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36. Unfolded RuBisCO [5 M urea, 10 mM HCl, 1 mM dithiothreitol (DTT, pH 2] was initially labeled to hydrogen exchange equilibrium in tritiated water (~10 mCi/ml). To begin the exchange of hydrogen, we diluted RuBisCO (1:20) into conditions that do not permit folding (20 mM tris buffer, pH 8.0, 2 mM magnesium acetate, 2 mM potassium acetate, 1 mM DTT, 0.01% Tween-20, 22° ± 2°C, RuBisCO at 2 μM), with or without a small excess of $GroEL_{14}$. Free solvent tritium was immediately removed by centrifuging the RuBisCO solution through a Sephadex G-25 spin column [<1 min, 0.5 ml through a 1 cm x 5 cm column (*37*)] equilibrated with the nonpermissive refolding buffer. After hydrogen exchange for the desired time, free tritium was removed by a second spin column. The tritium label remaining bound was counted by liquid scintillation and computed in terms of the number of hydrogens per RuBisCO molecule not yet exchanged. For this calculation, 100% recovery of the known initial RuBisCO was assumed. Control experiments showed that GroEL does not account for any of the bound label; therefore, the analysis does not require the separation of GroEL from the labeled substrate protein. To avoid tritium contamination of samples, it is necessary to remove the initial free tritium (~10^{10} cpm/ml) by a large factor (~10^8), and it is advisable to spatially separate experimental operations to avoid minuscule splash and volatility problems, which accounts for the data spread seen in our early data. In the absence of tritium contamination, accuracy is at the level of a few percent. RuBisCO was prepared as described before (*38*). GroEL and GroES were overexpressed in *E. coli* and purified as described before (*39*). Protein concentration was measured spectrophotometrically at 280 nm using extinction coefficients of 9600 M^{-1} cm^{-1} per GroEL monomer, 1200 M^{-1} cm^{-1} per GroES monomer (calculated from sequence), and 67,100 M^{-1} cm^{-1} for RuBisCO (*38*). GroEL and GroES concentrations were confirmed by quantitative amino acid analysis.
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Viral Clearance Without Destruction of Infected Cells During Acute HBV Infection

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Viral clearance during hepatitis B virus (HBV) infection has been thought to reflect the destruction of infected hepatocytes by $CD8^+$ T lymphocytes. However, in this study, HBV DNA was shown to largely disappear from the liver and the blood of acutely infected chimpanzees long before the peak of T cell infiltration and most of the liver disease. These results demonstrate that noncytopathic antiviral mechanisms contribute to viral clearance during acute viral hepatitis by purging HBV replicative intermediates from the cytoplasm and covalently closed circular viral DNA from the nucleus of infected cells.

We have previously reported that HBV-specific cytotoxic T lymphocytes can abolish HBV gene expression and replication in the liver of transgenic mice by a noncytopathic mechanism mediated by inflammatory cytokines (*1*). On the basis of these observations, we postulated that viral clearance during human HBV infection may be primarily due to this antiviral process rather than the destruction of infected cells (*2*). The previous studies had two important limitations, however. First, the transgenic mice are not infected by the virus, so the observations were limited to biochemical aspects of viral replication and gene expression, and they excluded viral entry and spread. Second, for unknown reasons, the mice do not produce the episomal covalently closed circular (ccc) HBV DNA species (*3*) that serves as the viral transcriptional

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template (*4*), and therefore must be eliminated for viral clearance to occur. Accordingly, confirmation of the hypothesis that clearance of HBV reflects noncytopathic antiviral effects of the immune response requires analysis of these events in the liver of infected animals that produce cccDNA. Chimpanzees are ideal for these purposes because they are the only species other than humans that is infectible by HBV (*5*) and they are known to mount a cellular immune response to HBV similar to that observed in acutely infected humans (*6*).

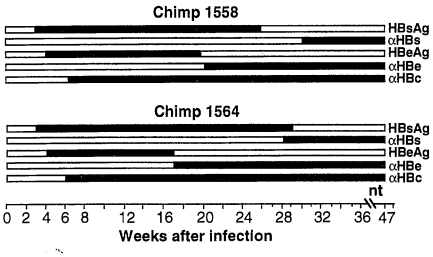

Fig. 1. Serum HBsAg, anti-HBs (αHBs), anti-HBc, HBeAg, and anti-HBe were measured as described (*8*). Shading in the horizontal bars represents a positive response for the corresponding parameter.

Two healthy, young adult, HBV-seronegative chimpanzees (chimp 1558 and chimp 1564) were inoculated intravenously with infectious serum containing $\sim 5 \times 10^7$ genome equivalents of HBV DNA (*ayw* subtype) (*7*) derived from transgenic mice that replicate the virus at levels comparable to patients with chronic hepatitis (*3*). Both chimpanzees developed typical cases of acute, self-limited HBV infection that were documented by virological, immunological, histopathological, and molecular analyses of serum specimens and liver biopsies that were obtained on a weekly basis throughout the course of the infection. Hepatitis B surface antigen (HBsAg) became detectable in the serum during week 3 and remained positive until weeks 26–29, coinciding with the appearance of antibody to HBsAg (anti-HBs), which remained for the duration of the study (Fig. 1). Hepatitis Be antigen (HBeAg) appeared on week 4 and remained until weeks 17–20, coinciding with the appearance of antibody to HBeAg (anti-HBe), which persisted. Antibodies to the hepatitis B core antigen (HBcAg) provided the first serological evidence of an immune response to the virus, appearing at weeks 6–7 and remaining thereafter. The relative kinetics and magnitude of these serological parameters show that both chimpanzees developed a typical case of acute HBV infection (*5*), proving that the transgenic mice produce infectious HBV.

The self-limited nature of the infection was further illustrated by quantitative analysis of viral DNA and viral antigens in the serum and liver (*8*) (Fig. 2). Serum and liver viral DNA content peaked at week 8 after inoculation in both chimps, decreasing rapidly thereafter. HBsAg and HBcAg appeared in the serum and liver slightly after the viral DNA but persisted for several weeks after most of the viral DNA was eliminated. More than 75% of the hepatocytes were $HBcAg^+$ at the peak of the infection, thereby establishing the minimum number of hepatocytes infected in both animals. Both chimps displayed the classical biochemical and histological profiles of acute viral hepatitis (Fig. 2). In keeping with the noncytopathic nature of HBV, there was no evidence of liver cell injury at the height of viral replication (week 8). After weeks 10–12, however, serum alanine aminotransferase (sALT) activity (a marker of hepatocellular necrosis) (*9*) began to rise, slowly at first, peaking on weeks 16 (chimp 1564) and 21 (chimp 1558), and returning to baseline by weeks 22–24. The sALT profile reflected the histological appearance of the liver. For chimp 1558, very few inflammatory cells or necroinflammatory foci were detectable in the liver before weeks 16–18, at which time the number of inflammatory foci (Fig. 3) and apoptotic hepatocytes (illustrated in Fig. 3 and quantitated in Fig. 2, gray bars in bottom panels) increased in concert with the sALT profile. In keeping with the histological evidence of inflammation, intrahepatic CD3 and CD8 RNA were barely detectable during weeks 12–14 and increased strongly during weeks 16–18 (Fig. 4). The CD4 RNA also increased at these time points, but it started from a higher baseline because of the presence of CD4 RNA in intrahepatic macrophages (*10*) and natural killer T cells (*11*) that are abundant in the uninflamed liver. The histological features of the liver disease in chimp 1564 were virtually

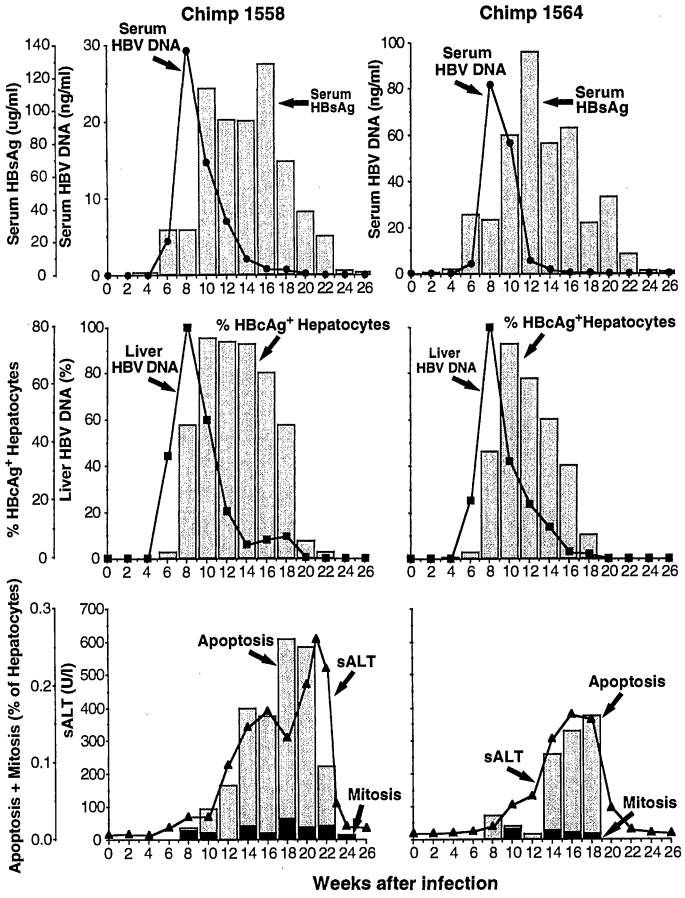

Fig. 2. (Upper panels) A 40-μl sample of serum was used to quantify HBV DNA by dot blot analysis (closed circles), as described (*1*). Serum HBsAg (gray bars) was measured as described (*8*). **(Middle panels)** Liver HBV DNA (closed squares) was monitored by Southern blot analysis (Fig. 4) and was quantified by phosphorimaging analysis, with the Optiquant image analysis software (Packard, Meriden, CT). Liver HBV DNA was also quantified by direct competitive PCR (*8*). Liver HBV DNA was normalized to the size of each liver biopsy by dividing the Southern blot– and PCR-derived HBV DNA values by the amount of the housekeeping gene L32 RNA (obtained by phosphorimaging analysis) detected in the same sample by ribonuclease (RNase) protection assay (Fig. 4). Virtually identical profiles were obtained by both techniques, and the indicated values represent the average (expressed in percentage of the peak value) of Southern blot– and PCR-derived HBV DNA values after normalization. Detection and quantitation of HBcAg-positive hepatocytes (gray bars) were carried out as described (*8*) and expressed as a percentage of the total number of hepatocytes counted. **(Bottom panels)** Liver function was evaluated by analysis of serum alanine aminotransferase (sALT) (closed triangles) activity as previously described (*1*). The number of apoptotic hepatocytes (gray bars) was calculated by quantitative morphometric analysis and expressed as a percentage of the total number of hepatocytes (*8*). The number of mitotic hepatocytes displaying the characteristic cytological features of mitosis (black bars in the bottom panels) was also calculated by quantitative morphometric analysis and expressed as a percentage of the total number of hepatocytes. Selected liver sections from weeks 17–19 in both animals were stained and found not to contain hepatocytes positive for proliferating cell nuclear antigen (PCNA) (*8*).

identical with those shown in Fig. 3 for chimp 1558.

Surprisingly, neither the surge in T cell RNA on weeks 16–18 (Fig. 4) nor the cytodestructive parameters discussed above (Figs. 2 and 3) were associated with the disappearance of serum and liver HBV DNA in the animals (Fig. 2) that occurred several weeks earlier. Indeed, the HBV DNA content in the serum and liver decreased by up to 90% between weeks 8 and 12, with little or no biochemical or histological evidence of liver disease at these time points (Figs. 2 and 3). These results indicate that something other than destruction of the hepatocytes was responsible for the reduction of viral DNA. This is underscored by the unchanging number of $HBcAg^+$ hepatocytes when the viral DNA was disappearing from the liver (Fig. 2). For example, the viral DNA content decreased by a factor of 10 to 20 in the liver of both chimps between weeks 8 and 14, at which time the number of $HBcAg^+$ hepatocytes either increased or remained unchanged. Collectively, these results demonstrate that clearance of more than 90% of the viral DNA did not require the destruction of 90% of the infected cells. An alternative scenario can be envisaged in which a small fraction of the infected cells make most of the viral DNA, and the destruction of these cells eludes detection. Although this is formally possible, it is not consistent with the uniformity of HBcAg staining from cell to cell that we observed in the liver of both animals (*12*). Furthermore, there is no precedent for this notion in other hepadnavirus models; in fact, there is evidence that the opposite is true, that is, hepadnaviral gene expression is uniform on a cell-to-cell basis in woodchuck hepatitis virus (*13*)– and duck hepatitis B virus (*14*)–infected livers.

The results also show that the viral DNA is more sensitive to noncytolytic clearance mechanisms than HBcAg, suggesting that the turnover of HBcAg is slower than that of viral DNA in infected cells. If so, the elimination of HBcAg from the liver could be due to its turnover in the absence of replenishment in living hepatocytes or it could be due to the destruction of $HBcAg^+$ hepatocytes. Although the similar kinetics of HBcAg clearance and liver disease in these animals supports the latter hypothesis, it may not be correct for the following reasons. First, relatively few hepatocytes appear to have been destroyed during the infection because the regenerative response (black bars in bottom panels of Fig. 2) throughout the infection was minimal even though the number of $HBcAg^+$ hepatocytes decreased from 75% to zero. Second, the abrupt decrease in $HBcAg^+$ hepatocytes between weeks 18 and 20 in chimp 1558 and between weeks 16 and 18 in chimp 1564 was not accompanied by a commensurate increase either in sALT activity or in the number of apoptotic or regenerating hepatocytes. Thus, even the reduction of hepatocellular HBcAg content during acute HBV infection may be primarily due to noncytolytic mechanisms rather than the destruction of infected cells.

As mentioned above, we have previously

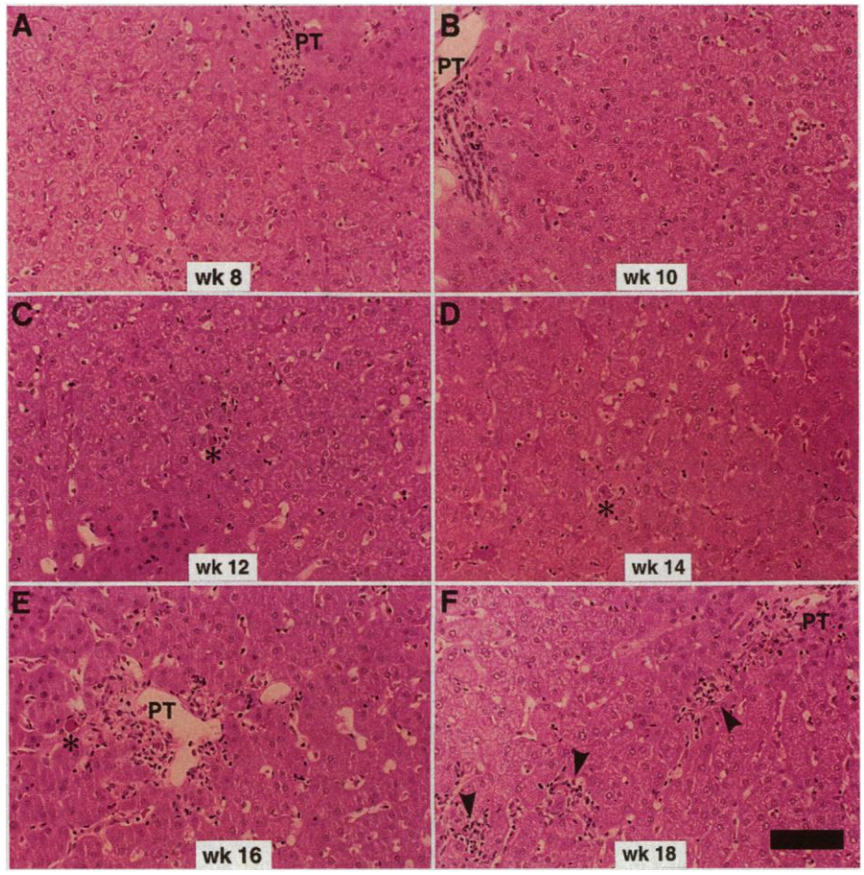

Fig. 3. Histopathological features of viral hepatitis in chimpanzees. Hepatic needle biopsies obtained from chimp 1558 at the indicated weeks were stained with hematoxylin and eosin as described (*8*). Necroinflammatory foci (arrowheads) and apoptotic hepatocytes (asterisks) are indicated. In (**A**) and (**B**) the hepatic parenchyma is entirely normal. A few mononuclear cells are seen in rare portal tracts (PT) as shown at the top of the panels. In (**C**) and (**D**) the hepatic parenchyma contains isolated, rare, small collections of inflammatory cells around individual apoptotic hepatocytes (asterisks). Note that most of the hepatocytes are histologically normal. Panels (**E**) and (**F**) display mononuclear infiltrates in the portal tracts and increased numbers of necroinflammatory foci (arrowheads) and apoptotic hepatocytes (asterisks). Most of the hepatocytes remain histologically normal. Original magnification ×200. Scale bar, 1 cm = 10 μm.

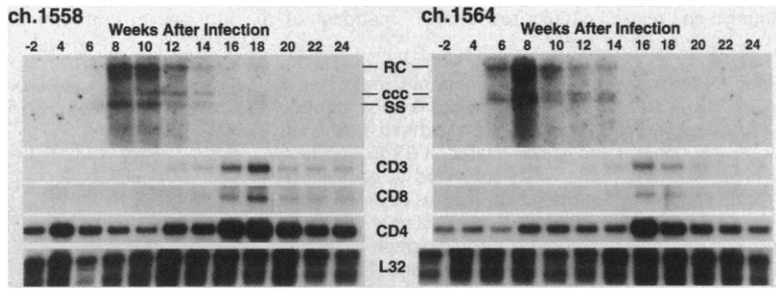

Fig. 4. Total liver nucleic acid was extracted (*3*) from each biopsy and divided in two samples. One sample was treated with RNase A, and HBV DNA was measured by Southern blot analysis as described (*3*). Bands corresponding to the relaxed circular (RC), covalently closed circular (ccc), and single-stranded (SS) linear HBV DNA forms are indicated. The other sample was not treated with RNase A and was subjected to RNase protection analysis with human CD3, CD8, CD4, and L32 subclones in the pGEM-4 transcription vector as described (*27*). All results should be compared with the L32 signal, a housekeeping gene included to normalize for the amount of total RNA included in each lane. All reactions shown in this figure were performed in the same experiment.

reported that the single-stranded and relaxed circular double-stranded HBV DNA replicative intermediates are eliminated from the cytoplasm of HBV transgenic hepatocytes as a result of the noncytopathic antiviral effects of certain inflammatory cytokines (*1*, *2*, *15*). We have not determined, however, if the cccDNA is susceptible to noncytopathic control because it is not produced by the transgenic mice. This is a critical issue because, as the transcriptional template of the virus, the cccDNA is required for initiation and maintenance of HBV infection and because it must be eliminated for viral clearance to be permanent and complete. Because of its circular minichromosome structure (*4*, *16*, *17*) and its long-term stability in the absence of replenishment during antiviral therapy (*18*), clearance of cccDNA has been thought to involve the destruction or turnover of infected cells (*19*). Although we do not dispute that notion, the results presented below demonstrate that hepatocellular cccDNA content can also be controlled noncytopathically during acute viral hepatitis.

The single-stranded and relaxed circular replicative DNA intermediates display characteristic electrophoretic mobilities that are readily distinguished from the cccDNA by Southern blot analysis. In addition, the cccDNA is stable to heat denaturation under conditions that reduce the relaxed circular DNA to the single-stranded form. We took advantage of these properties to monitor the clearance of these different forms of the viral genome from the liver. All HBV DNA forms, including the cccDNA, first appeared in the liver between 6 and 8 weeks after inoculation, reached maximal levels on week 8, and subsided very rapidly thereafter. The ccDNA disappeared with comparable but slightly delayed kinetics relative to the other replicative intermediates (Fig. 4). The species identified as cccDNA in the chimp liver displayed the same electrophoretic mobility of cccDNA from human infected liver and was not denatured at 85°C (Fig. 5), so it must represent the viral episome. Therefore, like the single-stranded and relaxed circular replicative DNA intermediates (which are located inside viral capsid particles in the cytoplasm of the hepatocyte) (*20*), the results presented in Fig. 4 demonstrate that the viral cccDNA episome, which is located in the nucleus of the cell (*20*), is also susceptible to noncytolytic control.

The viral DNA content of the liver decreased long before the surge in T cell mRNA (Fig. 4), which was temporally associated with the liver disease (Figs. 2 and 3). If the T cell response to HBV is similar to that occurring in most other viral infections (*21*), it is likely to be induced during the first 2 weeks after infection. If this is correct, the delayed (16 weeks) influx of T cells into the liver could reflect the absence of processed viral antigen at the surface of infected hepatocytes during active viral replication. If so, the early noncytopathic control of HBV replication in these animals may reflect primarily the influx of non–T cells, perhaps natural killer (NK) cells, that recognize infected cells in the absence of class I expression (*22*). This is especially intriguing because class I expression required for T cell antigen recognition is minimal in hepatocytes except in the context of an inflammatory response (*23*). Furthermore, viral clearance in HBV transgenic mice is triggered by interferon γ and tumor necrosis factor α that are produced not only by activated $CD4^+$ (*24*) and $CD8^+$ T cells (*1*) but also by NK cells (*25*) in the liver. In keeping with these observations, in ribonuclease protection assays we have demonstrated that interferon γ and tumor necrosis factor α are induced in the chimpanzee livers during the noncytopathic pre–T cell phase of viral clearance (*12*). Clearly, additional studies are needed to elucidate the role played by the innate and the adaptive immune response in viral clearance and viral pathogenesis during acute viral hepatitis in these animals.

In summary, the results presented in this study demonstrate that at least 90% of the viral DNA is eliminated from the liver during a typical HBV infection by noncytolytic processes that precede and are independent of the immune elimination of infected hepatocytes that probably supplements these noncytopathic antiviral events to fully control the infection. They also demonstrate that the viral cccDNA, a form thought to have a long half-life under noninflammatory conditions (*18*, *19*), is also susceptible to noncytolytic control. Finally, they suggest that different populations of inflammatory cells may be responsible for early viral clearance and late viral pathogenesis during HBV infection. This tissue-sparing, noncytolytic antiviral process can be viewed as a host survival strategy to control infections of vital organs that would otherwise be destroyed if the only way to eliminate the infections was to kill all of the infected cells. By down-regulating viral antigen expression, the same process could also function as a viral evasion strategy and contribute to viral persistence. Indeed, both scenarios might be correct, and they could even be operative at the same time in the same individual in view of the recent discovery that traces of HBV can persist for several decades after complete serological and clinical recovery from acute viral hepatitis (*26*). If so, the noncytolytic process described here should be strongly favored during evolution and possibly extend to other pathogens, because it provides a strong survival advantage for both virus and host.

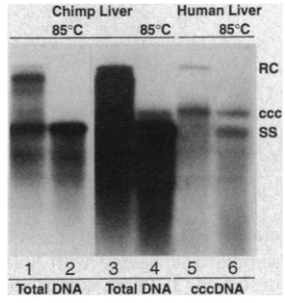

Fig. 5. Total DNA extracted from a liver biopsy obtained at week 9 (chimp 1558) was treated with RNase A and divided into two samples. One sample (lanes 1 and 3) was not heated, whereas the other sample (lanes 2 and 4) was heated at 85°C for 5 min, cooled on ice for 2 min, immediately subjected to gel electrophoresis, and analyzed by Southern blot analysis as described in the legend to Fig. 4. Lanes 1 and 2 and 3 and 4 represent different exposure times of the same autoradiograph to display the cccDNA signal in the unheated sample (lane 1) and the heated sample (lane 4). Note that the cccDNA band is resistant to heat denaturation, as opposed to the relaxed circular form, which was reduced to single-stranded DNA upon heating. The migration pattern of the cccDNA band detected in the chimp liver biopsy was compared with the pattern of the cccDNA band detected in a chronically infected human liver (lanes 5 and 6) that was analyzed in the same Southern blot after extraction of non–protein bound DNA (*3*) and subjected to heat denaturation, as indicated. Bands corresponding to the relaxed circular (RC), covalently closed circular (ccc), and single-stranded (SS) linear HBV DNA forms are indicated.

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7. Both chimpanzees were housed at Bioqual Laboratories (Rockville, MD), an American Association for Accreditation of Laboratory Animal Care International accredited institution under contract to the National Institute of Allergy and Infectious Diseases (number N01-AO-52705). Animals were handled according to human use and care guidelines specified by the Animal Research Committees at the National Institutes of Health and The Scripps Research Institute. Chimpanzees 1558 and 1564 were inoculated with $\sim 2 \times 10^7$ and $\sim 5 \times 10^7$ HBV genome equivalents in 0.5 ml of serum from transgenic mouse lineages 1.3.32 and 1.3.46, respectively. Before inoculation and each week thereafter, blood was obtained by venipuncture and liver tissue was obtained by needle biopsy.
8. Serum HBsAg, anti-HBs, anti-HBc, HBeAg, and anti-HBe were measured by solid-phase enzyme-linked immunosorbent assay (ELISA) (Auszyme for HBsAg, Ausab for anti-HBs, Corzyme for anti-HBc, and Abbott-HBe EIA for HBeAg and anti-HBe; Abbott Laboratories, Abbott Park, IL). HBsAg and anti-HBs concentrations were quantified by reference to internal standards (Bureau of Biologics, Bethesda, MD, and World Health Organization, respectively). Serum and liver HBV DNA were analyzed and quantified by direct competitive polymerase chain reaction (PCR). A pair of highly conserved diagnostic primers located within the HBV envelope coding region that yielded a PCR product of 441 base pairs (HBV 246S, 5′-

GAGTCTAGACTCGTGGTGGA and HBV 687AS, 5′-CGAACCACTGAACAAATGGC, *ayw* subtype, Galibert sequence) were used under conditions previously described (*26*). A novel Eco RI site was introduced by creating a point mutation at nucleotide 482 (T to G) with the GeneEditor in vitro site-directed mutagenesis system (Promega, Madison, WI). The competitor plasmid was linearized by Hind III digestion, and different concentrations of competitor plasmid (from 0.3 fg to 1 fg) were used to contaminate chimp serum DNA extracts. After PCR, the amplified fragments were subjected to Eco RI digestion, electrophoresed through 1.8% agarose gels in the presence of of ethidium bromide (0.1 μg/ml), and examined on an ultraviolet transilluminator. The amplified competitor plasmid produced two bands of 205 and 236 nucleotides, respectively, which migrated as a single band under the conditions used. Serum HBV DNA was quantified by comparing the relative intensity of the band corresponding to the competitor plasmid with the band obtained by amplification of serial dilutions of chimp serum DNA extracts. Hepatic needle biopsies, obtained on a weekly basis from anesthetized animals, were fixed in 10% neutral-buffered formalin, paraffin-embedded, sectioned (3 μm), and stained with hematoxylin and eosin, as previously described (*1*). The intracellular distribution of HBcAg and PCNA was assessed exactly as described (*1*). Quantitation of HBcAg-positive hepatocyte nuclei (wide bars) was carried out by counting a minimum of 10,000 hepatocytes with a 20× objective. Although HBcAg was detectable in both cytoplasms and nuclei, the results reflect nuclear staining. Apoptotic hepatocytes were identified as cells containing one or more of the following features: nuclear chromatin margination, pyknotic nuclei, and cytoplasmic fragmentation. The number of apoptotic hepatocytes was also calculated on selected liver sections by TUNEL assay that was performed according to the manufacturer's instructions (Oncor, Gaithersburg, MD). Similar numbers of apoptotic hepatocytes were obtained by the two independent techniques.

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