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TOPIC HIGHLIGHT

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The woodchuck as an animal model for pathogenesis and therapy of chronic hepatitis B virus infection

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Abstract

This review describes the woodchuck and the woodchuck hepatitis virus (WHV) as an animal model for pathogenesis and therapy of chronic hepatitis B virus (HBV) infection and disease in humans. The establishment of woodchuck breeding colonies, and use of laboratory-reared woodchucks infected with defined WHV inocula, have enhanced our understanding of the virology and immunology of HBV infection and disease pathogenesis, including major sequelae like chronic hepatitis and hepatocellular carcinoma. The role of persistent WHV infection and of viral load on the natural history of infection and disease progression has been firmly established along the way. More recently, the model has shed new light on the role of host immune responses in these natural processes, and on how the immune system of the chronic carrier can be manipulated therapeutically to reduce or delay serious disease sequelae through induction of the recovery phenotype. The woodchuck is an outbred species and is not well defined immunologically due to a limitation of available host markers. However, the recent development of several key host response assays for woodchucks provides experimental opportunities for further mechanistic studies of outcome predictors in neonatal- and adult-acquired infections. Understanding the virological and immunological mechanisms responsible for resolution of self-limited infection, and for the onset and maintenance of chronic infection, will greatly facilitate the development of successful strategies for the therapeutic eradication of established chronic HBV infection. Likewise, the results of drug efficacy and toxicity studies in the chronic carrier woodchucks are predictive for responses of patients chronically infected with HBV. Therefore, chronic WHV carrier woodchucks provide a well-characterized mammalian model for preclinical evaluation of the safety and efficacy of drug candidates, experimental therapeutic vaccines, and immunomodulators for the treatment and prevention of HBV disease sequelae.

Key words: Woodchuck; Woodchuck hepatitis virus; Hepatitis B virus; Neonatal-acquired infection; Adult-acquired infection; Resolution; Chronicity; Humoral immune response; Cellular immune response; Antiviral therapy; Immunotherapy; Combination therapy; Hepatocellular carcinoma

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INTRODUCTION

Infection of adult humans with the hepatitis B virus (HBV) results characteristically in self-limited hepatic disease with recovery based on serological and clinical parameters. Progression to chronic HBV infection occurs infrequently in infected adults, but HBV infections often persist in unvaccinated infants born to HBV-carrier mothers. Chronic HBV infection can lead to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) later in life. Estimates indicate that more than 2 billion people worldwide have serological evidence of previous or current HBV infection, with at least 350 million chronic carriers, and an overall mortality rate from HBV-induced liver disease of 1.2 million deaths per year[1]. Although highly effective vaccines are licensed and have been in use since the early 1980's to prevent HBV infection in neonates and adults, the large reservoir of chronic HBV

carriers currently remaining could benefit immensely from the timely development of effective antiviral and/or immunotherapies that cure the infection or reduce the risk of disease progression.

Evidence from HBV-infected humans, and from animal models of HBV (i.e., HBV-transgenic mice, chimpanzees, pekin ducks, and woodchucks), indicate that the success or failure of humoral and cellular immune responses to the virus determine the initial outcome of acute HBV infection (i.e., as self-limited versus chronic), and that defective responses appear to play a role in the progression of chronic HBV infection (i.e., to chronic hepatitis, cirrhosis, and possibly HCC)[2-10]. Self-limited infections by HBV involving successful immune responses represent by far the more favorable outcome. Chronic HBV infections, where immune responses have failed or are sub-optimal for virus clearance, represent a daunting challenge to successful therapy against a background of continuing disease progression. Current treatment strategies for chronic HBV infection are suboptimal when compared to the curative process observed in self-limited HBV infection. Understanding the prevention and pathogenesis of HBV infection has advanced greatly through clinical studies in humans, and through experimental studies in the chimpanzee model of HBV infection; however, neither of these models is well-suited for the routine testing of therapeutic strategies for treatment of chronic HBV infection.

Woodchuck hepatitis virus (WHV) is a naturally occurring hepadnavirus of the Eastern woodchuck (*Marmota monax*) (Figure 1). WHV was described initially in 1977 at the Penrose Zoo in Philadelphia in a colony of woodchucks where high rates of chronic hepatitis and HCC had been observed[11]. Several strains of WHV have been identified since then, which are all very closely related genetically[12-16], but which may induce differing proportions of chronic infections in neonatal woodchucks[17]. WHV, and another HBV-like virus, the duck hepatitis B virus (DHBV)[18-20] have been used most extensively in the modeling of HBV infection and antiviral therapy (for previous reviews see[21-24]).

Research using the woodchuck began in 1978 and it was developed further into a laboratory model by 1980 when a woodchuck breeding colony was established at Cornell University. Early progress in model development at the Georgetown and Cornell Universities involved: (1) the production and validation of reagents and assays for WHV and for disease markers, (2) the characterization of infectious WHV inocula that induced predictably high rates of chronic infection when inoculated in neonatal woodchucks, and (3) basic studies of the natural history of virologic responses and tumor development associated with experimental infection of neonatal and adult woodchucks. Since 1988, the neonatal chronic WHV infection model has been applied primarily in the testing of antiviral nucleoside analogues for chronic HBV infection (for previous reviews see[10,25-30]).

Early studies in woodchucks also involved the testing of conventional vaccines for the prevention of acute, self-limited WHV infection in neonatal and adult woodchucks[28], and also for prevention of the chronic outcome and HCC in the vaccinated neonates challenged with higher doses of inoculum to enable breakthrough infections[31,32]. Immunomodulation of acute and chronic WHV infections using immunosuppressive drugs, such as cyclosporine A[33,34], was performed to gain an initial understanding of the role of the woodchuck immune response in the outcome and maintenance of WHV infection. The focus of investigations using the woodchuck has ranged widely since 1980, with flexible emphasis on both model development and model application in many areas of HBV research. These included viral and disease pathogenesis, and the prevention and treatment of HBV infection and disease sequelae (including HCC) using vaccines, antiviral drug candidates, and immunomodulators alone and in combination. The purpose of this review is to highlight the woodchuck as an animal model for pathogenesis and therapy of chronic HBV infection.

Figure 1 Eastern woodchuck (Marmota monax).

NATURAL HISTORY OF WHV INFECTION AND DISEASE

Experimental infection of woodchucks with WHV is a well-accepted model for many aspects of the pathogenesis of human HBV infection[7,10,26-29,35-38]. Recent studies of the host response of woodchucks to WHV infection and therapy have revealed numerous parallels to the immunopathogenesis of HBV infection. Certain immune markers in woodchucks cannot be analyzed currently to the same extent as those in mice and in humans. However, the patterns and profiles of those immune responses measured thus far in the woodchuck model are highly consistent with the underlying immunologic mechanisms defined in humans.

Experimental infection of neonatal or adult woodchucks with WHV7P1[17], a well characterized inoculum of WHV, produces predictable proportions of acute, self-limited (i.e., resolved) infections versus chronic infections. This mimics the effects of age on outcome of HBV infection in humans[3,4]. In adult woodchucks, WHV7P1 infections result mainly in resolution, with less than 5% of woodchucks progressing to chronicity[17] (Figure 2).

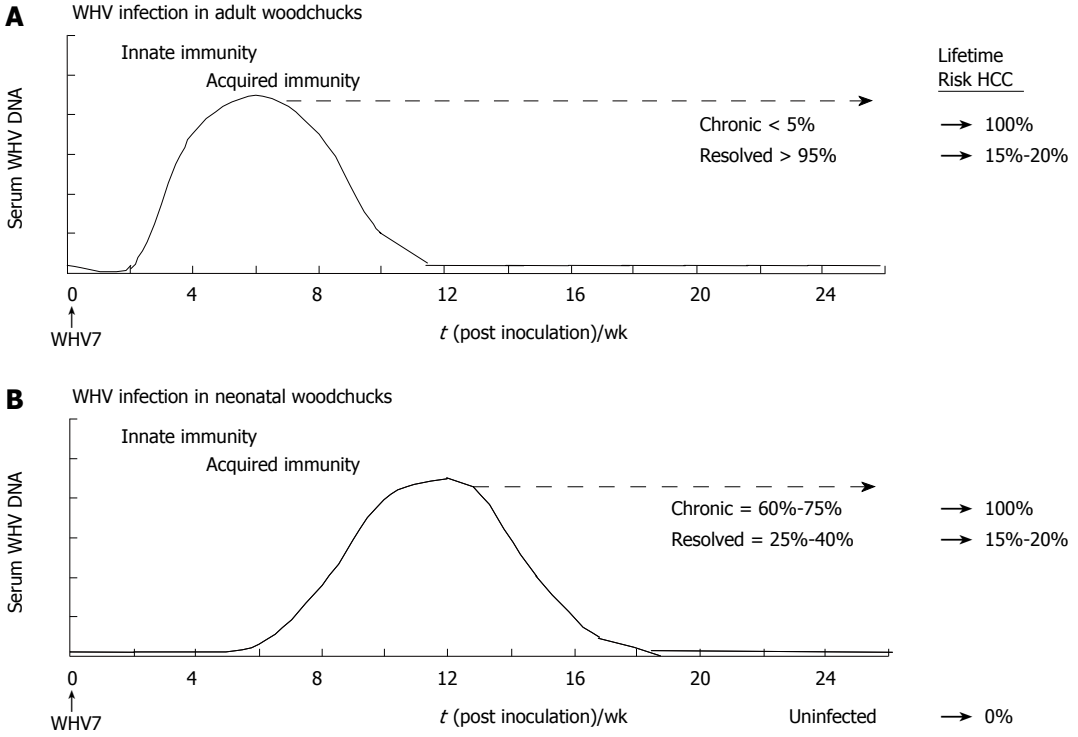

Figure 2 Schematic profiles for serum viremia in adult and neonatal models of experimental WHV infection. **A**: Adult woodchucks. Adult woodchucks born to WHV-negative dams are infected with 1 x 10^7 woodchuck infectious doses 50% of a defined WHV inoculum by the intravenous route. The proportions of chronic and resolved outcomes of adult woodchucks usually are less than 5% and more than 95%, respectively. The lifetime risk for the development of HCC in established chronic and resolved WHV infections is 100% and 15%-20%, respectively; **B**: Neonatal woodchucks. Neonatal woodchucks born to WHV-negative dams are infected with 5 x 10^6 woodchuck infectious doses 50% of a defined WHV inoculum by the subcutaneous route. The proportions of chronic and resolved outcomes range between 60%-75% and 25%-40%, respectively. The lifetime risk for the development of HCC in established chronic and resolved WHV infections is 100% and 15%-20%, respectively. HCC in uninfected, WHV-negative woodchucks is not observed. Approximate time intervals for the development of innate and acquired immunity are shown.

However, transient suppression of cellular immune responses with cyclosporine A (CsA) during the incubation and acute phase of adult WHV infections results in 92% of these infections progressing to the chronic outcome; with CsA given only during the incubation period and very early acute stage (0 to 4 wk post infection), the result is up to 50% chronic outcomes in adult WHV infections[33,34]. This shows the importance and timing of early immune responses in the resolution of acute WHV infection. Experimental immunosuppression, however, does not necessarily mimic natural processes associated with the progression to chronic infection.

Most chronic HBV infections occur as a result of neonatally-acquired infection[39-41]. Experimental infection of neonatal woodchucks with WHV7P1 usually results in a 60%-75% frequency of chronic carriers and a 25%-40% frequency of naturally recovered infections[17] (Figure 2). Viral and host response kinetics are relatively uniform when neonatal woodchucks are inoculated with WHV7P1 in the spring of the year, thus enabling statistical modeling of serologic and hepatic responses using samples collected in successive years. Such features also enable co-temporal comparisons of early acute phase immune responses before the self-limited and chronic outcomes become evident serologically, which can help to differentiate and identify the underlying mechanisms involved in the onset versus maintenance of chronic WHV infection[42-46] (Figure 3).

Chronic WHV infection involves life-long active viral replication and inevitable disease progression to chronic hepatitis and HCC[35,47-50]. In chronically infected woodchucks, there is no naturally occurring e-antigen to anti-e seroconversion and associated step-down of viral replication (i.e., as is commonly seen in chronic HBV infection; e.g.,[51-54]). In general, the high viral replication and high surface antigen and e-antigen loads present in the chronic WHV carrier appear to play a role in the maintenance of immunologic tolerance, and are associated with disease progression to HCC[43-45,55-57].

Self-limited WHV infection involves a relatively complete shut down of viral replication and a nearly complete clearance of virus from the system with full recovery. It has been suggested that trace amounts of residual WHV genomes often detected in long-term recovered woodchucks in liver, serum, and in peripheral blood mononuclear cells (PBMC), could actually represent an alternate form of persistent viral infection[58-65]. Residual HBV DNA has been documented also for humans recovered from self-limited HBV infection (e.g.,[66-71]). In woodchucks, even recovery from acute WHV infection incurs a discernable lifetime risk of HCC (5%-20%) when compared to control seronegative woodchucks (i.e., uninfected with WHV); however, this risk is significantly lower compared to the lifetime risk of HCC in chronic

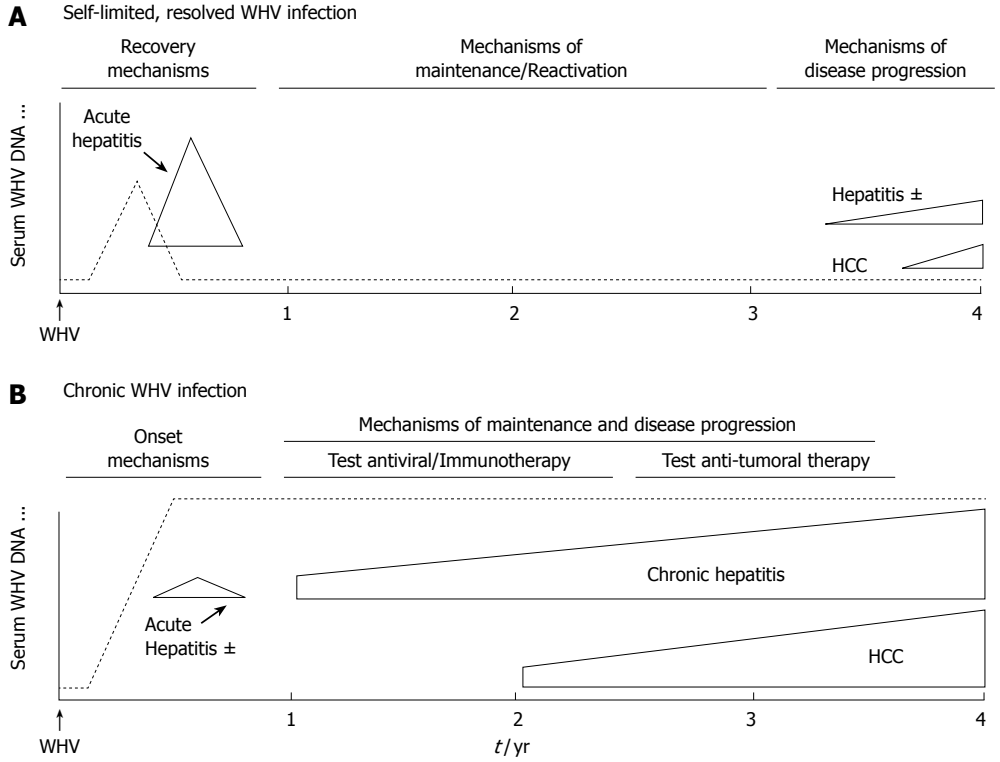

Figure 3 Schematic profiles for viremia, acute hepatitis, and disease progression in the neonatal model of experimental WHV infection. **A**: Self-limited, resolved WHV infection; **B**: Chronic WHV infection. Neonatal woodchucks born to WHV-negative dams are infected experimentally at 3 d of age with 5 x 10^6 woodchuck infectious doses 50% of a defined WHV inoculum by the subcutaneous route. Approximate time intervals for self-limited acute hepatitis and progressive chronic hepatitis are shown. Use of the neonatal WHV infection model enables co-temporal comparisons of acute self-limiting and chronic outcome of WHV infections as they develop. Comparison of acute phase events at early time points before or near the times when self-limited, resolved and chronic outcomes begin to segregate based on serologic criteria allows the definition of immune mechanisms involved in progression toward recovery versus chronicity. Comparison at later time points enables the investigation of mechanisms that are important in the maintenance of the established WHV infection state and that lead to disease progression and tumor development. During this time the established chronic WHV carrier woodchucks are used mainly for the testing of antiviral nucleosides, immunotherapeutic strategies, and for the prevention of onset and development of HCC.

WHV carrier woodchucks, which is essentially 100%[58,72] (Figure 2). Such results provide direct experimental evidence for the carcinogenicity of WHV and, by analogy, for HBV where chronic infection also is associated with HCC.

In a recent study, we examined the reactivation of WHV replication and the generation of infectious WHV in long-term resolved adult woodchucks during experimental immunosuppression with CsA (Menne *et al*, unpublished data). Administration of CsA to serologically recovered woodchucks with evidence of residual WHV DNA in liver and PBMC, and with durable recall cellular immune responses of PBMC to WHV antigens, resulted in a transient reactivation of WHV replication during CsA treatment. This supports the idea that replication-competent WHV (and by analogy, HBV) can persist for many years after recovery from acute viral hepatitis, possibly as part of a continuing process. That is, the virus may be controlled by virus-specific immune responses that are primed continuously by trace amounts of virus and viral antigens. In any case, the presence of long-term recall cellular immune responses with mutual persistence of residual WHV covalently closed circular DNA (WHV cccDNA) is significant to the durability of recovery responses over the long term for the stable control of replication and shut down of the infectious process. The apparent lack (or need) of such immune responses with the apparent loss of WHV cccDNA is significant to the extent of viral immune clearance possible in recovery. One implication from the above studies is how much a relatively successful antiviral and/or immunotherapy for chronic HBV or WHV infection will improve the prospects for disease outcome beyond that observed in natural recovery from infection.

MOLECULAR VIROLOGY STUDIES

WHV is classified as a member of the genus *Orthohepadnavirus*, family Hepadnaviridae[73]. The genetic organization of WHV is similar to that of HBV and other mammalian hepadnaviruses, and their biological properties and replicative strategies are essentially the same[74]. Filaments and spherical particles are found in the serum of WHV-infected woodchucks which are composed of the envelope protein of the virus. Complete virions are 42 to 45 nm in diameter and are composed of an exterior envelope protein (WHV surface antigen; WHsAg), an inner nucleocapsid or core protein (WHcAg), and, within the nucleocapsid, the DNA genome[75,76]. The replicative cycle of WHV seems to be identical to that of HBV[75-78]. The role of cccDNA as the template for viral transcription, the mechanism of replenishment of the cccDNA pool,

and the control of this pathway by surface antigen, have been investigated mainly using DHBV. For some studies, full-length clones of the WHV genome, cut and ligated to form a supercoiled cccDNA, have been used for *in vivo* molecular studies since direct injection into the hepatic parenchyma of woodchucks results in productive WHV infection[79]. Only a brief overview is provided below for background purposes.

During infection, HBV enters the hepatocyte, but the mechanism is poorly understood. No hepatocyte receptor has yet been defined for HBV, although studies suggest that the virus-cell recognition may be mediated all or in part by specific sequences located in the pre-S1 region of the large envelope protein. However, with numerous other potential envelope recognition sites for the cell suggested from *in vivo* neutralization studies with monoclonal antibodies, and the fact that antibodies elicited by vaccines to only the small envelope protein provide protective immunity, we are a long way from understanding the mechanisms of antibody-mediated neutralization of HBV attachment, entry, and uncoating during infection. It is known that the circular, partially double-stranded DNA genome makes its way to the nucleus where the partial DNA strand (i.e., positive strand) is completed via the endogenously linked virion reverse transcriptase-DNA polymerase, and the now fully circularized double strand is then ligated into a cccDNA. The cccDNA serves as the key template for viral mRNA transcription via the cellular RNA polymerase Ⅱ. One of the viral mRNAs (slightly larger than the genome length transcript) becomes encapsidated into maturing core particles along with the virion polymerase, where it is then reverse transcribed into the viral negative strand DNA *via* the RNA-dependent DNA polymerase activity of the encapsidated enzyme. The viral polymerase then uses its DNA-dependent DNA polymerase activity to partially complete the positive strand DNA to about 50%-75%, and this non-covalently closed circularized DNA is found in mature virions of HBV and WHV. Envelope acquisition occurs at the endoplasmic reticulum (ER) and mature virions are secreted from hepatocytes. Hepadnaviruses are not directly cytotoxic to infected cells.

Amplification and replenishment of cccDNA in the nucleus of the infected hepatocyte occurs when a portion of the maturing core particles complete positive strand DNA synthesis and are cycled back to the nucleus (i.e., instead of through the ER) where the new double strand DNA is processed into cccDNA. In HBV, most immunostaining of core is found in the nucleus, whereas in WHV, the core staining is primarily cytoplasmic, and not detected in the nucleus. This suggests a process of newly synthesized cytoplasmic core particles carrying out reverse transcription, partial or complete positive strand synthesis, and occasional re-entry into the nucleus for amplification of cccDNA (alternatively, cytoplasmic core staining may reflect incoming virus, but this seems far less likely). For HBV, cytoplasmic cores may go undetectable by immunostaining, and the denser staining of core particles within the nucleus may reflect maturation of HBV core particles there, with exit to the ER for envelope acquisition via a different cellular pathway. In established carrier woodchucks, WHV virions often circulate in 10- to 100-fold greater concentrations than do HBV virions in human chronic carriers. This may relate to the differential immuno-localization of core particles in the two models.

Transition of viral DNA to RNA during the life cycle of WHV has similarities to that of retroviruses[75,78], but integration of viral DNA into the host genome is not, however, essential for replication of hepadnaviruses, as is the case with retroviruses. Persistence of episomal cccDNA in infected hepatocytes is considered stable and this is problematic for its removal from the system, which appears to require elimination of the infected hepatocyte. It therefore represents the main target for attaining complete eradication of hepadnavirus from the system. When hepadnaviral DNA does integrate into host cell DNA, it is usually truncated and rearranged, and can target any number of sites in cellular DNA[80,81], some or all of which may be important in hepatocarcinogenesis. Morphological and molecular virological studies of the liver have shown that virtually 100% of hepatocytes become infected after experimental WHV infection[82]. Although replicative forms are cleared rapidly during recovery, WHV cccDNA persisted in a certain proportion of woodchucks long after evidence of WHV replication had ceased. That said, recovery is indeed durable and protective against disease progression in the vast majority of cases. On the other hand, persistence of the episomal cccDNA in chronic HBV (and WHV) infections remains a major conundrum in attempts to clear the virus via various therapeutic approaches (see below).

HBV generally is considered a hepatotropic virus, but hepadnavirus DNA can also be detected in extrahepatic tissues. For example, DHBV is often found replicating in the pancreas of ducks. HBV and WHV appear to infect the lymphatic system, although the exact significance of this observation is not well understood[58,60-63,65,83-86]. Some studies suggest that WHV replication and spread in the lymphatic compartment can proceed independently, even before infection of the liver[60,86]. Quiescent (non-replicating) WHV DNA molecules in PBMC from chronic WHV carriers can be activated to form replicative intermediates by stimulation of PBMC with lipopolysaccharide (LPS)[62]. The cell-free supernatants from LPS-stimulated WHV carrier PBMC (but not those from the unstimulated carrier PBMC) contain newly replicated infectious WHV that induce-acute hepatitis in WHV-susceptible adult woodchucks[84].

WHV quiescence versus replication in the lymphatic compartment may vary depending on the state of the host lymphatic target cell (i.e., resting, dividing, circulating in blood, within lymphatic tissue, *etc.*). WHV DNA can be detected in bone marrow cells as early as one month post neonatal WHV infection, but the first signs of WHV replication in PBMC, lymph nodes, and spleens occur during the acute stage of hepatic infection[83]. During recovery lymphatic WHV replication subsides to a quiescent state (or approaches complete elimination). In chronic infections, WHV replication also becomes quiescent in circulating PBMC, but often continues in the

spleen[83], and the quiescent WHV in PBMC often can be activated upon *ex vivo* stimulation using LPS, as indicated above[62,84]. More recent published studies indicate that long-term recovered woodchucks can also harbor infectious DNA in PBMC[58].

From the above, woodchucks recovering from acute WHV infection and those progressing to chronicity seem to have similar PBMC infection profiles, and in both cases the PBMC respond robustly in proliferation assays to polyclonal mitogens such as ConA, PHA, and LPS[44,55,87-89]. Even with similar PBMC WHV DNA profiles, the PBMC proliferative responses to viral antigens are generally more robust in the recovery outcome compared to the chronic outcome[44,55,56,87-90]. Thus, immune response function in viral infection does not appear to be affected adversely by the ongoing lymphatic infection. In fact, lymphatic infection by WHV, either acutely or in chronic WHV carriers, does not result in any lymphadenopathy, lymphopenia, lymphoma, or generalized immunodeficiency enabling opportunistic infections. As with natural recovery, therapy of chronic WHV infection presumes to target all reservoirs and molecular forms of the virus in both the lymphatic system and liver.

IMMUNOLOGICAL STUDIES

Resolution of experimental WHV infection in both neonatal and adult woodchucks involves a self-curative process with appropriate virus-specific immune responses in the periphery and liver (Figure 3). Natural recovery perhaps represents a benchmark for the possible induction of antiviral and/or immunotherapeutic effects in chronic WHV carriers. Specific activation of humoral and cellular immune responses is a prerequisite for viral clearance during acute HBV infection in adult patients, as reported in numerous studies[2-4,9,91]. However, the kinetic development of these responses during the early incubation and acute stages of adult HBV infection, and their influence on the course and outcome of infection, are less well characterized in humans, since patients usually do not present with clinical symptoms immediately after HBV transmission (except for a few rare cases involving known exposure times; e.g.,[92]).

Studies of self-limited WHV infection reveal numerous virus and host response patterns analogous to self-limited HBV infection[61,79,82,83,85,88,89,93-105]. In general, resolution of WHV infection in both the neonatal and adult settings is characterized by: (1) a transient peak of WHV DNA and antigen detection in serum and liver during the acute phase of infection, (2) timely and appropriate cell-mediated immunity (CMI) to viral antigens, (3) acute viral hepatitis with limited liver injury, (4) a transient peak and subsequent normalization of serum aminotransferases, and (5) seroconversion to virus-neutralizing antibodies, all leading to a substantial clearance of virus and viral antigens from the blood and liver. The humoral immune response to viral antigens (i.e., WHcAg and WHsAg) during resolution is associated with the development of robust titers of anti-core (anti-WHc), and of virus-neutralizing, protective, anti-surface antibodies (anti-WHs) with the onset and waning of the acute phase, all usually within several weeks after experimental infection[17,42].

In adult woodchucks, the CMI associated with recovery is characterized by activation of PBMC detected by *in vitro* stimulation of PBMC with WHsAg, WHcAg, and synthetic peptides of both antigens[87-90,93,94,106]. The successful PBMC response to WHcAg is associated co-temporally with viral clearance from serum, and this has been mapped extensively to several key epitopes of the WHcAg[87,93]. In fact, immunization with the dominant WHcAg epitope sequence between amino acids 97 to 110 significantly dampens acute WHV infections in adult woodchucks following experimental challenge with WHV, when compared to infections in unvaccinated control woodchucks[87]. Mapping of the PBMC responses to WHsAg and WHV x antigen (WHxAg) during the acute phase of resolution have been in progress.

The CMI to viral antigens during the acute phase in neonatal woodchucks experimentally infected with cWHV8P1 (from which neonates resolve more frequently) is similar to that in resolving adult woodchucks, which is independent of the WHV inoculum used[44]. Robust PBMC responses to WHcAg, WHsAg, and WHxAg, and to several non-overlapping core peptides, are associated temporally with the clearance of WHV DNA and WHsAg from serum. Detailed analysis of the WHcAg-specific PBMC responses revealed a broad recognition of several WHcAg epitopes representing apparently distinct regions of this antigen. Similar to adult WHV infections, neonatal woodchucks develop PBMC responses to important WHcAg peptides (residues 97 to 110, residues 100-113)[44].

In the liver, the CMI during resolution of adult WHV infection is characterized by moderate to marked hepatic inflammation and liver injury involving increased CD3- (cluster of differentiation 3) positive T lymphocyte accumulation, and apoptosis and regeneration of hepatocytes[96,97]. These events are accompanied by marked elevations of CD3, CD4, and CD8 mRNA expression and increased expression of the T-helper lymphocyte (Th)-type 1 cytokine mRNAs interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), and of the IFN-γ -inducible oligoadenylate synthetase (2'-5'-OAS) mRNA[96,97]. *In vitro* testing of cell-mediated killing of hepatocytes revealed activation of both FasL- (ligand for the apoptosis-inducing factor Fas) and perforin-dependent pathways during resolution, and a comparative analysis demonstrated that acute hepatitis, but not established chronic WHV infection, is associated with elevated hepatocyte killing as a consequence of increased activation of the perforin-dependent pathway[95]. Further acute phase studies of adult self-limiting WHV infections are in progress to better define the kinetic interrelationships between mRNA expression in liver and PBMC mRNA expression *ex vivo* or following *in vitro* stimulation with antigens.

Neonatal WHV infections can be studied prospectively, in proportionate and adequate numbers of woodchucks, as the dichotomy in outcome proceeds dynamically in real time toward recovery versus chronicity. As with recovering WHV infections in adult woodchucks, resolution of

neonatal WHV infection is associated with moderate hepatic inflammation and liver injury and accumulation of CD3-positive T-lymphocytes[42,43]. Hepatic inflammation is characterized further by significant accumulation of CD3, CD4, and CD8 mRNAs, with elevated expression of the Th-type 1 cytokine mRNAs IFN-γ and TNF-α, and of the intracellular transcription factor STAT4 (signal transactivator of transcription) and T-bet (T box expressed in T lymphocytes) mRNAs, and also of Fas ligand and perforin mRNAs. When taken together, the results indicate that both non-cytolytic and cytolytic clearance of WHV-infected hepatocytes is occurring[43,45,46]. The results from the above studies suggest further that early virus-specific CMI in both the periphery and liver play a pivotal role in resolution of acute WHV infection in neonatal and adult woodchucks, and that the responses to WHcAg and to selected core peptides are instrumental in controlling viral infection.

Although immune responsiveness has been well-characterized in the periphery and liver of established HBV chronic carriers during chronic hepatitis, the actual acute phase responses associated with the early onset of chronic HBV infection are less well understood. Lack of immune responsiveness to HBV antigens in some HBV carriers may ensue with the establishment of the carrier state and have little to do with the early onset at a time when other individuals may recover normally. Moreover, chronic hepatitis is defective by definition, when compared with the acute hepatitis that results in recovery, because the chronic inflammation is incapable of clearing the hepatic infection and lends itself only to progressive liver disease. Adult patients presenting with acute hepatitis B are often well into the infection and only rarely progress to chronic infection. Studies of the early onset of chronic HBV infection in humans after neonatal transmission have obvious limitations. While studies in established chronic HBV carriers show defective immune responses associated with tolerance and chronic hepatitis and a failure to clear the infection[2-4,9,91], it is unclear whether such deficient responses are representative of the primary acute phase responses that predispose to the chronic outcome. Understanding how the chronic infection first becomes established kinetically near the time of the acute stage of infection could lead to the identification of important cause-effect relationships that will facilitate the rational development of therapies for successfully treating established chronic infections.

For testing the hypothesis that chronic WHV infection develops due to a diminished host response to acute infection, co-temporal comparisons were performed in the neonatal WHV infection model[7,17,42-46,107-109]. Using a bank of control and WHV-infected liver specimens that were obtained surgically at two acute phase time points of neonatal WHV infection (wk 8 and 14) and that were assigned to recovered or carrier woodchucks once outcome was known based on later serological profiles, the early onset of the chronic WHV carrier state (compared to co-temporal resolving infections) was characterized by: (1) higher acute phase viral loads in liver (at wk 8 and 14 post infection), (2) diminished acute hepatitis (at wk 14), (3) detectable but significantly diminished hepatic inflammation (at wk 14), and (4) reduced liver injury (at wk 14)[42,43,45,46]. This was associated further with: (1) absent or suboptimal intrahepatic accumulation of CD3, CD4, and CD8 mRNAs, and (2) reduced expression of Th-type 1 cytokine mRNAs, especially IFN-γ and TNF-α, along with the key Th-type 1 transcription factor T-bet[43,45,46]. This represented an early primary deficiency in the Th-type 1 response in liver to acute WHV infection, and was not associated with any local antagonistic Th-type 2 immunoregulation[45].

Studies in the peripheral blood using serial measurements of PBMC responses in neonatal woodchucks experimentally infected with WHV7P1 or cWHV8P1 have shown thus far that all neonates with resolving infections had robust acute phase PBMC responses to WHcAg and to the key epitope of this antigen (core residues 97-110), with the majority of woodchucks also responding to WHsAg and WHxAg[44]. In contrast, prospective carriers responded less frequently or not at all, with only about one-third responding to WHcAg, and among these, only about half responded to the key core epitope and to other WHV antigens. Detailed mapping of the PBMC responses to WHcAg revealed that the epitopes recognized were localized to distinct regions of this antigen and were different from those recognized during resolving WHV infections[44]. In the prospective carriers with minimal acute phase PBMC responses to WHcAg, viremia and antigenemia developed later, and viral and antigen loads were lower compared to those seen in prospective carriers without any evidence of virus-specific PBMC responses[44]. In any case the levels of viremia and antigenemia in these prospective carriers were much higher than in neonates with resolving WHV infections[44]. Interestingly, the fact that virus-specific PBMC responses were undetectable in the majority of prospective carriers indicates an early genesis for the CMI defect commonly observed later in established chronic WHV infection[55,56,87-90,94]. Further studies to correlate the molecular immunologic responses of PBMC based on leukocyte surface marker and cytokine mRNA expression with outcome of neonatal WHV infection are in progress.

Established chronic HBV infection is associated with increased viral load and risk of severe liver disease sequelae[2-4,9,91]. Chronic HBV infections resulting from neonatal transmissions are characterized by T cell immunotolerance to viral antigens throughout most of life until end-stage disease, but may exhibit occasional exacerbations of liver disease before this time[51,110-114]. T cell proliferative responses to viral antigens in adult-acquired chronic HBV infections can be variable during disease progression, but are usually less responsive, except during periodic transient flare reactions and with seroconversion to anti-e antibodies (e.g.,[52-54,115,116]).

Studies in woodchucks indicate that viral antigen-specific CMI during established chronic WHV infection is also defective, similar to that observed in HBV infection[45,55,56,87-89,94]. In the neonatal woodchuck, following an occasional, early and transient, but suboptimal acute hepatitis, WHV chronic carriage is characterized for some

time with minimal chronic persistent hepatitis, and little or no liver injury based on serum enzyme markers up through at least 15 mo post infection[46]. This progresses subsequently to more active hepatitis and liver injury just before or at the time of HCC onset and tumor growth[46,47,49,50,55,57]. PBMC remain essentially immunotolerant to WHV antigens throughout all of the chronic phase of neonatal WHV infection, including end stage disease[55,56,87,89,90,94]. The baseline expression of Th-type 1 cytokines in liver that is usually observed during the chronic phase can sometimes increase above normal with progressive chronic hepatitis (usually with increased TNF-α and less IFN-γ)[45,97], but without affecting clearance of the infection. Less is known of the CMI in documented adult-acquired chronic WHV infections, but the apparent greater degree of chronic hepatitis in this setting may suggest some exception to the fully tolerant state as leading to and maintaining the chronic infection[96]. Indeed, some leukocyte surface and cytokine markers become elevated in liver during acute hepatitis in adult woodchucks that eventually become chronic carriers, although to a lesser extent than seen during the acute phase of woodchucks that resolve.

THERAPEUTIC STUDIES

Antiviral drugs

Woodchucks with experimentally induced chronic WHV infection have been used successfully in the empiric screening and preclinical assessment of antiviral drugs being developed for treatment of chronic HBV infection (for previous reviews see[21,24,25,30,35,117]). Current strategies aim to suppress viral replication in liver and the concentration of viral DNA in serum during chronic HBV infection (i.e., reduce viral load) by treatment with nucleoside and nucleotide analogues. As indicated above, it has been difficult to target the viral cccDNA directly in this process, and so potent inhibition of viral replication is the main means to reduce viral load in blood and tissues, and perhaps diminish replenishment of cccDNA indirectly, until cells harboring this intermediate can turnover or be eliminated by immune responses. Accordingly, lifelong therapy with antiviral drugs is currently the accepted procedure, even though this often results in the selection of drug-resistant mutants (i.e., mutations of the polymerase gene), which has been observed and modeled in the woodchuck (e.g.,[118-122]).

Before testing in woodchucks, potential drug candidates are screened for antiviral activity against HBV in the 2.2.15 cell system, a HepG2 cell line that is engineered to produce HBV constitutively[123]. Drugs with significant antiviral activity *in vitro* also have been tested in a HBV-transgenic mouse model designed and validated for this purpose[124]. However, drug efficacy in an *in vivo* infection model is most usually assessed in the woodchuck model. Most nucleoside analogues with intermediate antiviral activity *in vitro* against HBV had comparable antiviral activity against WHV in woodchucks, but some exceptions exist. For example, fialuridine (D-FIAU) had modest activity *in vitro*, and potent antiviral activity in woodchucks; however, this was associated with a marked and delayed hepatotoxicity characterized by microvesicular steatosis and mitochondrial injury[125], similar to the unfortunate hepatotoxic effects this drug had in humans, where it was first tested[126].

In recent years, numerous nucleoside and nucleotide analogues designed to inhibit HBV replication were tested in the woodchuck model (e.g.,[125,127-140]. Some of these nucleoside analogues had demonstrated antiviral efficacy in chronic WHV carrier woodchucks, such as lamivudine (3TC, Epivir)[21,120,141-143], adefovir dipivoxil (ADV, Hepsera)[144,145], and entecavir (ETV, Baraclude)[139,146], and are now approved by the FDA for treatment of chronic HBV infection. Other nucleoside analogues, also having activity in woodchucks against WHV, are used for the treatment of human immunodeficiency virus (HIV), such as tenofovir disoproxil fumarate (TDF, Viread)[147] and emtricitabine (FTC, Coviracil)[148-150], and still others are in advanced clinical testing, such as telbivudine (LdT)[151-153], valtorcitabine (val-LdC)[151-153], and clevudine (L-FMAU)[55,56,122,150,154-156]. FDA approval of these drugs for treatment of chronic HBV infection is expected in the near future (in fact, telbivudine was approved most recently). Table 1 summarizes the antiviral activities of these second and third-generation nucleosides in chronic WHV carrier woodchucks that were reported in selected studies. We note here in these experiments that viral recrudescence following cessation of drug is often a function of being unable to completely suppress viral replication sufficiently during a given treatment, or significantly enough over time in order to allow cells containing cccDNA to turnover or be eliminated by the immune response.

Lamivudine is a moderately potent antiviral drug in woodchucks and is without toxicity during daily, oral administration for up to 24 wk[21,141,157], and even longer[142]. The average reduction in serum WHV DNA after 4 or 12 wk of treatment with different doses (1, 5, or 15 mg/kg bodyweight) was approximately 2.5 and 1.5 logs, respectively. The average time to recrudescence of viral replication after drug withdrawal was within 1 to 2 wk. In woodchucks, lamivudine also has been shown to act synergistically both with alpha-interferon and with famciclovir[141,157]. An antiviral activity comparable to lamivudine has been reported for adefovir[144]. Daily oral administration of adefovir for 12 wk with doses of 5 and 15 mg/kg resulted in a reduction in serum viremia of 1.7 or 2.5 logs, respectively. Viral recrudescence after drug withdrawal occurred within 6 wk. No toxicity associated with administration of adefovir was observed. The antiviral activity of tenofovir in woodchucks[147] is comparable to those of lamivudine and adefovir. The reduction in serum WHV DNA observed after 4 wk of daily, oral treatment with tenofovir doses of 5 and 15 mg/kg was 1.5 or 1.2 logs, respectively. After drug withdrawal viral recrudescence occurred within 1 to 4 wk and treatment was without any evidence of drug-associated toxicity.

A higher antiviral activity on chronic WHV infection was reported for emtricitabine[149]. Daily oral treatment for 4 wk with doses of 10 or 30 mg/kg reduced serum viremia by 3.2 and 4.9 logs, respectively. Recrudescence of viral replication occurred within 1 to 2 wk after drug withdrawal.

Table 1 Antiviral activities of second and third-generation nucleosides and nucleotides in the woodchuck model of chronic HBV infection

Antiviral drug	Oral dose (mg/kg per day)	Treatment duration (wk)	Follow up duration (wk)	Serum WHV DNA reduction (log)	Time to viral recrudescence (wk)	Drug-associated toxicity	Other viral markers	Ref.
Lamivudine	1	24	24	1.5	within 1-2	none	WHV RI red. (3-fold) no WHV RNA red. no serum WHsAg red.	[141]
	5	4	12	3.4	within 1	none	WHV RI red. (4-fold) no WHV RNA red. no serum WHsAg red.	[21]
	5	12	12	1.9	within 1-2	none	WHV RI red. (3-fold) no WHV RNA red. no serum WHsAg red.	[157]
	15	4	12	5.4	within 1	none	WHV RI red. (12-fold) no WHV RNA red. no serum WHsAg red.	[21]
Adefovir	5	12	6	1.7	within 6	none		[144]
	15	12	6	2.5	within 6	none		[144]
Entecavir	0.02	12	12	7-8[1]	within 2-10	none	WHV RI red. in individual animals to undetectable levels	[139]
	0.1	12	12	7-8	within 6-10	none	WHV RI red. in most animals to undetectable levels	[139]
Tenofovir	5	4	12	1.5	within 1-4	none	no WHV RI red. no WHV RNA red. no serum WHsAg red.	[147]
	15	4	12	1.2	within 1-4	none	no WHV RI red. no WHV RNA red. no serum WHsAg red.	[147]
Emtricitabine	3	4	12	1.4	within 1-2	none	WHV RI red. (3-fold) no WHV RNA red. no serum WHsAg red.	[149]
	10	4	12	3.2	within 1-2	none	WHV RI red. (13-fold) no WHV RNA red. no serum WHsAg red.	[149]
	30	4	12	4.9	within 1-2		WHV RI red. (80-fold) no WHV RNA red. no serum WHsAg red.	[149]
	20[2]	4	4	1.4	within 1-2	none		[148]
	30[2]	4	4	1.8	within 1-2	none	WHV RI red. (2-fold)	[148]
Telbivudine	10	4	8	8	within 4-8	none	serum WHsAg red.	[151-153]
Valtorcitabine	10	4	8	4-6	within 1-8	none		[151-153]
Clevudine	3	4	12	9.2	within 2-10	none	WHV RI red. (28-fold) no WHV RNA red. serum WHsAg red. (2-fold)	[154]
	10	4	12	8.2	within 8-12[3]	none	WHV RI red. (68-fold) WHV RNA red. (2.7-fold) serum WHsAg red. (4-fold) WHV cccDNA red. (2-6-fold or to undetectable levels)	[154]

[1]Two of 6 woodchucks with modest reduction in serum WHV DNA of approximately 2.0 logs were not included. [2]Dosage was given twice daily by intraperitoneal administration. [3]One woodchuck had suppressed serum WHV DNA at the end of the study. WHV RI, hepatic WHV DNA replicative intermediates; WHV RNA, intrahepatic WHV RNA; red., reduction.

A dose of emtricitabine of 3 mg/kg in this study was less efficacious, but was comparable to those observed with 20 and 30 mg/kg, administered twice daily, intraperitoneally, for 4 wk[148]. There was no toxicity associated with this drug treatment. A more remarkable antiviral activity was obtained with valtorcitabine in chronic WHV carrier woodchucks after daily oral administration for 4 wk with a dose of 10 mg/kg[151-153]. In this case, serum WHV DNA became reduced by 4 to 6 logs with no evidence of drug-associated toxicity at the dose used. The time to recrudescence of viral replication after drug withdrawal was as little as 1 wk, but extended to 8 wk in many of the animals. Entecavir had an even higher antiviral activity in woodchucks[139]. Daily oral administration of entecavir for 12 wk at a dose of 0.02 mg/kg resulted in a reduction in serum viremia of 7 to 8 logs in 4 of 6 treated woodchucks (2 of the 6 treated woodchucks had only a modest antiviral effect). Recrudescence of viral replication after drug

withdrawal occurred in as little as 2 wk, but was extended to 10 wk in several of the animals. Administration of entecavir at a dose of 0.1 mg/kg reduced serum viremia by 7 to 8 logs and viral recrudescence was observed within 6 to 10 wk. No toxicity associated with drug treatment was reported.

The most potent antiviral drugs that have been tested so far in woodchucks are telbivudine and clevudine. Daily oral administration of telbivudine for 4 wk at a dose of 10 mg/kg resulted in an 8 log reduction in serum viremia and viral recrudescence was observed within 4 to 8 wk after drug withdrawal[151,152]. Daily oral administration of clevudine for 4 wk at doses of 3 or 10 mg/kg reduced serum viremia by 9.2 and 8.2 logs, respectively[154]. With the lower dose of clevudine viral recrudescence after drug withdrawal was observed within 2 to 10 wk. The higher dose delayed viral recrudescence and serum WHV DNA concentrations reached pretreatment levels within 8 to 12 wk, and in one woodchuck, serum viremia was still suppressed at the end of the study. No toxicity was associated with the above short-term treatments using either telbivudine or clevudine.

The above studies in the woodchuck model demonstrate that a significant antiviral effect on chronic WHV infection could be achieved with all drugs. The relative antiviral efficacy against WHV, at the doses administered and for the duration of treatment used, was clevudine $\geqslant$ telbivudine $\geqslant$ entecavir > valtorcitabine $\geqslant$ emtricitabine $\geqslant$ tenofovir = adefovir = lamivudine. A prolonged suppression of WHV replication after drug withdrawal was achieved with clevudine, telbivudine, entecavir, and valtorcitabine, and the magnitude of these responses was often associated indirectly with transient or sustained reductions in WHV cccDNA potentially enabling some turnover of residually infected cells. The favorable safety and efficacy profile obtained thus far in the woodchuck model using relatively short-term treatments with clevudine, telbivudine, and valtorcitabine suggest that these drugs should be of value in the long-term control of chronic HBV infection in humans and support their continued clinical development.

The preclinical evaluation of antiviral drugs for treatment of lamivudine-resistant HBV infection has been modeled in woodchucks by the experimental induction of lamivudine-resistant WHV with nine or more months of lamivudine treatment, followed by continued therapy with lamivudine along with the new drug candidate of interest[122,145]. Prolonged treatment with lamivudine led to the establishment of drug-resistant WHV mutants, characterized mainly by mutations in the B domain of the WHV polymerase gene (i.e., HBV mutations occur in B and C domains). Supplemental daily oral treatment of these circulating B domain mutants with adefovir or clevudine (10 mg/kg per day, 12 wk and 7 wk, respectively) demonstrated that both drugs could suppress replication of these lamivudine-resistant WHV mutants. In a different study, lamivudine-resistant mutants of WHV were found to be cross-resistant to treatment with clevudine[122]. Studies are in progress using engineered lamivudine-resistant mutants of WHV that mimic the additional polymerase C domain mutants observed in human HBV patients treated with lamivudine.

In addition to the testing of drugs in woodchucks for antiviral effects, applications also have been extended to the testing of entecavir, clevudine, or lamivudine for efficacy against disease progression[30,142,146,155]. Extended lamivudine treatment of woodchucks with chronic WHV infection delayed the development of HCC and significantly extended survival of woodchucks in one study[142]. In that study, twenty 8-mo-old chronic WHV carrier woodchucks were treated throughout the rest of their lifetime with lamivudine (5 mg and then 15 mg/kg, orally, daily). Twenty placebo control WHV carrier woodchucks were included for comparison. Serum WHV DNA decreased by 4 to 5 logs in lamivudine-treated carrier woodchucks, with an antiviral effect that was sustained for more than one year with continued treatment. Importantly, there was a significant delay in time to onset of HCC and death due to HCC among lamivudine-treated woodchucks compared to placebo controls. In another study of lamivudine in WHV carrier woodchucks, no delay in hepatocarcinogenesis was observed with treatment, most likely because drug treatment began when woodchucks were at an older age, was of shorter duration, and less of an antiviral effect on serum WHV DNA was observed[120]. In both studies, lamivudine resistance developed that was associated with a high frequency of mutation in the WHV polymerase gene B domain[118,121].

In another study, long-term oral treatment with entecavir[146] in 8-mo-old woodchucks at 0.5 mg/kg per day for 8 wk, and then with a weekly dose of 0.5 mg/kg for 14 or 36 mo, produced sustained antiviral responses in half of the woodchucks treated for 14 mo, and in 80% of the woodchucks treated for 36 mo (i.e., reduced serum viremia of 5 to 8 logs). Here, the drug-treated woodchucks had marked reduction in viral load and did not develop HCC during the next 2 years follow-up. Compared in this case with historical controls, entecavir treatment significantly delayed the development of HCC and prolonged survival.

In another study, clevudine was administered orally to chronic WHV carrier woodchucks at 10 mg/kg per day for 32 wk[30,55,56,155] starting at 1 to 2 years of age. Half of the clevudine-treated woodchucks and half of the placebo recipients then received 4 doses of a conventional WHsAg vaccine (alum-adsorbed, formalin-inactivated WHsAg) during the next 16 wk. Combination treatment with clevudine and vaccine resulted in a sustained antiviral effect with reductions in serum viremia of more than 8 logs in many cases (Figure 4), and prevented the development of HCC altogether in up to 38% of treated woodchucks. In a subset of the woodchucks studied, where clevudine or placebo treatment was initiated at 1 year of age (and the data analyzed independent of combination with WHsAg vaccine), the development of HCC in clevudine-treated woodchucks was delayed significantly and long-term survival after 4 years likewise was increased significantly compared to woodchucks that did not receive clevudine. These studies show that chemotherapy with antiviral drugs can delay and reduce disease progression in chronic carrier woodchucks, and also show the correlation between

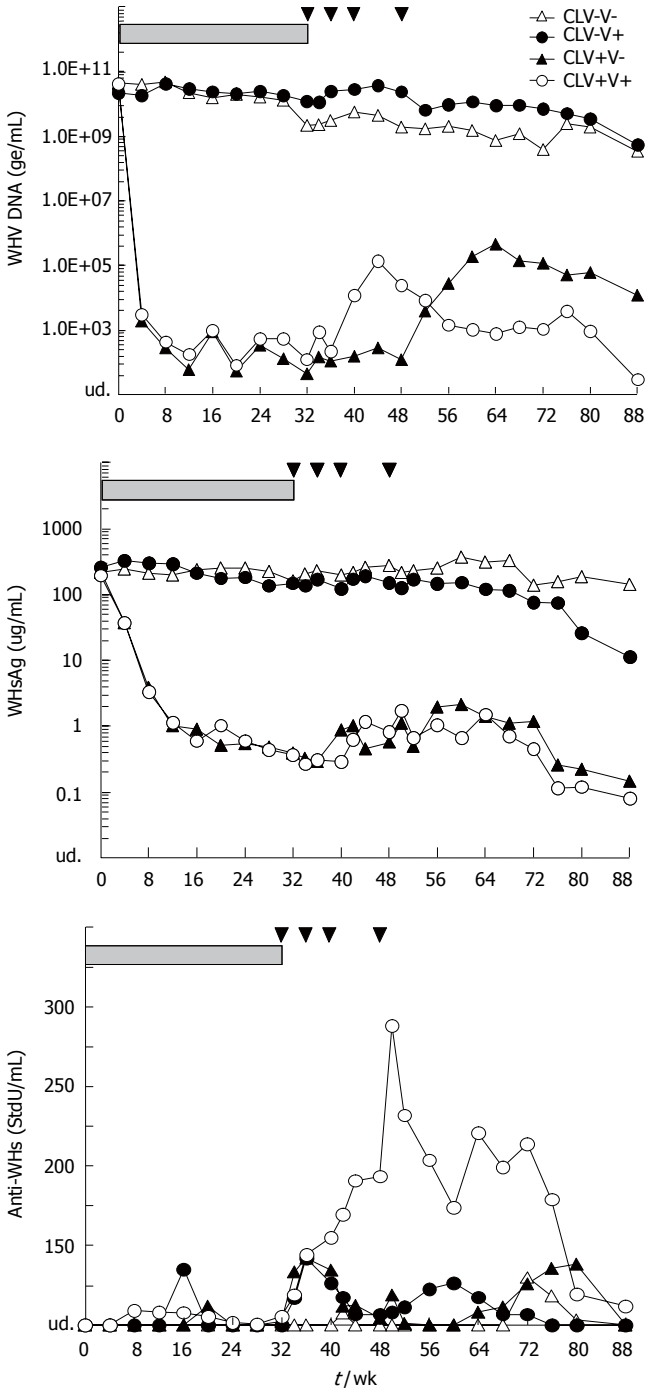

Figure 4 Combination treatment with clevudine and WHsAg vaccine suppresses serum viremia and antigenemia and induces a humoral response in chronic WHV carrier woodchucks. Changes in serum WHV DNA, serum WHsAg, and anti-WHs antibodies of chronic carriers in response to treatment with placebo (CLV-V-), vaccine (CLV-V+), drug (CLV+V-), and combination of drug and vaccine (CLV+V+) are shown. Horizontal bars denote the period of clevudine (CLV) administration for 32 wk. Arrowheads represent the 4 immunizations (V) using 50 μg doses of an alum-adsorbed, formalin-inactivated WHsAg vaccine at wk 32, 36, 40, and 48. WHVge, WHV genomic equivalents (virion or WHV DNA-containing particles).

reduced viral load and reduced disease progression, with noteworthy implications for HBV therapy in humans.

In addition to nucleoside or nucleotide analogues, various other compounds of organic and plant origin have been tested in woodchucks for their antiviral activity (e.g.,[141,158-161]), but these will not be discussed in detail in this review. Direct testing of anti-tumor agents against established HCC in woodchucks is also possible[162-164], but has not been fully developed to date.

Immunotherapy

The main goal of basic immunological studies described above in neonatal and adult WHV-infected woodchucks is to identify and differentiate factors that cause and maintain chronic infection from those that result from chronic carriage. By better defining cause-effect relationships, it should be possible to develop and test rational immunotherapies that can induce immune responses in the established chronic carrier that mimic those in recovery from WHV infection. In this way, it should be possible to enhance the immune elimination of cells harboring viral cccDNA (and/or control its level and expression), as occurs with successful immune responses leading to recovery.

As indicated in the sections above, chronicity as an outcome of neonatal WHV infection appears to result from a failed or suboptimal primary immune response relatively early during the acute phase of infection in the periphery and in the liver. The onset of chronic infection (compared to resolution) is characterized by deficiencies in the CD8-positive cytolytic T lymphocyte (CTL) response, and reduced expression of Th-type 1 cytokines and intracellular transcription factors, minimal acute hepatitis, and humoral and cellular immunologic tolerance to viral antigens[42-46]. Negative immunoregulation of the intrahepatic Th-type 1 response by excessive intrahepatic Th-type 2 immune responses is not a defining factor in this outcome[45,46]. Chronicity then appears to develop due to reduced immune-mediated clearance of infected hepatocytes by both non-cytolytic and cytolytic processes[45,46]. The above studies indicate further that early induction of immune tolerance may be a factor in the onset of chronic neonatal WHV infection, and a similar mechanism may be involved in the onset of chronic HBV infection in unvaccinated infants born to HBV-carrier mothers. This may include the deletion of higher affinity virus-specific T cells by negative selection of precursor T cells in the thymus (central tolerance), or clonal anergy or exhaustion of virus-specific T cells that escaped early negative selection in the thymus, which are then rendered unresponsive due to higher viral and antigen loads (peripheral tolerance).

Several studies have used WHV-naïve woodchucks for testing experimental vaccines, including conventional and DNA vaccines, and adjuvants, for later therapeutic vaccination of chronic WHV carrier woodchucks. In these studies antibody responses against WHsAg or WHcAg were induced[31,55,87,165-172], and partial or full protection against viral infection and disease by challenge with WHV was observed[31,87,165,166,168-172]. A few studies also determined that cellular immune responses were induced in addition to the humoral responses[87,168-171].

Unlike when WHV-naive woodchucks are immunized, the detection of free anti-WHs in serum of WHV carriers vaccinated with WHsAg is more problematic due to an excess of WHsAg in the serum samples. However, positive

Table 2 Immunotherapeutic approaches in the woodchuck model of chronic HBV infection

Treatment	Outcome	Additional results	Ref.
Vaccination			
WHsAg vaccine/adjuvant	Anti-WHs response	CMI to WHsAg	[55,56,155]
WHsAg vaccine/adjuvant	Anti-WHs response (antibodies mainly directed against preS region)		[173]
WHsAg vaccine/Th peptide epitope	Anti-WHs response Transient serum WHV DNA red. in a few animals (1 log)	Two woodchucks died	[174]
Cytokines			
IFN-α (adenoviral vector)	Transient serum WHV DNA red. (1 log)	Transient WHV RI red. (1 log)	[181]
IFN-α (adeno-associated viral vector)	Transient serum WHV DNA red. (2 logs) Sustained serum WHV DNA red. in 2 animals		[182]
IFN-γ (adenoviral vector)	No antiviral effect		[181]
Adoptive immunotransfer			
Liver transplantation	Serum WHV DNA red.	WHV RI red., WHV RNA red.	[188]
Combination treatment			
Lamivudine + WHsAg vaccine/ Th peptide epitope	No additional benefit beyond lamivudine-induced antiviral effect	CMI to WHsAg/WHcAg	
Lamivudine + β-galactosidase (adenoviral vector)	Transient but sustained serum WHV DNA red. (> 1 log) in addition to lamivudine-induced antiviral effect	WHV RI red., WHV cccDNA red., WHV RNA red.	[191]
Clevudine + β-galactosidase/+ IFN-γ/+ IFN-α (adenoviral vector)	Transient but sustained serum WHV DNA red. in addition to clevudine-induced antiviral effect	WHV RI red.	[156]
Clevudine + emtricitabine + IFN-γ (adenoviral vector)	No additional benefit beyond clevudine + emtricitabine-induced antiviral effect	Increased liver inflammation with IFN-γ	[150]
Clevudine + WHsAg vaccine	Anti-WHs response Sustained serum WHV DNA red. (> 6 to 8 log)	WHV cccDNA red., CMI to WHsAg/WHcAg Delay in onset of disease progression	[55,56,155]

WHV RI, hepatic WHV DNA replicative intermediates; WHV cccDNA, covalently closed circular WHV DNA, WHV RNA, intrahepatic WHV RNA; red., reduction.

signals for anti-WHs can often be detected by enzyme immunoassay under these conditions, even though it may be complexed in native serum. This is because of the exchange of bound anti-WHs between WHsAg in the sample solution, and the WHsAg adsorbed to the solid phase assay matrix. Unvaccinated WHV carriers rarely if ever show detectable anti-WHs of this nature, even though they may have some complexed anti-WHs in serum. Thus, the vaccination of WHV carriers with WHsAg most likely increases the levels of anti-WHs in complex, which enables its subsequent detection (at generally low levels) in the various enzyme immunoassay formats. Assays able to detect anti-WHs in complex with WHsAg are being developed to better study such responses.

One approach to immunotherapy is to modulate the deficient humoral and cellular immune responses of chronic HBV carriers by conventional vaccination (Table 2). In one study chronic WHV carrier woodchucks received up to 6 immunizations with a serum-derived WHsAg vaccine that was adsorbed to aluminum salt and contained monophosphoryl lipid A[173]. Following immunization, all of the carrier woodchucks developed an antibody response against WHsAg that was directed mainly against the WHV preS region, but there was little in the way of positive CMI to the antigen used in the vaccine. Despite the induction of anti-WHs antibodies, serum levels of WHV DNA and WHsAg in vaccinated carriers remained unchanged. This was consistent with another study in which four doses of an alum-adsorbed, formalin-inactivated WHsAg vaccine were administered[55,56,155]. In the latter study, CMI to WHsAg and WHsAg peptides was detected in the majority of vaccinated carriers, but, again, there was little effect on serum viral load (Figures 4 and 5). Therapeutic vaccination of chronic WHV carrier woodchucks with a serum-derived WHsAg in combination with an experimental adjuvant (i.e., a peptide carrying a Th epitope from sperm whale myoglobin) induced anti-WHs antibody responses and minor transient reductions in serum WHV DNA in a few of the vaccinated carriers[174]. Caution in the use of this therapeutic WHsAg vaccine was recommended, however, since some of the carriers died during the vaccinations. Such adverse effects could have been related to the experimental adjuvant and/or to liver disease present in the woodchucks at entry into the study. The results from these studies indicate that immunization of chronic WHV carrier woodchucks with WHsAg can partially induce (or boost) B cell responses to WHsAg. Additional modulation, however, seems necessary for inducing a response profile that resembles that observed during resolution of WHV infection.

Another approach to immunotherapy of chronic HBV infection involves direct reconstitution of the deficient Th-type 1 immune responses in the liver to mimic natural recovery from infection. Cytokines such as IFN-γ and TNF-α have been reported to have direct, non-cytolytic antiviral effects in HBV transgenic mice[8,175-177]. However,

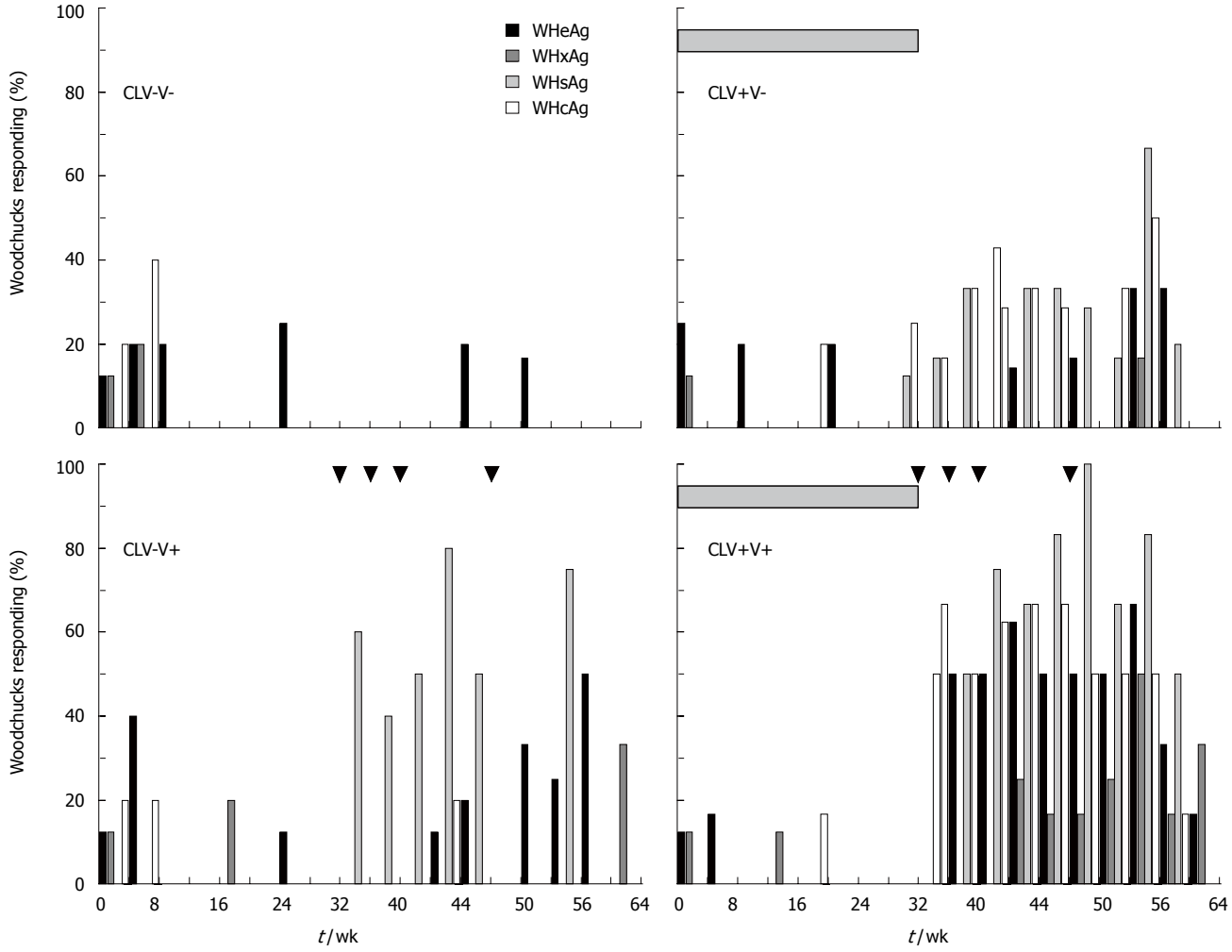

Figure 5 Combination treatment with clevudine and WHsAg vaccine enhances and expands the pattern of cell-mediated immune responses to WHV antigens in chronic WHV carrier woodchucks. Changes in the PBMC responses to WHsAg, WHcAg, WHeAg, and WHxAg of chronic carriers in response to treatment with placebo (CLV-V-), vaccine (CLV-V+), drug (CLV+V-), and combination of drug and vaccine (CLV+V+) are shown. Horizontal bars denote the period of clevudine (CLV) administration for 32 wk. Arrowheads represent the 4 immunizations (V) using 50 μg doses of an alum-adsorbed, formalin-inactivated WHsAg vaccine at wk 32, 36, 40, and 48.

increased expression of these cytokines can occur in established chronic WHV carriers with progressing chronic hepatitis and liver injury[45,96,97,178], but with little concurrent reduction in viral replication. This indicates that additional responses would be important to developing a more complete therapeutic effect resembling recovery. Recent studies have shown that woodchuck IFN-γ (and TNF-α) does not significantly deplete WHV RNA or WHV DNA replicative intermediates *in vitro* in virus-infected primary hepatocytes from chronic carriers[179]. Other studies in primary hepatocyte cultures from established WHV carriers suggest that expression of IFN-γ from a transfected plasmid (and also of TNF-α) can induce partial host response profiles with similarity to recovering liver, and also impair a later step in viral replication by a non-cytolytic mechanism that is probably mediated by TNF-α[180].

The effects of woodchuck IFN-α and IFN-γ on WHV replication were determined *in vivo* in a recent study in chronic WHV carrier woodchucks using an adenoviral vector for the expression of these cytokines[181]. Following vector administration directly into the liver, a slight but transient reduction in intrahepatic WHV DNA replication and in serum WHV DNA of about 1 log was obtained with the IFN-α expressing vector. The intrahepatic expression of IFN-γ, however, had no effect on WHV, thus leading to the conclusion that hepatocytes of chronic WHV carrier woodchucks may be functionally altered in their response to IFN-γ or resistant to this cytokine. In another study, the administration of woodchuck IFN-α using an adeno-associated viral vector for intrahepatic delivery of this cytokine into chronic WHV carrier woodchucks had a significant antiviral effect in that serum WHV DNA was reduced by 2 logs on average (range 1.5 to 4 logs)[182]. The antiviral effect observed was transient in the majority of woodchucks, but two woodchucks appeared to have sustained suppression in serum WHV DNA concentration. The results from these studies indicate that *in vivo* therapeutic gene delivery to augment the deficient Th-type 1 cytokine responses in liver may restore some of the failed antiviral and immunologic functions in human chronic HBV infection.

Another approach to immunotherapy of chronic HBV infection involves the restoration and stimulation

of higher-affinity Th and CTL clones in the periphery (or locally in the liver). Rather than to supplement a specific cytokine deficiency, it may be possible to reconstitute a complete and successful cellular immune response to acute infection by transfer of autologous or histocompatible T cell clones. The efficacy of the latter approach has been demonstrated in recent clinical studies of lymphocompatible bone marrow or PBMC transplantation from HBV-recovered or anti-HBV immunized donors into chronic carrier recipients (e.g.,[183-187]). Studies of cell-based therapies in chronic WHV carrier woodchucks involving adoptive lymphocyte transfer from vaccinated or WHV-resolved donors are in progress using neonatal-infected carrier woodchucks made lymphocompatible with their sires by co-injection of parental bone marrow and/or lymphocytes at birth. Later, after the neonates become established WHV carriers, they are re-administered parental lymphocytes therapeutically that were primed in the parent by immunization or recovery from acute WHV infection (Menne *et al*, unpublished data). Recently, another approach involving adoptive immunotransfer *via* liver transplantation from vaccinated WHV-naïve woodchucks into chronic carrier woodchucks was tested[188]. Following vaccination of donor woodchucks with DNA plasmids encoding WHcAg, WHsAg, and WHsAg in combination with a plasmid expressing IFN-γ livers were transplanted into recipient woodchucks, and the therapeutic effect determined. Two of 3 recipient carriers demonstrated a reduction in serum WHV DNA below the limit of detection by Southern hybridization analysis immediately following transplantation that lasted for up to 7 wk. WHV DNA in serum samples was detected when a more sensitive PCR assay was used. Nevertheless, the reductions in serum viremia were consistent with parallel reductions in intrahepatic levels of WHV RNA and DNA replicative intermediates.

Combination therapy

The high viral and antigen loads in serum during the chronic phase of infection are believed to maintain immunologic tolerance in established carrier woodchucks[55,56]. In some cases, treatment with antiviral drug alone may unmask host immune responses as seen during treatment of adult-acquired chronic HBV infection with lamivudine[189,190]; however, such responses appear sub-optimal for bringing about a complete recovery phenotype. To facilitate the emergence of the host immune response from a tolerant state maintained by high antigen load, combination therapy with a nucleoside analogue followed by modulation of the deficient immune responses represents a promising approach. Such an approach might even be able to improve upon natural recovery by creating optimal conditions for more rapid and complete eradication of viral cccDNA from the system.

In one study, chronic WHV carrier woodchucks were treated with lamivudine at a relatively high daily dose of 200 mg/kg given orally for 23 wk[143]. At the time, WHV DNA and WHsAg serum levels had declined by 3 to 5 logs or 1 log, respectively, woodchucks were vaccinated with three doses of a serum-derived WHsAg in combination with a peptide carrying a Th epitope from sperm whale myoglobin. In contrast to a previous study[174], therapeutic vaccination did not induce detectable anti-WHs antibody responses in carriers; the levels of viremia and antigenemia remained nearly unchanged from that achieved by drug treatment, and they returned to pretreatment levels following drug withdrawal. One important finding of this study was that the combination of lamivudine and vaccine, but not treatment with drug alone, induced CMI to WHsAg and WHcAg, presumably by shifting the cytokine profile from Th-type 2 to that of Th-type 0/1.

In another study chronic WHV carrier woodchucks received lamivudine treatment for 6 mo, again at a relatively high dose (200 mg/kg per day, oral) in order to reduce serum WHV DNA by 1 to 3 logs, and were then superinfected with an adenoviral vector expressing β-galactosidase[191]. Compared to control woodchucks, combination treatment resulted in further reductions of serum WHV DNA (10-20-fold) in the majority of woodchucks. The vector itself induced local immune responses in liver, and a bystander antiviral effect was observed on intrahepatic WHV DNA, WHV cccDNA, and WHV RNA that correlated with the inflammatory responses involving increased intrahepatic expression of woodchuck leukocyte markers and cytokines. The suppression of WHV replication was transient, but prolonged compared to woodchucks receiving lamivudine monotherapy. Similar results were obtained following superinfection of chronic WHV carriers with adenoviral vectors expressing IFN-γ, TNF-α, or β-galactosidase in combination with orally administered clevudine at 10 mg/kg per day[156]. Adenovirus superinfection led to declines in the intrahepatic WHV DNA levels, but a long-term benefit of combination treatment over clevudine alone was not observed. However, in contrast to monotherapy with lamivudine[191], recrudescence of WHV replication was deltayed until 14 wk after withdrawal of clevudine.

The antiviral effect of a combination of two nucleoside analogues in addition to an adenoviral vector expressing IFN-γ also has been tested in chronic WHV carrier woodchucks[150]. Woodchucks received clevudine and emtricitabine simultaneously at daily oral doses of 10 mg/kg and 30 mg/kg, respectively, for 8 wk, with two intravenous injections of the vector at wk 4 and 8. Combination treatment with clevudine and emtricitabine resulted in an antiviral effect on WHV replication, with reductions in serum viremia by 4 logs, and associated declines in intrahepatic levels of WHV DNA replicative intermediates and WHV cccDNA. The antiviral effect was sustained in a few woodchucks following drug withdrawal. The additional administration of the adenoviral vector led to increased liver inflammation, but enhancements of the antiviral effect compared to combination treatment with clevudine and emtricitabine were not observed.

In our study in chronically WHV-infected woodchucks described above[55,56,155], combination therapy with clevudine (10 mg/kg per day, oral, 32 wk), followed by 4 doses of a conventional WHsAg vaccine (alum-adsorbed, formalin-inactivated WHsAg), enhanced the virus-specific CMI to WHsAg, and resulted in additional

collateral responses to other viral antigens (Figures 4 and 5). Vaccination alone elicited low-level antibody responses to WHsAg in most woodchucks but did not affect serum WHV DNA or WHsAg levels compared to placebo-treated control woodchucks. Chronic WHV carrier woodchucks treated first with clevudine to reduce serum WHV DNA (> 6 to 8 log reduction) and WHsAg (> 50- to 500-fold reduction), and then vaccinated, developed a more robust anti-WHs antibody response. After vaccination, WHsAg-specific CMI was shown in both vaccinated groups, but was significantly enhanced in woodchucks treated initially with clevudine, and was broadened to include responses to WHcAg and to selected peptide epitopes of WHcAg and WHsAg.

Thus, the long-term drug treatment combined with therapeutic vaccination was shown to break humoral and cellular immune tolerance in treated WHV carrier woodchucks better than the component monotherapies, and to produce a more complete immune response profile resembling that in recovery from acute WHV infection, including an associated and marked reduction in the concentration of WHV cccDNA in liver. While the inclusion of vaccine after clevudine treatment did not result in a significant further antiviral effect beyond that of clevudine alone (i.e., clevudine is so potent that further antiviral effects would be difficult to measure), the combination therapy did have an additive benefit over the monotherapies in delaying the onset and occurrence of disease progression, including chronic hepatitis and HCC[30,155]. The results of this study suggest that the delay in the onset of chronic hepatitis and HCC is due to the uniformly high degree of suppression of viral load, especially the expression of viral antigens in serum and liver, any of which could act to maintain immune tolerance during chronic carriage. Longer term protection against the onset or development of HCC then appeared to be a function of the improved cellular and humoral immune responsiveness to viral antigens, which could no longer serve as endogenous tolerogens after reduction by drug.

CONCLUSIONS

The woodchuck animal model of chronic HBV infection has been valuable in determining the mechanisms of hepadnavirus replication and for studies of viral pathogenesis including associated disease sequelae and host immune responses. Continued modeling of early acute phase immune responses leading to resolution versus chronicity in the neonatal woodchuck may help to identify useful predictive markers of outcome that will facilitate the early identification of the carrier state, and the rational development of antiviral and/or immunotherapies for established chronic HBV infection. Colony-born woodchucks infected as neonates with well-characterized inocula also enable the evaluation of efficacy and toxicity of new types of prophylaxis or therapy under controlled experimental conditions in a relevant animal model within a reasonable time frame. Continued testing of new therapeutic approaches empirically and rationally in the woodchuck model will ultimately improve the chances for successful therapeutic eradication of established chronic HBV infection and its disease sequelae.

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