

Limited Hepatitis B Virus Replication Space in the Chronically Hepatitis C Virus-Infected Liver

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We compared the kinetics and magnitude of hepatitis B virus (HBV) infection in hepatitis C virus (HCV)-naive and chronically HCV-infected chimpanzees in whose livers type I interferon-stimulated gene (ISG) expression is strongly induced. HBV infection was delayed and attenuated in the HCV-infected animals, and the number of HBV-infected hepatocytes was drastically reduced. These results suggest that establishment of HBV infection and its replication space is limited by the antiviral effects of type I interferon in the chronically HCV-infected liver.

Genomic analysis of the host response to hepatitis C virus (HCV) infection in chimpanzees revealed that initiation of HCV infection induces many alpha/beta interferon (IFN- α/β)-stimulated genes (ISGs) in the liver (1, 2), yet HCV spreads throughout the liver. Together with the finding that ISG expression is maintained during chronic HCV infection (1, 3), this suggests that HCV presumably defeats the ISG-mediated antiviral functions in the infected hepatocytes. In contrast, acute hepatitis B virus (HBV) infection in chimpanzees does not induce any ISG expression in the liver (4), possibly reflecting avoidance of the antiviral activity of interferon, which strongly inhibits viral replication in HBV-transgenic mice and immortalized hepatocyte cultures (1, 2, 5–7). To determine whether establishment of HBV infection is sensitive to the antiviral effects of interferon in an authentic infection, we monitored the kinetics and magnitude of HBV superinfection in chronically HCV-infected chimpanzees and compared them with acute HBV infection in a HCV-naive control chimpanzee.

Three healthy, young adult HBV-seronegative chimpanzees, including ChAOA007 (8), which was also HCV seronegative, and two (Ch1614 [1, 3, 9] and Ch1625 [4, 10]) which were chronically HCV infected with HCV genotypes 1b and 1a, respectively, were used in this study. The animals were handled according to humane use and care guidelines specified by the Animal Research Committees at the National Institutes of Health and The Scripps Research Institute. They 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. The animals were inoculated with an HBV-positive serum from Ch5835 that had been previously inoculated with a monoclonal HBV isolate (genotype D, ayw subtype [GenBank accession no. V01460]) (11) contained in HBV-transgenic mouse serum (12) as described previously (1, 2, 8). The HCV-naive control animal (ChAOA007) and the chronically HCV-infected animals (Ch1614 and Ch1625) were all infected with 10^7 genome equivalents (GE) of this HBV inoculum (1, 3, 8), and viremia was monitored by HBV-specific real-time quantitative PCR (qPCR) of total DNA isolated from weekly serum samples as described previously (4, 6, 8, 13). As shown in Fig. 1A, control animal ChAOA007 displayed a typical acute resolving HBV infection course (5–8), reaching maximal serum HBV DNA titers of $3.9 \times$

10^7 GE/ml 6 weeks after inoculation (Fig. 1A, red line, and Table 1). HBV DNA was rapidly cleared from the serum of this animal (Fig. 1A), associated with a highly synchronized episode of acute hepatitis as measured by elevated serum aminotransferase activity (ALT) (yellow shaded area) and HBsAg/anti-HBs seroconversion in week 13 as previously described (8, 9, 14). In contrast, as shown in Fig. 1B, the onset of HBV infection was strongly delayed and its magnitude strongly attenuated in the two chronically HCV-infected chimpanzees, Ch1614 and Ch1625, reaching maximal HBV DNA titers of only 1.4×10^5 and 5.2×10^3 during weeks 13 and 24 after inoculation (Fig. 1B and Table 1). This was further reflected in the lack of detection of serum HBeAg and the corresponding antibody in these animals and minimal elevation of serum ALT activity over the 38 and 54 weeks that Ch1614 and Ch1625 were monitored, respectively (Fig. 1B). Furthermore, antibodies against HBV core and envelope proteins emerged much later in the chronically HCV-infected animals (Fig. 1B) than in the control animal (Fig. 1A). Nevertheless, the low-level HBV infection was ultimately cleared, and the animals remained serum HBV DNA negative for the remainder of the study (weeks 38 and 54 for Ch1614 and Ch1625, respectively). Together, these results show that HBV infection in the chronically HCV-infected animals, Ch1614 and Ch1625, was delayed 2 and 4 months, and viral peak levels were reduced 2 and 4 logs, respectively, compared to that of the control animal receiving the same dose inoculum (ChAOA007; Fig. 1A). Importantly, HBV superinfection did not affect the underlying chronic HCV infection, since HCV viremia levels did not change significantly during the time of HBV superinfection (Fig. 1B).

Consistent with previous reports (1, 3, 10, 15), chronic HCV infection in Ch1614 and Ch1625 was associated with a strong intrahepatic IFN- α/β response reflected by induced oligodeoxy-

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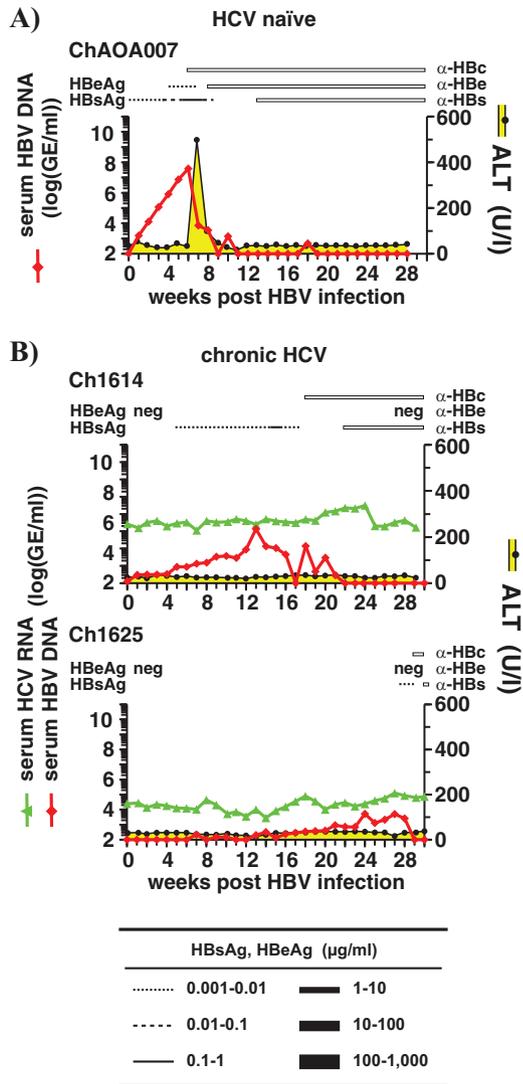


FIG 1 Course of acute HBV infection in HCV-naïve (A) and chronically HCV-infected (B) chimpanzees after experimental intravenous inoculation with a single HBV dose of 10^7 genome equivalents (GE) of HBV. Serum HBV DNA (red lines) and HCV RNA (green lines) levels are shown as log(GE/ml). Serum ALT activity (yellow shaded area) is shown as units/liter (U/l). Horizontal bars represent serum HBe and HBs antigen levels, and the open horizontal bars represent the presence of anti-HBc, anti-HBe, and anti-HBs antibodies. The protein concentration of each antigen is reflected by the thickness of the horizontal bar as indicated in the legend.

late synthase (OAS) gene mRNA levels in the liver as determined by OAS-specific quantitative reverse transcription (RT)-qPCR analysis of intrahepatic RNA as described previously (4, 11, 16). Indeed, intrahepatic OAS RNA levels were 29- and 15-fold induced prior to HBV superinfection (Fig. 2, week 0) compared to OAS mRNA levels determined in a liver biopsy specimen obtained prior to HCV infection. Although the absolute induction of OAS expression fluctuated throughout the course of HBV superinfection (Fig. 2), the average induction remained at similar levels in each chimpanzee, suggesting that the HBV infection did not influence the HCV-induced IFN- α/β response. In keeping with that hypothesis, intrahepatic OAS mRNA expression was not induced by acute HBV infection in control animal ChAOA007 (Fig. 2) or

in chimpanzees infected with a 1,000-fold-higher (ChAOA006) or -lower (Ch1622) dose of HBV (Fig. 2, Table 1, and Asabe et al. [8]), consistent with our previous finding that HBV apparently does not induce ISGs in the infected liver (4, 12, 17). Collectively, these results are compatible with the hypothesis that HBV spread in the chronically HCV-infected animals was controlled by IFN- α/β -induced noncytopathic antiviral mechanisms that we have previously shown to efficiently control HBV replication in HBV-transgenic mice (6, 7).

Next, we wanted to determine whether the attenuated serum HBV DNA levels observed in the HBV-superinfected animals were also reflected in the intrahepatic levels of HBV DNA replicative intermediates. Intrahepatic total DNA was isolated from liver biopsy specimens obtained at the peak of HBV viremia in the control and superinfected animals, and HBV DNA copy numbers per nanogram of total liver DNA were determined by HBV-specific qPCR exactly as previously described (13, 16). Indeed, the intrahepatic HBV DNA level (GE/ng of total liver DNA) at the peak of HBV superinfection in chronically HCV-infected Ch1614 was reduced to the same degree as the peak serum HBV DNA level compared to the corresponding peak levels observed in control animal ChAOA007 that was inoculated with the same dose of HBV (Table 1). The peak intrahepatic HBV DNA level during HBV superinfection in Ch1625 was detectable but too low for accurate quantification (Table 1). The lower peak intrahepatic HBV DNA level in Ch1625 is consistent with the serum HBV DNA peak level, also being almost 2 logs lower in Ch1625 than in Ch1614 (Table 1). Quantification of the intrahepatic HBV and HCV RNA levels by RT-qPCR (as described previously [18]) in total liver RNA isolated from liver biopsy specimens in Ch1614 and Ch1625 at the respective weeks of peak serum HBV DNA levels revealed that the copy numbers of HBV and HCV RNA/ng total liver RNA were similar in these animals (Table 1). Given that HBV replicative intermediates can accumulate to several hundred copies per hepatocyte during acute HBV infection in chimpanzees (13, 16), these numbers are compatible with the hypothesis that the ability of HBV to initiate infection and spread is restricted to a very small fraction of hepatocytes in the chronically HCV-infected liver.

To determine the frequency of HBV- and HCV-infected or coinfecting cells in the liver, we used a multiplex *in situ* hybridization (ISH) system (QuantiGene ViewRNA; Affymetrix, Santa Clara, CA) to simultaneously monitor HCV and HBV infection at the cellular level exactly as we have recently described for HCV- and HBV-infected patients (19). This system is characterized by an extremely low background, as demonstrated by the absence of any HBV or HCV RNA signal in the livers of ChAOA006 and ChAOA007 before HBV inoculation (Fig. 3A, week 0) or of Ch1614 before HCV inoculation (Fig. 3A, pre-HCV). Bright-field microscopic examination of $\geq 1,000$ cells revealed that the liver of chronically HCV-infected Ch1614 contained $\sim 7\%$ HCV RNA-positive cells prior to HBV superinfection (Fig. 3A, pre-HBV) and that that number was not significantly different ($\sim 7.5\%$) at the peak of HBV superinfection (Fig. 3B, week 13). The HCV signal intensity and percentage of HCV-positive cells are consistent with our recent analysis of liver biopsy specimens from chronically HCV-infected patients with similar serum and liver HCV RNA levels (19). Furthermore, assuming 30 to 40 pg of total RNA per hepatocyte, 6.4 GE/ng of total intrahepatic HCV RNA in Ch1614 (Table 1) would translate into an average of not more than 0.25 copies of HCV RNA per cell in the liver of Ch1614, similar to other

TABLE 1 Infection characteristics at peak of acute HBV infection

Chimp	Inoculum (GE)	No. of wks after inoculation	GE/ml serum		Liver				
			HBV ^a	HCV ^b	HBV			HCV	
					GE/ng DNA	GE/ng RNA	% pos ^c	GE/ng RNA	% pos ^c
ChAOA006 ^d	10 ¹⁰	5–6	3.3 × 10 ¹⁰	NA ^e	86,000	4,246	100	NA	NA
ChAOA007	10 ⁷	6	3.9 × 10 ⁷	NA	250	194	18	NA	NA
Ch1622 ^d	10 ⁴	8	2.5 × 10 ⁷	NA	110	151	NT ^f	NA	NA
Ch1614	10 ⁷	13	1.4 × 10 ⁵	6.2 × 10 ⁵	0.6	14	<0.04	6.4	7.6
Ch1625	10 ⁷	24	5.2 × 10 ³	3.7 × 10 ⁴	<0.5	<1	NT	1	NT

^a HBV DNA genome equivalents (GE)/ml serum.

^b HCV RNA genome equivalents (GE)/ml serum.

^c Percentage of HBV or HCV RNA-positive hepatocytes determined by *in situ* hybridization.

^d Course of infection described in Asabe et al. (8).

^e NA, not applicable.

^f NT, not tested.

HCV-infected chimpanzees (3) and consistent with a percentage of <10% of HCV RNA-positive hepatocytes in Ch1614 (Fig. 3). ISG (IFI27) mRNA-specific fluorescent ISH (FISH) revealed that virtually all hepatocytes expressed high levels of IFI27 mRNA in the chronically HCV-infected liver (data not shown), consistent with the strong induction of OAS mRNA as measured by RT-qPCR (Fig. 2). While HBV infection in positive-control animal ChAOA006 (Table 1) (8) spread to virtually all hepatocytes (Fig. 3B and Table 1), only ~18% of the hepatocytes in ChAOA007 were HBV RNA positive (Fig. 3B and Table 1) when the infection was abruptly terminated by the immune system, preventing it from spreading to all hepatocytes, as described in Asabe et al. (8). Importantly, however, compared to the ~18% HBV-positive hepatocytes in the HCV-naive animal ChAOA007 (Fig. 3B, Table 1), only very few cells in several fields of ≥1,000 cells (<0.04%) were HBV RNA positive in the liver of Ch1614 at the peak of HBV superinfection (Fig. 3B, Table 1). Furthermore, the HBV RNA signal did not overlap the HCV RNA (Fig. 3B), suggesting that all of the hepatocytes were relatively resistant to HBV infection in the HCV-infected liver irrespective of whether they were HCV positive or negative. Interestingly, the per-cell HBV RNA signal in the chronically HCV-infected animal was much stronger than the

per-cell HCV RNA signal (Fig. 3B, white and black arrows), consistent with similar amounts of total intrahepatic HCV and HBV RNA distributed to >100-fold-more HCV-positive than HBV-positive cells (Table 1). In addition, the per-cell HBV RNA signal in the chronically HCV-infected liver seemed to be at least as strong as the per-cell HBV signal in the HCV-naive animal (Fig. 3B, ChAOA007), consistent with the relative levels of total HBV RNA and the percentage of HBV-positive cells at the peak of HBV infection in the HCV-naive and chronically infected liver (Table 1). These results suggest that the few HBV-infected hepatocytes in the chronically HCV-infected liver are fully permissive for HBV replication.

It is tempting to speculate that HBV infection is limited to hepatocytes that do not express ISG proteins or that express only a subset of ISG proteins that do not inhibit HBV replication. While our observation that an HBV RNA-positive cell was also positive for IFI27 mRNA (data not shown) would suggest that IFI27 has no antiviral effect on HBV replication, a comprehensive analysis of the ISG protein expression profile in HBV-negative and -positive cells would be required to identify the ISG protein expression profile that determines HBV resistance or permissiveness at the cellular level. Unfortunately, the very low frequency of HBV-positive cells in the dually infected liver, the limited amount of biopsy material obtained from these animals, and the need for protease digestion of liver tissue during tissue processing prior to FISH precludes such an analysis.

Taken together, the current results demonstrate that initiation of HBV infection is severely limited in the HCV-infected liver. The finding that the few HBV-positive cells present in the liver of the HBV-superinfected chimpanzee were HCV negative suggests that HBV superinfection of HCV-infected cells is unlikely to occur *in vivo* even though there is no apparent interference between the two viruses when replicating within the same cell *in vitro* (20, 21). While we cannot exclude the possibility that HBV superinfection of HCV-positive cells is directly inhibited by HCV *in vivo*, the finding that most of the HCV-negative cells apparently are also refractory to HBV infection suggests that mechanisms other than direct viral interference contribute to the restriction of HBV infection in the chronically HCV-infected liver. Interestingly, however, the few HBV-positive hepatocytes seem to be fully permissive for HBV replication. Overall, these results are consistent with the hypothesis that the HCV-induced IFN- α/β response creates

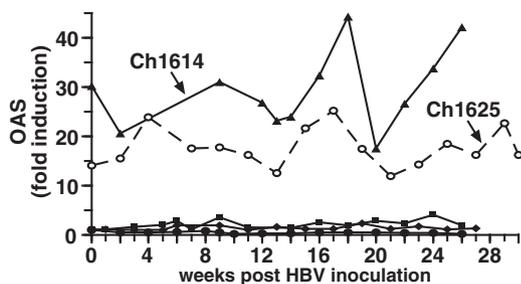


FIG 2 Intrahepatic induction of oligoadenylate synthase gene (OAS) mRNA expression during acute HBV infection in HCV-naive and chronically HCV-infected chimpanzees. Fold induction of OAS mRNA expression in the chronically HCV-infected animals (Ch1614 and Ch1625) is calculated against intrahepatic OAS mRNA levels measured in a liver biopsy specimen obtained prior to HCV infection. OAS mRNA induction prior to HBV superinfection was measured in liver biopsy samples obtained prior to HBV infection (week 0). Fold induction of OAS expression in the control animals (ChAOA006, ChAOA007, and Ch1622) is calculated against the OAS levels in liver biopsy specimens obtained prior to HBV infection (week 0).

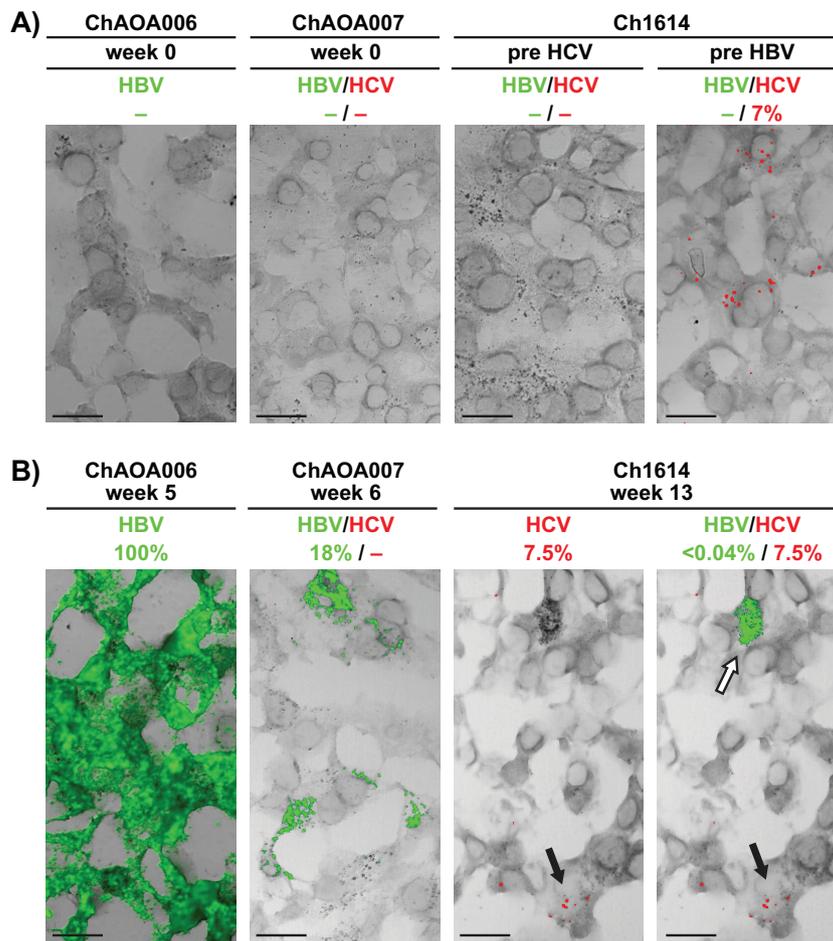


FIG 3 Analysis of HBV and HCV infection at the cellular level in the liver of HBV- and/or HCV-infected chimpanzees by multiplex *in situ* hybridization. (A) Simultaneous detection of HBV RNA (green) and HCV RNA (red) before HBV inoculation of ChAOA006 and ChAOA007 (week 0) and Ch1614 (pre-HCV and pre-HBV). (B) Simultaneous detection of HBV RNA (green) and HCV RNA (red) at the peak of HBV infection in the HCV-naive positive-control animals ChAOA006 (week 5) and ChAOA007 (week 6) and in the HBV-superinfected animal Ch1614 (week 13). The percentage of HBV and/or HCV RNA-positive cells in the liver tissue is shown above each panel (–, no FISH signal). Black and white arrows point to HCV RNA- and HBV RNA-positive cells, respectively. Scale bar, 20 μ m.

an antiviral state that restricts the initiation and spread of HBV infection to those cells whose ISG expression profile does not preclude HBV infection. The precise source of the cytokines that trigger the ISG response in the HCV-infected liver remains to be determined and might either be the HCV-infected hepatocytes and/or other liver-resident or recruited cells, such as NK cells (22, 23), dendritic cells (24), or macrophages (25, 26). In conclusion, our results are compatible with the hypothesis that restriction of the HBV infection space by the ISG response produced during chronic HCV infection might explain, at least partially, the apparent dominance of HCV over HBV in coinfecting patients (27–30).

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