Woodchuck Hepatitis Virus Is a More Efficient Oncogenic Agent than Ground Squirrel Hepatitis Virus in a Common Host

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Chronic infection with hepatitis B viruses (hepadnaviruses) is a major cause of hepatocellular carcinoma (HCC), but the incubation time varies from 1 to 2 years to several decades in different host species infected with indigenous viruses. To discern the influence of viral and host factors on the kinetics of induction of HCC, we exploited the recent observation that ground squirrel hepatitis virus (GSHV) is infectious in woodchucks (C. Seeger, P. L. Marion, D. Ganem, and H. E. Varmus, *J. Virol.* 61:3241–3247, 1987) to compare the pathogenic potential of GSHV and woodchuck hepatitis virus (WHV) in chronically infected woodchucks. Chronic GSHV infection in woodchucks produces mild to moderate portal hepatitis, similar to that observed in woodchucks chronically infected with WHV. However, HCC developed in GSHV carriers about 18 months later than in WHV carriers. Thus, although both viruses are oncogenic in woodchucks, GSHV and WHV differ in oncogenic determinants that can affect the kinetics of appearance of HCC in chronically infected animals.

Persistent hepatitis B virus (hepadnavirus) infection in humans and animals is associated with the development of hepatocellular carcinoma (HCC) (28). HCC occurs in ca. 5% of individuals chronically infected with human hepatitis B virus, mainly during the fourth to sixth decade after infection (1). HCC has also been observed in animals chronically infected with two other hepadnaviruses: ground squirrel hepatitis virus (GSHV) and woodchuck hepatitis virus (WHV) (16, 27). In captive ground squirrels (*Spermophilus beecheyi*) naturally infected with GSHV, HCC occurs after a relatively long latency period of at least 4 to 5 years (17). In contrast, HCC appears in woodchucks (*Marmota monax*) chronically infected with WHV as early as 1 year after infection; by the fourth year, over 90% of infected animals have succumbed to complications caused by HCC (22). During the same time period, HCC has not been observed in uninfected woodchucks (22).

The difference in the rate of HCC development in chronically infected ground squirrels and woodchucks raises the question of whether viral or host determinants account for the observed discrepancy between the two systems. The functional domains of the GSHV and WHV genomes are identically organized, and the protein-coding regions predict amino acid homologies ranging from 71 to 92% (24). The close genetic relationship between the two viruses has been further substantiated by constructing genetic recombinants between the cloned genomes of WHV and GSHV and by demonstrating infectivity of some of the chimeras in ground squirrels and woodchucks (25, 25a). The genetic analysis of the GSHV and WHV genomes has not yet provided any clues to the different kinetics of HCC development observed in ground squirrels or woodchucks infected with their indigenous viruses. Hence, the identification of host or viral

determinants that dictate the rate of HCC development depends on the possibility of propagating GSHV and WHV in the same host species. In the course of experiments aiming at the identification of the genetic determinants that direct species specificity of GSHV and WHV, we unexpectedly discovered that GSHV is infectious in woodchucks (25). Thus, we were able to compare WHV and GSHV directly for pathogenic potential in the woodchuck. Our results demonstrate that the two viruses differ significantly in their potential to induce HCC in chronically infected animals, despite similar levels of virus production and similar preneoplastic inflammatory disease. Our findings support the idea that the oncogenic potential of hepadnaviruses depends on specific determinants present in viral genomes.

MATERIALS AND METHODS

Experimental animals and virus. Woodchucks used for this study were born in captivity from dams negative for serologic markers specific for WHV infection. The dams had become pregnant in a woodchuck breeding colony maintained at Cornell University. Three-day-old woodchucks were inoculated subcutaneously with 100 μ l of serum from carrier animals at a dilution of 1:10. WHV-containing serum was obtained from woodchuck 7, which had naturally acquired WHV infection and ultimately developed HCC (22). GSHV-containing serum was derived from a ground squirrel infected with cloned GSHV DNA (23) or from a woodchuck infected with cloned GSHV DNA (woodchuck 887). Serum samples for serologic analysis were obtained following inoculation at monthly intervals for 1 year and subsequently every 3 months. Liver biopsy specimens were obtained under general anesthesia induced with ketamine-xylazine and maintained with isoflurane. Hepatic tissues were fixed in 10% buffered Formalin and embedded in paraffin, and sections were stained with hematoxylin-eosin.

Serologic assays. Serologic tests were done after infection at monthly intervals for 9 months and subsequently at

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TABLE 1. Inoculation of woodchucks with GSHV^a

Group	Anti-GSHc	GSHsAg		Anti-GSHV surface protein
		Acute	Chronic	
1 (<i>n</i> = 14)	14	9	4	8
2 (<i>n</i> = 15)	15	14	13	2

^a Serologic response following neonatal infection of woodchucks with GSHV. The table summarizes the occurrence of viral markers in the sera of woodchucks inoculated with GSHV after 1 year. Animals in group 1 received serum from a ground squirrel; those in group 2 received serum from a woodchuck infected with GSHV. The assays for the determination of anti-GSHc antibodies, GSHsAg, and anti-GSHV surface protein antibodies were performed as described in Materials and Methods.

3-month intervals. Radioimmunoassays developed previously for WHV-specific serologic markers were used to detect the presence of GSHV surface antigen (GSHsAg) (6), anti-GSHV core (GSHc) antibody (21), and anti-GSHV surface protein antibody (7) in serum.

Isolation and characterization of viral and hepatic DNA. Virion DNA was extracted from serum of infected woodchucks with phenol after incubation with proteinase K. Purified DNA from 9 μ l of serum was denatured with alkali, spotted on nitrocellulose filters (Schleicher & Schuell), and hybridized to ³²P-labeled WHV or GSHV DNA probes. DNA from biopsied liver specimens was isolated after incubation with proteinase K by phenol extraction. DNA samples (15 μ g) were subjected to electrophoresis through agarose gels and then transferred to nylon membranes (Amersham) and hybridized to ³²P-labeled WHV DNA in hybridization buffer (0.45 M NaHPO₄ [pH 7.2], 0.9 mM disodium EDTA, 6% sodium dodecyl sulfate, 9% formamide, 0.9 mg of bovine serum albumin per ml) (Sigma) at 65°C. Films were exposed to Kodak XAR-5 film with intensifying screens (Du Pont) at -70°C.

RESULTS

Woodchucks chronically infected with GSHV. To establish a cohort of woodchucks chronically infected with GSHV, we inoculated 29 newborn woodchucks with GSHV-containing serum. One group of woodchucks (group 1; *n* = 14) received serum from a ground squirrel, the other group (group 2; *n* = 15) received serum from a woodchuck. Both animals were previously infected with cloned GSHV DNA by direct hepatic injection (23). The concentration of viral DNA in the serum sample from the GSHV-infected woodchuck was ca. fivefold elevated compared with that in the serum sample from the GSHV-infected squirrel (data not shown). To monitor the course of infection, we tested sera from infected woodchucks for the presence of antibodies directed against GSHc and GSHV surface proteins and for the occurrence of surface antigenemia (GSHsAg). All woodchucks developed anti-GSHc antibodies after infection with GSHV-containing sera, and 17 animals became chronic carriers of GSHsAg (Table 1). In most animals, anti-GSHc antibodies and GSHsAg appeared 2 to 5 months after inoculation, an incubation period similar to that observed after infection of woodchucks with WHV (22; data not shown).

A cohort of woodchucks (*n* = 16) chronically infected with WHV served as a control group for the GSHV-infected woodchucks (15). As previously described, this group was derived from 36 3-day-old woodchucks inoculated with infectious woodchuck serum (WHV-7 [22]); 27 of these animals developed persistent WHV surface protein antigen-

emia. Between 6 and 12 months after infection, 11 WHV surface antigen-positive carriers randomly selected from 10 litters were killed as a part of a previously reported study of the natural history of WHV infection (15). The remaining 16 chronic WHV carriers from nine separate litters were monitored for the development of HCC, serving as a cohort for direct comparison with woodchucks chronically infected with GSHV.

Tests for GSHsAg or WHV surface antigen remained positive in all woodchucks examined during the observation period of 51 months or until animals died or were euthanized. Although sAg levels varied among different animals, no significant differences could be detected between the two cohorts (data not shown). This was confirmed by an analysis of virion DNA levels present in sera of GSHV- and WHV-infected woodchucks 26 months after inoculation (Fig. 1A). The sera of all animals tested contained virion DNA, albeit at different amounts, ranging from ca. 10 to 140 ng/ml of serum. A comparison of the results obtained with the two cohorts revealed that the concentration of GSHV DNA was on average ca. twofold greater than that of WHV DNA.

The potential of GSHV to replicate in woodchucks was further examined by an analysis of intracellular DNA intermediates purified from liver samples of six woodchucks infected with either GSHV (1675, 2001, 2006) or WHV (1640, 1667, 1671). To distinguish the GSHV and WHV genomes, purified liver DNA from the infected animals was digested with the restriction endonucleases *Bam*HI and *Xba*I, producing a 3.3-kbp fragment from GSHV DNA and 2.9- and 0.4-kbp fragments from WHV DNA (14, 24). The anticipated 3.3-kbp DNA species were identified by the Southern blotting procedure in DNA samples prepared from GSHV-infected animals (Fig. 1B). They represent the linearized form of covalently closed circular DNA, the template for the transcription of the viral RNAs. The DNA samples from the WHV-infected animals showed only the 2.9-kbp DNA fragment since the 0.4-kbp fragment was obscured by faster-migrating heterogeneous DNA species (Fig. 1B). The latter represent DNA intermediates characteristic of hepadnavirus replication (18, 29). The autoradiograph showed that covalently closed circular DNA in the livers of GSHV- and WHV-infected animals accumulated to similar levels. Faster-migrating forms appeared to be more abundant in DNA samples from WHV-infected animals compared with the samples prepared from GSHV-infected woodchucks.

In summary, our data demonstrated that GSHV can cause persistent infection in woodchucks. Moreover, the levels of GSHV and WHV expression in woodchuck livers were similar. Therefore, we conclude that woodchucks are permissive for the replication of both WHV and GSHV.

WHV and GSHV induce HCC with different kinetics. From previous studies, we expected that hepatomas in woodchucks infected with WHV would appear 12 to 24 months after infection (22). To rapidly identify woodchucks with small tumors, we examined the livers of the chronically infected animals by ultrasound imaging at 2- to 3-month intervals, beginning 12 months after infection. Hepatic tumors were recognized in WHV-infected woodchucks as early as 1 year postinoculation; after 2 years, seven animals had detectable liver masses. During the same time period, none of the GSHV-infected animals showed evidence of hepatic mass lesions (data not shown).

To verify the results obtained from ultrasound imaging, we performed laparotomies on all animals 26 months after infection (with the exception of animals 1657 and 1665, which were biopsied 18 and 19 months postinoculation,

FIG. 1. GSHV and WHV DNAs present in sera and liver of chronically infected woodchucks. (A) Quantitative analysis of virion DNA in serum. Virion DNA was purified from 9 μ l of serum obtained 26 months after infection from 16 woodchucks infected with GSHV and from 15 woodchucks infected with WHV. DNA samples were dotted on nitrocellulose filters and hybridized to a GSHV- and WHV-specific probe labeled with 32 P. Serial dilutions of cloned GSHV and WHV DNAs (14, 24) served as standards for determination of the amount of DNA present in sera. (B) GSHV and WHV DNA from livers of infected woodchucks. Liver samples were obtained from GSHV-infected animals 1675, 2001, and 2006 (lanes 1 to 3) and from WHV-infected animals 1640, 1667, and 1671 (lanes 4 to 6) 2 years and 2.5 years (1671, lane 6) postinoculation. Before electrophoresis through 1% agarose gels, 10 μ g of DNA was cleaved with the restriction endonucleases *Xba*I and *Bam*HI. DNA was transferred to nylon membranes and hybridized with GSHV- and WHV-specific 32 P-labeled probes. The positions expected for linear duplex DNA of 3.3 and 2.9 kbp are indicated with arrows. *Hind*III-cleaved lambda DNA served as a molecular weight marker.

respectively). A total of 18 masses were identified in seven WHV-infected woodchucks (Table 2). The size of the tumors ranged from 5 to 53 mm in diameter. As anticipated from ultrasound imaging, no larger tumors were detected in the remaining WHV-infected woodchucks. Small nodules, less than 5 mm in diameter, also were detected in three of the WHV-infected woodchucks with HCC (data not shown). The small nodules in WHV-infected woodchucks were reminiscent of preneoplastic lesions that appear in the livers of rats during the course of experimental hepatocarcinogenesis (9, 10). Such lesions have been described previously in chronic WHV infection (22).

In contrast to the WHV-infected woodchucks, only two of the GSHV-infected animals had neoplastic lesions detectable during laparotomy (data not shown). Animal 2009 had a tumor nodule of 5 mm in diameter but died of complications following laparotomy. The other animal (1689) had a nodule 3 mm in diameter, which was resected, but then developed a large tumor 43 months postinoculation (see below).

From 27 to 51 months postinoculation, tumor masses were identified in the livers of all remaining WHV-infected woodchucks by ultrasound imaging and were confirmed as HCCs by postmortem examinations. By 51 months, a total of 41 hepatic tumors, ranging in mean diameter from 5 to 53 mm,

developed in 16 WHV carriers. All the neoplasms greater than 10 mm in diameter were classified histologically as HCC, primarily carcinomas of the trabecular and clear cell type. The smaller neoplastic nodules sometimes had trabecular characteristics, but most were classified as benign adenomas. During the period from 26 to 51 months postinoculation, 14 hepatic tumors were detected in 6 of the 14 remaining GSHV carriers, with sizes ranging from 5 to 40 mm in diameter (Table 2). After 51 months, eight surviving GSHV carrier woodchucks had no evidence of hepatic neoplasia based on ultrasound images of their livers.

Taken together, our results indicate that the rate of HCC development in woodchucks chronically infected with WHV was higher than that in woodchucks infected with GSHV. The median time for diagnosis of HCC in WHV-infected woodchucks was 32 months, compared with a projected 55 months in GSHV carriers (Fig. 2). From the number of HCCs that developed during the 51-month observation period (41 in WHV carriers and 14 in GSHV carriers), we infer that the risk of HCC development in WHV carrier woodchucks is ca. threefold higher than that of GSHV carriers within this time period.

Viral DNA in HCC induced by WHV and GSHV. Integration of viral DNA sequences into chromosomal DNA is

TABLE 2. HCC in WHV- and GSHV-infected woodchucks^a

Animal no.	No. of HCC	Diam (mm)	Mos postinoculation
WHV-infected animals			
1657	1	10	18
1665	3	49, 22, 13	19
1640	4	15, 12, 7, 5	26
1667	2	53, 22	26
1669	2	53, 10	26
1670	5	8, 6, 6, 5, 5	26
1671	1	38	26
1649	1	23	32
1654	3	33, 25, 5	41
1652	5	33, 10, 15, 9, 5	45
1653	3	48, 25, 5	45
1484	1	19	47
1659	2	19, 17	49
1660	2	13, 5	49
1658	3	23, 18, 12	50
1661	3	11, 11, 5	51
GSHV-infected animals			
1675	1	40	31
1689	1	33	43
1691	1	28	46
2006	3	17, 10, 8	50
1695	4	15, 10, 8, 5	51
2010	4	23, 20, 9, 8	51

^a HCC development in GSHV- and WHV-infected woodchucks. The table shows the number of HCCs identified during open liver biopsies and postmortem examinations. The diameters of the tumors and the time of identification are indicated. Not included in the list are eight GSHV-infected animals that were not diagnosed with HCC during the observation period of 51 months.

characteristic of hepadnavirus-induced HCC (11). To investigate whether GSHV sequences, like WHV sequences, integrated into host DNA, we examined DNA extracted from WHV-induced HCCs in woodchucks 1640, 1667, and

FIG. 2. Cumulative incidence of HCC in woodchucks infected with WHV and GSHV. The figure shows the percentage of woodchucks diagnosed with HCC after open liver biopsy or postmortem examination in relation to the time postinoculation (months) (see Table 2). One GSHV carrier died of unknown causes 1 year postinoculation, and two GSHV-infected animals died following open liver biopsy at 26 months postinoculation, reducing the number (*n*) of GSHV-infected woodchucks to 14.

FIG. 3. Qualitative analysis of GSHV and WHV DNAs from HCCs. DNA was isolated from HCC tissues obtained from WHV-infected woodchucks 1640, 1667, and 1671 (A) and from GSHV-infected woodchucks 1675 and 1689 (B). Purified DNA was incubated with the restriction endonucleases *Hind*III (A, lanes 1 to 3), *Pvu*II (A, lanes 4 to 6; B, lanes 1 and 2), and *Eco*RI (B, lanes 3 and 4), and DNA fragments were separated on 0.8% agarose gels. The positions expected for linearized CCC DNA of 3.3 kbp are indicated with arrows. *Hind*III-cleaved lambda DNA served as a molecular weight marker.

1671 and from GSHV-induced tumors in woodchucks 1675 and 1689 for virus-specific sequences (Fig. 3). DNA samples prepared from tumor tissues were incubated with the restriction endonuclease *Hind*III, *Pvu*II, or *Eco*RI. Whereas the WHV genome has no recognition site for *Pvu*II, both genomes bear single recognition sites for the other two endonucleases. Slowly migrating DNA fragments were present in all samples analyzed (Fig. 3A and B), indicating that WHV and GSHV sequences were integrated into cellular DNA. As expected, such fragments were not detected in DNA samples prepared from nonneoplastic liver tissue (Fig. 1B). All DNA samples from WHV-induced tumors (Fig. 3A) and one of the two samples from the GSHV-associated neoplasms (Fig. 3B, T1675, lanes 1 and 3) contained DNA species representing replicative DNA intermediates as shown in Fig. 1B. Based on the number of slowly migrating DNA species, the DNA samples analyzed contained one to three integrated viral DNA fragments per genome.

In summary, the analysis of DNA obtained from tumor tissue demonstrated that both WHV and GSHV induce HCCs containing viral DNA sequences integrated into chromosomal DNA, in accord with previous findings with hepadnavirus-induced neoplasms (2, 3, 8, 11).

Hepatitis in WHV- and GSHV-infected animals. To inves-

FIG. 4. Portal hepatitis in woodchucks chronically infected with WHV and GSHV. Liver biopsy of chronic WHV and GSHV carriers (1652, 2006) at 26 and 27 months postinoculation (A and C) showing mild mononuclear cell inflammation of portal tract. Postmortem liver specimens of the same woodchucks at 45 (1652) and 50 (2006) months showing marked portal hepatitis (B and D). At the time of postmortem examination, both woodchucks had HCC (Table 2). (Photographed at $\times 400$.)

tigate whether WHV infection causes more severe hepatitis than GSHV infection and to determine whether a correlation exists between the severity of hepatitis and the incidence of HCC, we made histologic examinations of liver specimens obtained from woodchucks chronically infected with WHV and GSHV. Nonneoplastic liver specimens were obtained 26 months after infection by open surgical biopsy from 15 WHV- and 16 GSHV-infected woodchucks. The severity of hepatitis was determined by assessing the degree of mononuclear infiltrates of the portal tract and the parenchyma, the severity of hepatocellular necrosis, the expansion of portal zones, and the degree of focal proliferation of bile ducts. Based on these criteria, no significant differences between the samples obtained from the two cohorts infected with GSHV and WHV could be detected. In most cases, hepatitis was mild, with patchy, partial involvement of the portal tracts. Moderate degrees of hepatitis, characterized by more diffuse lesions and with focal expansion of portal tracts with mononuclear infiltrates, were observed in woodchucks from both groups. Postmortem liver specimens of woodchucks infected with WHV and GSHV generally showed marked portal hepatitis, as exemplified by the samples shown in Fig. 4. Thus, based on the histologic analyses of liver from chronically infected woodchucks, GSHV and WHV induced chronic hepatitis of similar severity.

DISCUSSION

The major finding of this investigation was that the kinetics of tumor development in persistent hepadnavirus infection depends on one or more viral determinants. WHV and GSHV replicate with comparable efficiency in woodchucks, and infection with either agent causes mild to moderate hepatitis (Fig. 4). Despite these similarities, WHV induces HCC in woodchucks more rapidly and perhaps more frequently than GSHV (Fig. 2). WHV-infected animals developed approximately three times the number of tumors detected in GSHV-infected woodchucks within the 51-month observation period (Table 2). While histologically confirmed HCC developed in one-half of the WHV-infected woodchucks within 32 months postinoculation, HCC was detected in only 1 of 14 GSHV carriers (7%) in that period. Based on results obtained after 51 months of observation, it can be expected that the rate of tumor formation in GSHV-infected woodchucks will increase during the fifth year postinoculation and that after 6 years the majority of animals may have developed HCC (Fig. 2).

Previous observations made with captive ground squirrels indicated that HCC occurs in ca. 20% of GSHV-infected animals that are at least 5 years of age (17). Our results indicate that GSHV-infected woodchucks develop HCC at an earlier age. HCC in woodchucks was identified as early as

31 months after infection. However, a direct comparison of these two studies is hazardous, since in our experiments woodchucks were infected with GSHV as neonates, whereas captive ground squirrels may have acquired GSHV naturally at different ages.

The mechanism responsible for HCC induction during hepadnavirus infection is still enigmatic. The relatively long latency period between initial infection and HCC development indicates that virus replication per se is not sufficient for cellular transformation. HCC formation probably depends on secondary events that alter the genetic program of the infected hepatocyte. Since HCCs often contain viral DNA integrated into the chromosome, insertional mutagenesis leading to oncogene activation may play a role in HCC development (11, 20). This notion has been substantiated by the identification of a small number of WHV-induced hepatomas with viral integration sites near the proto-oncogene *c-myc* (13, 19). It is conceivable that the WHV genome is a better substrate for integration into the host chromosome, thereby increasing the chance for the activation of proto-oncogenes. Since viral DNA intermediates accumulate in GSHV- and WHV-infected livers at similar levels (Fig. 1A and B), any possible difference in the frequency of integration must be caused by variations between the nucleotide sequences of the two viral genomes. For instance, some variations may influence the efficiency of DNA integration by providing substrates for cellular enzymes, such as topoisomerase I, required for illegitimate recombination, as recently proposed by Hino et al. (12) for the integration of HBV into cellular DNA.

An alternative model recently proposed by Chisari et al. (4) explains HCC formation as a function of hepatic injury caused by the expression of viral envelope components. These researchers observed a direct correlation between the severity of liver injury and the development of liver tumors in transgenic mice expressing HBsAg. In our study, such a correlation is not apparent, since GSHV and WHV infections seem to cause similar degrees of hepatitis and yet induce HCC with significantly different kinetics. However, we cannot exclude the possibility that certain WHV-encoded polypeptides accumulate to higher levels in individual hepatocytes than do their GSHV-derived counterparts and thereby cause cell injury and eventually malignant transformation.

It is also possible that the differences in oncogenic potential of WHV and GSHV can be attributed to direct effects of viral gene products. The X gene product, for example, functions as a transactivator of viral promoters and hence could play a role in tumorigenesis by activating the promoters of cellular transforming genes (5, 26). Although the X proteins of GSHV and WHV are among the least conserved proteins encoded by the two genomes (71% amino acid homology [24]), they both display transcriptional activities similar to those described for the HBV-encoded X protein (5, 22a, 26). It is conceivable that differences between the two proteins influence the specificity of each product for certain promoters, some of which may play a role in HCC development.

Nevertheless, understanding the mechanism responsible for the differences in oncogenicity between WHV and GSHV depends on the identification of the responsible determinants on the WHV genome. The possibility of propagating genetic recombinants between the two viruses in ground squirrels and woodchucks provides an experimental system for such an analysis. The identification of such

determinants might be an important step toward clarification of the mechanism of hepadnavirus-induced liver cancer.

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