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Abstract

Background & Aims: Hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) is a unique episomal replicative intermediate responsible for persistent infection of hepatocytes. Technical constraints have hampered the direct study of cccDNA maintenance and clearance mechanisms in patients. The aim of this study was to develop a sensitive and specific assay quantifying cccDNA in biopsy samples from chronic hepatitis B patients during different natural history phases and in patients undergoing antiviral therapy.

Methods : Intrahepatic cccDNA levels were quantified by a specific real-time PCR assay. Ninety eight liver biopsy samples from patients in the major phases of the natural history of chronic hepatitis B and 32 pairs of samples from patients receiving adefovir dipivoxil (ADV) therapy were assessed.

Results : cccDNA was detected, at levels ranging over three orders of magnitude, in patients in different phases of the natural history of chronic hepatitis B. cccDNA levels were strongly correlated with levels of total intracellular HBV DNA and serum HBV DNA. Fourty eight weeks of ADV therapy resulted in a significant 0.8 log decrease in cccDNA copies/cell. Changes in cccDNA were correlated with a similar reduction in serum HBsAg titer, but not with a decrease in the number of HBV antigen-positive cells during ADV treatment.

Conclusion : cccDNA persists throughout the natural history of chronic hepatitis B, even in patients with serological evidence of viral clearance. Long term ADV therapy significantly decreased cccDNA levels by a primarily non-cytolytic mechanism.

Introduction

Chronic hepatitis B virus (HBV) infection remains a major health problem affecting approximately 400 million people worldwide[1]. Although HBV replication is only mildly cytopathic, cellular immune responses directed against the virus can produce substantial liver damage and result in chronic hepatitis, cirrhosis, and hepatocellular carcinoma[1]. Chronic hepatitis B (CH-B) encompasses a broad spectrum of disease and can be divided into several phases of natural history which are distinguishable by serological and virological markers [2]. One goal of antiviral therapy is to accelerate patients with chronic active disease into later natural history phases wherein viral replication abates and the incidence of serious liver disease is significantly reduced.

Chronic infection is believed to be maintained by a replicative form of HBV DNA termed cccDNA. During infection, HBV cccDNA accumulates in cell nuclei where it persists as a stable episome and acts as a template for the transcription of viral genes[3,4]. Viral transcripts are translated into capsid and polymerase proteins and subsequently encapsidated and retro-transcribed into new partially double-stranded viral genomes. DNA-containing nucleocapsids can be enveloped and secreted from the cell as mature virus or cycled back to the nucleus to maintain the cccDNA pool[5]. Considering the long half-life of hepatocytes, the limiting factor in eliminating infection is thought to be the clearance of cccDNA reservoirs from infected cells [6,7]. Two immune mechanisms have been proposed to mediate cccDNA clearance: 1) a cytolytic mechanism by which infected cells are eliminated and replaced by cells from an uninfected lineage[8,9] and 2) a non-cytolytic, cytokine-induced “curing” of infected cells[10,11]. There is evidence from animal models supporting each mechanism, and while these mechanisms are not mutually exclusive, it is currently unclear how infection is resolved in patients.

Despite the crucial role of cccDNA during persistent infection and the importance of understanding clearance mechanisms, few data have been collected from patients[12,13]. Indeed, our current understanding of cccDNA has been obtained primarily through studies of the woodchuck and duck hepatitis B virus models[7,8,14,15]. Historical obstacles to the study of HBV cccDNA have been 1) the requirement for liver biopsies, which are difficult to collect, especially from patients in quiescent natural history phases, and 2) the lack of sensitive, specific, and quantitative methods for detection of cccDNA from biopsies. In this study, we report the development of a novel real-time PCR assay that allowed us to quantify levels of cccDNA in biopsies collected from CH-B patients. To gain insight on the persistence of cccDNA and clearance mechanisms, we studied patients in different phases of the natural history of CH-B and a group of patients participating in a placebo-controlled trial of the recently approved nucleotide analog adefovir dipivoxil (ADV)[16,17]. These studies have provided novel insight into cccDNA persistence and have major implications for the monitoring of CH-B and antiviral therapy.

Materials and Methods

Patients

Biopsies were collected from a total of 98 CH-B patients corresponding to the relevant phases of the natural history including 63 patients with HBeAg+ active CH-B, 18 patients with HBeAg- CH-B (presumed precore mutant), 10 inactive carriers (HBsAg+, HBeAg-, antiHBeAb+), and 7 HBsAg seroconverters (serologically resolved patients with HBsAg-, anti HBsAb+, HBeAg-, antiHBeAb+). Thirty-two patients were also part of a phase III clinical trial evaluating the antiviral efficacy of ADV (10 patients received placebo, 22 received 10 mg or 30 mg of ADV daily)[16]; baseline biopsies were included in the natural history study. Biopsies were stored at –80° C until experimental analysis.

Development and validation of quantitative cccDNA and total HBV DNA PCR assays

Selective primers for cccDNA amplification (targeted across the single-stranded (SS) gap region of relaxed circular (RC) HBV DNA) and non-selective primers for total HBV DNA amplification (targeted to a double-stranded region of HBV DNA) were designed using oligo5 software (MedProbe, Olso, Norway) (Figure 1A.). To enhance the specificity of cccDNA detection, Plasmid-safe DNase (Epicentre, Madison, WI) was used to degrade RC and SS forms of viral DNA prior to PCR. Selective and non-selective primers were able to efficiently amplify HBV contained in a closed circular plasmid regardless of DNase treatment (Fig. 1B). RC virion-associated HBV DNA extracted from the serum of a viremic patient could be amplified by both primer sets, however cccDNA primers were approximately 100-fold less efficient (Fig. 1C). Plasmid-safe DNase treatment of virion-associated DNA prevented amplification, confirming that this DNase degraded RC forms of HBV DNA. Selective and non-selective primers were able to amplify HBV DNA extracted from a liver biopsy of a CH-B patient, regardless of Plasmid-safe DNase treatment (Fig 1D). The use of Plasmid-safe DNase was also validated by Southern blot analysis of intrahepatic WHV DNA (Fig 1E). Replicative intermediates and cccDNA were extracted from frozen woodchuck liver samples as described in detail[7]. After cell lysis, samples were divided in two, one for cccDNA extraction and one for total DNA purification. Briefly, cccDNA was isolated from the supernatant after precipitation of protein-DNA-detergent-complexes by 0.25 volumes of 2.5M KCL. The supernatant was extracted twice with phenol, phenol-chloroform prior to ethanol precipitation. Total DNA was digested with Proteinase K prior to phenol extraction and ethanol precipitation. Twenty µg of total viral DNA were digested using the Plasmid-safe DNase. Twenty µg of DNA corresponding to : i) total viral DNA, ii) total viral DNA digested with the plasmid-safe DNase, iii) cccDNA preparation, were subjected to agarose gel electrophoresis and Southern blotting. As shown on the autoradiogram, the DNase efficiently

degraded replicative DNA intermediates but not cccDNA. The pattern of migration of viral DNA was similar in the total DNA digested with the Plasmid-safe DNase and in the cccDNA preparation. Note that the cccDNA preparation specifically enriches the extract for low molecular weight DNA and does not represent the same amount of starting cells as in the total DNA lanes.

Quantification of cccDNA and total intracellular HBV DNA in patient biopsies

DNA was extracted from biopsies using the MasterPure DNA purification kit (Epicentre). Before cccDNA amplification, aliquots of each DNA were treated with plasmid-safe DNase. Real-time PCR was performed in a LightCycler (Roche, Grenoble, France) using a 20 µl reaction volume containing 20ng of DNA (for cccDNA quantification, a volume equivalent to 20ng prior to DNase treatment), 3 mM $MgCl_2$, 0.5 µM of forward and reverse primers, 0.2 µM of 3'-fluorescein (FL) labeled probe, and 0.4 µM of 5'-Red640 (R_{640}) labeled probe. Forward and reverse primers were 5'-CTCCCCGTCTGTGCCTTCT-3' (NCCC1 nt 1548-1566) and 5'-GCCCCAAAGCCACCCAAG-3' (CCCAS2 nt 1903-1886) for cccDNA amplification, respectively and 5'-CTCGTGGTGGACTTCTCTC-3' (2RC/CCS nt 256-274) and 5'-CTGCAGGATGAAGAGGAA-3' (2RC/CCAS nt 421-404) for total intrahepatic HBV DNA amplification, respectively. FRET hybridization probes were 5'-GTTCACGGTGGTCTCCATGCAACGT-FL-3'and5'-R_{640}-AGGTGAAGCGAAGTGCACA CGGACC-p-3' for cccDNA quantification and 5'CACTCACCAACCTCCTGTCCTCCAA -FL-3' and 5'- R_{640}-TGTCCTGGTTATCGCTGGATGTGTCT-3' for total intrahepatic HBV DNA quantification. Amplification of total HBV DNA was performed as follows: 95°C for 10 minutes, then 45 cycles of 95°C for 10 seconds, 58°C for 10 seconds, and 72°C for 15 seconds. Amplification of cccDNA was performed as follows: 95°C for 10 minutes, then 45 cycles of 95°C for 10 seconds, 58°C for 5 seconds, 10 seconds at 63°C, 72°C for 20 seconds. β-globin amplification was performed using the LightCycler β-globin control kit (Roche).

Serial dilutions of a plasmid containing an HBV monomer (pHBVEcoRI) served as quantification standards. Liver biopsies from uninfected patients were analyzed as negative controls. The detection limits were 3 x 10^{-4} and 2 x 10^{-4} copies/cell for the cccDNA and of total HBV DNA assays, respectively. Patient samples were independently analyzed in duplicate at three different sites (Lyon, France; Melbourne, Australia; Hamburg, Germany). Eighty two of 130 samples were analyzed at three sites and 129/130 samples were analyzed at two sites. Despite some variability, there was a significant correlation in the cccDNA and total intracellular DNA results obtained at each site ($P \leq 0.001$, for all pairwise comparisons). Results from the three sites were integrated by averaging cccDNA and total intracellular HBV DNA values (in $\log_{10}$ copies/cell) for each patient. Biopsies from the ADV study were analyzed while blinded to treatment assignment. To ensure cccDNA amplification signals were not due to HBV DNA integrated into host cell genomes, an HBV-Alu-specific PCR assay was performed on 13 extracted liver biopsy specimens as previously described [18].

Immunostaining of liver samples for HBV core antigen

Immunostaining for HBsAg and HBcAg was performed using a Dako Auto-Stainer and the Dako Envison+ system with peroxidase and 3,3'-diaminobenzidine chromogen (Dako, Carpenteria, CA). Primary antibodies were rabbit polyclonal antibody to HBcAg (Dako), used at a 1:600 dilution and monoclonal antibody to HBsAg (Zymed, San Francisco, CA), used at a 1:2000 dilution. The percentage of cells containing the chromogen, if present, was estimated in the ranges of 0%, ≤5%, 5-25%, 26-50%, 51-75%, and ≥75%.

Detection of HBsAg titers in serum samples

HBsAg titers in patient sera were quantified using an ELISA assay (Monolisa AgHBs plus, Biorad France) with purified HBsAg as standard (Hytest, Finland).

Statistical analysis

Statistical comparisons were performed using SAS v8.1 (SAS Institute Cary, NC USA). Two-group comparisons were performed using Wilcoxon rank-sum tests; comparisons of more than 2 groups were made using Kruskal-Wallis tests. All pair-wise comparisons were made by Pearson correlation. Analyses of the predictive value of baseline parameters on changes in cccDNA were made by linear regression after modeling cccDNA changes as a function of treatment (ADV vs. placebo) and the potential predictive factor. *P* values <0.05 were considered significant.

Results

cccDNA levels during different phase of the natural history of chronic hepatitis B.

We developed and validated a real-time PCR assay for the specific detection of cccDNA and total intracellular HBV DNA in liver biopsies. Using this assay, a panel of 98 patients in different phases of the natural history of CH-B [1,2] were analyzed. cccDNA and total intracellular HBV DNA were measured and normalized to per cell values (using the cellular β-globin gene) at three independent laboratories and the results were integrated. Median intrahepatic cccDNA levels in patients with hepatitis B e antigen positive (HBeAg+) CH-B (1.4 copies/cell) were significantly higher than in HBeAg- patients (0.01 copies/cell; *P*<0.001), or inactive carriers (0.02 copies/cell; *P*<0.001) (Fig. 2*A*). Patients with evidence of hepatitis B surface antigen (HBsAg) clearance had extremely low levels of cccDNA (0.002 copies/cell; *P*<0.001, compared to HBeAg+ patients). Similarly, median total intracellular HBV DNA levels in HBeAg+ patients (155 copies/cell) were significantly higher compared to HBeAg- patients (0.6 copies/cell; *P*<0.001), inactive carriers (2 copies/cell) (*P*<0.001), and HBsAg- patients (0.19 copies/cell; *P*<0.001) (Fig 2*B*). Levels of cccDNA and total

intracellular HBV DNA were both highly correlated with viral load (HBV DNA) in serum of these patients (P<0.001, data not shown).

Effect of 48 weeks of adefovir dipivoxil therapy on cccDNA levels in patients.

We next determined the effect of 48 weeks of ADV therapy on cccDNA and total intrahepatic HBV DNA in 32 patients (22 treated, 10 placebo) with HBeAg+ CH-B. Our integrated analysis revealed median changes in cccDNA and total intrahepatic HBV DNA in ADV-treated patients of -0.80 and -1.63 $\log_{10}$ copies/cell, respectively (Fig 3 *A* and *B*). This equates to an 84% reduction of cccDNA and 98% reduction of total intracellular HBV DNA by the end of treatment. Reductions in cccDNA and total intrahepatic HBV DNA were highly significant compared to placebo patients who had median changes of +0.32 and -0.0001 $\log_{10}$ copies/cell in cccDNA and total intrahepatic HBV DNA, respectively (P=0.002, cccDNA, treated vs. placebo; P<0.001 total intracellular HBV DNA, treated vs. placebo). To ensure that the integrated results were not biased by the results from a single laboratory, we analyzed each site's data independently (Fig. 3 *D* and *E*). Although absolute values for cccDNA and total intracellular HBV DNA varied between sites, the magnitudes of DNA loss were very similar and supported the integrated findings. Median changes in serum HBV DNA were –4.7 vs. –0.6 $\log_{10}$ copies/mL in ADV-treated and placebo patients, respectively (P<0.001) (Fig. 3*C*). Therefore, the decay rate of HBV replicative forms during ADV therapy can be ranked as follows: serum HBV DNA > total intracellular HBV DNA > cccDNA (Fig 3F).

We also measured changes in HBsAg titers in the serum of ADV treated patients. At baseline, patients in the ADV and placebo groups had similar HBsAg titers (medians of 332,599 and 217,378 ng/mL, respectively, p=0.48). After 48 weeks, patients receiving placebo had a median -0.09 $\log_{10}$ change in HBsAg titer (p=0.85 vs. baseline). However, patients receiving ADV therapy demonstrated a median 73% reduction in serum HBsAg (median change of -0.58 $\log_{10}$ ng/mL); this change was significant compared to baseline

(p<0.001) and compared to changes in placebo (p<0.001). Changes in HBsAg were significantly and positively correlated with changes in intrahepatic cccDNA (p=0.008), total intracellular HBV DNA (p=0.002), and serum HBV DNA (p<0.001).

Interestingly, patients who underwent HBeAg seroconversion had significantly lower baseline levels of intracellular cccDNA compared to patients that remained HBeAg+ (p=0.034). However, patients receiving ADV underwent similar declines in cccDNA, regardless of whether they underwent HBeAg seroconversion (n=5, -0.94 $\log_{10}$ copies/cell) or not (n=17, -0.75 $\log_{10}$ copies/cell) (p=0.91).

Immunohistochemical staining of biopsies for HBV antigens

For patients in the ADV study, immunohistochemical staining was performed to determine the number of hepatitis B core antigen (HBcAg) and HBsAg positive cells in each biopsy (data not shown). In most biopsies, only a minority of the cells stained positive for HBcAg or HBsAg and positive cells tended to cluster together. There was a significant correlation between cccDNA copy number and the number of HBcAg+ cells (P=0.015, data not shown). A stronger correlation existed between the level of total intrahepatic HBV DNA and number of HBcAg+ cells (P<0.001, data not shown). Normalization of cccDNA levels to the number of infected cells using HBcAg as a marker (instead of total cell number using β-globin) yielded a median copy number of 33 copies per infected cell which is consistent with copy numbers derived from animal hepadnavirus models[7,8]. Although 48 weeks of antiviral therapy significantly decreased cccDNA levels, the number of infected (positive-staining) cells did not change significantly (data not shown), indicating a decrease in cccDNA copy number per cell rather than a loss of a proportion of infected cells.

Correlation of cccDNA changes with baseline and post-therapy parameters

Using the cccDNA and clinical data available for patients in the ADV study, we examined whether baseline parameters were predictive of cccDNA loss, and if changes in cccDNA

during therapy correlated with changes in other clinical parameters. Baseline ALT, liver histology (histological activity index), and viral load, using linear regression models were not predictive for cccDNA loss (Table 1). Changes in cccDNA were highly correlated with changes in total intrahepatic HBV DNA (P<0.001) as well as with changes in serum HBV DNA (P=0.008). We also examined if changes in cccDNA correlated with changes in ALT levels, liver histology, or the number of cells staining positive for either HBcAg, or HBsAg; none of these correlations were significant.

Discussion

In this report, we describe the development of a novel and selective real-time PCR assay for quantifying cccDNA and total HBV DNA in liver biopsies from CH-B patients. This technique allowed, for the first time, an analysis of cccDNA levels in significant numbers of patients in different phases of the natural history of CH-B as well as a longitudinal analysis of patients undergoing antiviral therapy during a placebo-controlled study. This is noteworthy as these findings are likely derived from the last placebo-controlled trial in CH-B patients[19].

Our cross-sectional natural history study revealed that cccDNA levels were significantly higher in HBeAg+ patients compared to HBeAg- patients, inactive carriers, and patients who underwent HBsAg clearance. This result is consistent with our observations of greater levels of intrahepatic HBV replicative intermediates and serum HBV DNA in HBeAg+ patients (compared to the later groups) and with previous reports of viral load in HBeAg+ and HBeAg- patients[20]. Surprisingly, cccDNA levels in HBeAg- patients, who are infected with precore mutant HBV and have active liver disease, were not significantly different (P=0.68), than those in inactive carriers who have no evidence of ongoing liver disease. This is potentially attributable to the natural variation in HBV replication levels observed in patients infected with pre-core mutant HBV[21], and the relatively small sample size for the inactive

carrier group (Figure 2*A* and 2*B*). However, these data could also indicate that the primary difference between a patient with HBeAg- active hepatitis and an inactive carrier is due to immunological rather than virological factors [2,22].

The observation that cccDNA remained detectable in all HBeAg- patients explains why viral reactivation has been observed, to varying frequencies, in all these patient groups. Indeed, fluctuating levels of viral replication and hepatitis are common in patients with precore mutant infection[22]**.** Viral reactivation and hepatitis flares have also been observed, albeit less frequently, in inactive carriers during long-term natural history studies[23-25]. Even patients with serological evidence of HBV clearance can develop active disease during periods of severe immune suppression such as transplantation, cancer chemotherapy, or steroid use[26,27]**.** The observed low-level persistence of cccDNA also explains the occurrence of *de novo* HBV infection in liver transplant patients who received grafts from donors that previously resolved infection[28]**.** Overall, our observations from the natural history study are consistent with the theory that resolution of hepadnaviral infection does not occur through "sterilizing immunity" but rather by consistent control of viral replication by the host immune system[29].

Clinical trials of nucleoside analogs have indicated that short-term therapy is followed by viral recrudescence in most CH-B patients[30,31]. Studies performed in animal hepadnavirus models suggested that the long half-life of viral cccDNA is responsible for viral persistence and reactivation following drug cessation[6,32,33]. Here we show, for the first time, that antiviral therapy with a potent HBV polymerase inhibitor significantly reduces cccDNA in CH-B patients. Importantly, reductions in cccDNA were correlated with, and similar in magnitude to, reductions in serum HBsAg titer. The parallel change in HBsAg provides further evidence that transcriptionally-active cccDNA is being depleted during therapy.

The rate of cccDNA loss was nearly an order of magnitude lower than that of intracellular HBV replicative intermediates and several orders of magnitude lower than the rate of serum

HBV DNA loss (Figure 3). Factors contributing to the relatively slow loss of cccDNA during therapy potentially include 1) the existence of cccDNA as a chromatinized episome[4] 2) the asymmetric nature of hepadnaviral replication which protects cccDNA from being directly depleted by chain-terminating polymerase inhibitors[3,6,14], 3) the preferential use of mature viral nucleocapsids to replenish cccDNA pools during periods of reduced replication[5,6] and 4) the potential for hepatocyte re-infection by residual circulating virus[34].

Results from our study potentially explain those of kinetic analyses of viral load decay during potent antiviral therapy. Two main phases of viral load decline have previously been identified: a first phase of rapid viral decline (attributed to the inhibition of viral production in infected cells) and a second phase of slow decline (hypothesized to represent the clearance of infected cells)[35]. The slow rate of cccDNA clearance that we observed is likely to contribute to the slower second phase kinetic**.** The observed longevity of cccDNA also reinforces the prediction that long-term antiviral therapy will be required to control HBV replication unless a vigorous host immune response is mounted[36]. In this respect, our results are reminiscent of studies of HIV proviral DNA[37,38]. Fortunately, treatment of CH-B patients with potent nucleos/tides such as lamivudine and adefovir dipivoxil can result in partial restoration of immune responses which are necessary for the durable host-mediated control of infection[39,40] (Cooksley et al., submitted).

Mechanisms for clearance of viral cccDNA are being actively debated. Based on results of acute hepadnaviral infections in chimpanzees and woodchucks, it is unclear whether the primary clearance mechanism of hepadnavirus infection is mediated by a non-cytolytic TH-1 immune response[10,41] or by immune-mediated cell killing, followed by hepatocyte cell division[9,42,43,44]. While both mechanisms are likely to factor into viral clearance, our data support the former mechanism. Immunohistochemical staining did not identify a significant correlation between loss of cccDNA and the disappearance of viral antigen expressing cells,

suggesting that cccDNA loss during antiviral therapy occurred at a similar rate in most cells, rather than by the clearance or destruction of a subset of infected cells. We hypothesize that decreases in intrahepatic cccDNA were primarily due to the potent suppression of viral DNA synthesis which would effectively deplete the pool of mature cytoplasmic nucleocapsids available for conversion into cccDNA[33,34]. Our analyses also indicated that cccDNA clearance during antiviral therapy was not significantly correlated with baseline indicators of host-mediated hepatocyte lysis (e.g. high baseline ALT levels and high baseline histological activity index). This also suggests that the clearance of cccDNA during antiviral therapy may be occurring primarily through a mechanism other than infected cell killing. Based on a mathematical modeling using cccDNA data derived from patients in the ADV sub-study, it would take approximately 14.5 years to completely clear a chronically HBV infected human liver of intracellular cccDNA[45]. However, since intracellular cccDNA remained detectable in patients that resolved chronic hepatitis B (HBsAg seroconverters, Figure 2A), it may not be necessary to completely eradicate cccDNA to establish long-term control of HBV infection. Despite the widespread availability of quantitative PCR technology, it is not currently practical to measure cccDNA in biopsies during routine clinical practice. However, monitoring cccDNA may provide an endpoint if a threshold can be determined, below which host-mediated control is likely to establish control of viral replication. Further longitudinal studies are ongoing to investigate such endpoints.

In conclusion, our study of HBV cccDNA in the livers of chronically-infected patients provides new insight on the persistence of cccDNA throughout the natural history of CH-B, and on the kinetics of cccDNA clearance during potent antiviral therapy. Our observations, together with previously published clinical studies [16, 17, 30, 31, 35, 36] further support the argument for long-term antiviral therapy using agents that are both potent and have a low incidence of viral resistance. In this regard, additional clinical studies focusing on virological

outcomes are ongoing. The development of rational combination regimens may enhance antiviral response and further minimize resistance, thus fostering the progressive clearance of cccDNA reservoirs and enhanced anti-HBV immune responses.

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Figure Legends

Figure 1. Design and validation of a selective assay for HBV cccDNA amplification. ***A,*** PCR primers for amplification of cccDNA are targeted to opposite sides of the single-stranded gap regions of relaxed circular (RC) HBV DNA. ***B,*** Both non-selective primers and selective cccDNA primers are able to efficiently amplify a circular HBV encoding plasmid in the absence or presence of Plasmid-safe DNase. ***C,*** Relaxed circular HBV DNA derived from patient sera can be detected using either non-selective or selective cccDNA primers; however amplification with the cccDNA specific primers is approximately 100-fold less efficient. After digestion with Plasmid-safe DNase, virion-derived RC DNA was not detected with either primer pair. ***D,*** Amplification of extracted liver DNA using either non-selective or selective cccDNA primers allows the detection of nuclease-resistant cccDNA. *E,* Southern blot analysis were performed using woodchuck liver samples, after extraction of replicative

intermediates and cccDNA according to two DNA extraction procedures (see Material and Methods section).

Figure 2. cccDNA and total intracellular HBV DNA levels in patients belonging to distinct natural history groups. ***A,*** cccDNA levels in patients with chronic active HBeAg+ hepatitis B (HBeAg+), chronic active HBeAg- hepatitis B (HBeAg-), inactive carriers patients (Inactive) and patients that have cleared HBV infection (HBsAg-). Each point represent a single patient with the bars indicating median values. LLOD: lower limit of detection. ***B.*** Total intracellular HBV DNA levels in the same groups of patients.

Figure 3. Effect of 48 weeks of adefovir dipivoxil therapy on cccDNA, total intracellular HBV DNA and serum HBV DNA levels. ***A,*** Change in cccDNA levels between baseline and week 48 in 22 ADV-treated patients and 10 placebo (PBO) patients, integrated results. Each point represents the baseline to week 48 change of a single patient with the bars indicating median values. ***B,*** Change in total intracellular HBV DNA levels between baseline and week 48 in 22 ADV-treated patients and 10 PBO patients, integrated results. ***C,*** Change in serum HBV DNA levels between baseline and week 48 in ADV-treated and PBO patients. ***D,*** Change in cccDNA levels between baseline and week 48 in ADV-treated and placebo patients as reported by individual analysis sites (Lyon, Hamburg, Melbourne), bars represent median values and error bars indicate interquartile ranges. ***E,*** Change in total intracellular HBV DNA levels between baseline and week 48 in ADV-treated and PBO patients as reported by individual analysis sites. ***F,*** Comparison of rates of serum HBV DNA, total intracellular HBV DNA and cccDNA loss in ADV-treated and PBO patients.

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Table 1: Correlation of changes in cccDNA copy number with baseline and post-treatment parameters.

Correlation	P Value
Baseline viral load	0.15
Baseline HAI	0.97
Baseline ALT	0.86
Change in Serum HBV DNA	0.008
Change in Total Intracellular HBV DNA	<0.001
Change in ALT	0.76
Change in HAI	0.709
Change in number of cells positive for nuclear HBcAg	0.48
Change in number of cells positive for cytoplasmic HBcAg	0.70
Change in number of HBsAg-positive cells	0.59

Figure 1

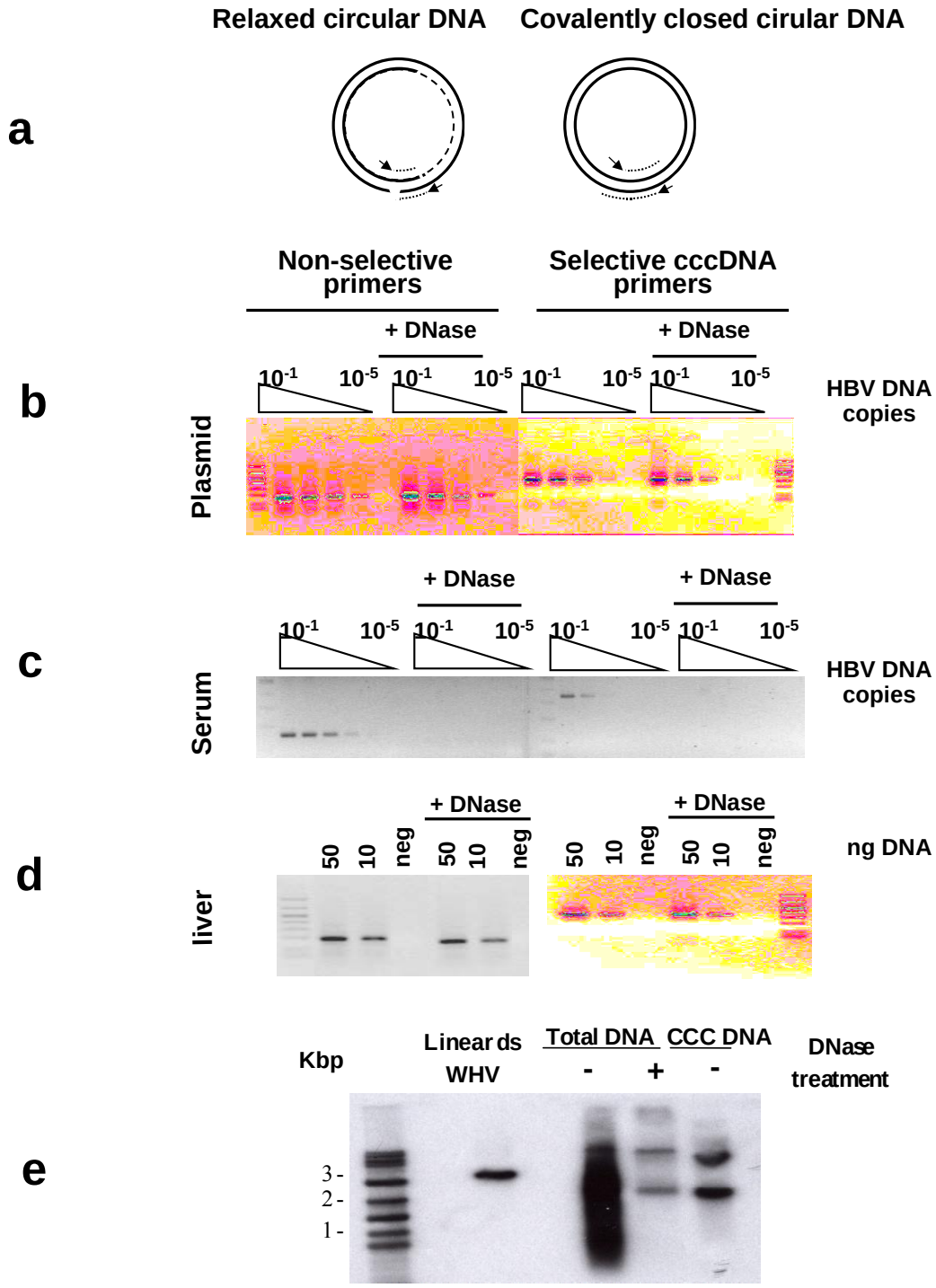
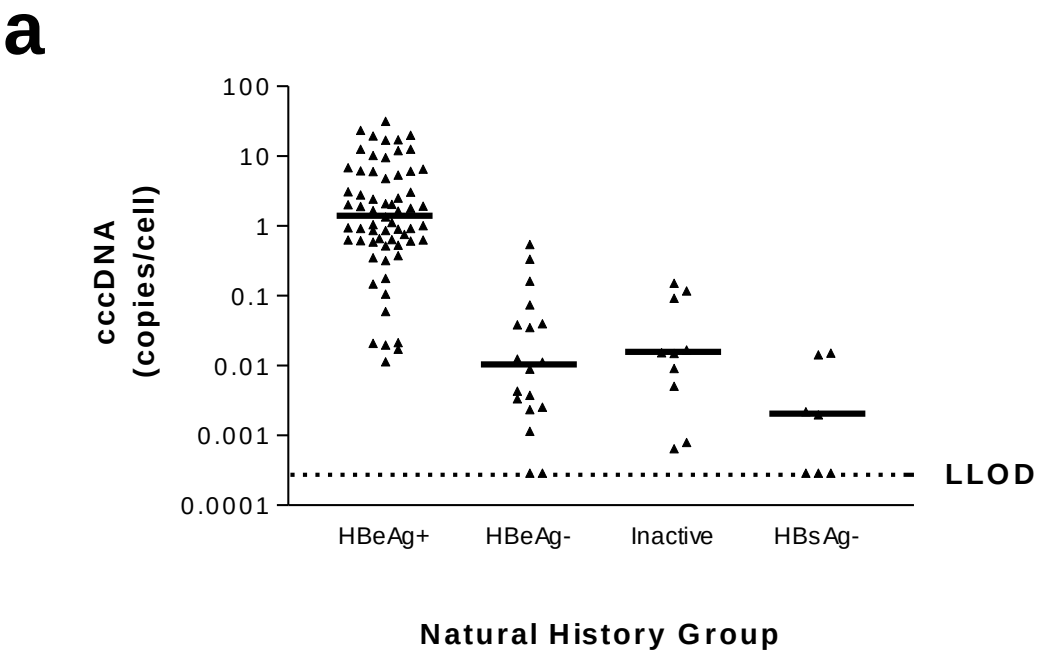

a
Relaxed circular DNA
Covalently closed cirular DNA
b
Non-selective primers
Selective cccDNA primers
+ DNase
+ DNase
10-1 10-5 10-1 10-5 10-1 10-5 10-1 10-5
HBV DNA copies
Plasmid
c
+ DNase
+ DNase
10-1 10-5 10-1 10-5 10-1 10-5 10-1 10-5
HBV DNA copies
Serum
d
+ DNase
+ DNase
50 10 neg 50 10 neg 50 10 neg 50 10 neg
ng DNA
liver
e
Kbp
Linear ds WHV
Total DNA
CCC DNA
DNase treatment
- + -
3 -
2 -
1 -

Figure 2


a
cccDNA (copies/cell)
100
10
1
0.1
0.01
0.001
0.0001
LLOD
HBeAg+
HBeAg-
Inactive
HBsAg-
Natural History Group

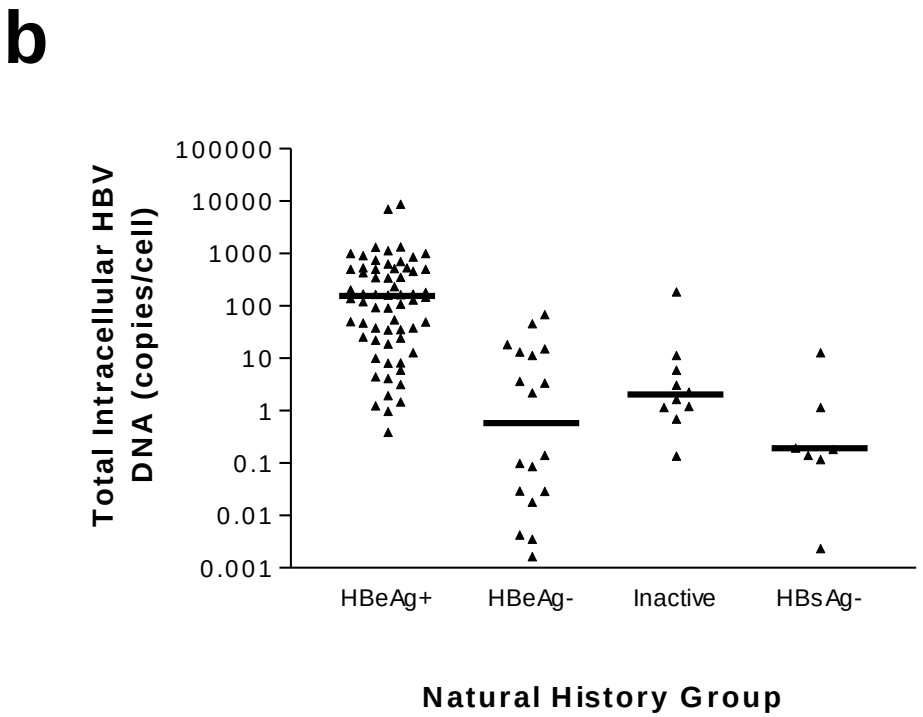
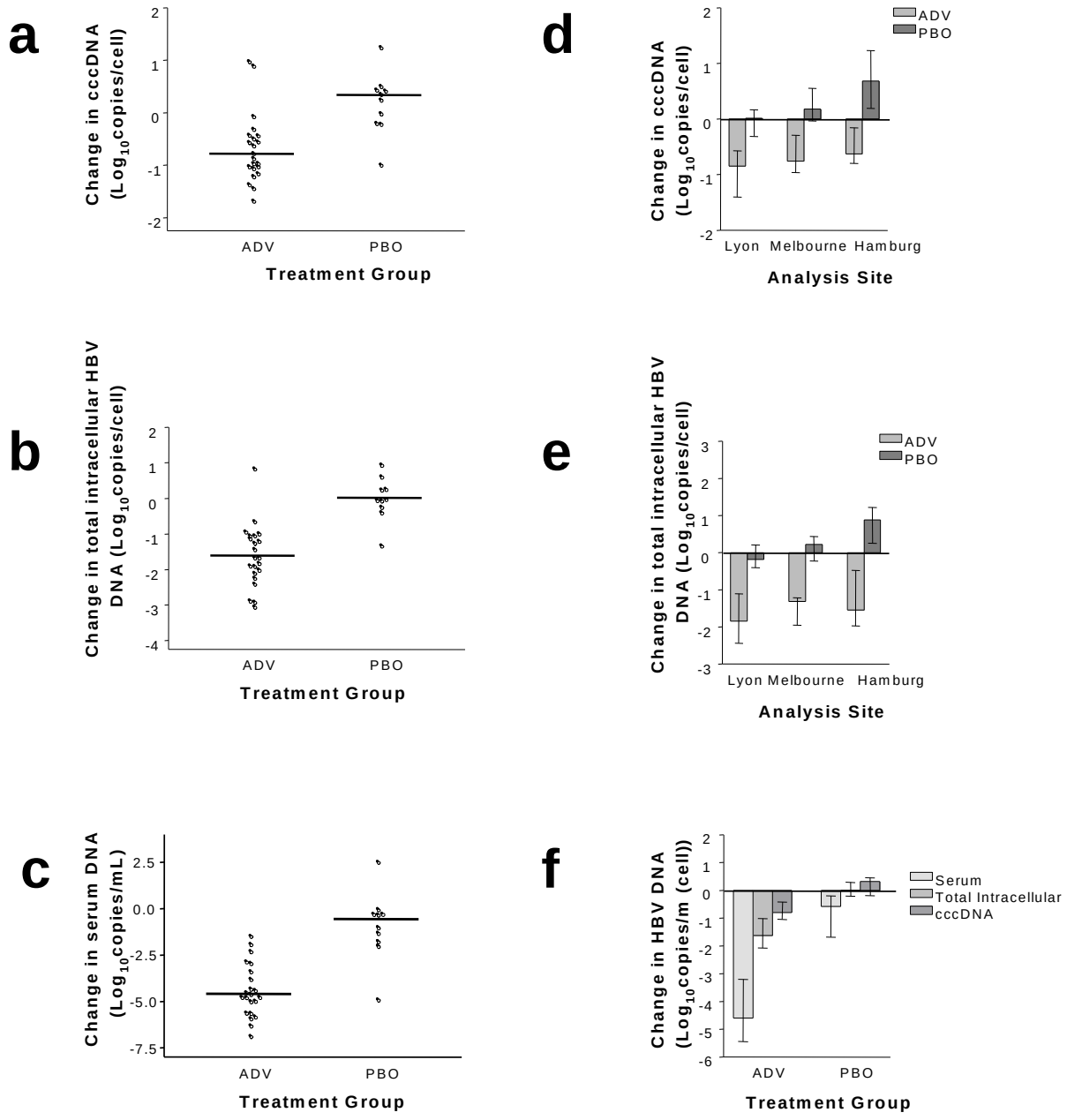

b
Total Intracellular HBV DNA (copies/cell)
100000
10000
1000
100
10
1
0.1
0.01
0.001
HBeAg+
HBeAg-
Inactive
HBsAg-
Natural History Group

Figure 3


a
Change in cccDNA (Log10copies/cell)
2
1
0
-1
-2
ADV
PBO
Treatment Group
d
Change in cccDNA (Log10copies/cell)
ADV
PBO
Lyon
Melbourne
Hamburg
Analysis Site
b
Change in total intracellular HBV DNA (Log10copies/cell)
2
1
0
-1
-2
-3
-4
ADV
PBO
Treatment Group
e
Change in total intracellular HBV DNA (Log10copies/cell)
ADV
PBO
3
2
1
0
-1
-2
-3
Lyon
Melbourne
Hamburg
Analysis Site
c
Change in serum DNA (Log10copies/mL)
2.5
0.0
-2.5
-5.0
-7.5
ADV
PBO
Treatment Group
f
Change in HBV DNA (Log10copies/m (cell))
2
1
0
-1
-2
-3
-4
-5
-6
Serum
Total Intracellular
cccDNA
ADV
PBO
Treatment Group