs) large envelope protein. Quantification was performed by densitometry and normalized to transfection efficiency and loading. Means and standard deviations are given relative to the WT, based on results from three independent experiments. Significant differences compared to the WT are marked by an asterisk.

cipitated HBV virions and HBV envelope proteins from the supernatant of transfected cells by using a specific antibody recognizing the pre-S1 region which is not affected by the sG145R or sP120T mutations (33). All combination constructs with the sG145R mutations showed a reduction in large envelope (L-HBs) protein as evidenced by Western blot analysis (Fig. 5A), although the reduction was less pronounced than that in the HBsAg assay (compare to Fig. 2B). Similarly, the sP120T mutation also impaired detection of L-HBs in the supernatant (Fig. 5B). However, no difference could be detected in sP120T constructs with the rtM204I or the rtL180M/rtM204V mutations, in contrast to the results from the HBsAg assay (compare to Fig. 4B).

ADV and TDF are effective antiviral compounds in HBV mutants combining immune escape and LAM resistance in HBeAg-positive and -negative strains. Bock et al. had reported that the HBV mutants combining immune escape (sG145R, sP120T) and LAM resistance isolated from liver transplant patients with HBs-immunoglobulin treatment may even result in enhanced replication in the presence of LAM (4). We therefore systematically analyzed the replication of the various constructs by progeny DNA quantification from transfected cells with and without LAM (Fig. 6). Using an efficient inhibitory concentration for LAM around the EC_{50} in our model, only WT HBV had a suppressed HBV replication, while in all other clones containing the rtM204I or rtL180M/rtM204V muta-

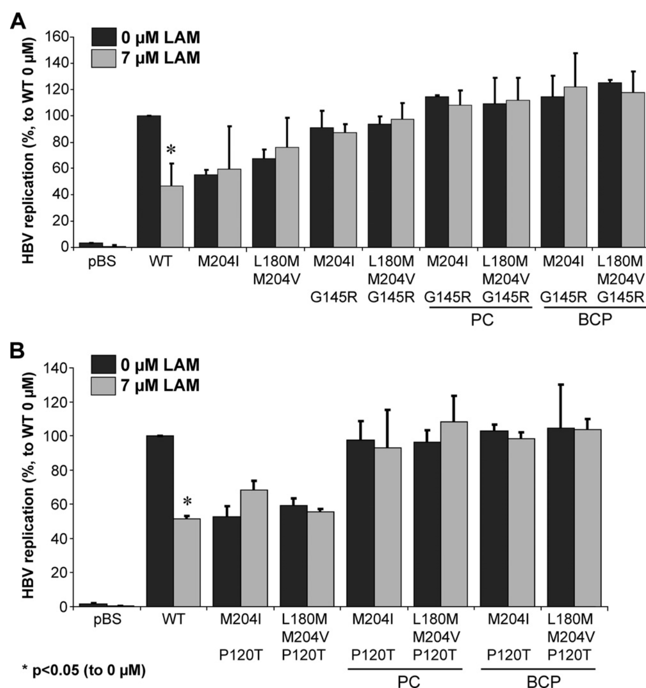

FIG. 6. Efficiency of LAM on viral suppression. Huh-7 hepatoma cells were transiently transfected with WT or mutant constructs as indicated. LAM was added to the culture medium at 7 μM (EC_{50} = 6.1 μM) as indicated. HBV progeny DNA levels were quantified as a measure of HBV replication and normalized to the protein concentration of the cell lysates and to the β-Gal transfection efficiency. Means and standard deviations are given relative to WT replication without antiviral treatment, based on results from three independent experiments. Significant differences ($P < 0.05$) compared to drug-free medium are marked by an asterisk. (A) Analysis of the sG145R combination mutants. (B) Analysis of the sP120T combination mutants.

tions, LAM did not affect the replicative activities of the constructs in vitro. Interestingly, this was true irrespective of the type envelope mutation (sG145R [Fig. 6A], sP120T [Fig. 6B]) or additional PC/BCP mutations.

At present, no systematic analysis exists for determining which antiviral compounds can be used in the case of HBV mutants with combined immune escape and LAM resistance (20). We hypothesized that nucleotide analogues would remain effective in these constructs with polymerase mutations conferring resistance to the nucleoside analogue LAM. Currently, ADV and TDF are the only nucleotide analogues approved by the FDA and European Medicines Agency (EMEA) for the treatment of chronic HBV infection (8, 9). Therefore, we tested the susceptibility of our constructs against ADV and TDF. As shown in Fig. 7A for sG145R mutants and in Fig. 7B for sP120T combination mutants, ADV significantly decreased the replication of the constructs. Interestingly, the efficiency of ADV in reducing HBV progeny DNA appeared to be slightly lower in sP120T constructs (Fig. 7B) than in WT or sG145R vectors (Fig. 7A). However, the progeny DNA assay may have some limitations in clones with very low replication levels (1), and the differences did not reach statistical significance.

Similarly, TDF efficiently inhibited HBV replication in WT, LAM-resistant, and combination mutants of sG145R or sP120T and LAM resistance (Fig. 7C and D). TDF was equally potent in HBeAg-positive and -negative strains, making this most recently approved antiviral drug an appealing possible "rescue" option for complex HBV mutants.

DISCUSSION

The emergence of immune escape variants of HBV is of exceptional clinical relevance, as these mutants may cause HBV reactivation even in anti-HBs-positive patients, can spread despite proper active/passive vaccination strategies, and may not be diagnosed due to failure of commercial HBsAg diagnostic assays (20). Furthermore, a recent report indicated that HBV strains vary in distinct compartments of chronically infected patients, revealing that immune escape sG145R mutants are frequently selected in peripheral blood leukocytes and may then serve as a source of reactivation or transmission (7). It is therefore important to understand the functional implications of immune escape mutations on the replication of HBV strains, especially on strains with common treatment-associated mutations, such as LAM resistance or HBeAg negativity (26).

Our study demonstrates that the sG145R and the sP120T mutations, both affecting the "a-determinant" domain of the HBs envelope protein, promote differential effects on the replication of LAM-resistant HBV mutants. Whereas the sG145R mutation can fully restore the impaired replication of LAM-resistant mutants to WT level, the sP120T mutation has no such effect. These results considerably advance prior findings by Torresi et al., who noted a partial restoration of HBV replication in clones combining envelope and polymerase mutations but did not systematically compare these compound variants (30). Our data indicate that after liver transplantation, patients who are treated with immunosuppressants, anti-HBs immunoglobulin, and LAM might be at considerable risk for selecting sG145R/LAM-resistant mutants with a high replicative capacity. This suggests that these patients require close monitoring during LAM therapy and might possibly benefit from newer nucleoside/nucleotide analogues with lower resistance rates.

It is not surprising that changes in the S gene may also affect HBV replication, because the sG145R mutation results in an rtW153Q polymerase exchange located in the "finger" subdomain of the polymerase protein. The LAM resistance mutations affect the YMDD motif of the "palm" subdomain of the polymerase, and this steric hindrance might be alleviated by compensatory changes in the "finger" or "thumb" subdomains (20, 29). The fact that only sG145R, and not sP120T, substitutions enhance replication of LAM-resistant clones might explain why sG145R mutations are much more frequently observed in the clinics than are sP120T mutations (32, 34).

An increasing percentage of patients with chronic HBV infection are affected by HBeAg-negative disease (11), which develops spontaneously during chronic infection through mutations in the PC or BCP region of the HBV genome (26). Patients with HBeAg-negative chronic HBV infection commonly have more advanced liver disease than do HBeAg-positive patients, and the likelihood of spontaneous remission is very low (5, 12). We and others had previously demonstrated that PC and BCP mutations enhance the replication of LAM-resistant HBV (6, 24). Interestingly, this was in principle now also confirmed for combination mutants with envelope and polymerase substitutions. However,

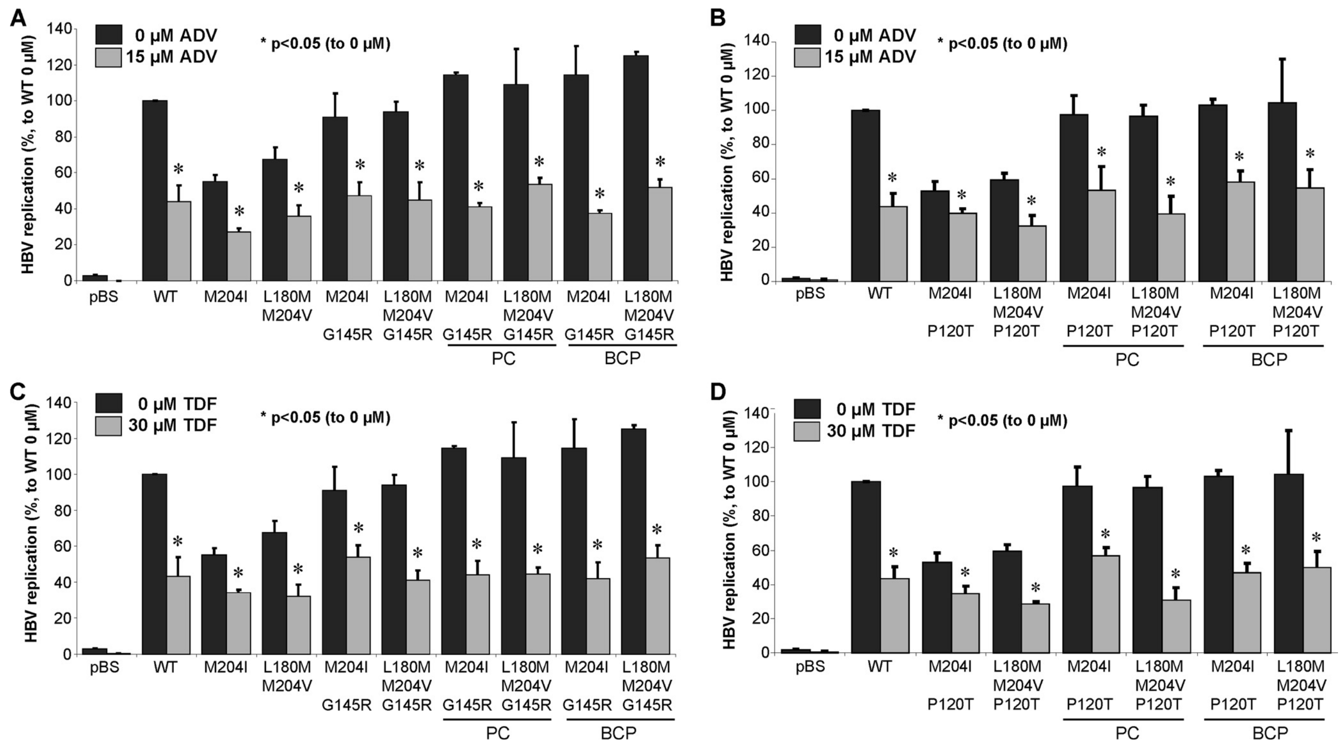

FIG. 7. Efficiency of ADV and TDF on viral suppression. Huh-7 hepatoma cells were transiently transfected with WT or mutant constructs as indicated. As indicated, ADV was added to the culture medium at 15 μM (EC_{50} = 11.5 μM), and TDF was added to the culture medium at 30 μM (EC_{50} = 22.7 μM). HBV progeny DNA levels were quantified as a measure of HBV replication and normalized to the protein concentration of the cell lysates and to the β-Gal transfection efficiency. Means and standard deviations are given relative to WT replication without antiviral treatment, based on results from three independent experiments. Significant differences ($P < 0.05$) compared to drug-free medium are marked by an asterisk. (A) Analysis of effects of ADV on the sG145R combination mutants. (B) Analysis of effects of ADV on the sP120T combination mutants. (C) Analysis of effects of TDF on the sG145R combination mutants. (D) Analysis of effects of TDF on the sP120T combination mutants.

only sG145R substitutions in combination with PC and BCP mutations were able to enhance replication above WT level, further corroborating the relevance of the sG145R/rtW153Q exchange for replication of HBV.

Interestingly, sG145R and sP120T mutants affected the HBsAg levels to different extents. HBV variants with the sG145R substitution expressed generally very low HBsAg levels, because the sG145R exchange directly alters the "a-determinant" epitope region (amino acids 124 to 147) (34). The sP120T substitution is located in the adjacent major hydrophilic region 2 (amino acids 120 to 123) and reduces the antigenicity to a comparatively lesser degree (28, 31). We performed additional experiments to distinguish if the reduced HBsAg were solely due to a detection failure by the commercial assay (based on polyclonal anti-HBs antibodies) or were also the result of an impaired secretion of HBs proteins. By Northern blot analysis, it was already clear that the intracellular transcription of the HBs gene was unaffected by the sG145R or sP120T mutation. In fact, we could demonstrate not only that the reduced HBsAg reflected a test failure, but also that a deficiency in HBV envelope and virion secretion contributed to the lower HBsAg release by these mutants. This finding is in full agreement with observations from the sG145R mutant without additional LAM resistance, as a deficiency in virion secretion and a decreased stability have been described before (16). Interestingly, the combination of rtM204I and sP120T, but not of rtL180M/rtM204V and sP120T, further reduced HBsAg levels. The most likely explanation is that the rtM204I mutation, simultaneously creating an sW196S/L/stop exchange in the envelope protein, by itself alters HBs antigenicity, in contrast to the rtM204V/sI195M substitution (20, 31).

We confirmed that all constructs containing LAM resistance mutations (rtM204I or rtL180M/rtM204V) were resistant to LAM in vitro, irrespective of the additional envelope or core gene mutations. However, in contrast to a previous study (4), LAM was not able to enhance the replication of these constructs. The exact reason for this discrepancy with the previous study (4) is unknown, but the two experimental approaches differed considerably. We used a LAM concentration (7 μM) around the EC_{50} determined for our assay system (6.1 μM), as this might allow us to most accurately measure significant effects on viral replication. In fact, WT replication was reduced to about 50% under this condition, and rtM204I or rtL180M/rtM204V mutants remained unaltered, as anticipated due to their resistance phenotype. In Bock et al.'s study that reported increased replication of sP120/rtL180M/rtM204V and sG145R/rtL180M/rtM204V mutants in the presence of LAM, the applied LAM concentration reduced WT replication by about 90%, whereas replication of rtL180M/rtM204V mutants was also reduced by 70% instead of resistance (4). The very high LAM concentration in their cell culture system could have possibly mediated nonphysiological side effects on the transfectants, which might have biased their results.

The detection of complex HBV mutants in clinical practice, e.g., combined HBeAg negativity, immune escape, and LAM resistance, represents a serious challenge to the hepatologist in order to adjust the antiviral therapy (10, 22). We therefore investigated possible treatment options by testing the susceptibility of these mutants to other antivirals in vitro. Notably, all constructs remained fully susceptible to the two approved nucleotide analogues ADV and TDF, supporting the current "road map" concept of a switch/add-on therapy with drugs that have a nonoverlapping resistance profile (10, 22). Consequently, we did not test the efficacy of entecavir, as recent data revealed that about 50% of LAM-resistant patients develop entecavir resistance during 5 years of therapy (27) and entecavir is not generally recommended in patients with existing LAM resistance anymore (10). Given the superiority of TDF to ADV with respect to viral suppression in clinical trials (18), our data in vitro suggest that TDF can serve as an efficient rescue drug in cases of complex HBV mutants combining LAM resistance and immune escape envelope mutations, an observation which needs to be clinically confirmed.

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REFERENCES

1. **Amini-Bavil-Olyaee, S., U. Herbers, S. R. Mohebbi, F. Sabahi, M. R. Zali, T. Luedde, C. Trautwein, and F. Tacke.** 2009. Prevalence, viral replication efficiency and antiviral drug susceptibility of rtQ215 polymerase mutations within the hepatitis B virus genome. J. Hepatol. **51:**647–654.
2. **Amini-Bavil-Olyaee, S., U. Herbers, J. Sheldon, T. Luedde, C. Trautwein, and F. Tacke.** 2009. The rtA194T polymerase mutation impacts viral replication and susceptibility to tenofovir in hepatitis B e antigen-positive and hepatitis B e antigen-negative hepatitis B virus strains. Hepatology **49:**1158–1165.
3. **Bock, C. T., B. Buerke, H. L. Tillmann, F. Tacke, V. Kliem, M. P. Manns, and C. Trautwein.** 2003. Relevance of hepatitis B core gene deletions in patients after kidney transplantation. Gastroenterology **124:**1809–1820.
4. **Bock, C. T., H. L. Tillmann, J. Torresi, J. Klempnauer, S. Locarnini, M. P. Manns, and C. Trautwein.** 2002. Selection of hepatitis B virus polymerase mutants with enhanced replication by lamivudine treatment after liver transplantation. Gastroenterology **122:**264–273.
5. **Brunetto, M. R., F. Oliveri, B. Coco, G. Leandro, P. Colombatto, J. M. Gorin, and F. Bonino.** 2002. Outcome of anti-HBe positive chronic hepatitis B in alpha-interferon treated and untreated patients: a long term cohort study. J. Hepatol. **36:**263–270.
6. **Chen, R. Y., R. Edwards, T. Shaw, D. Colledge, W. E. Delaney, H. Isom, S. Bowden, P. Desmond, and S. A. Locarnini.** 2003. Effect of the G1896A precore mutation on drug sensitivity and replication yield of lamivudine-resistant HBV in vitro. Hepatology **37:**27–35.
7. **Datta, S., R. Panigrahi, A. Biswas, P. K. Chandra, A. Banerjee, P. K. Mahapatra, C. K. Panda, S. Chakrabarti, S. K. Bhattacharya, K. Biswas, and R. Chakravarty.** 2009. Genetic characterization of hepatitis B virus in peripheral blood leukocytes: evidence for selection and compartmentalization of viral variants with immune escape G145R mutation. J. Virol. **83:**9983–9992.
8. **Dienstag, J. L.** 2009. Benefits and risks of nucleoside analog therapy for hepatitis B. Hepatology **49:**S112–S121.
9. **Di Marco, V., and A. Craxi.** 2009. Chronic hepatitis B: who to treat and which choice of treatment? Expert Rev. Anti. Infect. Ther. **7:**281–291.
10. **European Association for the Study of the Liver.** 2009. EASL clinical practice guidelines: management of chronic hepatitis B. J. Hepatol. **50:**227–242.
11. **Funk, M. L., D. M. Rosenberg, and A. S. Lok.** 2002. World-wide epidemiology of HBeAg-negative chronic hepatitis B and associated precore and core promoter variants. J. Viral Hepat. **9:**52–61.
12. **Hadziyannis, S. J., and D. Vassilopoulos.** 2001. Hepatitis B e antigen-negative chronic hepatitis B. Hepatology **34:**617–624.
13. **Hawkins, A. E., R. J. Gilson, N. Gilbert, T. G. Wreghitt, J. J. Gray, I. Ahlers-de Boer, R. S. Tedder, and G. J. Alexander.** 1996. Hepatitis B virus surface mutations associated with infection after liver transplantation. J. Hepatol. **24:**8–14.
14. **Henke-Gendo, C., S. Amini-Bavil-Olyaee, D. Challapalli, C. Trautwein, H. Deppe, T. F. Schulz, A. Heim, and F. Tacke.** 2008. Symptomatic hepatitis B reactivation despite reduced viral fitness is associated with HBV test and immune escape mutations in an HIV-coinfected patient. J. Infect. Dis. **198:**1620–1624.
15. **Jammeh, S., H. C. Thomas, and P. Karayiannis.** 2007. Replicative competence of the T131I, K141E, and G145R surface variants of hepatitis B virus. J. Infect. Dis. **196:**1010–1013.
16. **Kalinina, T., A. Iwanski, H. Will, and M. Sterneck.** 2003. Deficiency in virion secretion and decreased stability of the hepatitis B virus immune escape mutant G145R. Hepatology **38:**1274–1281.
17. **Karlmark, K. R., R. Weiskirchen, H. W. Zimmermann, N. Gassler, F. Ginhoux, C. Weber, M. Merad, T. Luedde, C. Trautwein, and F. Tacke.** 2009. Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. Hepatology **50:**261–274.
18. **Marcellin, P., E. J. Heathcote, M. Buti, E. Gane, R. A. de Man, Z. Krastev, G. Germanidis, S. S. Lee, R. Flisiak, K. Kaita, M. Manns, I. Kotzev, K. Tchernev, P. Buggisch, F. Weilert, O. O. Kurdas, M. L. Shiffman, H. Trinh, M. K. Washington, J. Sorbel, J. Anderson, A. Snow-Lampart, E. Mondou, J. Quinn, and F. Rousseau.** 2008. Tenofovir disoproxil fumarate versus adefovir dipivoxil for chronic hepatitis B. N. Engl. J. Med. **359:**2442–2455.
19. **Michitaka, K., F. Akbar, and M. Onji.** 2007. Prevention of hepatitis B virus infection by vaccination: progress and problems. Hepatol. Res. **37:**673–675.
20. **Sheldon, J., and V. Soriano.** 2008. Hepatitis B virus escape mutants induced by antiviral therapy. J. Antimicrob. Chemother. **61:**766–768.
21. **Sloan, R. D., S. Ijaz, P. L. Moore, T. J. Harrison, C. G. Teo, and R. S. Tedder.** 2008. Antiviral resistance mutations potentiate hepatitis B virus immune evasion through disruption of its surface antigen a determinant. Antivir. Ther. **13:**439–447.
22. **Sorrell, M. F., E. A. Belongia, J. Costa, I. F. Gareen, J. L. Grem, J. M. Inadomi, E. R. Kern, J. A. McHugh, G. M. Petersen, M. F. Rein, D. B. Strader, and H. T. Trotter.** 2009. National Institutes of Health consensus development conference statement: management of hepatitis B. Hepatology **49:**S4–S12.
23. **Tabor, E.** 2006. Infections by hepatitis B surface antigen gene mutants in Europe and North America. J. Med. Virol. **78**(Suppl. 1):S43–S47.
24. **Tacke, F., C. Gehrke, T. Luedde, A. Heim, M. P. Manns, and C. Trautwein.** 2004. Basal core promoter and precore mutations in the hepatitis B virus genome enhance replication efficacy of lamivudine-resistant mutants. J. Virol. **78:**8524–8535.
25. **Tacke, F., C. Liedtke, S. Bocklage, M. P. Manns, and C. Trautwein.** 2005. CREB/PKA sensitive signalling pathways activate and maintain expression levels of the hepatitis B virus pre-S2/S promoter. Gut **54:**1309–1317.
26. **Tacke, F., M. P. Manns, and C. Trautwein.** 2004. Influence of mutations in the hepatitis B virus genome on virus replication and drug resistance—implications for novel antiviral strategies. Curr. Med. Chem. **11:**2667–2777.
27. **Tenney, D. J., R. E. Rose, C. J. Baldick, K. A. Pokornowski, B. J. Eggers, J. Fang, M. J. Wichroski, D. Xu, J. Yang, R. B. Wilber, and R. J. Colonno.** 2009. Long-term monitoring shows hepatitis B virus resistance to entecavir in nucleoside-naive patients is rare through 5 years of therapy. Hepatology **49:**1503–1514.
28. **Tian, Y., Y. Xu, Z. Zhang, Z. Meng, L. Qin, M. Lu, and D. Yang.** 2007. The amino acid residues at positions 120 to 123 are crucial for the antigenicity of hepatitis B surface antigen. J. Clin. Microbiol. **45:**2971–2978.
29. **Torresi, J.** 2002. The virological and clinical significance of mutations in the overlapping envelope and polymerase genes of hepatitis B virus. J. Clin. Virol. **25:**97–106.
30. **Torresi, J., L. Earnest-Silveira, G. Civitico, T. E. Walters, S. R. Lewin, J. Fyfe, S. A. Locarnini, M. Manns, C. Trautwein, and T. C. Bock.** 2002. Restoration of replication phenotype of lamivudine-resistant hepatitis B virus mutants by compensatory changes in the "fingers" subdomain of the viral polymerase selected as a consequence of mutations in the overlapping S gene. Virology **299:**88–99.
31. **Torresi, J., L. Earnest-Silveira, G. Deliyannis, K. Edgtton, H. Zhuang, S. A. Locarnini, J. Fyfe, T. Sozzi, and D. C. Jackson.** 2002. Reduced antigenicity of the hepatitis B virus HBsAg protein arising as a consequence of sequence changes in the overlapping polymerase gene that are selected by lamivudine therapy. Virology **293:**305–313.
32. **Trautwein, C.** 2004. Mechanisms of hepatitis B virus graft reinfection and graft damage after liver transplantation. J. Hepatol. **41:**362–369.
33. **Villet, S., G. Billioud, C. Pichoud, J. Lucifora, O. Hantz, C. Sureau, P. Deny, and F. Zoulim.** 2009. In vitro characterization of viral fitness of therapy-resistant hepatitis B variants. Gastroenterology **136:**168–176.
34. **Weber, B.** 2005. Genetic variability of the S gene of hepatitis B virus: clinical and diagnostic impact. J. Clin. Virol. **32:**102–112.