



# The Chimpanzee Model for Hepatitis B Virus Infection

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Even before the discovery of hepatitis B virus (HBV), it was known that chimpanzees (*Pan troglodytes*) are susceptible to human hepatitis viruses. The chimpanzee is the only primate animal model for HBV infections. Much like HBV-infected human patients, chimpanzees can develop acute and chronic HBV infections and consequent hepatitis. Chimpanzees also develop a cellular immune response similar to that observed in humans. For these reasons, the chimpanzee has proven to be an invaluable model for investigations on HBV-driven disease pathogenesis and also the testing of novel antiviral therapies and prophylactic approaches.

Chimpanzees are the most important animal model for HBV research because they are the only immunocompetent nonhuman host fully susceptible to HBV infection. Shortly after the discovery of the Australia antigen and its relation to hepatitis (Blumberg et al. 1968; Prince 1968; Gocke and Kavey 1969), the presence of HBsAg was shown in the sera of chimpanzees (Lichter 1969; Maynard et al. 1971). However, it was not known whether chimpanzees were native hosts of HBV or whether they contracted HBV as a result of transmission from humans. In the early 1970s, it was shown that chimpanzees were fully susceptible to infection with HBV present in human plasma (Maynard et al. 1972; Barker et al. 1973). Much later, HBV strains indigenous to chimpanzees and other nonhuman primates were discovered (Norder et al. 1996; Lanford et al. 1998, 2000; Warren et al. 1999; Grethe et al. 2000; Hu et al. 2000, 2001; MacDonald et al. 2000; Takahashi et al. 2000, 2001; Vartanian et al. 2002; Sall et al. 2005; Du-

pinay et al. 2013). In 1977, the HBV-associated delta antigen was identified in patients (Rizzetto et al. 1977). Subsequently, studies with the chimpanzee showed that delta antigen is a serological marker of a separate transmissible agent, hepatitis delta virus (HDV) (Rizzetto et al. 1980a,b). Since then, the chimpanzee has also served as an experimental model for HDV natural history and pathogenesis (reviewed in Gerin 2001). In this review, I will focus on the chimpanzee model for HBV infections and its impact on our understanding of HBV pathogenesis and the control of the HBV pandemic.

## RECOGNITION OF INFECTIOUS (VIRAL) HEPATITIS IN CHIMPANZEES

A report by Soper and Smith in the late 1930s was most likely the first to provide evidence that a human hepatitis virus could infect nonhuman primates. Hepatitis was apparently transmitted to persons receiving a yellow-fever vaccine, pro-

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duced in rhesus monkeys (Soper and Smith 1938). In 1961, Hillis documented a small outbreak of hepatitis among chimpanzee handlers at a U.S. Air Force Base, who were in contact with animals arriving from Africa (Hillis 1961). Chimpanzees also became ill from what appeared to be an “unknown” virus at the time. It was suspected that they might have acquired this “human” infection when they were inoculated with human blood, with the intent to protect them from human diseases, shortly after they were captured (Hillis 1961, 1963; Deinhardt et al. 1972). It was recognized that the infectious agent spread between cages, as well as to veterinary personnel, and that the most common transmission was by the fecal–oral route. Although not known at the time, most of these infections were most likely caused by hepatitis A virus (HAV), but infections with HBV might also have occurred. In particular, a chimpanzee developed fulminant hepatitis shortly after arrival at the Delta Regional Primate Research Center (Covington, LA) and two handlers developed severe hepatitis 1–2 months later (Smetana 1965; Smetana and Felsenfeld 1969). Although never confirmed, this was probably one of the earliest observed transmissions of HBV from a nonhuman primate to a human. Similarly, in studies in the 1950s, six chimpanzees developed hepatitis after inoculation with human serum obtained from students at the Willowbrook State School (New York, NY) for mentally disabled children (reviewed in Deinhardt 1976). It was later shown that HBV was endemic among the students at that school. With the identification and characterization of HBV in the 1960s and 1970s (Blumberg 1964; Dane et al. 1970; Millman et al. 1970), it became possible to detect HBV infections with serological tests. In 1969, Hirschman et al. (1969) showed the presence of Australia antigen in chimpanzees. One of the first well-documented cases of transmission of HBV from a chimpanzee to a human was reported in 1970 when a child suffering from liver failure and subjected to cross-circulation with a chimpanzee became positive for HBsAg (Rivers and Keeling 1970). It was later found that the chimpanzee was HBsAg positive for at

least 1 year before the cross-circulation event, although the animal did not show signs of any disease (Rivers and Keeling 1970). Nonetheless, it remained obscure for years, whether spontaneous viral hepatitis in chimpanzees was of human or animal origin (Smetana and Felsenfeld 1969; Grethe et al. 2000). Sequence analysis finally showed that HBV is also indigenous to chimpanzees and other nonhuman primates (Vaudin et al. 1988; Grethe et al. 2000). In 1972 and the years following, it was conclusively shown that HBV sera–negative chimpanzees were susceptible to infection with human HBV isolates (Maynard et al. 1972; Barker et al. 1973, 1975a,b; Markenson et al. 1975). These crucial experiments paved the way for future prospective studies of HBV infections, vaccine development, and therapeutic interventions as discussed in the next sections.

#### ESTABLISHING THE CHIMPANZEE AS AN HBV MODEL SYSTEM

Several studies using limited dilutions of inocula containing different subtypes of human HBV showed an inverse relationship between inoculum size and incubation period (ranging from 2 to 20 wk) (Barker et al. 1975b; Shikata et al. 1977; Tabor et al. 1983b,c). Infectivity titers ranging from  $10^7$  to  $10^8$  chimpanzee infectious doses (CID)<sub>50</sub>/ml were measured in patient-derived inocula (Tabor et al. 1983b). Later, when real-time quantitative polymerase chain reaction (PCR) allowed for precise measurements of HBV DNA, it was reported that inocula from chronic HBV carriers, containing as little as three genome equivalents (GE) of HBV DNA, could infect a chimpanzee (Hsia et al. 2006; Komiya et al. 2008). Together with a recent demonstration that one GE of HBV DNA, obtained from a previously infected animal, was sufficient to reproducibly infect chimpanzees (Asabe et al. 2009), these studies showed that HBV does not produce viral DNA-containing particles that are defective. Several studies showed that acute HBV infections can also be induced in chimpanzees following intrahepatic injection of recombinant HBV DNA (Will et al. 1982, 1983, 1985), or after inoculation with

HBV produced in tissue culture cells (Acs et al. 1987; Sureau et al. 1988) or in HBV transgenic mice (Guidotti et al. 1999).

Those early studies also showed that acute HBV infection is associated with hepatitis and liver disease, as determined histologically and by elevated serum alanine aminotransferase (sALT) and/or serum aspartate levels (summarized in Tabor et al. 1983b). Interestingly, the severity of disease depended on the inoculum (i.e., subtypes), but not the dose of the inoculum (Tabor et al. 1983c). For example, inoculation of three chimpanzees with an HBV strain that caused fulminant hepatitis in human patients also resulted in severe hepatitis in chimpanzees (Ogata et al. 1993). A recent study also showed that low-dose inoculation, with as little as one GE of HBV, resulted in levels of serum and intrahepatic HBV markers, and was cleared with similar kinetics as inoculation with a very high dose of  $10^{10}$  GE of HBV (Asabe et al. 2009).

Chronic HBV infection, as defined by serum HBsAg positivity for >6 mo, occurred at a similar frequency (5%–10%) in chimpanzees inoculated with different HBV subtypes (Shikata et al. 1980; Tabor et al. 1983b,c), as described for human patients (Hoofnagle 1981). Despite the similarities of the course of HBV infection in human patients and chimpanzees, the severity of hepatitis consistently appeared to be milder in HBV-infected chimpanzees (Barker et al. 1973, 1975a).

Initial efforts to expand HBV infection to other nonhuman primate systems failed. Reports that HBV could also be transmitted to, and serially passaged in, rhesus monkeys could not be confirmed (London et al. 1972). Similarly, a more recent report showed HBV replication in a Barbary macaque (*Macaca sylvanus*) after transfection of cloned HBV DNA, but serial passage and persistent infection in these animals was not observed (Gheit et al. 2002; Dupinay et al. 2013). In contrast, gibbons (genus *Hylobates*) at the International Center for Gibbon Studies (Santa Clarita, CA) were positive for markers of ongoing or past HBV infection and phylogenetic analysis of the isolated HBV sequences suggested that the gibbons, most likely, had been infected by transmission of HBV

from humans (Lanford et al. 2000). This observation is consistent with two previous studies, providing serological evidence of HBV infection in gibbons inoculated with human HBsAg–positive saliva or semen (Bancroft et al. 1977; Scott et al. 1980). Finally, it was shown that a *Macaca fascicularis* colony from Mauritius Island was naturally infected with a human HBV isolate (Dupinay et al. 2013). Macaques might become an alternative model to chimpanzees for the study of therapeutic approaches against HBV infections (Bukh et al. 2013).

### THE CHIMPANZEE MODEL AS A “SAFETY TEST” TO PREVENT HBV INFECTIONS IN HUMANS

The detailed characterization of the course of HBV infections in chimpanzees, together with its reproducibility, made it possible to use this model to test for the presence of HBV in human serum and blood-derived products. For example, chimpanzee blood was used to show that inactivation of HBV in blood products with heat was only partially successful (Shikata et al. 1978; Hollinger et al. 1984; Purcell et al. 1985; Lelie et al. 1987). Over the next 15 years, many different inactivation strategies were used, including urea/formalin treatment (Tabor et al. 1983a), UV-irradiation alone and in combination with Tween 80 and  $\beta$ -propiolactone (Stephan et al. 1981; Prince et al. 1983a,b), chloroform treatment (Feinstone et al. 1983), exposure to Tween 80 and ether at 4°C (Prince et al. 1984a), glutaraldehyde or ethyl alcohol treatment (Kobayashi et al. 1984), exposure to heat (Hollinger et al. 1984; Kobayashi et al. 1984; Purcell et al. 1985; Lelie et al. 1987), photochemical treatment (Alter et al. 1988; Lin et al. 2005), ion exchange chromatography (Zolton et al. 1985), various disinfectants (Prince et al. 1993), and also antibodies to HBV (Tabor et al. 1980a; Brummelhuis et al. 1983).

These efforts were instrumental in containing the spread of HBV by blood transfusion and blood- or serum-derived products, including clotting factors, immunoglobulins, and HBV vaccines, which were derived from plasma of chronic carriers of HBV (see next section).

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Nevertheless, it was also recognized that non-A non-B (NANB) hepatitis infection could delay or prevent HBV infection in chimpanzees and lead to ambiguous results in tests for blood-derived products and vaccines (Brotman et al. 1983).

### THE CHIMPANZEE MODEL IN HBV VACCINE AND DRUG DEVELOPMENT

It was observed early on that chimpanzees previously exposed to HBV were protected from reinfection with high doses of HBV (Wilson and Logan 1975). Cross-challenging studies indicated that animals developing hepatitis following inoculation of HBsAg from one subtype were protected from challenge with another subtype (Murphy et al. 1974; Maynard et al. 1975; Gerety et al. 1979). Moreover, HBsAg immunization could trigger long-lasting cellular, as well as humoral responses (Ibrahim et al. 1974; Trépo et al. 1975). Together with the observation that immune function was directly related to infection outcome (Wilson and Logan 1975), these studies provided the rationale for vaccine development. Chimpanzee studies were instrumental as they allowed for both vaccine efficacy and safety testing. Thus, with the exception of certain polio vaccine studies in the 1950s (Sabin 1955a,b), the characterization of HBV infection and vaccine development probably reflects the first large-scale use of the chimpanzee model in biomedical research (McAuliffe et al. 1980; Prince et al. 1985).

The first-generation HBV vaccine was developed in the 1970s and was based on purification of HBsAg from the plasma of healthy HBV carrier patients (reviewed in McAuliffe et al. 1980). Evaluation of vaccine safety and efficacy mostly relied on studies in chimpanzees (Hilleman et al. 1975; Purcell and Gerin 1975; Buynak et al. 1976; Gerety et al. 1979; and reviewed in McAuliffe et al. 1980; Tabor et al. 1982; Prince et al. 1985), and these efforts greatly contributed to the licensing and use of the first plasma-derived HBV vaccine in the United States (Immunization Practices Advisory Committee 1982). Despite the success of the first-generation HBV

vaccine, there remained a certain risk of contamination of the vaccine with trace amounts of infectious HBV and/or other blood-borne diseases. Thus, alternative vaccines were developed and tested in chimpanzees. These included synthetic peptides (Itoh et al. 1986; Neurath et al. 1986; Emini et al. 1989), antibodies targeting HBV (Stephan et al. 1984; Hong et al. 2004; Kim et al. 2008a,b), HBV proteins produced in cell culture (Tabor et al. 1981), live recombinant viruses expressing HBV proteins (Moss et al. 1984; Lubeck et al. 1989), DNA immunization (Davis et al. 1996; Prince et al. 1997), the use of other viral proteins as immunogens (Prince et al. 1984b; Iwarson et al. 1985; Murray et al. 1987), and recombinant HBsAg produced in yeast. The latter was licensed in 1986 and rapidly replaced the plasma-derived vaccine. In recent years, modified recombinant vaccines were also tested in the chimpanzee model, further emphasizing the importance of this model in HBV vaccine development (Wahl et al. 1989; Fujisawa et al. 1990; Kuroda et al. 1991; Davis et al. 1996; Ogata et al. 1997, 1999; Page et al. 2001; Payette et al. 2006; Kamili et al. 2009; Kamili 2010).

Although the current recombinant vaccines are extremely efficient in preventing new infection, they do not provide any benefit to the millions of people that are chronic carriers and at greatly enhanced risk for developing liver cirrhosis and hepatocellular carcinoma (Prince 2001). Currently available therapeutic treatments for chronic HBV infection, while reducing the viral load, are very rarely curative (Kwon and Lok 2011; Sonneveld and Janssen 2011). Thus, different therapeutic vaccination strategies are under development with the hope of achieving viral clearance by boosting the immune response to HBV (reviewed in Liu et al. 2014). A series of therapeutic vaccination studies has been performed, over the years, in chimpanzees. These included administration of the interferon inducer poly(I/C) (Purcell et al. 1976), DNA-vaccine/boost protocols (Pancholi et al. 2001; Shata et al. 2006), treatments with therapeutic antibodies (Eren et al. 2000, 2006; Dagan and Eren 2003), genetic immunization using a recombinant retroviral vector-express-

ing HBV core protein (Sällberg et al. 1998), and, recently, immune modulation through Toll-like receptor (TLR)-7 activation (Lanford et al. 2013). Although some of these experiments resulted in prolonged reduction of viral markers and improved immune functions, none were curative, indicating that alternative strategies involving a combination with antiviral treatments may be required to cure chronic HBV infections (Bertoletti and Gehring 2013).

### STUDYING HBV PATHOGENESIS IN THE CHIMPANZEE MODEL

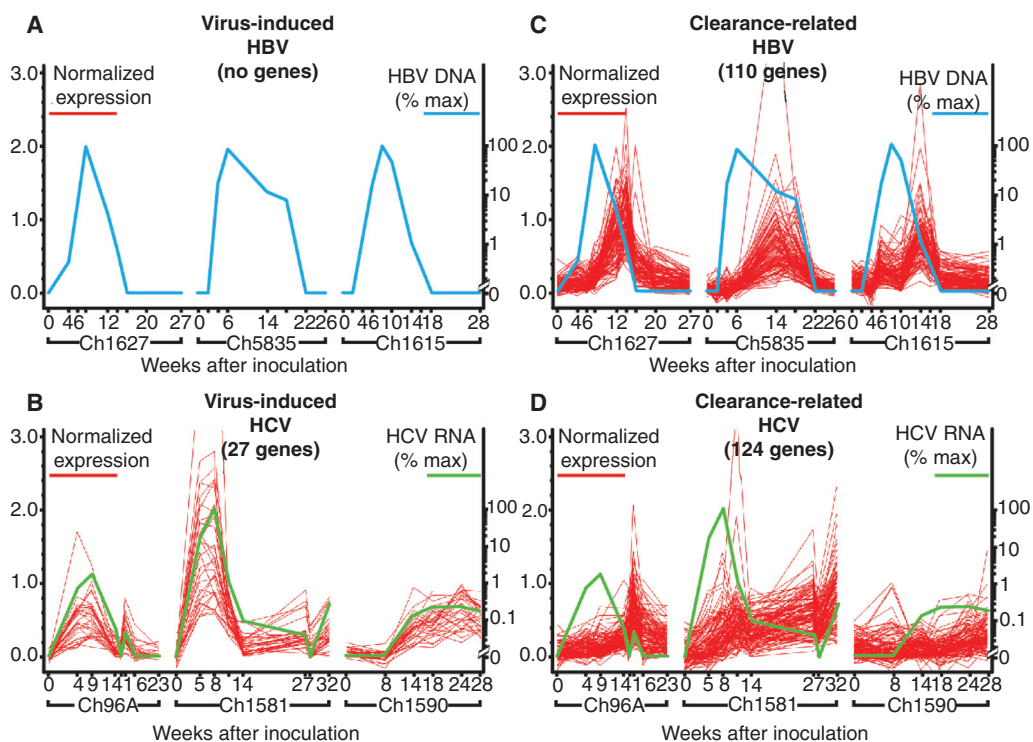
The ability to obtain serial liver biopsy samples from HBV-infected chimpanzees allows for investigations of viral and host parameters during the course of acute and chronic HBV infections. For example, studies in the 1980s described the detection of HBV DNA replicative intermediates, as well as covalently closed circular DNA (cccDNA) in infected livers (Shouval et al. 1980; Monjardino et al. 1982; Ruiz-Opazo et al. 1982a,b). Similarly, viral transcripts were identified and characterized to determine their 5' and 3' ends (Cattaneo et al. 1983, 1984). Other studies investigated immunopathogenesis in the infected liver using histological and ultrastructural methods (Karasawa et al. 1985a, 1985b; Schaff et al. 1992; Falcon et al. 1993). Besides serological time course studies of viral antigens and their corresponding antibodies, most of these studies were descriptive in nature (Barker et al. 1975b; Takahashi et al. 1979; Tabor et al. 1980b; Klinkert et al. 1986). However, with the recognition that chimpanzee (Patr) class I major histocompatibility complex (MHC) molecules and human leukocyte antigen (HLA) supertypes in humans share overlapping peptide-binding specificities, it became possible to investigate, in detail, the T-cell response to HBV in chimpanzees (Bertoni et al. 1998; McKinney et al. 2000; Sidney et al. 2006, 2007). Real-time quantitative PCR, specific for HBV DNA and cellular genes, as well as DNA microarray analyses, enabled design and execution of comprehensive longitudinal studies on host–virus interactions using chimpanzee sera and tissues from liver biopsies. These advances also permit-

ted investigations on HBV DNA integration in the liver of chronically infected chimpanzees (Mason et al. 2009).

### Absence of an Innate Response by HBV-Infected Cells

Expression of interferons, a hallmark of many viral infections, activates a cellular innate immune response characterized by transcriptional induction of a large number of genes called interferon-induced genes (ISGs). ISGs, in turn, encode proteins that exert a variety of intracellular antiviral functions to limit virus production and spread (Samuel 1991; Wieland and Chisari 2005). Longitudinal, intrahepatic expression profiling in chimpanzees showed that many ISGs are strongly induced during the spread of hepatitis C virus (HCV) infection, suggesting that HCV is highly visible to the innate immune system (Fig. 1B) (Bigger et al. 2001; Su et al. 2002; Wieland and Chisari 2005; Chisari et al. 2010). Surprisingly, however, intrahepatic gene expression profiling in acutely HBV-infected chimpanzees revealed that HBV does not induce detectable ISGs or other cellular gene expression as it spreads throughout the liver (Fig. 1A) (Wieland et al. 2004a, 2014; Wieland and Chisari 2005). This observation indicated that, unlike HCV, HBV acts like a “stealth virus,” which is invisible to the innate immune system (Wieland et al. 2004a; Wieland and Chisari 2005). The reason might lie in the mechanism of HBV replication. cccDNA is localized in the cell nucleus, whereas most known innate sensor proteins are cytoplasmic, the viral transcripts are capped and polyadenylated, resembling the structure of normal cellular transcripts, and the replicating genome is sequestered within viral capsids in the cytoplasm of infected cells (Summers 1988; Seeger and Mason 2000; Ganem and Prince 2004). Thus, the typical widespread expansion of HBV in the liver may reflect its ability to evade detection mechanisms that would result in interferon production, to which the virus can be exquisitely sensitive, as it has been shown in HBV transgenic mice (Guidotti et al. 1996; Wieland et al. 2000).





**Figure 1.** Liver gene expression profile during HBV and HCV infection (Wieland et al. 2004a). (A) Genes correlated with viremia in all acutely HBV-infected chimpanzees. No genes correlated positively or negatively with the level of intrahepatic HBV DNA during the time course of acute HBV infection in all three animals. (B) Intrahepatic gene expression correlated with viremia in three HCV-infected chimpanzees (Su et al. 2002). (C) Liver gene expression profile associated with viral clearance in all three acutely HBV-infected chimpanzees, and (D) that associated with clearance in HCV-infected chimpanzees (Su et al. 2002). Gene identities in HBV- and HCV-infected animals are described in Wieland et al. (2004a) and Su et al. (2002), respectively. Blue and green lines show the intrahepatic HBV DNA or serum HCV RNA as a percentage (% max) of the corresponding peak levels, respectively. For optimal visualization, gene expression levels (red lines) are shown after normalization to the 10th and 90th percentiles for each gene. Values on the x axis represent weeks after inoculation with HBV. (From Wieland et al. 2004a and Su et al. 2002; adapted, with permission, from the National Academy of Sciences © 2004 and 2002, respectively; and from Chisari et al. 2010; with permission from Elsevier Masson SAS © 2010.)

### Mechanisms of HBV Clearance

Many studies have shown that the peripheral blood CD4<sup>+</sup> and CD8<sup>+</sup> T-cell response to HBV is vigorous and multispecific in patients with acute hepatitis who ultimately clear the virus, whereas these are relatively weak in persistently infected patients with chronic hepatitis (Ferrari et al. 1990; Bertolotti et al. 1991; Penna et al. 1991; Rehermann et al. 1995; Tsui et al. 1995). Furthermore, HBV-specific T cells are detectable in the livers of chronically infected

patients, where they probably contribute to disease pathogenesis but, for some reason, are unable to control or cure the infection (Ferrari et al. 1987; Bertolotti et al. 1997; Maini et al. 2000; Rehermann 2000). Interestingly, a study examining a relationship between the number of intrahepatic HBV-specific CD8 T cells, extent of liver disease, and levels of HBV replication in chronically infected patients indicated that inhibition of virus replication could occur independently of liver damage. This study also showed that the functionality of HBV-specific

CD8 T cells was more important to the control of HBV replication than their absolute number (Maini et al. 2000). Indeed, in the HBV transgenic mouse model, adoptively transferred HBsAg-specific CTL clones not only kill engaged HBV-positive hepatocytes, but also secrete interferon (IFN)- $\gamma$ , which noncytopathically inhibits HBV gene expression and replication in the rest of the hepatocytes (Moriyama et al. 1990; Ando et al. 1994; Guidotti et al. 1994, 1996). Furthermore, in the HBV transgenic mouse model, HBV replication is inhibited by any stimulus that induces IFN- $\gamma$  and/or IFN- $\alpha/\beta$  in the liver (Guidotti et al. 1996; Franco et al. 1997; Cavanaugh et al. 1998; Kakimi et al. 2000; McClary et al. 2000a, 2000b; Paschetto et al. 2000; Wieland et al. 2000; Isogawa et al. 2005b). This raises the possibility that HBV infections can be controlled by several arms of the immune response and perhaps explains why HBV infections are almost always self-limiting in immunologically competent adults.

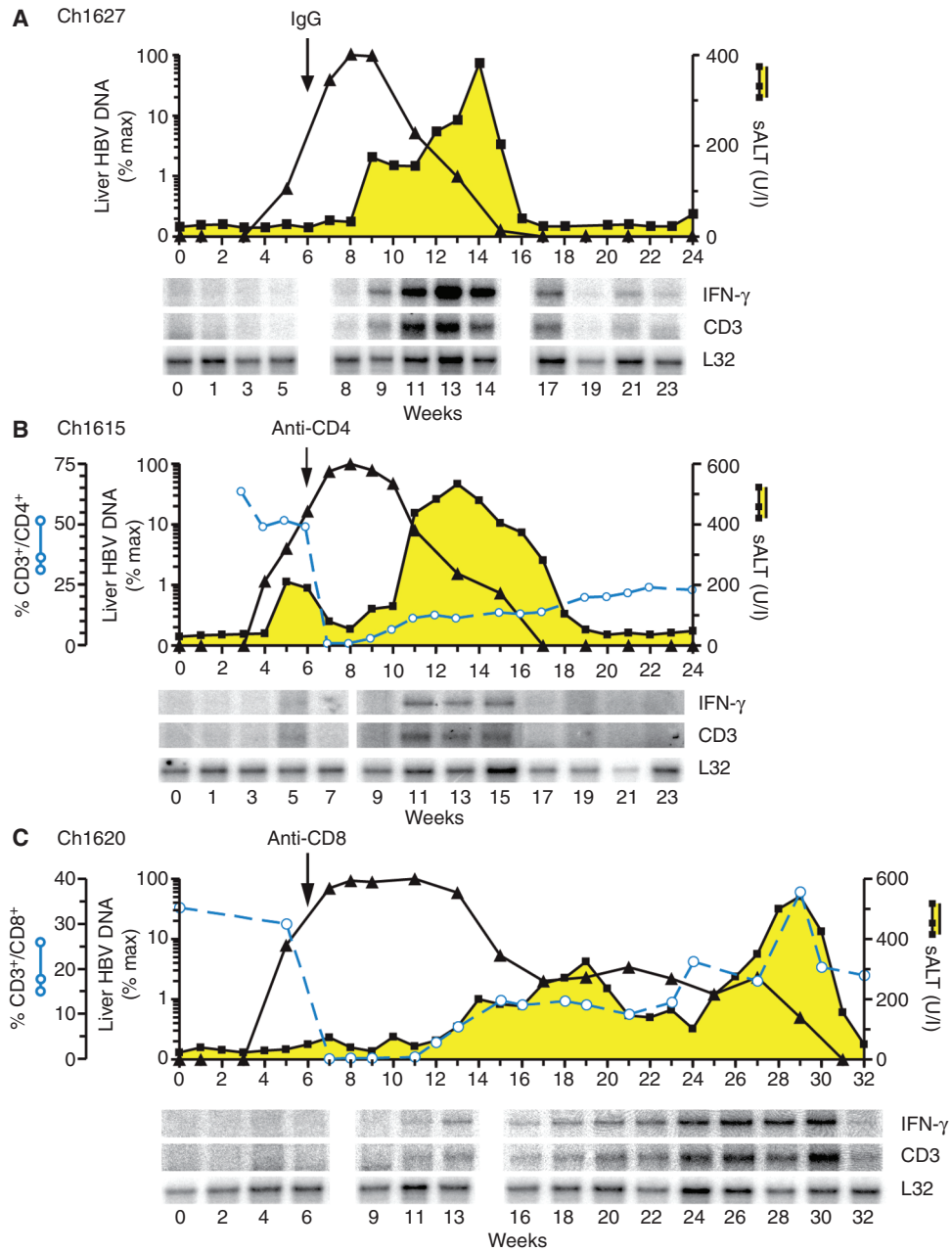
The chimpanzee model made it possible to characterize, in more detail, the cells and mechanisms responsible for viral clearance and liver disease during acute HBV infections. For example, monoclonal antibody-mediated T-cell depletion studies showed that CD4<sup>+</sup> T-cell depletion at the peak of infection did not significantly affect the kinetics of HBV clearance and liver disease during acute HBV infection (Fig. 2A,B) (Thimme et al. 2003). In contrast, depletion of CD8<sup>+</sup> T cells at the peak of infection profoundly altered the duration and outcome of an acute HBV infection (Fig. 2C) (Thimme et al. 2003). In the absence of CD8<sup>+</sup> T cells (wk 8–11), infection remained at peak levels and increases in sALT activity were delayed until wk 13, when CD8<sup>+</sup> T cells started to reappear. Furthermore, the time span from the first phase of clearance (wk 15) to eventual termination of the infection was markedly prolonged (Fig. 2C). Importantly, during wk 29, the CD8<sup>+</sup> cell numbers returned to baseline levels, which coincided with a surge in sALT activity and termination of the infection. Interestingly, the initial phase of viral clearance in all three animals was associated with intrahepatic CD3 and IFN- $\gamma$  expression but low sALT activity, suggesting that reduction

of HBV levels in this phase might be mostly driven by noncytopathic mechanisms similar to the observations in an earlier study in chimpanzees (Guidotti et al. 1999).

Intrahepatic gene expression profiling of these and other acutely HBV-infected animals confirmed that the early phase of clearance of HBV (Fig. 1C) (Wieland et al. 2004a) and, parenthetically, also for HCV (Fig. 1D) (Su et al. 2002), was temporally associated with the appearance of CD3, CD8, and IFN- $\gamma$  mRNA. It also showed that other T-cell-derived and IFN- $\gamma$ -stimulated genes were induced in this phase, suggesting that some of them might contribute to noncytopathic inhibition of HBV replication (Wieland et al. 2004a; Wieland and Chisari 2005). Indeed, although HBV replicative intermediates decreased as much as 50-fold from peak levels during this time, there was little or no attendant liver disease, despite the fact that virtually 100% of the hepatocytes were infected (Guidotti et al. 1999; Thimme et al. 2003; Wieland et al. 2004b), suggesting that noncytopathic mechanisms were active during this early phase of viral clearance. Interestingly, HBV cccDNA also decreased eightfold during the same time period, suggesting that cccDNA could, at least partially, be eliminated from hepatocytes by a noncytolytic mechanism (Guidotti et al. 1999; Wieland et al. 2004b). Thus, the chimpanzee model showed that the mechanism of CD8-dependent cytopathic and noncytopathic clearance of HBV, which was first discovered in the HBV transgenic mouse model (Guidotti et al. 1996), might be operative in the context of a natural HBV infection (Guidotti et al. 1999; Thimme et al. 2003; Wieland et al. 2004b; Murray et al. 2005).

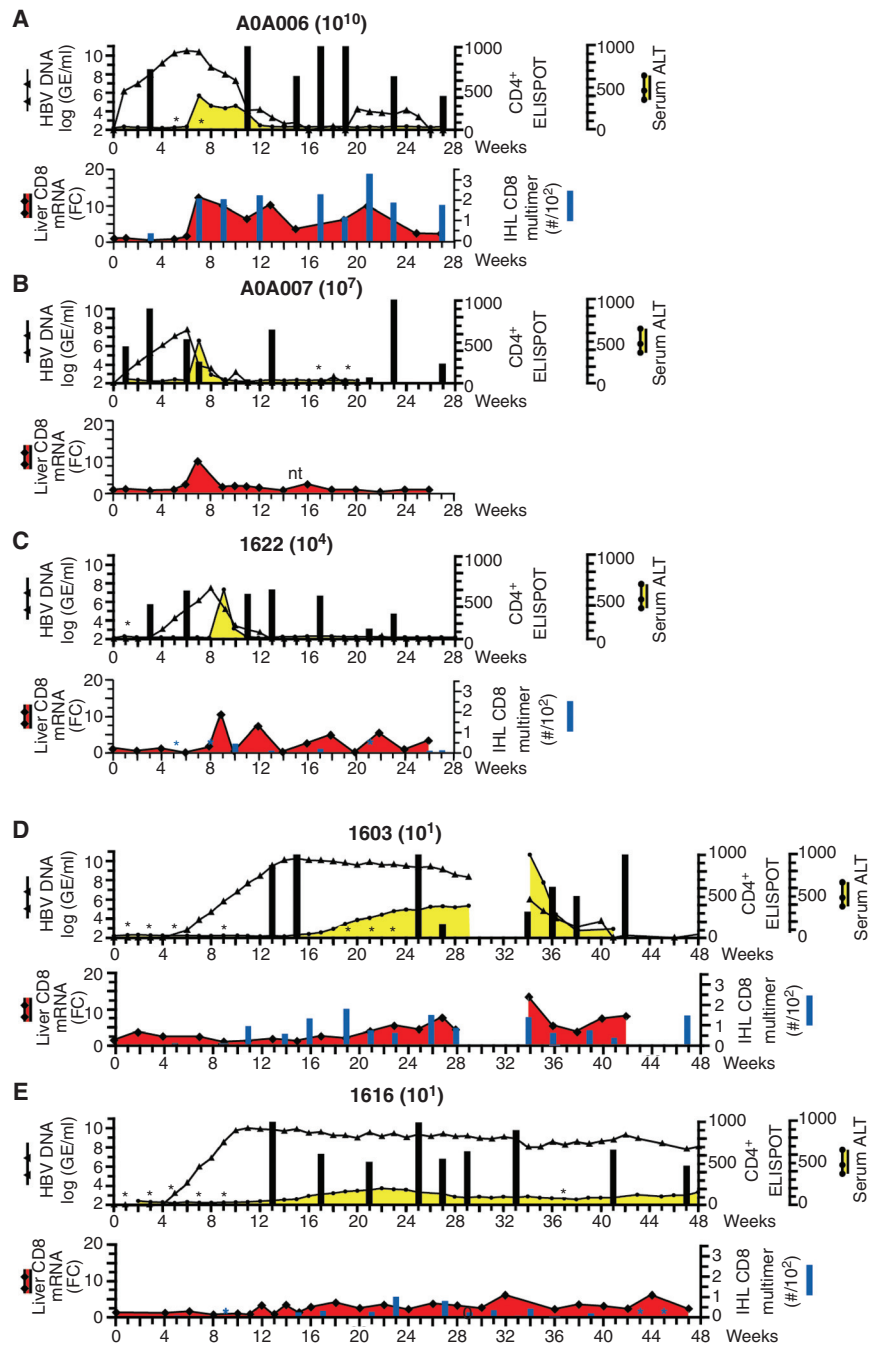
### Mechanisms of HBV Persistence

Neonatal tolerance to HBV is believed to be responsible for viral persistence following mother-to-infant transmission (Chisari and Ferrari 1995). Tolerance might be induced by the HBV precore protein (HBeAg), which has the capacity to cross the placenta, and, in HBV transgenic mice, induce neonatal tolerance (Milich et al. 1990). On the other hand, the basis for the



**Figure 2.** Course of acute HBV infection in chimpanzees after experimental inoculation with HBV in the presence or absence of CD4<sup>+</sup> and CD8<sup>+</sup> cells (Thimme et al. 2003). All animals were inoculated with 10<sup>8</sup> genome equivalents (GE) of HBV intravenously in wk 0 and a monoclonal antibody (see below) in wk 6. (A) Chimpanzee (Ch.) 1627 was injected with irrelevant control antibody (IgG). (B) Chimpanzee 1615 was injected with a CD4-specific monoclonal antibody. (C) Chimpanzee 1620 was injected with a CD8-specific monoclonal antibody. Intrahepatic HBV DNA levels in the liver of each animal. sALT activity (black squares and yellow area) is expressed in units per liter. The number of CD3<sup>+</sup> CD4<sup>+</sup> or CD3<sup>+</sup> CD8<sup>+</sup> T cells is expressed as a percentage of the total number of CD3<sup>+</sup> T cells in the peripheral blood. Vertical arrows indicate the time of antibody treatment. Total RNA isolated from liver biopsy samples was analyzed for the expression of CD3, IFN- $\gamma$ , and L32 by a ribonuclease (RNase) protection assay (Thimme et al. 2003). The L32 signals reflect the amount of RNA used in the assay. (From Thimme et al. 2003; adapted, with permission, from the *Journal of Virology*, American Society for Microbiology © 2003.)





**Figure 3.** Peripheral CD4<sup>+</sup> T-cell responses against HBV core protein and intrahepatic CD8<sup>+</sup> T-cell responses in chimpanzees infected with a dose range of HBV as described in Asabe et al. (2009).  $10^{10}$  (A),  $10^7$  (B),  $10^4$  (C), and  $10^1$  (D,E) genome equivalents (GE). The upper panel in each part represents the serum HBV DNA as a black line and sALT as a yellow shaded area and the results of peripheral CD4<sup>+</sup> T-cell ELISPOT assays are overlaid as black bars. Cryopreserved peripheral blood mononucleated cells (PBMCs) were thawed and stimulated in vitro with HBV core protein and the numbers of IFN- $\gamma$ -producing cells were determined by ELISPOT assay as described in Asabe et al. (2009). (Legend continues on following page.)

blunted immune response, which is characteristic for adult onset chronic HBV infections, is not well understood (reviewed in Guidotti and Chisari 2006; Rehmann 2013). Potential contributing factors to HBV persistence in adults include viral escape mutants with altered B- and T-cell epitopes that can evade the immune response (reviewed in Wieland and Chisari 2005; Guidotti and Chisari 2006), specific inhibition of the adaptive immune response by viral proteins (Brunetto et al. 1991; Hu et al. 1999; Reignat et al. 2002; Chen et al. 2004; Ganem and Prince 2004), and the size of the inoculum as described below.

### *The Size of the Viral Inoculum Contributes to the Outcome of HBV Infections*

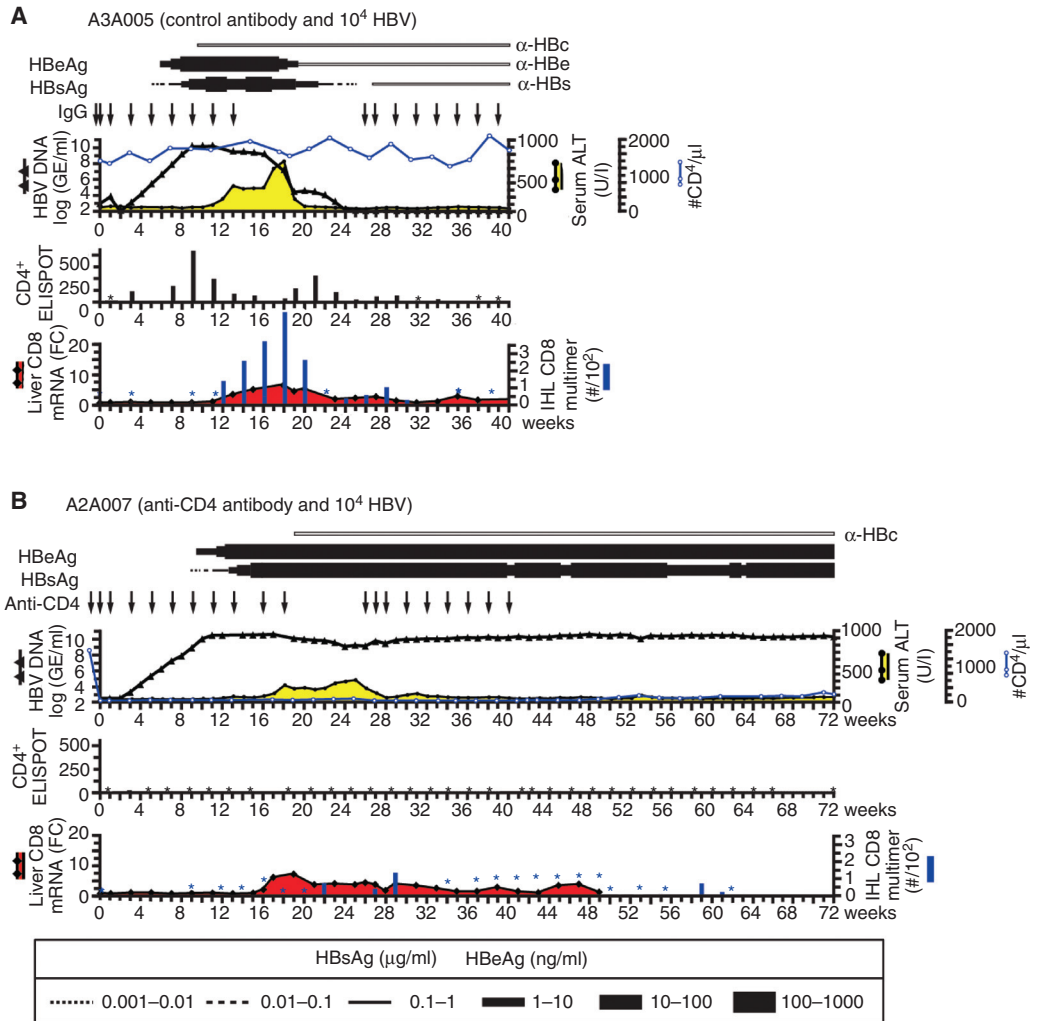
HBV infections in chimpanzees using a wide dose range of an inoculum showed that the size of the inoculum can influence the outcome of HBV infections (Asabe et al. 2009). Animals inoculated with  $10^{10}$ ,  $10^7$ , and  $10^4$  GE of HBV developed acute HBV infection and cleared the virus within 8–30 wk after its first detection (Fig. 3A–C). The incubation time was inversely correlated with the inoculum dose, but clearance followed the same pattern as previously observed in several other animals that had been inoculated with  $10^8$  GE (Fig. 2A,B) (Guidotti et al. 1999; Thimme et al. 2003). In contrast, two animals inoculated with 10 GE became chronically infected (Fig. 3D,E), with one of them (like many chronically infected humans) ultimately clearing the virus in the context of an acute disease flare-up 42 wk postinfection (p.i.), whereas the other remained infected for 55 wk p.i. when the study was terminated. This suggested that a viral dose  $>10^4$  GE favors

clearance (Fig. 3A–C), whereas doses between  $10^4$  and 1 GE favor persistent infections (Fig. 3D,E). Acute infections were associated with early  $CD4^+$  T-cell priming, either before or at the onset of detectable viral spread, which coincided with a sharply synchronized influx of HBV-specific  $CD8^+$  T cells into the liver and a corresponding increase in sALT activity and histological evidence of acute viral hepatitis. Interestingly, animals that developed chronic or prolonged infection showed the first detectable peripheral  $CD4^+$  T-cell response after the virus had infected almost all hepatocytes in the liver (Fig. 3) (Asabe et al. 2009). This correlated with an uncoordinated influx of HBV-specific  $CD8^+$  T cells into the liver and a correspondingly asynchronous increase in sALT activity (Asabe et al. 2009).

### *Early Priming of the $CD4$ T-Cell Response Is Required for Viral Clearance*

The above results suggested that an early  $CD4^+$  T-cell response to HBV infection is required to induce an effective  $CD8^+$  T-cell response, which is able to clear the infection. Indeed, inoculation of an animal that was depleted of  $CD4^+$  cells before inoculation with a virus dose of  $10^4$  GE (Fig. 4A,B) resulted in persistent HBV infection (Asabe et al. 2009). Importantly, as shown in Figure 2B,  $CD4^+$  T-cell depletion, using the same antibody at the peak of HBV infection, had no impact on the outcome of infection (Thimme et al. 2003). Together, these results suggest that the timing of  $CD4^+$  T-cell priming relative to the kinetics of viral spread is important in determining the magnitude and quality of the subsequent  $CD8^+$  T-cell response to HBV and, therefore, the outcome of HBV

**Figure 3.** (Continued) The data are shown as number of spots at each time point minus the number of spots before inoculation per million PBMCs. The lower panel shows the total number of intrahepatic HBV-specific  $CD8^+$  T cells per  $10^2$  total  $CD8^+$  T cells as filled blue bars (right axis) and fold induction (FC) of intrahepatic  $CD8$  messenger RNA (mRNA) compared with two preinoculation time points as a shaded red area (left axis). Intrahepatic lymphocytes were expanded antigen-nonspecifically in vitro and tested with all the corresponding Patr/peptide multimer complexes as described in Asabe et al. (2009). \*Tested and negative. nt, not tested. (From Asabe et al. (2009); reproduced/amended, with permission, from the American Society for Microbiology © 2009; and from Chisari et al. (2010); with permission, from Elsevier Masson SAS © 2010.)



**Figure 4.** Course of HBV infections, peripheral  $\text{CD4}^+$  T-cell responses against HBV core protein, and intrahepatic  $\text{CD8}^+$  T-cell responses in chimpanzees without (A), or with (B)  $\text{CD4}$  immunodepletion as described in Asabe et al. (2009). Serum HBV DNA levels are shown as a black line and sALT as a yellow shaded area. Horizontal bars represent serum HBe and HBs antigen levels and the open horizontal bars represent the presence of anti-HBc, -HBe, and -HBs antibodies. The amount of each protein is reflected by the thickness of each bar as indicated in the legend. The numbers of  $\text{CD4}^+$  T cells per  $\mu\text{L}$  of whole blood were shown as closed squares (top panel, right axis). Arrows on the top panels represent injections of control antibody (A), or anti- $\text{CD4}$  antibody (B). Peripheral  $\text{CD4}^+$  T-cell  $\text{IFN-}\gamma$  ELISPOT assays against HBV core protein (middle panel) and detection of intrahepatic HBV-specific  $\text{CD8}^+$  T cells (bottom panel, left axis) were as described in Figure 3, except that freshly prepared cells were used instead of using cryopreserved cells. Fold induction (FC) of intrahepatic  $\text{CD8}$  mRNA compared with two preinoculation time points is shown as a shaded red area (bottom panel, right axis). \*Tested and negative. (From Asabe et al. 2009; reproduced/amended, with permission, from the American Society for Microbiology © 2009; and from Chisari et al. (2010); with permission, from Elsevier Masson SAS © 2010.)



infection. It is conceivable that CD4<sup>+</sup> T-cell priming, which occurred before detectable viremia in some animals infected with a high dose of HBV, could have been triggered by subviral particles that are present at a large molar excess relative to infectious virions in the inoculum (Fig. 3B,C) (Asabe et al. 2009). In contrast, the reduced amount of subviral particles present in low-dose inocula might not be sufficient to induce an early CD4<sup>+</sup> T-cell response. As a consequence, CD8<sup>+</sup> T-cell priming might occur in the liver, resulting in T-cell inactivation, tolerance, or apoptosis (Bertolino et al. 2002; Crispe 2003; Isogawa et al. 2005a).

### CONCLUDING REMARKS

Given the restricted host range of HBV, the chimpanzee model system has been, and still is, critical for the study of HBV infections. Even before HBV was characterized at the molecular level, chimpanzee infection experiments have been crucial in recognizing that hepatitis could be attributed to diverse viral agents transmitted by different routes of infection. With the molecular characterization of HBV, use of the chimpanzee model immediately led to greatly improved safety of blood products used by human patients. Most importantly, the chimpanzee model system enabled the quick development and testing of a very effective prophylactic vaccine, which is still in use today. Besides the immediate impact of those studies on public health, by preventing spread of HBV infection, the chimpanzee model also provided unique opportunities to perform longitudinal studies of the virus–host interactions during acute HBV infection. Those studies revealed that HBV acts like a stealth virus early in infection, remaining undetected and spreading until the onset of the adaptive immune response several weeks later. However, HBV can be controlled when properly activated HBV-specific CD8<sup>+</sup> T cells enter the liver, recognize antigen, kill infected cells, and secrete IFN- $\gamma$ , which triggers a broad-based cascade that amplifies the inflammatory process and also has noncytopathic antiviral activity against HBV. But, absence of proper CD4<sup>+</sup> T-cell priming early in infection,

as observed in low-dose infections, induces functionally impaired CD8<sup>+</sup> T-cell responses, resulting in the establishment of persistent infections. Efforts to restore an effective CD8<sup>+</sup> T-cell response in the chimpanzee have not yet been successful. Funding constraints, National Institutes of Health (NIH) restrictions on the use of the chimpanzee model, and the high costs involved with this animal model will increasingly limit its use for biomedical research and antiviral drug testing. Whether alternative animal models for HBV infections, such as the woodchuck, can replace the chimpanzee model remains to be seen.

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