Nucleic Acid Polymer REP 2139 and Nucleos(T)ide Analogues Act Synergistically Against Chronic Hepadnaviral Infection *In Vivo* in Pekin Ducks

Jonathan Quinet,[1] Catherine Jamard,[1] Madeleine Burtin,[1] Matthieu Lemasson,[2] Sylviane Guerret,[3] Camille Sureau,[2] Andrew Vaillant,[4] and Lucyna Cova [ID][1]

Nucleic acid polymer (NAP) REP 2139 treatment was shown to block the release of viral surface antigen in duck HBV (DHBV)-infected ducks and in patients with chronic HBV or HBV/hepatitis D virus infection. In this preclinical study, a combination therapy consisting of REP 2139 with tenofovir disoproxil fumarate (TDF) and entecavir (ETV) was evaluated *in vivo* in the chronic DHBV infection model. DHBV-infected duck groups were treated as follows: normal saline (control); REP 2139 TDF; REP 2139 + TDF; and REP 2139 + TDF + ETV. After 4 weeks of treatment, all animals were followed for 8 weeks. Serum DHBsAg and anti-DHBsAg antibodies were monitored by enzyme-linked immunosorbent assay and viremia by qPCR. Total viral DNA and covalently closed circular DNA (cccDNA) were quantified in autopsy liver samples by qPCR. Intrahepatic DHBsAg was assessed at the end of follow-up by immunohistochemistry. On-treatment reduction of serum DHBsAg and viremia was more rapid when REP 2139 was combined with TDF or TDF and ETV, and, in contrast to TDF monotherapy, no viral rebound was observed after treatment cessation. Importantly, combination therapy resulted in a significant decrease in intrahepatic viral DNA (>3 log) and cccDNA (>2 log), which were tightly correlated with the clearance of DHBsAg in the liver. *Conclusion:* Synergistic antiviral effects were observed when REP 2139 was combined with TDF or TDF + ETV leading to control of infection in blood and liver, associated with intrahepatic viral surface antigen elimination that persisted after treatment withdrawal. Our findings suggest the potential of developing such combination therapy for treatment of chronically infected patients in the absence of pegylated interferon. (HEPATOLOGY 2018;67:2127-2140).

The hepatitis B virus (HBV) causes chronic liver infection in more than 248 million persons worldwide,[1] which leads to liver fibrosis, cirrhosis, and development of hepatocellular carcinoma.[2] The disappearance of the hepatitis B surface antigen (HBsAg) from the blood (HBsAg loss) is

considered the best indicator of the establishment of functional control over HBV infection which endures in the absence of therapy.[3-5] Currently approved treatments include nucleos(t)ide analogs (NUCs) that block the maturation of HBV by inhibiting the viral polymerase and interferon (IFN)-based therapy to improve host immune control of HBV infection.[6,7] However, although NUCs suppress HBV DNA and control the progression to fibrosis, they rarely result in HBsAg loss, and IFN-based therapy can only achieve HBsAg loss in a small fraction of treated patients,[8,9] indicating the need for new therapies capable of directly targeting HBsAg clearance from the blood.

Nucleic acid polymers (NAPs) are broad-spectrum antiviral agents[10] that, in HBV infection, inhibit the release of subviral particle (SVP)-derived HBsAg from hepatocytes.[11,12] NAPs use the sequence-independent properties of phosphorothioated oligonucleotides as amphipathic polymers, which naturally accumulate in the liver,[13] to interfere with an as yet uncharacterized host process essential for the assembly and/ or secretion of SVPs.[10,12] This effect is independent from any direct immunostimulatory activity of NAPs[14] and blocks the release of HBsAg not only from infected hepatocytes, but also from cells with integrated HBV DNA.[15] Because SVPs represent >99.99% of circulating HBsAg,[16] inhibition of SVP release is an efficient means to clear HBsAg from the circulation. The ability of NAPs to clear circulating HBsAg has been validated in several proof-of-concept clinical trials in hepatitis B e antigen (HBeAg)-positive, HBeAg-negative, HBV monoinfected, and HBV/HDV (hepatitis D virus) coinfected patients.[15,17,18] Clearance of HBsAg in these trials has been accompanied not only by improved immune function, but also by a dramatic improvement in the antiviral effects of IFN-based immunotherapy, which has led to the establishment of functional control of HBV and HDV infection in a significant proportion of patients.[15,17,18]

Infection of Pekin ducks with duck hepatitis B virus (DHBV) is a well-recognized surrogate model of HBV infection previously used to evaluate novel anti-HBV approaches such as NUCs, peptide nucleic acids, and therapeutic DNA vaccines.[19-21] This model is well suited for examining the capacity of investigational agents to establish functional control of HBV infection given that neonatal DHBV infection of Pekin ducks results in a chronic infection of the liver, including the establishment of a reservoir of covalently closed circular DNA (cccDNA)[22] in infected cells, and has an abundant amount of SVPs in the circulation,[23] similar to HBV infection in humans. DHBV shares with human HBV several important features such as genome organization and a replication pathway. Despite the overall similar genome structure compared with HBV, DHBV has a cryptic X open reading frame and codes for only two DHBsAg-bearing envelope proteins: the large and small DHBsAg proteins. The DHBV model was extensively used to demonstrate the key role of cccDNA in the persistence of hepadnaviral infection.[19] The chronic DHBV infection model also allowed the mechanistic study of the viral rebound after removal of NUCs. However, this model also has limitations, given that monitoring of host immune responses remains difficult in the absence of commercial tools allowing the analysis of duck innate, humoral, and cellular immune responses, and there is no development of fibrosis or liver inflammation in this model. Interestingly, the antiviral activity of NAP monotherapy observed in the chronic DHBV model closely emulates the effects of NAPs in HBV infection in clinical trials,[11,13,15,17,18] validating the preclinical performance of NAP-based therapy in DHBV-infected ducks as a reliable predictor of the effects of NAPs in HBV-infected patients.

ARTICLE INFORMATION:

From the [1]Institut National de Santé et Recherche Médicale (INSERM) U1052, Lyon, France; [2]Institut National de la Transfusion Sanguine (INTS), Paris, France; [3]Novotec, Lyon, France; [4]Replicor Inc., Montreal, Quebec, Canada.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Lucyna Cova, Ph.D.
INSERM U1052
151 cours A. Thomas
69003 Lyon, France
E-mail: lucyna.cova@inserm.fr
Tel: +33 4 72 68 19 81; or

Andrew Vaillant, Ph.D.
Replicor Inc.
6100 Royalmount Avenue
Montreal, Quebec, Canada, H4P 2R2
E-mail: availlant@replicor.com
Tel: +1 514-733-1998

In the current study, ducks with previously established chronic DHBV infection were treated with combination therapy comprising the NAP REP 2139, tenofovir disoproxil fumarate (TDF), and entecavir (ETV). The goal of this study was to evaluate the potential benefit of combination therapy on on-treatment antiviral performance and the establishment of function control of DHVB infection after treatment withdrawal.

Materials and Methods

REP 2139 PREPARATION

REP 2139 formulated as a calcium chelate complex and was prepared as previously described[13] and used to dose ducks as described below.

VIRAL INFECTION AND THERAPEUTIC PROTOCOL

Chronic DHBV infection was established by intravenous inoculation of 3-day-old Pekin ducklings with a pool of viremic serum (2×10^{11} viral genome equivalents [vge]/mL) as described.[20] Four-week-old chronically infected ducks were randomly assigned into five groups: normal saline (NS; intraperitoneal); REP 2139 (10 mg/kg intraperitoneal once-daily [QD]); TDF (15 mg per oral QD); REP 2139 + TDF; and REP 2139 + TDF + ETV (1 mg per oral QD). The use of REP 2139, TDF, and ETV in combination was included based on the demonstration of accelerated HBV-DNA response with combined TDV and ETV versus ETV alone in some patient populations.[24] After 4 weeks of daily treatment, all animals were monitored during an additional 8 weeks to assess the rebound of viral replication after treatment cessation. At the end of follow-up, all necropsy liver samples were snap-frozen in liquid nitrogen and preserved at $-80°C$ until analysis.

Animals were housed at the National Veterinary School of Lyon (VetAgro Sup, Marcy L'Etoile, France), and all experimental protocols were approved by the Animal Care and Ethics Committee of VetAgro Sup.

QUANTIFICATION OF SERUM AND LIVER DHBV DNA

Viremia was assessed by detection of DHBV DNA in duck serum using qPCR as described.[13] Briefly, DNA extraction was performed on 100 μL of duck serum using the High Pure Viral Nucleic Acids kit (Roche Diagnostics, Meylan, France). For intrahepatic DHBV DNA analysis, total DNA extraction was performed on deep-frozen liver samples using the Nucleo-Spin Tissue kit (Macherey-Nagel, Hoerdt, France). DHBV DNA was amplified in the LightCycler 480 (Roche Diagnostics, Bâle, Switzerland) instrument as described.[13] In brief, 1× SYBR Green I Master mix (Roche Diagnostics, France) containing 20 pmol of each primer, 5′CTGACGGACAACGGGTCTAC-3′ (position 1596-1615) for the forward primer and 5′-GGGTGGCAGAGGAGGAAGT-3′ (position 1728-1746) for the reverse primer, was used for DHBV DNA amplification. The reaction started by denaturation at 95°C for 1 minute 30 seconds, followed by 40 PCR amplification cycles (denaturation at 95°C for 13 seconds, annealing and elongation at 63°C for 18 seconds). For detection of viral cccDNA, digestion of relaxed circular DNA by Plasmid Safe ATP-dependant DNase (Epicentre, Madison, WI) was first performed as described.[25] cccDNA was then amplified using specific primers and established conditions.[13,26] The number of vge per cell was calculated as described.[15]

cccDNA was further analysed in autopsy liver samples by Southern blotting. Total liver DNA was extracted from liver samples according to the procedures described.[20] Hybridization was carried out as previously described.[27] Radiolabeled probe was generated by *in vitro* transcription of PCR amplified DHBV DNA that included the T7 promoter for specific detection of the plus strand of DHBV DNA. Hybridization signal intensity was measured by phosphorimager analysis (Fujifilm BAS 1800-II) and quantitated by comparison to one-half dilutions of 660 pg of 3-kilobase-pair DHBV DNA.

DETECTION OF DHBsAg BY ENZYME-LINKED IMMUNOSORBENT ASSAY

Serum samples collected on days 1, 14, and 28 of treatment and weeks 4 and 8 of follow-up were tested to determine the levels of DHBsAg by enzyme-linked immunosorbent assay (ELISA) as described.[11] In brief, 96-well microtiter plates (High Binding, Greiner Bio One, Dutscher) were coated in duplicates with 100 μL of a 1:100 dilution of serum samples; the high-titer positive controls for DHBsAg, and normal duck serum negative controls were included on each plate. Plates were covered

with plastic film and incubated at 37°C overnight. Thereafter, plates were washed and bound DHBsAg was detected with a 1:1,000 dilution in blocking solution of primary anti-DHBV preS mouse 1H1 monoclonal antibody.[28] This was followed by incubation with a 1:1,250 dilution in blocking solution of horseradish peroxidase (HRP)-conjugated goat anti-mouse polyclonal antibody (Life Technologies, France). The visualization of bound HRP was performed by incubation for 30 minutes with 3,3′,5,5′-tetramethylbenzidine substrate (Single Solution kit; Life Technologies, France) and optical density (OD) values were read at 450 nm using a MultiScan (Thermo Scientific, USA). Background OD_{450} values determined from uninfected duck serum were subtracted from all observed OD_{450} values obtained from infected ducks in the study.

DETECTION OF ANTI-DHBsAg ANTIBODIES BY ELISA

Anti-DHBsAg antibodies were determined on serum samples from each duck at each time point using a previously described direct ELISA test.[26,29] Briefly, 96-well microtiter plates (High Binding, Greiner Bio One, Dutscher) were coated in duplicates with 300 ng of purified bacterially expressed DHBVpreS polypeptide and incubated overnight at room temperature. Following washes, bound antibodies were revealed with goat antiduck immunoglobulin G secondary antibody conjugated to the alkaline phosphatase (KPL, Gaitheburg). The reaction was revealed with paranitrophenylphosphate, and OD values were read at 405 nm using a MultiScan (Thermo Scientific).

DHBsAg IMMUNOHISTOCHEMISTRY

Necropsy liver samples were formalin-fixed, wax-embedded, and 5-μm-thickness sections were analyzed for DHBsAg expression using 1H1 monoclonal antibody[28] and revealed with HRP-conjugated goat anti-mouse polyclonal antibody as previously described.[11] All sections were counterstained with hematoxylin. Sections of liver tissue from each duck were examined under code.

STATISTICAL ANALYSIS

Where indicated, statistical analyses of differences between means were conducted using the Student's t test. Differences between evaluated means were considered statistically significant when $P \leq 0.05$.

Results

SAFETY AND TOLERABILITY

Administration of all dosing regimens were generally well tolerated, with no significant change in weight gain observed compared to the NS group (Fig. 1). Onset of mild weakness was observed in some ducks receiving REP 2139 during the last 2 weeks of treatment. Ascites at the injection point were noted in some animals from NS and REP 2139 groups that received treatment by intraperitoneal administration. Euthanasia before the end of therapy for ducks in all treatment groups was attributed to the complications arising from the repeated manipulations over the 4 weeks of daily administrated treatment and to coincidental mortality previously observed during long-term experimentation in the Pekin duck model.[29,30]

EFFECTS OF TREATMENT ON SERUM DHBsAg AND VIRAL DNA

Reductions in duck HBsAg (DHBsAg) during treatment occurred in all treatment groups (Fig. 2). In NS-treated animals, DHBsAg persisted during
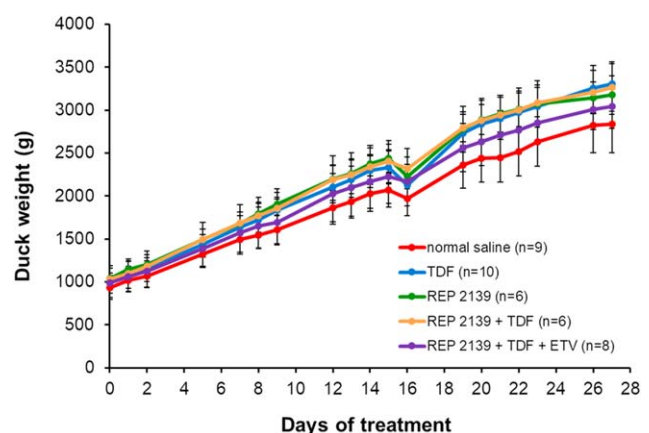

FIG. 1. Body weight gain during 28 days of treatment with various combinations of REP 2139, TDF, and ETV. Mean values ± SD are plotted for each treatment group. No statistically significant differences between treatment groups and NS group were observed.
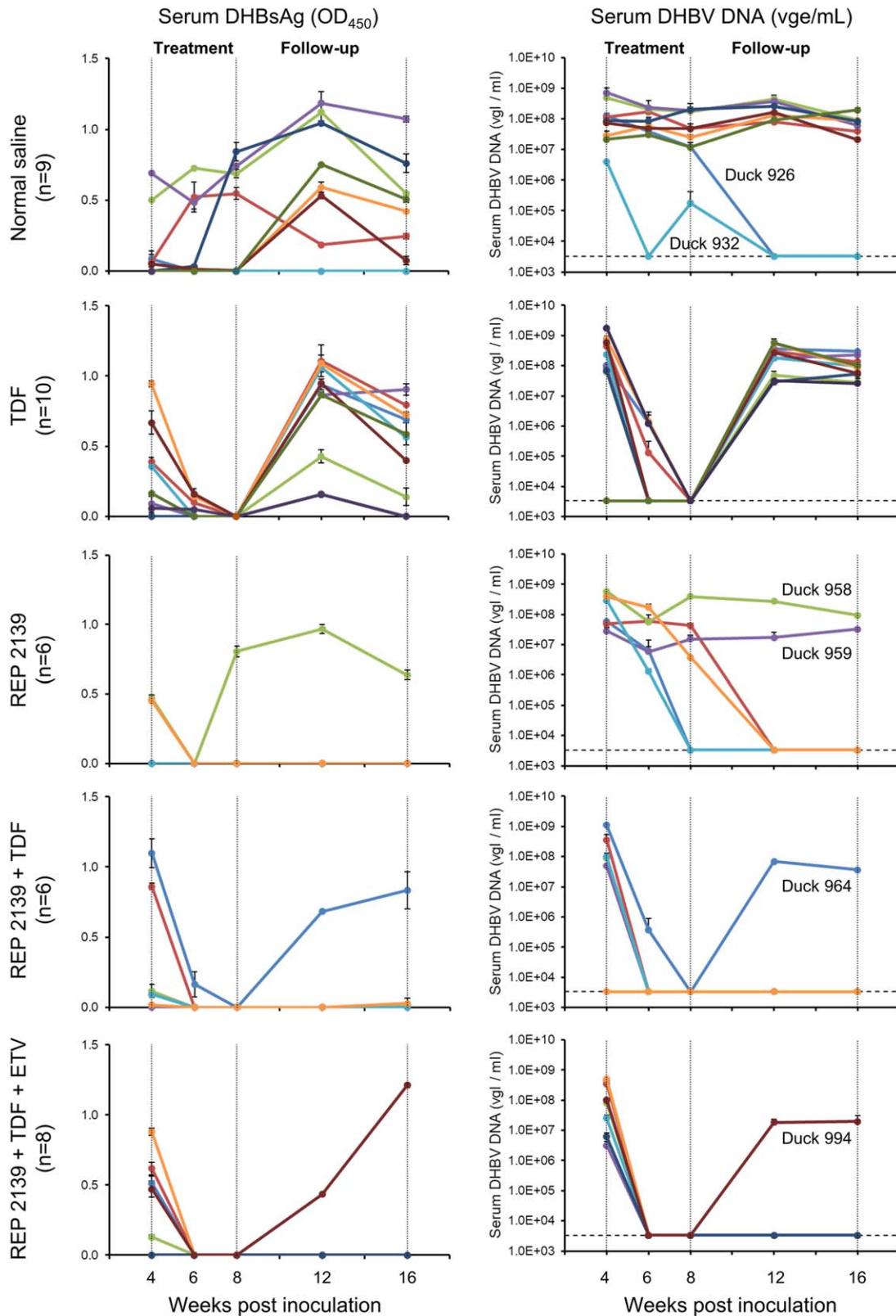
FIG. 2. Changes in serum DHBsAg and DHBV DNA during treatment and follow-up. Mean ± SD of duplicate measurements are presented for serum DHBsAg (left column) and DHBV DNA (right column) taken at the start of treatment (4 weeks postinoculation), mid-treatment (6 weeks postinoculation), end of treatment (8 weeks postinoculation), 4 weeks after discontinuing treatment (12 weeks postinoculation), and 8 weeks after discontinuing treatment (16 weeks postinoculation). Serum DHBsAg values have had background reactivity (from uninfected duck sera) removed. Lower limit of detection of DHBV DNA (3.30×10^3 vge/mL) is indicated by horizontal dashed lines. Individual sampling points where no detectable DHBV DNA was present were set to 3.30×10^3 vge/mL for analysis purposes. Individual ducks either establishing functional control in the NS group or rebounding in REP 2139 treatment groups are identified.

treatment and follow-up in 7 of 9 ducks. In 2 ducks in this group (926 and 932), DHBsAg was not detected. In the TDF monotherapy group, DHBsAg reductions were observed during treatment only, but after treatment cessation, DHBsAg rebounded in all 10 treated animals within the first 4 weeks of follow-up. Serum DHBsAg decrease was more pronounced in the presence of REP 2139, becoming undetectable in all but one animal by the second week of treatment. In the treatment groups receiving REP 2139 combined with TDF or TDF and ETV, DHBsAg remained persistently supressed in all but 1 animal in each of these groups up to the end of follow-up, indicating a sustained control of antigenemia.

Serum DHBV DNA persisted throughout the dosing phase and follow-up in the NS control group, except for ducks 926 and 932 whose viremia became controlled during follow-up (these animals also had no detectable DHBsAg), suggesting spontaneous clearance of infection (Fig. 2). Viremia became well controlled during TDF monotherapy, but rebounded to pretreatment levels in all ducks within 4 weeks after treatment cessation and persisted to the end of follow-up. With REP 2139 monotherapy, 2 nonresponder ducks (958 and 959) exhibited high viremia during the treatment and follow-up periods. In the remaining animals from this group, DHBV DNA drops were observed in 2 animals during the treatment, but were decoupled from DHBsAg reductions, with DHBV DNA persisting when DHBsAg declined to undetectable levels during treatment and follow-up. Four weeks after treatment cessation, 2 additional animals from the REP 2139 monotherapy group established control of viremia, which persisted to the end of follow-up. Interestingly, an accelerated reduction in viremia relative to that generally observed with TDF alone (i.e., at week 6) was observed during combination therapy with REP 2139 + TDF and REP 2139 + TDF + ETV, in 5 of 6 and 8 of 8 animals, respectively. This trend in accelerated DHBV DNA response with REP 2139 and TDF was more pronounced and approached statistical significance with REP 2139 in combination with TDF and ETV (Supporting Fig. S1). After treatment withdrawal, DHBV DNA remained persistently controlled in all but 2 nonresponder animals (964 and 994) in these two groups at the end of follow-up. Thus, in contrast to TDF monotherapy, no rebound in viremia was observed after treatment cessation with REP 2139 + TDF or REP 2139 + TDF + ETV in all except 2 animals from these two groups (Fig. 2).

Individual DHBsAg and DHBV-DNA values for all ducks throughout the study are presented in Supporting Table S1.

EFFECTS OF TREATMENT ON SERUM ANTI-DHBsAg ANTIBODIES

Free anti-DHBsAg antibodies (anti-DHBs) were present in some ducks from the NS, REP 2139, and REP 2139 + TDF + ETV groups before the start of treatment (Supporting Fig. S2). During the treatment, a rise in anti-DHBsAg antibodies was observed in several animals from REP 2139 monotherapy and combination therapy groups, although these antibodies dropped to the baseline level during the follow-up indicating the absence of the sustained effect on seroconversion. Thus, anti-DHBs levels were not correlated with reduction or absence of detectable DHBsAg during therapy or maintenance of functional control of DHBV infection during follow-up.

IMPACT ON INTRAHEPATIC VIRAL DNA

Total liver DHBV DNA and cccDNA (Fig. 3A) was assessed at the end of 8-week follow-up from livers taken during necropsy. In the 2 ducks in the NS group (926 and 932) achieving control of serum DHBV DNA during follow-up, marked decreases in total liver DHBV DNA (>3 log) and cccDNA (~2 log) were also observed in liver, confirming the self-resolution of infection. Livers of all other animals in this group had significant liver DHBV DNA and cccDNA levels at the end of follow-up. In the TDF-monotherapy group, all animals exhibited high total DHBV-DNA and cccDNA levels at the end of follow-up. In contrast, in the group receiving REP 2139 monotherapy, a marked decrease in DHBV DNA (>3 log) and cccDNA (>2 log) were observed in animals who maintained persistent suppression of serum DHBV DNA and DHBsAg during follow-up. In 2 animals (958, 959) DHBV DNA was not supressed during treatment or followup, consistent with abundant DHBsAg and viremia present in these animals. Importantly, marked decreases in intrahepatic DHBV DNA (>3 log) and cccDNA (>2 log) were observed in 5 of 6 animals receiving REP 2139 + TDF and 7 of 8 receiving REP 2139 + TDF + ETV therapy, consistent with suppression of serum DHBsAg and DHBV DNA in these animals.
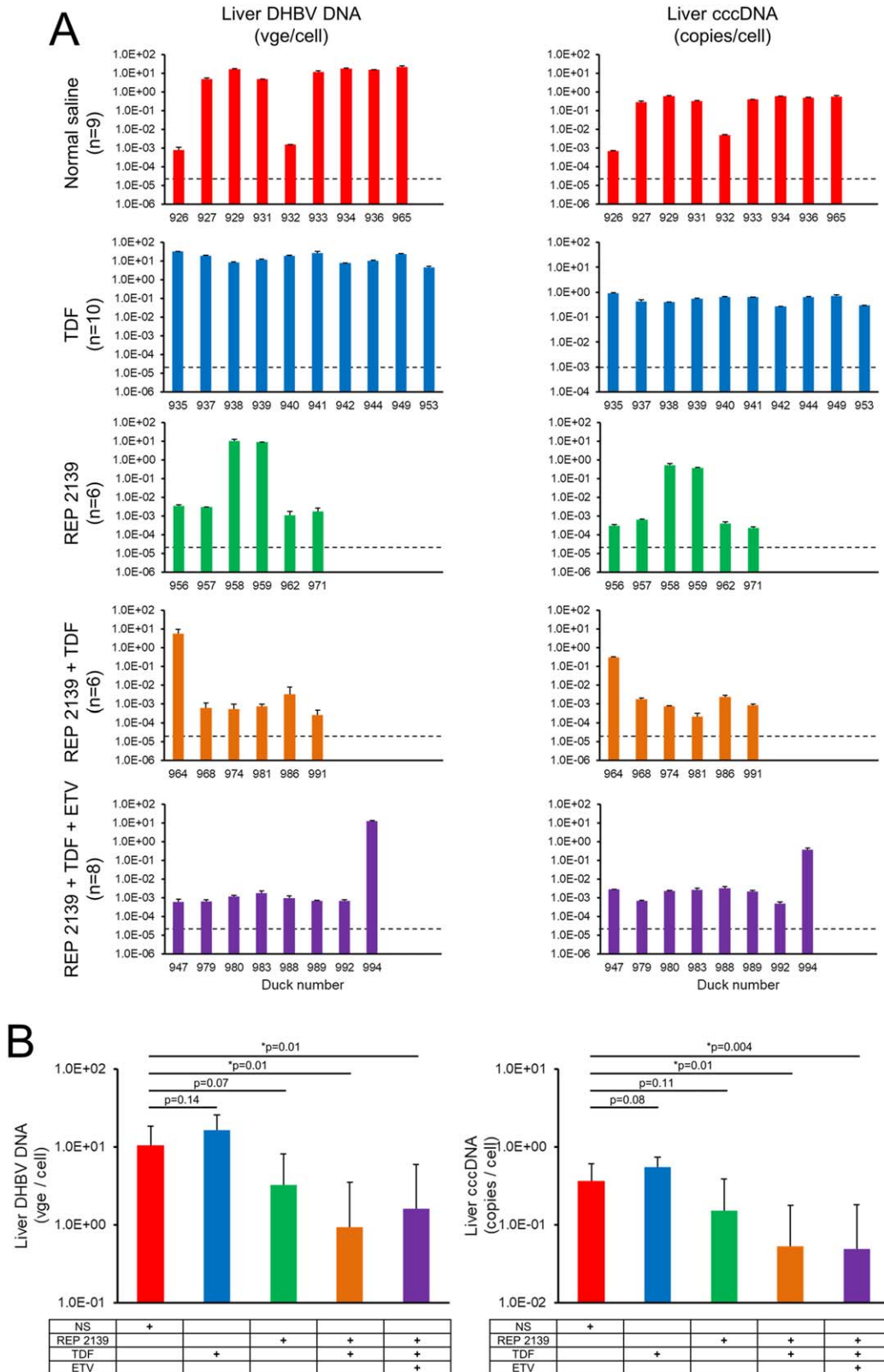
FIG. 3. Liver DHBV DNA and cccDNA at the end of follow-up. (A) Individual measurements for each animal are presented. Mean ± SD of duplicate measurements for each individual animal are presented for liver DHBV DNA (left column) and liver cccDNA (right column) taken from liver tissue harvested 8 weeks after discontinuing treatment. Lower limit of detection of 2.1×10^{-5} vge/cell (for liver DHBV DNA) and 2.1×10^{-5} copies/cell (for liver cccDNA) are indicated in (A) by horizontal dashed lines. (B) Group mean values are presented ± SD and P values for the differences between the means of the NS and treatment groups. Reductions in liver DHBV DNA (left) and cccDNA (right) relative to the NS group were significant ($P < 0.05$) for all REP 2139 combination treatment groups. Asterisks (*) indicate statistically significant difference between viral DNA and ccDNA in NS versus treatment groups ($P < 0.05$) as determined by t test.
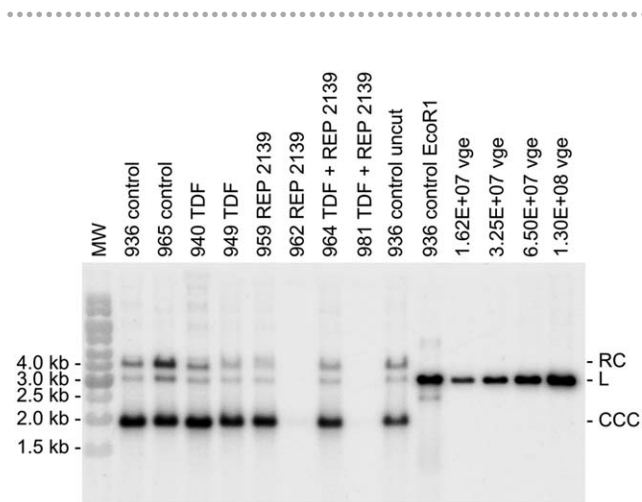
FIG. 4. Detection of DHBV cccDNA by Southern blotting assay. DNA was isolated from approximately 300 mg of liver tissue using the Hirt extraction technique. DHBV DNA was detected using a radiolabeled RNA probe specific for the detection of the plus strand. Signals are from approximately 4.20E+06 liver cells. Controls of cloned linear (L) DHBV DNA, corresponding to 1.62E+07, 3.25E+07, 6.50E+07, and 1.30E+08 vge, were used for quantification by Phosphorimager. The cccDNA copy numbers for samples 936, 965, 940, 949, 959, 962, 964, and 981 were estimated at 1.6, 1.8, 1.8, 1.2, 1.1, 0.03, 1.0, and 0.01 vge/cell, respectively. To validate the authenticity of cccDNA, extract from sample 936 was digested with single cutter EcoR1 restriction enzyme resulting in conversion to linear (L) DHBV DNA. Abbreviations: CCC, covalently closed circular DNA; RC, relaxed circular DNA; L, linear DNA; MW, molecular weight.

Analysis of mean total liver DHBV DNA and cccDNA levels between treatment groups (Fig. 3B) revealed no significant difference in suppression of liver DHBV and cccDNA between NS, TDF, and REP 2139 groups. By contrast, significant decreases of mean total DHBV DNA and cccDNA versus normal saline- or TDF-treated animals was observed for REP 2139 + TDF and REP 2139 + TDF + ETV groups, suggesting a synergistic antiviral effect with the combination of REP 2139 and TDF and or ETV.

To further examine the presence of cccDNA, we subjected to Southern blotting analysis the representative liver samples from controls and different treatment groups (Fig. 4). As expected, liver samples from 2 control ducks (936, 965) exhibited a high amount of cccDNA, in agreement with the results of qPCR. Note that cccDNA converts to a linear form upon digestion with the EcoRI restriction enzyme, which has a unique restriction site within the DHBV genome. Thus, following the digestion of liver DNA

(duck 936) with EcoRI, the cccDNA form disappeared and was converted to a single 3-kilobase (kb) band that migrated at the same position as the linearized, cloned DHBV genome used as a control. Analysis of liver samples from 2 ducks on TDF monotherapy (940, 949) showed, as expected, high cccDNA levels that were comparable to those of NS-treated controls. Accordingly, livers sample from the 2 nonresponder animals to REP 2139 (959) and REP 2139 + TDF (964) exhibited high cccDNA levels, comparable to those of the TDF monotherapy group, in agreement with the qPCR quantification. Interestingly, 2 ducks (962, 981) exhibiting only residual cccDNA by qPCR analysis following REP 2139 and REP 2139 + TDF treatment, respectively, had undetectable cccDNA in Southern blotting (Fig. 4). Southern blotting analysis thus clearly identifies and quantifies cccDNA at levels identical to those measured by qPCR.

SURFACE ANTIGEN CLEARANCE IN LIVER

To investigate the effect of treatment on intrahepatic surface antigen expression, we next examined all necropsy liver samples by immunohistochemistry. In the NS-treated group, all ducks, except the 2 who self-resolved their infection, exhibited a typical profile of chronic infection with detection of DHBsAg in 30%-100% of hepatocytes (Fig. 5; Table 1). TDF monotherapy also had no effect on surface antigen expression in liver, with 10 of 10 animals in this group having 30%-60% of hepatocytes DHBsAg positive. In the REP 2139 monotherapy group, intrahepatic surface antigen expression (60% and 62% of hepatocytes DHBsAg positive) was observed in the 2 animals (958 and 959) with serum DHBsAg and liver DHBV DNA persistent at the end of follow-up. However, the remaining 4 animals from this group had undetectable DHBsAg, as illustrated in Fig. 6 and presented in Table 1. Notably, DHBsAg-positive hepatocytes were not detected in any animals in the REP 2139 + TDF or REP 2139 + TDF + ETV groups except for the 2 nonresponder animals (964 and 994; Fig. 6; Table 1), consistent with persistent DHBV DNA in serum and liver in these animals (Figs. 2, 3, and 4).

Discussion

In this preclinical study, we evaluated the ability of REP 2139 alone or in combination with TDF or
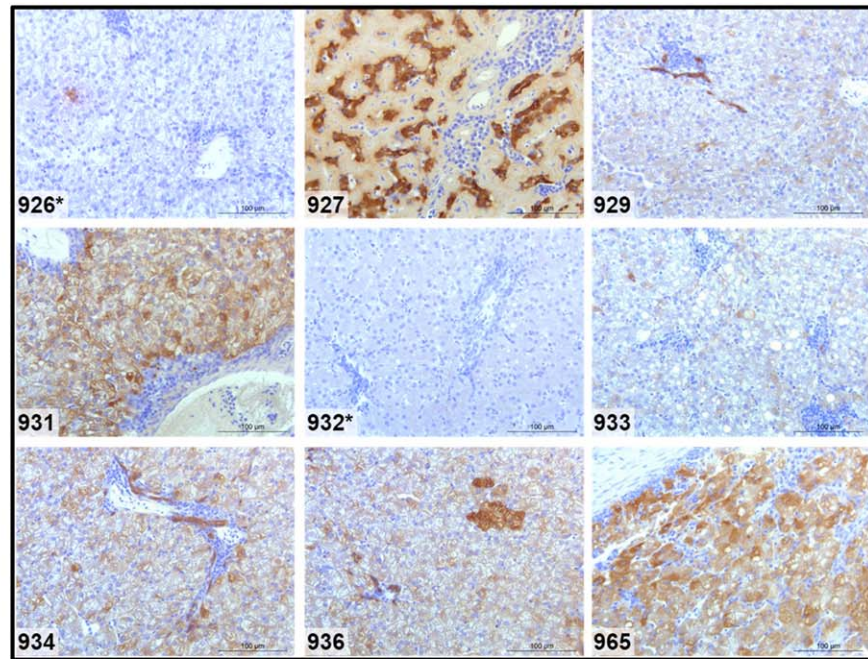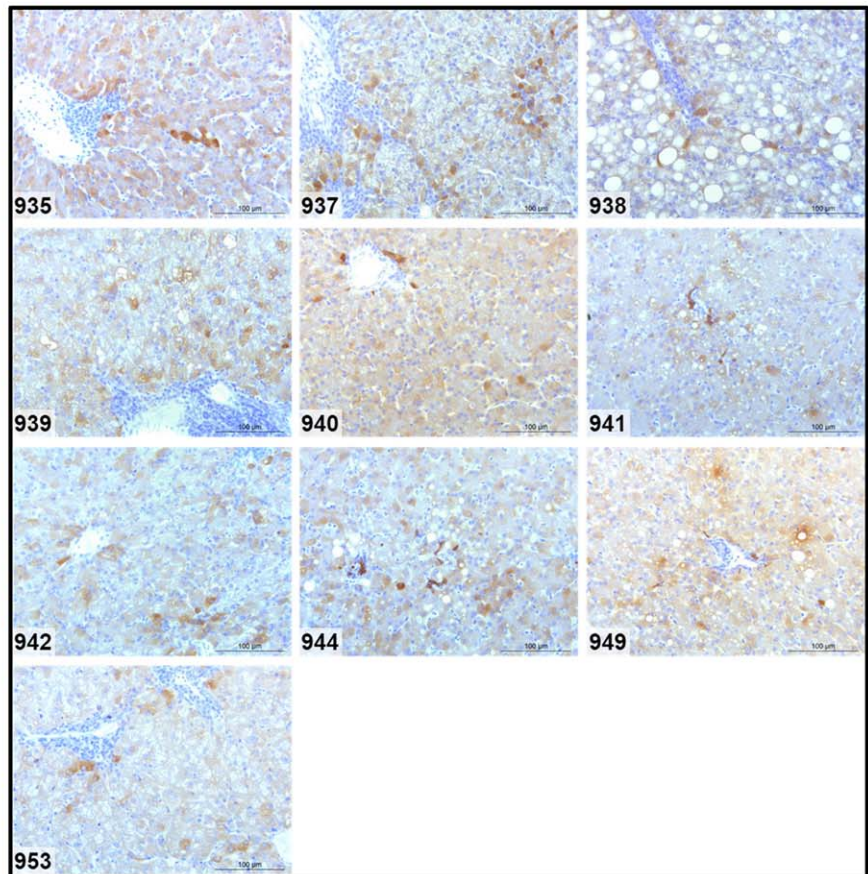
FIG. 5. Detection of DHBsAg in liver at the end of follow-up in the NS and TDF groups. Representative immunocytochemistry for DHBsAg in liver sections taken 8 weeks after discontinuing treatment are presented for the NS (top) and TDF (bottom) groups. Individual duck numbers are indicated in the bottom left corner of each photomicrograph. Scale bars represent 100 μm. *No DHBsAg detected.

TABLE 1. Evaluation of the Percentage of DHBsAg-Positive Hepatocytes at the End of Follow-up
Duck Number (% DHBsAg-Positive Hepatocytes)

Normal Saline	TDF	REP 2139	REP 2139 + TDF	REP 2139 + TDF + ETV
927 (100)	949 (59)	959 (66)	964 (39)	994 (42)
931 (77)	944 (56)	958 (62)	968 (0)	947 (0)
936 (49)	953 (51)	956 (0)	974 (0)	979 (0)
934 (44)	935 (48)	957 (0)	981 (0)	980 (0)
965 (39)	939 (48)	962 (0)	986 (0)	983 (0)
933 (38)	940 (44)	971 (0)	991 (0)	988 (0)
929 (28)	937 (40)			989 (0)
926 (1)	938 (40)			992 (0)
932 (0)	942 (37)			
	941 (31)			

TDF + ETV to control DHBV infection. Using the chronic DHBV infection model, we report herein the synergistic effect of REP 2139 and NUCs when used in combination, leading to a sustained control of serum antigenemia and viremia, which correlated with a significant decrease in intrahepatic viral cccDNA and, importantly, with the achievement of DHBsAg clearance in the liver at the end of follow-up. The improvement in control of DHBV replication in the liver was statistically significant when REP 2139 was combined with NUCs, but given the large dynamic ranges and the bimodal response, these effects are likely to be underestimated.

Treatment with REP 2139 alone or in combination with TDF or TDF and ETV was well tolerated given that no particular adverse effects or weight loss were observed during 4 weeks of daily treatment and 8 weeks of follow-up. Gross pathology analysis at autopsy revealed ascites in some animals in the REP 2139 group, but ascites were also observed in the NS group. Importantly, ascites were not observed in previous studies with REP 2139 or its progenitor, REP 2055 in ducks,[11] mice or nonhuman primates,[13] or in humans.[15,17,18] indicating that the ascites observed in this study were unlikely attributed to REP 2139 exposure, but more likely related to repeated manipulation and intraperitoneal administration of treatment to the ducks throughout the study.

All treatment groups included animals with little or no detectable serum DHBsAg at the start of treatment (Supporting Table S1). However, measurable DHBsAg was present in serum of all but 2 animals in the NS and TDF groups during treatment and follow-up (Fig. 2). DHBsAg was present in livers of these animals at the end of follow-up as assessed by immunohistochemistry (Figs. 5 and 6). Variability in pretreatment DHBsAg levels was not observed in our previous study with NAPs in the duck model[11] and is likely a result of a different immunological status of Pekin ducks used in this study compared to Aylesbury ducks used in previous studies.[11] Serum DHBsAg was generally not well correlated with levels of anti-DHBsAg antibodies (Supporting Fig. S2) throughout the study. In this regard, the presence of immune complexes in duck serum may, to some level, prevent anti-DHBsAg antibodies detection.[29,30] Importantly, serum DHBV-DNA levels at baseline were comparable in all but 2 animals from all groups (Supporting Table S1). Except for the 2 self-resolving ducks in the NS group, DHBV-DNA levels were persistent or rebounded to pretreatment levels in all ducks not receiving REP 2139, indicating the presence of a similarly well-established and chronic DHBV infection in all groups.

In this study, only animals having completed treatment and follow-up were analyzed. The loss of 4 of 10 animals in the REP 2139 group during the experiment, and the reduction of serum DHBsAg with TDF monotherapy, makes the statistical evaluation of DHBsAg reduction between groups and in the presence of REP 2139 difficult. Nonetheless, the elimination of detectable DHBsAg in all groups exposed to REP 2139 is consistent with the ability of NAPs to block the assembly/release of SVPs and to achieve clearance of serum DHBsAg as observed in previous studies,[11,13] Importantly, persistence of functional control of DHBV infection during the follow-up was only achieved in the presence of REP 2139. These effects have been shown to occur in the absence of any direct effect of NAPs on the immune response[11,14] and to be the consequences of DHBsAg elimination. Although both TDF and REP 2139 achieved serum DHBsAg reduction during the treatment below the detection threshold, the sustained control of DHBV

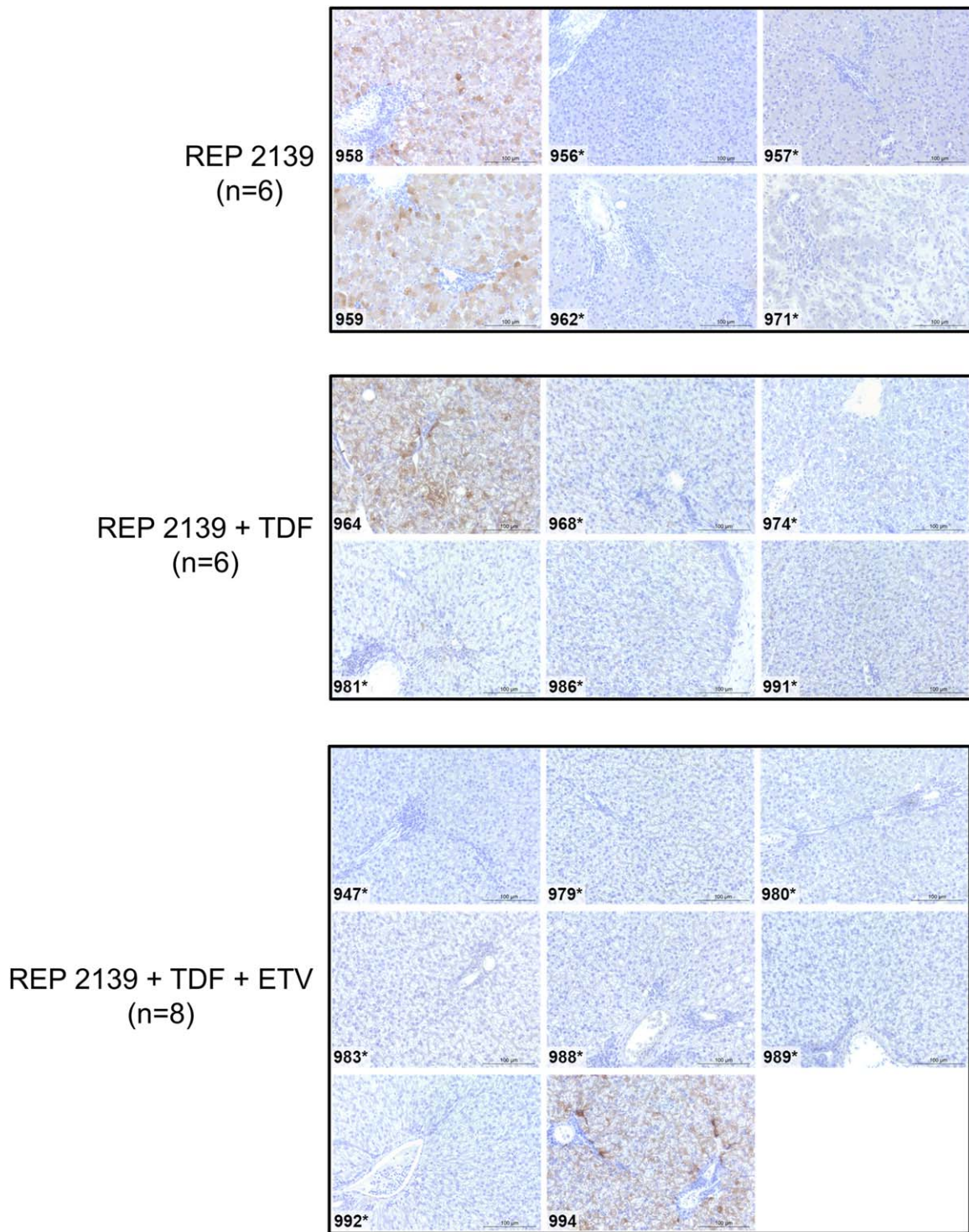**FIG. 6.** Detection of DHBsAg in liver at the end of follow-up in the REP 2139 groups. Representative immunocytochemistry for DHBsAg in liver sections taken 8 weeks after discontinuing treatment are presented for REP 2139 (top), REP 2139 + TDF (middle), and REP 2139 + TDF + ETV (bottom) groups. Individual duck numbers are indicated in the bottom left corner of each photomicrograph. Scale bars represent 100 μm. *No DHBsAg detected.

infection after treatment cessation clearly occurred with REP 2139 and not with TDF. Thus, in contrast to TDF monotherapy, there was no viral rebound after treatment cessation in a large majority of animals on REP 2139 mono and combination therapy.

Previous studies conducted in the DHBV infection model and in HBV patients suggest the selective targeting of subviral particles by NAPs.[11,15,17] The molecular targets underlying the antiviral effects of NAPs have not yet been elucidated; however, the remarkable similarities between SVP and high-density lipoprotein (HDL)[31] suggest that HDL metabolism may be somehow involved in SVP morphogenesis. Interestingly, an absence of antiviral activity of NAPs has been recently reported in rodent models of HBV infection.[32] This study hypothesized that the differences in HDL metabolism in mice and woodchucks, as compared to ducks and humans, may contribute to the absence of anti-HBV activity of NAPs in rodent species. The apolipoproteins involved in lipid metabolism may be also involved in the assembly of SVPs and may be targeted by NAPs, thus interfering with SVP assembly and secretion without affecting normal apolipoprotein metabolism. However, this hypothesis needs to be directly confirmed by further studies in avian and rodent models of hepadnaviral infection.

With regard to TDF effects on viral surface antigen, there is a clear disconnect between (1) the duck and woodchuck models of HBV infection in which TDF monotherapy reproducibly achieves reductions of hepadnaviral surface antigen during treatment[33,34] and (2) the situation in patients in whom TDF rarely achieves serum HBsAg reduction.[8,9] Thus, in the current study, TDF monotherapy resulted in the reduction of both DHBsAg and DHBV DNA during drug administration, an effect previously observed with TDF in the DHBV model[33] and in the woodchuck model of WHV infection.[34] The differences in the response to TDF in these models during the treatment compared to HBV-infected patients deserves further investigation. Importantly, in the current study, the antiviral effect of TDF monotherapy on DHBsAg was only transient, given that a rebound in serum surface antigen and viremia to pretreatment levels was observed in all 10 of 10 animals from this group at the end of follow-up, consistent with the maintaining of cccDNA pool and the presence of DHBsAg in livers.

Remarkably, the DHBsAg immunochemistry analysis at the end of follow-up revealed that the sustained control of infection in blood and liver of REP 2139-treated ducks was correlated with the clearance of DHBsAg

from the liver. By contrast, none of the animals from the TDF monotherapy group eliminated DHBsAg (30%-60% of hepatocytes exhibiting positive staining for DHBsAg), consistent with their high viremia, serum DHBsAg, and the presence of elevated DHBV DNA and cccDNA in the liver. The clearance of intrahepatic DHBsAg in the responders under combination therapy is consistent with the substantial reduction of cccDNA. This is consistent with a serum DHBsAg decrease being responsible for the break of immune tolerance that leads to clearance of viral cccDNA through cytolytic and noncytolytic pathways as documented in the chimpanzee model.[35,36] However, we were unable to explore these hypotheses because of the lack of duck-specific tools for immunological responses. Further studies aimed at elucidating the mechanisms underlying the establishment of control of DHBV infection in the liver following clearance of viral surface antigen in the blood by REP 2139 are warranted.

In the present study, when REP 2139 was used in combination with TDF or TDF and ETV, an greater reduction in serum DHBsAg levels was observed already at 2 weeks of therapy in a large majority of animals (5 of 6 and 7 of 8, respectively), without rebound posttreatment during 8 weeks of follow-up. Moreover, during treatment, DHBV DNA was cleared from the blood more quickly in the REP 2139 + TDF groups than in the REP 2139 or TDF monotherapy groups. Although not reaching statistical significance, this trend was more pronounced when REP 2139, TDF, and ETV were given in combination (Supporting Fig. S1). This synergistic effect on the kinetics of serum DHBV-DNA reduction may result from the combined effects of DHBsAg reduction by REP 2139 and viral RT inhibition to drive earlier and more pronounced clearance of DHBsAg. The accelerated HBV DNA response with combined ETV + TDF therapy versus ETV monotherapy has been demonstrated in some groups of patients,[24] but further investigation is required to explore whether an accelerated HBV-DNA suppression would translate into a clinical benefit in the presence of REP 2139.

Taken together, the present results suggests that REP 2139 in combination with NUCs may shorten the treatment duration required to establish functional control of infection, which may also occur at a higher frequency in comparison to REP 2139 monotherapy. Moreover, our findings show a synergistic effect of REP 2139 and NUCs, leading to a sustained and drastic decrease in viremia and circulating DHBsAg, correlated with a decrease in viral cccDNA and intrahepatic

viral surface antigen elimination, that persisted after treatment withdrawal. Previous and ongoing clinical trials with REP 2139 have included the use of immunotherapies.[15,17,18] However, based on this preclinical study, future clinical trials using only REP 2139 in combination with TDF or other NUCs should be considered to see whether the synergistic antiviral effect of REP 2139 and NUCs observed in the DHBV model translate into similar achievements in the clinical setting in the absence of pegylated IFN.

REFERENCES

1) Schweitzer A, Horn J, Mikolajczyk RT, Krause G, Ott JJ. Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. Lancet 2015;386:1546-1555.

2) Gish, RG, Given BD, Lai CL, Locarnini SA, Lau JY, Lewis DL, et al. Chronic hepatitis B: Virology, natural history, current management and a glimpse at future opportunities. Antiviral Res 2015;121:47-58.

3) Frenette CT, Gish RG. To "be" or not to "be": this is the question. Am J Gastroenterol 2009;104:1948-1952.

4) Moucari R, Lada O, Marcellin P. Chronic hepatitis B: back to the future with HBsAg. Exp Rev Anti Infect Ther 2009;7:633-636.

5) Geoffrey Dusheiko G, Wang B, Carey I. HBsAg loss in chronic hepatitis B: pointers to the benefits of curative therapy. Hepatol Int 2016;10:727-729.

6) Lok AS, McMahon BJ, Brown RS, Jr., Wong JB, Ahmed AT, Farah W, et al. 2016 Antiviral therapy for chronic Hepatitis B viral infection in adults: a systemic review and analysis. HEPATOLOGY 2016;63:284-306.

7) Lampertico P, Agarwal K, Berg T, Buti M, Janssen HL, Papatheodoridis G, et al. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. J Hepatol 2017;67:370-398.

8) Marcellin P, Heathcote EJ, Buti M, Gane E, de Man RA, Krastev Z, et al. Tenofovir disoproxil fumarate versus adefovir dipivoxil for chronic hepatitis B. N Engl J Med 2008;359:2442-2455.

9) Marcellin P, Ahn SH, Ma X, Caruntu FA, Tak, WY, Elkashab M, et al. Combination of Tenofovir Disoproxil Fumarate and Peginterferon a-2a Increases Loss of Hepatitis B Surface Antigen in Patients With Chronic Hepatitis B. Gastroenterology 2016;150:134-144.

10) Vaillant A. Nucleic acid polymers: broad spectrum antiviral activity, antiviral mechanisms and optimization for the treatment of hepatitis B and hepatitis D infection. Antiviral Res 2016;133:32-40.

11) Noordeen F, Scougall CA, Grosse A, Qiao Q, Ajilian BB, Reaiche-Miller G, et al. Therapeutic antiviral effect of the nucleic acid polymer REP 2055 against persistent duck hepatitis B virus infection. PLOS One 2015;10:e0140909.

12) Guillot C, Martel N, Berby F, Bordes I, Hantz O, Blanchet M, et al. Inhibition of hepatitis B viral entry by nucleic acid polymers in HepaRG cells and primary human hepatocytes. PLOS One 2017;12:e0179697.

13) **Roehl I**, Seiffert S, Brikh C, Quinet J, Jamard C, Dorfler N, Lockridge JA, Cova L, **Vaillant A**. Nucleic acid polymers with accelerated plasma clearance for the treatment of chronic HBV infection. Mol Ther Nuc Acids 2017;8:1-12.

14) Real CI, Werner M, Paul A, Gerken G, Schlaak JF, Vaillant A, et al. Nucleic acid-based polymers effective against hepatitis B virus infection don't harbour immune stimulatory properties in primary isolated blood or liver cells. Sci Reports 2017;7:43838.

15) Bazinet M, Pântea V, Cebotarescu V, Cojuhari L, Jimbei P, Albrecht J, et al. Safety and efficacy of REP 2139 and pegylated interferon alfa-2a for treatment-naive patients with chronic hepatitis B virus and hepatitis D virus co-infection (REP 301 and REP 301-LTF): a non-randomised, open-label, phase 2 trial. Lancet Gastroenterol Hepatol 2017;2:877-889.

16) Robinson WS, Lutwick LI. The virus of hepatitis, type B. N Eng J Med 1976;295:1169-1175.

17) Al-Mahtab M, Bazinet M, Vaillant A. Safety and efficacy of nucleic acid polymers in monotherapy and combined with immunotherapy in treatment naive Bangladeshi patients with HBeAg + chronic hepatitis B infection. PLOS One 2016;11:e0156667.

18) Bazinet M, Pantea V, Placinta G, Moscalu I, Cebotarescu V, Cojuhari L, et al. Update on safety and efficacy in the REP 401 protocol: REP 2139-Mg or REP 2165-Mg used in combination with tenofovir disoproxil fumarate and pegulated interferon alpha-2a in treatment naïve Caucasian patients with chronic HBeAg negative HBV infection. J Hepatol 2017;66:S256.

19) Cova, L. Present and future DNA vaccines for chronic hepatitis B treatment. Exp Op Biol Ther 2017;17:185-195.

20) Cova L, Zoulim F. Duck hepatitis B virus model in the study of hepatitis B virus. Methods Mol Med 2004;96:261-268.

21) Ndeboko B, Ramaurthy N, Lemamy GJ, Jamard C, Nielsen PE, Cova L. Role of cell-penetrating peptides in intracellular delivery of peptide nucleic acids targeting hepadnaviral replication. Mol Ther Nucleic Acids 2017;9:162-169.

22) Mason WS, Aldrich C, Summers J, Taylor JM. Asymmetric replication of duck hepatitis B virus DNA in liver cells: free minus-strand DNA. Proc Natl Acad Sci U S A 1982;79:3997-4001.

23) Franke C, Matschl U, Bruns M. Enzymatic treatment of duck hepatitis B virus: topology of the surface proteins for virions and non-infectious subviral particles. Virology 2007;359:26-136.

24) Lok AS, Trinh H, Carosi G, Akarca US, Gadano A, Habersetzer F, et al. Efficacy of entecavir with or without tenofovir disoproxil fumarate for nucleos(t)ide-naïve patients with chronic hepatitis B. Gastroenterology 2012;143:619-628.e1

25) Blanchet M, Sureau C. Infectivity determinants of the hepatitis B virus pre-S domain are confined to the N-terminal 75 amino acid residues. J Virol 2007;81:5841-5849.

26) Khawaja G, Buronfosse T, Jamard C, Abdul F, Guerret S, Zoulim F, et al. In vivo electroporation improves therapeutic potency of a DNA vaccine targeting hepadnaviral proteins. Virology 2012;433:192-202.

27) Le Mire MF, Miller, DS, Foster WK, Burrell CJ, Jilbert AR. Covalently Closed Circular DNA Is the Predominant Form of Duck Hepatitis B Virus DNA That Persists following Transient Infection. J Virol 2005;79:12242-12252.

28) Pugh JC, Di Q, Mason WS, Simmons H. Susceptibility to duck hepatitis B virus infection is associated with the presence of cell surface receptor sites that efficiently bind viral particles. J Virol 1995;69:4814-4822.

29) Rollier C, Sunyach C, Barraud L, Madani N, Jamard C, Trepo C, Cova L. Protective and therapeutic effect of DNA-based immunization against hepadnavirus large envelope protein. Gastroenterology 1999;116:658-665.

30) Thermet A, Buronfosse T, Werle-Lapostolle B, Chevalier M, Pradat P, Trepo C, et al. DNA vaccination in combination or

not with lamivudine treatment break humoral immune tolerance and enhances cccDNA clearance in the duck model of chronic hepatitis B virus infection. J Gen Virol 2008;89:1192-1201.

31) Gavilanes F, Gonzalez-Ros JM, Peterson DL. Structure of hepatitis B surface antigen. J Biol Chem 1982;257:7770-7777.

32) **Schöneweis K**, Motter N, Roppert PL, Lu M, Wang B, Roehl I, Glebe D, Yang D, Morrey JD, Roggendorf M, **Vaillant A**. Activity of nucleic acid polymers in rodent models of HBV infection. Antiviral Res 2017;8:26-33.

33) Noordeen F, Grosse A, Zhu Y, Borroto-Esoda K, Jilbert AR. Antiviral efficacy of NAs, TDF and FTC against persistent DHBV infection. In: Development of Antiviral Therapies for Chronic Hepatitis B Infection. The University of Adelaide, Adelaide, Australia; July 2009.

34) Menne S, Cote PJ, Korba BE, Butler SD, George AL, Tochkov IA, et al. Antiviral effect of oral administration of tenofovir disoproxil fumarate in woodchucks with chronic woodchuck hepatitis virus infection. Antimicrob Agents Chemother 2005;49:2720-2728.

35) Guidotti LG, Rochford CJ, Chung J, Shapiro M, Purcell MR, Chisari FV. Viral clearance without destruction of infected cells during acute HBV infection. Science 1999;284:825-829.

36) Guidotti LG, Isogawa M, Chisari FV. Host-virus interactions in hepatitis B infection. Curr Opin Immunol 2015;36:61-66.

Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.29737/suppinfo.