Review Article

Asymptomatic Hepadnaviral Persistence and Its Consequences in the Woodchuck Model of Occult Hepatitis B Virus Infection

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Abstract

Woodchuck hepatitis virus (WHV) is molecularly and pathogenically closely related to hepatitis B virus (HBV). Both viruses display tropism towards hepatocytes and cells of the immune system and cause similar liver pathology, where acute hepatitis can progress to chronic hepatitis and to hepatocellular carcinoma (HCC). Two forms of occult hepadnaviral persistence were identified in the woodchuck-WHV model: secondary occult infection (SOI) and primary occult infection (POI). SOI occurs after resolution of a serologically apparent infection with hepatitis or after subclinical serologically evident virus exposure. POI is caused by small amounts of virus and progresses without serological infection markers, but the virus genome and its replication are detectable in the immune system and with time in the liver. SOI can be accompanied by minimal hepatitis, while the hallmark of POI is normal liver morphology. Nonetheless, HCC develops in about 20% of animals with SOI or POI within 3 to 5 years. The virus persists throughout the lifespan in both SOI and POI at serum levels rarely greater than 100 copies/mL, causes hepatitis and HCC when concentrated and administered to virus-naïve woodchucks. SOI is accompanied by virus-specific T and B cell immune responses, while only virus-specific T cells are detected in POI. SOI coincides with protection against reinfection, while POI does not and hepatitis develops after challenge with liver pathogenic doses >1000 virions. Both SOI and POI are associated with virus DNA integration into the liver and the immune system genomes. Overall, SOI and POI are two distinct forms of silent hepadnaviral persistence that share common characteristics. Here, we review findings from the woodchuck model and discuss the relevant observations made in human occult HBV infection (OBI).

Keywords: Hepatitis B; Occult hepatitis B virus infection; Woodchuck model of hepatitis B; Woodchuck hepatitis virus; Secondary occult infection; Primary occult infection; Consequences of occult hepadnaviral infection; Hepatocellular carcinoma.

Abbreviations: AH, acute hepatitis; ALT, alanine aminotransferase; anti-HBc, antibodies against HBV core antigen; anti-HBs, antibodies against HBV surface antigen; anti-WHc, antibodies against WHV core antigen; anti-WHs, antibodies against WHV surface antigen; cccDNA, covalently closed circular DNA; CH, chronic hepatitis; ELISA, enzyme-linked immunosorbent assay; HBsAg, HBV surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFN, interferon; IL, interleukin; NAH, nucleic acid hybridization; NAT, nucleic acid testing; OBI, occult HBV infection; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; POI, primary occult infection; RT, reverse transcription; SIV, simian immunodeficiency virus; SOI, secondary occult infection; TNF, tumor necrosis factor; WHO, World Health Organization; WHsAg, WHV surface antigen; WHV, woodchuck hepatitis virus.

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Introduction

Occult hepatitis B virus (OBI) infection is a distinct form of hepatitis B virus (HBV) infection, identified using highly sensitive assays that detect small amounts of the HBV genome and its replicative intermediates. This silent form of HBV carriage is maintained by persistent virus replication progressing at very low levels. OBI is currently defined as the presence of HBV DNA in the absence of identifiable serum HBV surface antigen (HBsAg), as determined using standard clinical laboratory tests. Because of the inherent difficulties in identifying low levels of HBV in clinical situations, mainly due to insufficient sensitivity of the routine assays and the ethical issues related to serial sampling of seemingly healthy individuals, it was apparent that the employment of a relevant animal model would be highly advantageous for research on the properties, forms, and outcomes of OBI. From the early stages of research on OBI, the eastern North American woodchuck (*Marmota monax*), which is naturally prone to infection with woodchuck hepatitis virus (WHV), a close relative of HBV,[1,2] was applied.[3,4] This experimental system became an important source of new information on the natural course, pathogenicity, transmissibility, and epidemiological relevance of occult hepadnaviral persistence. This review will summarize findings that were central to the delineation of virological, molecular, and immunological characteristics and to the recognition of long-term pathological consequences of OBI in the woodchuck model. It will also relate and juxtapose the two forms of occult infection identified in WHV-infected woodchucks, i.e. secondary and primary occult infection, SOI and POI, respectively.

The woodchuck model of HBV infection

WHV was discovered in a colony of woodchucks in the Philadelphia Zoological Garden, where high rates of chronic

hepatitis and hepatocellular carcinoma (HCC) were noticed.[5,6] Subsequent studies have shown that WHV is very similar to HBV in terms of genome size (3308-3323 and 3182-3221 base pairs, respectively), nucleotide sequence (62–70%), overall genomic organization, replication strategy, the number, length, and functions of the transcribed viral proteins, and organs targeted (i.e. the liver and the immune system). WHV and HBV also share significant antigenic cross-reactivity and the timing of the appearance of viral antigens in the liver and circulation, as well as antibodies against them are similar. There also is strong compatibility in the induction of the innate and the virus-specific (adaptive) humoral and cellular immune responses, and in the progression and outcomes of liver disease where acute hepatitis (AH) can advance to chronic hepatitis (CH) and HCC.[5–9] Both infections similarly respond to anti-HBV and immunomodulatory agents, and the drug toxicity and pharmacokinetic profiles are compatible in infected woodchucks and humans.[10,11] For these and other reasons, the woodchuck-WHV infection has been acknowledged as the most appropriate model of HBV infection and HBV-induced liver disease, even surpassing other currently available natural and transgenic animal models. Nonetheless, there are some differences between WHV and HBV and the diseases caused. At the molecular level, the glycosylation pattern of the virus surface (envelope) proteins is different,[12] and there are slight discrepancies in the activities of individual enhancers and promoters.[13] Regarding the disease induced by these viruses, the major differences are that progression of CH to cirrhosis does not occur in woodchucks and that HCC develops at a high (80–90%) frequency in animals with serum WHV surface antigen (WHsAg)-positive CH, while HCC occurs at much lower (~5%) rates in patients with CH type B and cirrhosis.[6,14]

SOI after resolution of acute hepatitis

Prior to the era of sensitive nucleic acid testing (NAT), cases of persistent HBV infection in the absence of detectable serum HBsAg and transmission of HBV by blood and organ donations from serum HBsAg-negative individuals were only occasionally reported.[15–20] The availability of NAT for HBV DNA testing, first for research and then in the clinic, increased the frequency of OBI identification.[21–23] With time, transmission of HBV by blood transfusions and organ transplantation carrying trace amounts of virus was shown.[23–26] Severe consequences of OBI reactivation have been observed following immunosuppression or administration of cytotoxic drugs,[27–31] revealing the high clinical relevance of OBI. In this context, the recent World Health Organization (WHO) estimations indicated that up to 2 billion people have evidence of exposure to HBV.[32]

An early study documented the long-term persistence of HBV in sequential serum and peripheral blood mononuclear cell (PBMC) samples from individuals with spontaneous resolution of acute hepatitis type B. In the presence of detectable antibodies to HBsAg (anti-HBs) and HBV core antigen (anti-HBc), it was shown that HBV DNA was present in both these compartments for up to 70 months after an apparent complete recovery from AH type B.[18] HBV DNA was detected by nested polymerase chain reaction (PCR) with primers specific for different HBV genes followed by amplicon detection by nucleic acid hybridization (NAH) to recombinant HBV DNA as a probe (PCR-NAH). The sensitivity of this assay was less than 10 genome copies/mL, also called virus genome equivalents (vge). It was also shown that HBV DNA reactivity cosedimented with HBsAg in sucrose gradients and exhibited buoyant density of intact complete HBV virions or HBV core particles in cesium chloride gradients.[18] It was inferred that traces of infectious virus may persist in these convalescent and seemingly healthy individuals for years after termination of self-limited AH. These observations initiated a series of studies in the woodchuck-WHV model.

One of the first studies in woodchucks aimed to ascertain whether WHV persisted after recovery from self-limited AH, and if so, what the longevity of virus endurance was and where the reservoirs of virus replication were. It also was imperative to establish whether persisting virus retained infectivity and what the pathological consequences of such residual infection could be.[3] Detection of WHV DNA and WHV mRNA using WHV-specific PCR/NAH and reverse-transcriptase PCR (RT-PCR/NAH; sensitivity <10 copies/mL or μg of total RNA), respectively, uncovered that low levels of virus genome and its transcription persisted for the lifetime in the liver and in cells of the lymphatic system after resolution of experimentally induced AH. During lifelong follow-up, serum WHV DNA levels fluctuated from 10 to 100 vge/mL, with rare transient increases up to 10^3 vge/mL. The stealth nature of this silent infection is exemplified by time periods when WHV DNA was not detectable in serum but was in the liver and/or PBMC obtained at the time of serum collection. WHV DNA loads in the liver, PBMC, and lymphatic organs (i.e. bone marrow, spleen) were between 0.02 to 200 vge/10^4 liver cells and 0.005 to 0.5 vge/10^4 lymphoid cells. Importantly, WHV RNA was detected in serial liver biopsy and PBMC samples up to the end of life, indicating protracted virus replication. The ultimate evidence for productive replication and assembly of infectious virions long after recovery from a self-limited episode of AH was provided by administration of PBMC-derived inocula (either culture supernatant from *ex vivo* mitogen-stimulated PBMC or viable non-stimulated PBMC) to virus-naïve woodchucks, which caused AH capable of advancing to CH and HCC in some animals.[3] Serological markers of WHV infection (i.e. immunovirological indicators detectable in serum) were also monitored over the lifetime and while WHsAg was consistently negative, antibodies to WHV core antigen (anti-WHc; an equivalent of anti-HBc in HBV infection) coincided with WHV DNA detection after an acute episode of hepatitis. This form of WHV DNA-positive but serum WHsAg-negative and anti-WHc reactive infection that continued indefinitely after resolution of an episode of symptomatic infection was subsequently designated as SOI (Table 1).[3,33–35]

In addition to molecular detection of WHV, histological examination of serial liver biopsies collected over the lifetime indicated intermittent minimal-to-mild inflammation with periods of normal or nearly normal liver morphology. However, typical HCC, confirmed by histological examination, ultimately developed in some (~20%) animals with SOI (Table 1).[3] The results were startling since the data clearly showed that convalescence from a typical episode of AH does not prevent development of HCC. It was also found that the virus retained in the lymphoid cells after resolution of AH caused classical, serologically and histologically evident AH when transmitted to WHV-naïve woodchucks and that this advanced to CH and HCC in some animals.[3] It was concluded that the virus persisting during SOI remained infectious and liver pathogenic and retained its oncogenic potential. Thus, this study not only confirmed the existence of persistent occult hepadnaviral infection but

Table 1. Characteristics of primary and secondary occult hepadnavirus infection in the woodchuck model of hepatitis B

	Primary occult infection (POI) *Primary infection with <10^3 WHV virions*	Secondary occult infection (SOI) *Residual infection continuing after resolution of hepatitis or subclinical serologically evident infection*
Serology:		
WHsAg	Neg	Neg
anti-WHc	Neg	Pos
anti-WHs	Neg	Pos or Neg
WHV DNA load:		
Serum	<10^2 vge/mL	<10^2 vge/mL
PBMC	<10^3 vge/μg DNA	<10^3 vge/μg DNA
Liver	Neg → Pos	<10^3 vge/μg DNA
WHV-specific T cell response	Yes	Yes
WHV-specific B cell response	No	Yes
Longevity of persistence	Lifelong	Lifelong
Infectivity/transmissibility	Yes	Yes
Susceptibility to re-infection with WHV dose >10^3 virions	Yes	No
Spectrum of organs involved:		
Lymphatic system	Yes	Yes
Liver	No → Yes with time	Yes
Liver histology	Normal	Intermittent minimal to moderate inflammation with periods without alterations
HCC development	~20%	~20%

WHsAg, WHV surface (envelope) antigen; anti-WHc, antibodies to WHV core (nucleocapsid) antigen; anti-WHs, antibody to WHsAg; HCC, hepatocellular carcinoma; pos, positive; neg, negative; vge, virus genome equivalent.

also identified the natural history, possible pathological outcomes (i.e. HCC), and general virological characteristics of SOI. These findings provided a solid groundwork for further studies on virological and immunological properties of OBI, approaches to its diagnosis, and pathogenic relevance.

The presence of anticore antibodies alone indicates SOI

The common hallmark of an exposure to hepadnavirus is the presence of antibodies to hepadnavirus nucleocapsid (core) antigen (except WHV POI, see below and Table 1). In the case of WHV, the presence of anti-WHc in the absence of detectable serum WHsAg is a lifelong consequence of recovery from a self-limited episode of hepatitis, as summarized above. Regarding HBV infection, anti-HBc alone can be encountered in a window before the appearance of serum anti-HBs, after resolution of hepatitis B, or following a serologically silent (i.e. serum HBsAg-negative) exposure to HBV. However, the virological significance of this finding in regard to virus replication status and virus persistence was uncertain.[16,36–38] To investigate these issues, two groups of woodchucks were selected from animals injected intravenously (i.v.) with 10^{10} DNase-digestion-protected WHV virions of the same or very closely related inoculum. One group (n=6) developed primary serum WHsAg-negative infection not detectable by a sensitive enzyme-linked immunosorbent assay (ELISA), while the other group (n=7) developed a transient episode of typical, serum WHsAg-positive AH.[39] The origin of this divergent pattern was unclear but was most likely related to host milieu than to the virus itself, since the same inoculum caused both infection outcomes. In this regard, a swift and more effective innate response, a lower susceptibility of some animals to WHV infection, or a masking of low levels of circulating WHsAg by anti-WHs could be possible underlying factors. The woodchucks were followed for 11 to 64 months after virus inoculation.[39] The results indicated that the detection of anti-WHc in the absence of WHsAg was concomitant with the detection of WHV DNA in the serum, liver, and PBMC. Moreover, serial liver biopsies and PBMC collected during the WHsAg-nonreactive period in both groups demonstrated WHV replication intermediates, i.e. covalently closed circular DNA (cccDNA) and mRNA. In addition, electron microscopy on ultracentrifugated anti-WHc-positive sera from woodchucks, which were serum WHsAg-negative by ELISA, revealed singular 22 to 24 nm spherical and short tubular WHsAg particles. These findings demonstrated that the persistence of anti-WHc in the absence of conventionally detectable WHsAg in serum is an excellent surrogate indicator of ongoing low-level WHV infection. These findings were in line with other evaluations that demonstrated that the WHV residing within lymphoid cells of woodchucks with the anti-WHc alone profile was infectious to WHV-naïve animals.[3,39] Occult WHV infection persisting after a self-limiting episode of AH was also accompanied by T lymphocyte responses directed to different WHV antigenic epitopes that endure for very long time, if not for life (unpublished data).[40] This type of persistent T cell reactivity also existed in individuals with a past history of self-limited AH type B.[34,41–43]

Transmission of WHV from mothers with SOI to their offspring

The existence of lifelong SOI raised the question whether virus persisting in this form of infection is transmissible from mothers to the litter. Woodchuck offspring born to dams, who resolved experimentally induced AH, developed antibodies to WHV surface antigen (anti-WHs) and established SOI, were followed for up to 3.5 years after birth and investigated for molecular, serological, and histological evidence of WHV infection.[4] The data showed that all offspring (n=11) examined remained serum WHsAg and anti-WHc nonreactive as well as anti-WHs negative. However, all of them carried trace amounts of WHV DNA in sera and PBMC throughout the entire observation period. Importantly, WHV cccDNA and mRNA, indicative of active replication, were detected in PBMC and in organs of the immune system. The ability of immune cells to produce infectious WHV was confirmed by i.v. injection of culture supernatants from cells stimulated with a mitogen (lipopolysaccharide) to virus-naïve woodchucks. The injected animals developed typical serum WHsAg-reactive AH.[4]

However, serial liver biopsies obtained from some (n=4) of the offspring were, surprisingly, completely WHV negative, despite extensive testing by highly sensitive PCR/NAH assays with primers specific for different virus genes.[4] Thus, in these animals, the virus was solely expressed and actively replicating, albeit at very low levels, in the PBMC and cells in the lymphatic organs. In another case, liver biopsies collected for up to 19 months after birth were WHV DNA negative, but subsequent biopsies were WHV DNA and cccDNA reactive. In the remaining animals (n=6), all liver biopsies collected from 6 months after birth until the end of follow-up were WHV DNA positive. Histological examination showed normal hepatic tissue morphology in all offspring, except in those that were challenged with WHV doses known to be large enough to cause hepatitis (see below). The persistence of intact virions in the offspring studied, including those with liver WHV-negative infection, was confirmed by testing the physicochemical properties of circulating WHV DNA-reactive particles, with DNase digestion-protection assay, and by infection of virus-naive woodchucks with pools of serum and plasma from the offspring after their concentration by ultracentrifugation.

The above findings clearly showed that hepadnavirus was transmitted from mothers with SOI to their newborns, even though the mothers carried otherwise protective anti-WHs. The study also discovered the existence of a molecularly evident, serologically negative (i.e. WHsAg and anti-WHc nonreactive) persistent WHV infection. The data showed that WHV can exclusively persist in the cells of the immune system without engaging the liver, suggesting that the virus at low doses is preferentially, if not exclusively, lymphotropic. This also demonstrated the truly stealth nature of hepadnaviral infection, as the initiation of the liver infection can occur months or even years after the initial exposure to virus. This finding was recently confirmed in a study on the characteristics and long-term pathological consequences of POI (see below).[44]

Moreover, the low levels of persistent WHV in these offspring was not able to induce a protective immune response.[4] Thus, when three of the offspring were challenged with a large dose of WHV (also called, liver pathogenic dose; see below), they became serum WHsAg and anti-WHc positive and developed classical AH. This was clearly distinct from animals with SOI, which were protected against such challenge. This form of occult infection was later designated as POI (Table 1).

POI is caused by very small amounts of WHV

Interestingly, WHV in the offspring born to dams with SOI was solely confined to the lymphatic system in about 40% of offspring. This intriguing fact initiated a series of experiments to determine how this may happen and to develop a reproducible model for future investigations. Two approaches were used to study the nature of viral requirements that lead to the establishment of POI. The first was directed at revealing whether or not naturally acquired neonatal POI limited to the lymphatic system can be transferred to adult, immunocompetent woodchucks and, if so, whether the infection retained its molecular and serological characteristics through the passage. The second approach focused on establishing POI in adult woodchucks using a wild-type WHV as inoculum and examined how POI affects the host's susceptibility to and/or recovery from challenge with a dose of WHV that was large enough to cause hepatitis.

Serial passage of POI established after vertical transmission

Serum samples collected from an offspring that acquired POI from a mother with SOI were pooled and concentrated by ultracentrifugation, and the resulting pellet was i.v. injected into two healthy, adult woodchucks.[34] From one of these two woodchucks, the pellet recovered from concentrated plasma and isolated splenocytes were then separately i.v. administered to two other healthy adult animals.[34] All four of these animals developed serologically silent (i.e. serum WHsAg and anti-WHc negative) but WHV DNA positive infection that was restricted to the lymphatic system. However, approximately 5 months after inoculation, low levels of WHV DNA was evident in the liver of two of the animals.[34] In one case, liver histology showed minimal lymphomononuclear cell infiltrations and moderate proliferation of bile ducts, signifying marginal inflammation. WHV cccDNA was detected in the spleen and bone marrow, albeit at low levels, confirming virus replication within the immune system. Partial sequence analysis of WHV DNA isolated from the spleen and bone marrow of the woodchuck that received splenocytes from an animal infected with serum from the offspring with neonatal POI did not identify any unique mutations. Thus, the sequence analyzed was identical to that of WHV used to infect the mother of the offspring.[34] Furthermore, WHV challenge of the adult animals with POI with a large dose ($\sim 10^{10}$ virions) of the same virus that was used to inoculate the mother caused typical, serum WHsAg-positive, self-limited AH, indicating again that POI did not provide immune protection against WHV. Thus, this finding was identical to that identified in offspring born to mothers with SOI.[4] These results unequivocally proved that POI is an experimentally demonstrable entity that can be serially transmitted between immunocompetent hosts. Moreover, they confirmed that the virus persisting during POI maintained its molecular and virological properties and displayed preferable tropism towards cells of the immune system.

POI is initiated by WHV doses smaller than 1000 virions

To further characterize the viral requirements initiating POI, WHV-naïve, adult woodchucks were i.v. injected with titrated doses of WHV ranging from 10 to 10^7 DNase-digestion-protected virions that carried wild-type WHV DNA, as confirmed by sequencing.[34] The results showed that WHV doses equal to or less than 10^3 virions induced POI, while those greater than 10^3 virions caused serum WHsAg-positive infection coinciding with hepatitis. It was also noticed that WHV at amounts of 10^3 virions can cause either POI or serologically evident infection and AH, suggesting that the outcome of infection at this particular virus dose was host dependent.[34]

As previously shown, WHV replication was restricted to the cells of the lymphatic system in animals that developed POI, as confirmed by detection of WHV cccDNA in the bone marrow, isolated splenocytes, and PBMC.[34] Moreover, the WHV DNA sequences within the splenocytes and plasma in animals with POI were identical to that of the initial inoculum, indicating that the dose of the virus but not the existence of viral variants was responsible for the induction of POI. Similar to the woodchucks that developed POI after a naturally acquired neonatal infection, animals with experimentally induced POI were not protected from challenge with a large, liver pathogenic WHV dose, and they developed serum WHsAg-positive AH. Taken as a whole, this study identified virological requirements responsible for the commencement of POI. WHV quantities below or above 10^3 virions were found to be pivotal in determining whether POI or serologically overt infection followed by SOI was induced, respectively. Liver nonpathogenic doses consistently caused, at least initially, infection of the immune system. These findings were confirmed by using inocula derived from different animals with serum WHsAg-positive CH when low amounts of WHV (i.e. $<10^3$ virions) were i.v. injected.[44–46] Because of the considerable similarities between WHV and HBV, including the capability of HBV to infect the immune system,[16,47–49] these data justify the further investigation of infections caused by trace amounts of HBV in humans. Nonetheless, it should be noted that our findings in woodchucks with POI were in contrast to subsequent data indicating that one virion of HBV derived from an HBV transgenic mouse was able to cause serologically detectable chronic hepatitis in a chimpanzee.[50] Differences in the liver pathogenic potency between a single HBV isolate from a transgenic mice and authentic, intact WHV might explain this discrepancy.

Repeated exposure to small amounts of WHV does not culminate in hepatitis

We aimed to determine if multiple small amounts of WHV causing POI could be additive over time and result in serologically evident infection coinciding with hepatitis. To test this possibility, WHV-naïve, adult woodchucks were i.v. injected 12 times with a dose of 100 virions over two rounds of six injections given at weekly intervals.[46] Approximately 3 months after the last injection, the woodchucks were challenged with a liver pathogenic dose (1.1×10^{10} virions) of the same WHV inoculum and followed for an additional 6 months. Detailed examination of serological and molecular markers of WHV infection, histology of serial liver biopsies, and WHV-specific and generalized (mitogen-induced) T cell responses were performed. The results showed that multiple exposures did not result in serologically detectable infection (i.e. WHsAg and anti-WHc positive) or hepatitis, even though the cumulative dose was above the threshold known to induce WHV hepatitis (i.e. $>10^3$ virions). Nonetheless, the infection was accompanied by clearly identifiable WHV-specific T cell response that occurred, characteristically, in the absence of anti-WHc or anti-WHs antibodies, i.e. virus-specific B cell response.[40,45,46] As expected, the woodchucks were not protected against challenge with a liver pathogenic WHV dose. It was concluded that the WHV-specific T cell response is a very sensitive indicator of occult hepadnaviral persistence, even when this response occurred in the absence of anti-WHc. A similar finding was reported for humans.[43]

As indicated, the disparity between the presence of hepadnavirus-specific T cell reactivity and antivirus antibodies was associated with the lack of protection against infection with liver-pathogenic doses of WHV (i.e. $>10^3$ virions). This finding is consistent with data from studies on other asymptomatic viral infections, such as hepatitis C virus (HCV), simian immunodeficiency virus (SIV), and human immunodeficiency virus type 1 (HIV).[51–56]

Repeated contacts with small amounts of HBV may occur in both occupational and familial settings, such as close contacts of infected individuals, primary healthcare and social service providers, and in i.v. drug users.[18,22,23,36,41] The results from woodchucks summarized above suggest that trace amounts of HBV that infect unvaccinated individuals will unlikely cause serologically detectable infection (i.e. HBsAg and/or anti-HBc positive) or clinically evident hepatitis B but may lead to the establishment of POI and the development of HBV-specific T lymphocyte responses.

Pathological significance of lifelong WHV POI

The liver pathogenic relevance of hepadnaviral infection enduring as POI was investigated in a prospective study of woodchucks inoculated with WHV doses known to cause POI. These animals were followed throughout their natural lifespan or until they were challenged with a liver pathogenic dose of WHV. Thus, POI was induced by i.v. injection with 10 or 100 virions from two different WHV inocula.[44] As expected, all woodchucks (n=10) developed POI restricted to the lymphatic system.[44] Serum WHV DNA loads detected throughout the life of these animals were usually between <100 and 200 vge/mL, and the levels in PBMC were $<10^3$ vge/μg of cellular DNA. It was also found that after 2.5 to 3.3 years of infection, WHV DNA, mRNA, and/or cccDNA became detectable in the liver of these animals. Although the virus finally engaged the liver, liver histology remained normal throughout follow-up thereafter.[44] The engagement of the liver was preceded by a temporal increase in serum WHV load to $>1\times10^3$ vge/mL. As previously documented, this threshold defines whether occult or overt WHV infection will develop.[9,34]

The most notable finding of the study was that 20% of the woodchucks with POI developed multinodular HCC, as confirmed by histological examination. HCC developed 55 months after injection of 100 virions. Thus, the study documented that the virus persisting during POI maintained its oncogenic potential, and this highlighted the pathological importance of this seemingly innocuous form of hepadnaviral infection. In this context, it has been shown that seronegative, HBV DNA-reactive infection occurs in HBV endemic regions at rates of up to 7.6%.[57,58] This rate is most likely

underestimated due to infrequent testing for HBV DNA in asymptomatic individuals and the relatively low sensitivity of the commonly used HBV DNA detection assays. The results from the above study in woodchucks suggest that the role of HBV POI in the pathogenesis of HCC of unknown etiology should be considered and investigated.

Another one of our noteworthy findings was the demonstration of the infectivity and liver pathogenic properties of virus that persisted during POI. Hence, WHV retrieved after ultracentrifugation of pools of serum and plasma samples collected from either the liver WHV-negative or the liver WHV-positive phases of POI was i.v. injected to healthy woodchucks (n=5).[44] All animals established serum WHsAg-positive, anti-WHc-reactive infection with minimal to moderate hepatitis. Interestingly, three of the woodchucks developed HCC within 4.5 to 35 months post-infection, and two of the animals were injected with virus recovered from the liver WHV-negative phase of POI. In addition, when WHV DNA sequences identified in the liver virus-negative or the liver virus-positive phases of POI were compared to each other and to the sequence of WHV in inoculum used for induction of POI in these animals, the predicted amino acid sequences were essentially identical, indicating that a cell type-specific variant was not likely responsible for the initiation of the liver virus-positive phase of POI. Furthermore, WHV sequences from the liver-virus negative POI phase retained the same sequence in serum and liver when injected to healthy animals, but unique nonsynonymous variants were detected within PBMC. This may imply that WHV propagated more robustly in the immune cells than in the liver in this situation. Overall, the results revealed that WHV, regardless of whether it was retrieved from the liver WHV-negative or the liver WHV-positive phase of POI, initiated infection involving both the liver and the lymphatic system. This confirmed that the virus tropism to hepatocytes and immune cells is a natural property of the authentic, wild-type virus and not a consequence of a virus variant uniquely predisposed to infect a particular cell type. This result is in accordance with the *in vitro* infection study where WHV was subjected to serial passage in either woodchuck hepatocytes or lymphoid cells without the development of cell type-specific virus variants or changing virus *in vivo* infectivity.[59]

Occult infection coincides with WHV DNA integration into the host genome

Random integrations of HBV DNA into the liver genome have been well documented in advanced chronic hepatitis B and related HCC.[60,61] HBV genome integrations were also identified in the PBMC of patients with chronic hepatitis B.[49] In contrast, HBV DNA integrations in OBI have been rarely examined. However, there is some evidence that HBV DNA integrates into HCC and nonHCC liver DNA in HBsAg-negative patients regardless of anti-HBc detection.[60–65]

WHV DNA insertions have been found in HCC caused by WHV during either CH or SOI.[14,66] In WHV-induced CH, virus-host junctions have been most often detected near *myc* proto-oncogenes.[67–69] In regard to WHV POI, we recently identified multiple WHV DNA-host genome junctions in the liver and lymphatic organs, including bone marrow, spleen, and lymph nodes, using inverse-PCR designed to specifically detect WHV X gene or WHV preS region integrations.[44] WHV-host genome junctions were found whether HCC had developed or not and were most often between the WHV X gene and various host sequences.[44] This is consistent with the tendency of the HBV X gene to preferably integrate into the human genome.[63,70,71] To date, the integration sites identified in woodchucks with POI-associated HCC have not been located in the proximity of the *myc* oncogene sequence.

The immune system is an unvarying site of WHV infection

The role of the immune system as the reservoir and site of active WHV replication during both symptomatic and occult infections has been clearly shown through studies in the woodchuck model. In addition to highly sensitive, PCR/NAH-based techniques for the detection and quantification of WHV DNA, mRNA, and cccDNA, *in situ* PCR coupled with flow cytometric identification of cells carrying amplified WHV genome signals can be used to efficiently amplify WHV DNA within intact lymphoid cells.[72] This technique allows for the enumeration of WHV DNA-reactive cells. To ensure that the virus DNA was truly located within the cells, DNase-trypsin-DNase treatment to strip the cell surfaces of any potentially attached virions or virus DNA was performed prior to testing by *in situ* PCR. The results showed that a significant proportion of the lymphoid cells were WHV DNA reactive in serum WHsAg-positive AH or CH (3.4% to 20.4%, mean 9.6%). Interestingly, PBMC collected during SOI and POI were WHV DNA-reactive at a similar frequency, ranging from 1.1% to 14.6%, (mean 4.4%).[72] Thus, even though there were differences in terms of serological markers of SOI and POI, there was no difference in the average number of lymphoid cells carrying the virus. This was accompanied by comparable loads of WHV DNA in PBMC from SOI and POI (Table 1).[33–35,39,40,73–75]

Immune responses in occult WHV infections

Virus-specific T and B cell responses

WHV-specific T lymphocyte and antibody responses and profiles of cytokine expression in circulating lymphoid cells and serial liver biopsies were studied in both SOI and POI, including the time periods after challenge with liver pathogenic (>10^3 virions) or nonpathogenic (<10^3 virions) doses of WHV.[33,34,40,45,46,73] Since SOI is accompanied by anti-WHc and frequently by anti-WHs, this indicates that the WHV-specific B cell response remains operational in this form of WHV infection. WHV-specific T cells found during SOI also retained their ability to respond to WHV and its antigens,[40] similar to individuals with a past episode of self-limited AH type B.[36,41,43] The WHV-specific T cell reactivity in SOI is directed against multiple virus epitopes and remains detectable for years after resolution of AH, although it tends to wane over time. Interestingly, WHV-specific T cell responses, which generally arise at approximately 6 weeks post-infection, are preceded by an enhanced capacity of T lymphocytes to proliferate when exposed to nonspecific mitogens. This heightened state of lymphocyte activation occurs immediately after infection and has been shown to coincide with activation-induced apoptotic death of lymphocytes.[73] This generalized activation and lymphocyte death most likely contribute to a delay in the initiation of WHV-specific T cell response at the time when this response is critical for a swift elimination of invading virus.[40,45,46]

With respect to POI, surprisingly, the profiles of virus-specific and generalized (mitogen-induced) T cell proliferative responses to the initial and subsequent exposures to WHV were the same as those identified in animals infected with liver pathogenic doses or in those with SOI.[40,45,46] However, unlike SOI, neither anti-WHc nor anti-WHs were detected during POI, suggesting that infection with very small amounts of virus was insufficient to stimulate and maintain memory B cell response.

Cytokine expression in occult WHV infection

Immediately after exposure to either liver pathogenic or liver nonpathogenic doses of WHV, peripheral lymphocytes displayed a heightened proliferative response to non-specific mitogens (i.e. generalized T cell response) and exhibited similar profiles of enhanced expression of interferon (IFN)-α, interleukin (IL)-12, and IL-2.[40,45,46] Notably, the kinetics of the cytokine responses in circulating lymphoid cells in POI were undistinguishable from those seen during infection caused by liver pathogenic doses of WHV.[40,45,46]

Transcription of cytokines and immune cell marker genes was also quantified in serial liver biopsies collected during POI and after challenge of the animals with POI with a liver pathogenic or a liver nonpathogenic dose of WHV.[40,45,46] In general, the results showed that despite the absence of detectable WHV in the liver during POI, the liver transcription of IFN-α was upregulated, while that of tumor necrosis factor (TNF)-α, IFN-γ, IL-4, or CD3 was unaltered.

Reactivation of occult WHV infection

The clinical relevance of the residual hepadnaviral persistence became even clearer with experiments in woodchucks with SOI that were treated with immunosuppressive agents, such as cyclosporin A (CsA)[76] and dexamethasone (DEX) (Mulrooney-Cousins *et al.,* unpublished data). It was shown that after even short periods of immunosuppression, reappearance of serum WHsAg-positive infection ensued. In one study, woodchucks that seemingly had completely resolved neonatal WHV infection (i.e. cleared detectable serum WHsAg and WHV DNA and developed anti-WHs by 6 months of age) were followed for up to 6.5 years after birth and treated i.p. with CsA once daily for 12 weeks.[76] Following treatment, WHV DNA loads in the serum rose to $\sim 1\times10^5$ to 1×10^9 vge/mL by 4 to 6 weeks after the initiation of treatment in 75% of the animals tested. Also, serum WHsAg became detectable, and the antigen remained positive for about 4 months. An increase in serum levels of alanine aminotransferase (ALT) and sorbitol dehydrogenase (SDH) above normal values was evident in 25% of the woodchucks, indicating a transient liver injury.[3] A study with DEX was performed in woodchucks with SOI lasting for 2-3 years after resolution of experimental AH. Thus, these animals were consistently serum WHV DNA-positive at low levels (10-100 vge/mL), WHsAg-negative, and anti-WHc reactive. They were treated with 2 mg/kg of DEX daily by i.p. injections for 10 days. Serum WHV DNA loads in the treated animals increased up to 2.9×10^5 vge/mL early after treatment initiation. The liver samples collected immediately after the termination of treatment showed a 3-fold increase in WHV DNA load. A brief reappearance of serum WHsAg as well as a transient increase in serum SDH indicative of liver injury were also noticed (Mulrooney-Cousins *et al.,* unpublished data). Taken together, these data indicated that immunosuppressive agents used even for a short period of time may cause the reactivation of occult infection and re-establish hepatitis.

Several drugs and chemotherapeutic agents have been identified that have the ability to reactivate HBV in those who either spontaneously or due to antiviral treatment apparently completely cleared HBV infection.[27–31,77–82] Many case reports and retrospective studies have shown that there are a multitude of clinical outcomes from a very mild episode of hepatitis to fulminant hepatitis, liver failure, and death. The molecular mechanisms of reactivation of HBV infection from the pre-existing occult infection are not yet defined. The well characterized woodchuck models of SOI and POI offer a unique opportunity to fully delineate the pathogenesis of HBV reactivation in OBI, to evaluate whether new chemotherapies and immunosuppressive agents might reactivate OBI, and to determine ways to eradicate HBV persisting at levels not detectable by conventional assays.

Conclusions

The woodchuck model of hepatitis B has significantly contributed to identification of virological and immunological characteristics of occult HBV infection and its long-term pathological outcomes and to the recognition of the natural history and the basic biological properties of hepadnaviral infection in general. Reproducible experimental systems to study both POI (i.e. a serologically unapparent but molecularly evident, asymptomatic infection caused by traces of infectious virus) and SOI (i.e. a residual asymptomatic infection continuing after resolution of a serologically evident infection) were established in eastern North American woodchucks. In addition, the woodchuck model considerably contributed to the recognition of the pivotal role of the immune system in the persistent maintenance of hepadnaviral replication, even in the absence of the liver involvement, and in the production of infectious and liver pathogenic and oncogenic virus. The expertise gained during studies of the woodchuck-WHV model should serve to advance our understanding of other important aspects of OBI, including the mechanisms underlying its reactivation and the recurrence of potentially life-threatening hepatitis, the modes of clinically unapparent virus transmission, and to test the efficacy of novel approaches aimed at eradication of OBI.

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Conflict of interest

None

Author contributions

Writing the paper, selection of references, and preparation of the final version of the paper (PMMC, TIM).

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