Hepatocarcinogenicity of the woodchuck hepatitis virus
(hepatocellular carcinoma/precursor lesions/woodchuck hepatitis surface antigen/necroinflammation)

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ABSTRACT During investigations of the evolution of experimental laboratory infections of woodchucks (Marmota monax) with the woodchuck hepatitis virus (WHV), eight hepatocellular carcinomas (HCC) were observed, six in newborns and two in young adult animals, all within 17–36 months after infection. The absence of an external cocarcinogenic effect in the well-monitored woodchucks indicates the carcinogenicity of WHV and suggests the same for the genetically and biologically similar human hepatitis B virus (HBV). Laboratory infections of woodchucks with two strains of WHV, not reported here in detail, resembled human and chimpanzee HBV infections histologically and serologically. In these studies, eight woodchucks became carriers of surface antigen of WHV for >1 year. All eight woodchucks developed HCC, indicating a 100% risk of HCC in experimentally infected chronic WHV antigen carriers, which is analogous to the high risk of HCC in human hepatitis B surface antigen carriers. Histologically, the absence of cirrhosis in the examined pericarcinomatous tissue permits recognition of gradual transition from normal parenchyma to neoplastic nodules to HCC of rising anaplasia, indicating a continuum of increasingly more malignant neoplastic stages, as known for chemical carcinogenesis. The HCC developed in carrier woodchucks infected as newborns with only minor, if any, hepatitic changes but is associated with antigen-carrying hepatocytes and sometimes with hyperplastic nodules. This stage was preceded in infected adults by an early, acute, weeks-long hepatitis coinciding with the appearance of surface antigen. These findings are also analogous to typical HBV infection in human newborns and young adults, respectively. At the time of HCC development in all animals with adequate histologic material, an acute recent necroinflammation appeared around the tumor, associated with abnormal hematopoietic cells around and within the tumor. A promoting role in carcinogenesis of this necroinflammation of yet unestablished pathogenesis is being postulated, to be confirmed by determination of the status of the WHV DNA in the HCC and by prospective histologic study of the inflammatory reaction.

Recently, several viruses have been associated with the induction of carcinomas in humans on epidemiologic and virologic evidence. This includes the Epstein–Barr virus in Burkitt lymphoma, the papilloma virus in cervical carcinoma, the group of human T lymphotropic viruses in lymphomas, and the hepatitis B virus (HBV) in hepatocellular carcinoma (HCC) (1). Despite the strong evidence for the carcinogenicity of these viruses, the requirement of a cocarcinogen is not fully excluded, although through the years this essentiality has become less probable. In chronic HBV infections, a potential role of aflatoxin (2, 3) is not excluded to date. HBV is a member of the hepadnaviruses, which show great homologies in DNA and its gene products but limited cross-infectivity (4, 5). In the eastern woodchuck (Marmota monax), in the Beechey ground squirrel in California, and in Pekin ducks in China, hepatitis and HCC have been observed in animals infected with the woodchuck hepatitis virus (WHV) (6), the ground squirrel hepatitis virus (7), and the duck hepatitis B virus (8), respectively. The animals had been infected in the wild and subsequently observed in captivity. The incidence of HCC in the woodchucks ranged from 25% to 85% (5, 9). Laboratory infection with the animal hepadnaviruses has succeeded in all three species [WHV (10), ground squirrel hepatitis virus (11), duck hepatitis B virus (12, 13)] and has been associated with the characteristic markers of infection and with histologic hepatic alterations of varying severity. Particularly in the woodchucks the lesions corresponded to those described in wild infected animals (14). Here we report the development of HCC in six of six woodchuck hepatitis surface antigen (WHsAg) carriers infected in the laboratory at birth and in two of two carriers infected as adults.

MATERIALS AND METHODS

Experimental Animals. Woodchucks from two experimental sources were utilized. One consisted of offspring of females trapped in Tompkins County, New York, and kept in individual isolators. The dams, proven to be free of present or past WHV infection, had become pregnant in their native habitat or after mating in the laboratory animal facility at the College of Veterinary Medicine, Cornell University. The newborns were inoculated with either of two pools of WHV (woodchuck 7, woodchuck 9) prepared by combining several DNA polymerase-positive serum samples from each of two woodchucks with naturally acquired chronic WHV infection. The newborns received total doses (WCID50) 1 day after birth. They were weaned 8 weeks following infection and at the age of 6 months were separated and kept in groups of 2 or 3 in separate cages. A second source consisted of adult woodchucks, either wild-caught or colony-born, without evidence of ongoing or past WHV infection and maintained at the animal care facility of SEMA (Gaithersburg, MD) (National Institute of Allergy and Infectious Diseases). They were part of an investigation of the infectivity titer of one of the standard WHV challenge pools (woodchuck 8) and of the pattern of WHV-specific markers as related to the natural history of chronic WHV infection. A dilution of an inoculum containing 10^5.8 WCID50 was given to 23 woodchucks. The animals were infected as adults and kept in isolators. Blood samples were obtained at weekly intervals for >2 years. All woodchucks received aflatoxin-free laboratory animal chow for rabbits and tap water ad libitum.

Abbreviations: HCC, hepatocellular carcinoma(s); WHV, woodchuck hepatitis virus; WHsAg, woodchuck hepatitis surface antigen; HBV, hepatitis B virus; anti-WH, woodchuck hepatitis core antibody; anti-WHS, woodchuck hepatitis surface antibody.
Serologic Determinations for WHV Markers. Serologic tests were carried out on the offspring of seronegative dams at 1 month following infection, repeated monthly for 9 months, and subsequently repeated at 3-month intervals. WHSAg was determined by solid-phase sandwich radioimmunoassay with an 125I-labeled monoclonal antibody probe (15). Core antibody anti-WHc was assayed by competitive inhibition with a solid-phase radioimmunoassay utilizing an 125I-labeled anti-WHc probe (16), and surface antibodies anti-WHSs were assayed by double antibody immunoprecipitation of 125I-labeled WHSAG-immunoglobulin complexes with rabbit anti-woodchuck IgG (17).

Histologic Investigations. In the Cornell study, hepatic tissue (ca. 1.5 g) was obtained from the offspring by exploratory laparotomy under anesthesia 1 year after infection. This was repeated at 18 months of age on surviving woodchucks. One woodchuck (no. 432) died unexpectedly at 17 months of age and liver tissue was obtained at autopsy. In the Maryland study, liver specimens were obtained by needle biopsy performed weekly and then at less frequent intervals and from an autopsy specimen of one woodchuck (no. 111). Paraffin sections were stained with hematoxylin/eosin, Victoria blue [a modification of Shikata’s orcein stain (18)], Mallory’s trichrome, and, where applicable, by other routine stains. At least one investigator (H.P.) examined all histologic material included in a large amount of other experimental material, without knowledge of virologic findings.

Identification of Animals Examined in Detail. The newborn study included, besides un inoculated animals, chronic WHSAG carriers and woodchucks with markers of past infection (anti-WHc and anti-WHSs), some of which were transiently WHSAG positive (Table 1). In this entire group only 6 woodchucks became chronic WHSAG carriers, 3 males and 3 females, and they were the subject of the detailed histologic study. The other woodchucks did not have significant histological lesions at 1 year and subsequent time points. The 19 animals with virologic markers of past infection and 15 uninfected controls were followed concurrently with the carrier woodchucks.

In the Maryland study of 12 inoculated woodchucks, 10 were transiently WHSAG positive and were followed for 1 year (5), while 2 females, woodchuck 27 (infected at 3 years of age) and woodchuck 111 (infected at 9 months), became chronic WHSAG carriers and never developed anti-WHSs, as confirmed by serial serologic examinations; they were subjected to serial histologic studies of needle biopsy specimens. Their WHSAG titers increased with time but declined in the last weeks of study. Of the 4 animals with transient WHSAG, only 2 developed a mild acute hepatitis, which disappeared after 4 weeks.

Table 1. HCC as an outcome of experimental WHV infection

<table>
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<tr>
<th>Serology</th>
<th>Animals</th>
<th>Total no. with HCC %</th>
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<tr>
<td>WHSAG anti-WHc</td>
<td>anti-WHSs</td>
<td>Status</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>Chronic carrier</td>
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<td>-</td>
<td>+</td>
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<td>Uninfected</td>
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Woodchucks were infected with 10^4.6-10^6.7 WHV infectious doses. Eight animals (6 newborn, 2 adults) developed persistent WHV infections and all had histologic evidence of HCC between 17 and 36 months after experimental infection. No histologic evidence of HCC was detected in 19 animals that seroconverted to anti-WHSs (postinfection) or 15 concurrent uninfected controls of the newborn study. Uninfected and postinfection animals were followed from 18 to 57 months.

RESULTS

In the consolidated histologic analysis, several stages were distinguished:

(i) Acute hepatitis. Acute hepatitis was demonstrated in both woodchucks infected as adults. It developed in one animal 12 weeks after infection and 3 weeks after the appearance of WHSAG, became maximal within 2 weeks, and gradually subsided within 4 weeks. In the other, borderline lesions developed 5 weeks after infection, simultaneously with the rise of WHSAG, were maximal after 17 weeks, and gradually subsided. The lesion was histologically characterized by focal eosinophilic changes of the cytoplasm of the hepatocytes progressing to acidophilic bodies, activation of sinusoidal cells, varying amounts of lymphocytes and few plasma cells in the parenchyma, as well as extensive portal infiltration by mainly lymphocytes, with variable admixture of neutrophils and plasma cells. The inflammation extended sometimes into the immediate periportal parenchyma. At the height of the lesion, bile ductules surrounded by inflammatory cells had proliferated.

(ii) Minimal lesion. WHSAG carrier state. All 6 woodchucks infected as newborns showed at 1 year of age slight infiltration by lymphocytes restricted to the portal tracts. In the parenchyma, occasional variations of hepatocytes were noted with focal increase of sinusoidal cells (Fig. 1A). Scattered hepatocytes showed Victoria blue-positive cytoplasmic inclusions. Groups of hepatocytes up to 0.2 mm in maximal diameter differed from the surrounding parenchyma by larger size of their cytoplasm and by different staining qualities, either basophilia or more intense vacuolization and granulation, presumably from excess glycogen deposition. The inflammatory lesion appeared more conspicuous than in all but 3 of the other 19 transiently infected woodchucks.

In the two WHSAG carrier woodchucks infected as adults, late biopsy specimens obtained at different intervals after the 22nd week in woodchuck 27 and the 27th week in woodchuck 111 (excluding the terminal weeks) had essentially normal appearance except for portal lymphocytic infiltration, which, however, did not significantly differ from what is seen occasionally in control woodchucks. Victoria blue-positive cells could not be stained in the available specimens, but nodular variations as described before were encountered in occasional specimens after 7 weeks after the first WHSAG rise.

(iii) Pericancrenomaous hepatitis. In all eight animals, the hepatic reaction was conspicuously aggravated around the time HCC was recognized. The expression of the inflammation depended on the nontumorous tissue available for histologic investigation. The portal tract inflammation was uniformly more severe, with expansion of the portal tract by lymphocytes and their extension into the surrounding parenchyma corresponding to human chronic active hepatitis, in contrast to the preceding features, which barely could be designated as chronic persistent hepatitis. Moreover, there was focal loss of hepatocytes and increased sinusaloid cells (Figs. 1B and 2A), and atypical hematopoietic cells, mainly megakaryocytes, were noted. From the available material it appeared that the inflammatory reaction was more conspicuous in the vicinity of the HCC.

(iv) HCC and preneoplastic nodules. In the liver specimens obtained shortly preceding or simultaneous with detection of the HCC, distinct hepatic nodules of varying size (up to 2 cm in diameter) were recognized (Fig. 1C) that consisted of mostly basophilic hepatocytes in plates more than one cell thick. The hepatocytes were often larger than in the surrounding parenchyma, their cytoplasm was mostly basophilic, and the demarcation of the nodules was sharp, although transition of nontumorous hepatocytes to those in the nodules occurred in single hepatocytic plates. The surrounding parenchyma was compressed in places. Portal
tracts were absent even in larger nodules, but efferent veins were present (Fig. 1B). There was no inflammation in the nodules, in contrast to the surrounding parenchyma. Hepatocytes with Victoria blue-positive inclusions were recognized in the border zone as well as within the neoplastic nodules (Fig. 2C). Gradual transition from the neoplastic nodules to frank HCC was noted, again, in the same cell plate, with the cells in the nodules having more cytoplasm than those in the HCC, although the nuclei appeared of similar size (Fig. 2B). Connective tissue capsules did not form, either around the nodules or around the HCC. The HCC revealed trabeculae of mostly several cells. These cells had basophilic cytoplasm, often vacuolated because of fat droplets. The nuclei were large, varying in size, and often polychromatic. Mitoses were rare. Lumina in the center of the trabeculae contained proteic material. Sinusoidal cells were mostly increased but only few lymphocytes and occasional neutrophils were noted (Fig. 2D). Single and often bizarre-appearing hematopoietic cells were present, sometimes arranged in groups (Fig. 1D). The degree of anaplasia of the HCC varied throughout, being more conspicuous in their central portions rather than near the transition from neoplastic nodules.

**DISCUSSION**

Experimental acute viral hepatitis in woodchucks with features described in natural infections (14) supplements and extends results of previous studies (10, 19, 20). The histological and serological features of the acute hepatitis correspond to those in humans and particularly in chimpanzees (21) and were related to the age of the animal at time of infection as well as the presence of serum WHsAg, with only rare hepatocytic lesions in animals that only seroconverted without woodchuck hepatitis surface antigenemia.

Minimal hepatitis was observed in newborn and adult animals that became chronic WHsAg carriers for at least 1.5 years, in the adults after subsidence of a transient acute hepatitis. The most distinctive features of the later carrier state are mild lymphocytic infiltration in the portal tracts in routine histological sections and frequent Victoria blue-positive cytoplasmic inclusions that correspond to the HBsAg-loaded hepatocytes in "ground-glass" hepatocytes in human and chimpanzee HBsAg carriers. Thus, the minimal-lesion WHsAg carrier state in woodchucks corresponds histologically to the human "healthy" HBsAg carrier state (22). The latter develops more frequently in neonatal than in adult infections and also has a high risk for HCC.

The most significant observation reported here is the development of HCC in woodchucks infected with WHV in the laboratory as newborns or adults. Careful control of the laboratory conditions reasonably excludes a chemical cocarcinogen and provides strong evidence for the carcinogenicity of one type of hepnavirus. Together with the development of HCC in the other known forms of animal hepnaviruses and the genomic and biologic similarity of
WHV to human HBV, this strongly suggests that HBV also has a carcinogenic potential without requiring an external cocarcinogen, in support of the long-claimed association of HBV with HCC (23, 24). HCC appeared in 100% of 8 WHsAg carriers within 17–36 months but in none of 15 uninfected control animals. This represents the highest reported incidence of HCC risk of any carcinogen. Furthermore, HCC developed only in WHsAg carriers and not in any woodchucks with evidence of only past infection, reflected in serum anti-WHc and anti-WHS, in this series, although HCC has been detected in a small proportion of animals with past natural infection (5). These observations and the nearly complete restriction of HCC development to HBsAg carriers in the extensive Taiwan study of Beasley and Hwang (25), in which very few persons with HCC had evidence of only past HBV infection, point to the importance of chronic HBsAg expression in HCC development and to the practical conclusion that for screening for HCC, HBsAg is by far the most important marker of risk. This permits reduction of screening to HBsAg carriers and raises the possibility that in some human HCC with only markers of past infection, other factors may be operative, as assumed in Japan (26).

The absence of cirrhosis in the examined WHV-associated HCC facilitates the interpretation of the precursor lesions. Serial examinations demonstrated precancerous nodular lesions and gradual transition from normal to nodular parenchyma to HCC of increasing anaplasia. This stepwise transition corresponds to the well-investigated transitions in chemical carcinogenesis (27) but, although resembling chemical effects (14), is probably solely virus-induced. Variation in cell populations and nodules in nodules (28) are long known in chemical experimental carcinogenesis and have been found also in early human HCC (29).

Though an external chemical cocarcinogen can be reasonably excluded in the WHV-associated HCC, the histologic observations suggest an endogenous promoting factor in the form of a new wave of necroinflammation of hepatic character. Because of the limitations of available histologic material, the relation of the inflammation to HCC is less secure than the other data reported. The infiltration of the precanceromatous and carcinomatous tissue by abnormal hematopoietic cells, particularly megakaryocytes, had been reported in woodchuck HCC following natural infection (14). Their regular appearance in this study may also be related to the reported HBV DNA in free replicative form in lymphocytes of infected woodchucks, ascribing them clinical significance (30). More significant but less well identified is the reappearance of a severe necroinflammation around the time of the HCC development. It seems to spare the HCC itself, in which, in other observations of large tumors, necrosis has been explained by the HCC outgrowing its blood supply. The inflammation described here seems to be more marked around the HCC.

In chemical carcinogenesis, necrosis has been postulated as an essential step (31) but its role is not established, particularly whether it is a result of the HCC or, more...
probably, its promoter. It could be a promoting factor by inducing mitoses during which the integrated viral DNA may undergo additional changes (32). In humans, exacerbations in inactive carrier states are known (33), in part explained by exacerbations of the HBV infection and in part by superinfection with another virus, for which no evidence exists in our experiments. Integration of viral DNA into the host chromosomes in liver and HCC has been demonstrated in woodchucks (4, 5). Similar to humans, in chronic infection only WHV genomes with small deletions have been found, in contrast to HCC, in which disarrangement of WHV DNA and its flanking sequences has been described (34), raising the possibility of abnormal mobilization of growth factors localized in another chromosome (35). Determination of the status of the WHV DNA in the material of this study requires further investigation (30), and additional prospective investigations of incidence and distribution of necroinflammation during development of WHV-associated HCC should identify the promoting nature of the necroinflammation in the development of HCC. If confirmed, prevention of the promoting hepatitic attack may become an important clinical strategy.

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