Synergistic Hepatocarcinogenic Effect of Hepadnaviral Infection and Dietary Aflatoxin B\textsubscript{1} in Woodchucks


ABSTRACT

Interactive hepatadnaviral and chemical hepatocarcinogenesis was studied in woodchucks inoculated as newborns with woodchuck hepatitis virus (WHV), which is closely related to the human hepatitis B virus. When the woodchucks reached 12 months of age, aflatoxin B\textsubscript{1} (AFB\textsubscript{1}) was administered in the diet at dose levels of 40 \(\mu\)g/kg body weight/day for 4 months and subsequently 20 \(\mu\)g/kg body weight/day (5 days/week) for lifetime. WHV DNA was demonstrated by Southern blot hybridization in the serum and by PCR in the serum and/or liver tissue. The histo- and cytomorphology of the liver were investigated by light and electron microscopy. WHV carriers with and without AFB\textsubscript{1}, treatment developed a high incidence of preneoplastic foci of altered hepatocytes, hepatocellular adenomas, and hepatocellular carcinomas that appeared 6–26 months after the beginning of the combination experiment. Administration of AFB\textsubscript{1} to WHV carriers resulted in a significantly earlier appearance of hepatocellular neoplasms and a higher incidence of hepatocellular carcinomas compared to WHV carriers not treated with AFB\textsubscript{1}. Neither hepatocellular adenomas nor carcinomas (but preneoplastic foci of altered hepatocytes) were detected in woodchucks receiving AFB\textsubscript{1} alone, and no preneoplastic or neoplastic lesions were found in untreated controls. These results provide conclusive evidence of a synergistic hepatocarcinogenic effect of hepadnaviral infection and dietary AFB\textsubscript{1}. Except for the frequent presence of ground glass cells containing surface antigen filaments in the infected woodchucks, the phenotype of preneoplastic foci of altered hepatocytes was similar in WHV carriers with and without exposure to AFB\textsubscript{1}, and in animals treated with AFB\textsubscript{1} alone. Clear cell foci excessively storing glycogen and/or fat, amorphophilic cell foci crowded with mitochondria and peroxisomes, and mixed cell foci composed of various cell types including basophilic cells rich in ribosomes predominated. The cellular phenotype in neoplastic lesions varied from clear, amorphophilic, and mixed cell populations in highly differentiated adenomas and carcinomas to basophilic cell populations prevailing in poorly differentiated carcinomas. The striking similarities in altered cellular phenotypes of preneoplastic hepatocellular foci emerging after both hepadnaviral infection and exposure to AFB\textsubscript{1} suggest closely related underlying molecular mechanisms that may be mainly responsible for the synergistic hepatocarcinogenic effect of these oncogenic agents.

INTRODUCTION

HCC\textsuperscript{2} is one of the most frequent malignant neoplasms in human beings, causing at least 250,000 deaths annually worldwide (1). Chronic infection with the HBV and ingestion of foodstuffs contaminated with aflatoxins, particularly AFB\textsubscript{1}, are considered major risk factors for the development of HCC (2–4). Evidence of a pivotal role of chronic HBV infection in the etiology of HCC has been provided by seroepidemiological associations, indicating at least a 100-fold increased risk for chronic HBV carriers (5, 6), and by the discovery that HBV\textsuperscript{v} belongs to a family of closely related DNA viruses (hepadnaviridae) including the WHV, the ground squirrel hepatitis virus, and the duck hepatitis virus, all of which elicit acute and chronic hepatitis in their natural hosts and may eventually lead to HCC (7). The assumption that aflatoxins represent additional risk factors for human hepatocarcinogenesis is based on the demonstration of their strong hepatocarcinogenicity in various animal species (3), classical epidemiological studies (3, 8), and more recent molecular epidemiological approaches using mutational hot spots in codon 249 of the p53 gene (9–17) or certain urinary metabolites (1, 18) as markers for aflatoxin exposure. Although a frequent synergy of HBV and AFB\textsubscript{1} is suggested by these studies, conclusive evidence for this concept is lacking (1).

The oncogenic potential of the hepadnaviruses differs markedly (19). WHV appears to be more oncogenic (20) than HBV and other known hepadnaviruses (2, 19, 21). Tennant et al. (22) were the first to use the woodchuck model for studying the influence of AFB\textsubscript{1} on viral hepatocarcinogenesis, but the interpretation of their results was hampered by the reduced survival of the WHV-infected animals receiving AFB\textsubscript{1} (4). The investigations demonstrated, however, that AFB\textsubscript{1} is hepatocarcinogenic in woodchucks like in many other species (3). In ducks that are also sensitive to the hepatocarcinogenic effect of AFB\textsubscript{1}, the combined effect of chronic infection with duck hepatitis virus and AFB\textsubscript{1} administered i.p. did not result in an increased incidence of HCC, questioning a synergy between hepadnaviral infection and AFB\textsubscript{1} in this species (23–25). Only in transgenic mice that express the large envelope polypeptide of HBV and are prone to develop hepatocellular neoplasms was the incidence of HCA and HCC shown to increase significantly after i.p. administration of AFB\textsubscript{1} (26). However, the relevance of these findings for evaluating carcinogenicity of HBV and its claimed interaction with dietary AFB\textsubscript{1} is unclear (4).

In about 50% of HCC emerging in woodchucks chronically carrying WHV, insertion mutagenesis activating myc family genes has been demonstrated, but the answer to the main question of whether these viral integrations play a part in the process of neoplastic cell conversion remained open (2, 27). Integration of viral DNA in HCC of chronic WHV carriers (which were not exposed to AFB\textsubscript{1}) did not lead to p53 gene mutations (28). i.p. injection of AFB\textsubscript{1} into ground squirrel hepatitis virus-carrying ground squirrels resulted in mutations of the p53 gene in only one of the five HCC-bearing animals studied (28). In HBV transgenic mice, multiple oncogenes including c-myc and the tumor suppressor genes p53 and RB remained structurally and functionally intact during hepatocarcinogenesis (29). Most of these findings question rather than endorse the frequently claimed role of multiple genetic events in hepadnaviral hepatocarcinogenesis, but only a few appropriate experimental investigations on the possible mutagenic effect of an additional AFB\textsubscript{1} treatment in this process have hitherto been conducted.

Woodchucks chronically carrying WHV seem to be the most reliable model of viral hepatocarcinogenesis. We took advantage of this...
animal model and investigated the effect of dietary AFB,

SYNERGY OF HEPADNAVIRAL INFECTION AND DIETARY AFB,

MATERIALS AND METHODS

Woodchucks and WHV Inoculation. Twenty-four WHV carriers and 24

Table 1 Presence of WHV DNA in serum and/or liver tissue of woodchucks inoculated with WHV as newborns

<table>
<thead>
<tr>
<th>Animal code</th>
<th>WHV DNA in serum (spot blot) months postinoculation</th>
<th>WHV DNA (PCR)</th>
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<tbody>
<tr>
<td></td>
<td>8 12-15 18-20 25-31 35-38</td>
<td>Serum</td>
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| F1074       | +++a | + | + | + | ++
| M1272       | +++ | +++ | ++ | ++ | * |
| M1071       | +++ | + | ++ | + | * |
| F1172       | +++ | nd | ++ | + | + |
| M1242       | ++ | ++ | nd | + | + |
| F1076       | ++ | nd | ++ | + | + |
| F1252       | ++ | + | ++ | + | + |
| F1043       | +++ | ++ | ++ | + | + |
| M1122       | ++ | ++ | + | ++ | + |
| M1082       | ++ | + | ++ | + | + |
| M1072       | +++ | + | ++ | + | + |
| F1055       | +++ | ++ | ++ | + | + |

a, corresponds to $10^5$; ++, to $10^{6-7}$, and ++++, to $10^{8-9}$ genome equivalents.

Table 2 Presence of WHV DNA in serum and/or liver tissue of woodchucks inoculated with WHV as newborns and treated with AFB,

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a, corresponds to $10^5$; ++, to $10^{6-7}$, and ++++, to $10^{8-9}$ genome equivalents.
b, death of animal.
c, not determined.

Experimental Design and AFB,

Serological Assays and Detection of WHV DNA in Sera and Tissue. WHsAg and antibodies to WHsAg and WHV core antigen in woodchuck sera were tested by an ELISA, essentially performed as described earlier (31). WHV DNA in the serum was detected by spot hybridization with the use of a [32P]dCTP-labeled DNA-probe containing the complete core gene of WHV.
Table 3  Incidence of liver tumors observed by repeated ultrasound examination of the
woodchucks chronically infected with the WHV with and without dietary administration
of AFB1 (AFB1)

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<th>Duration of AFB1 treatment (months)</th>
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<th>WHV</th>
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* One animal without a liver tumor died spontaneously at an earlier time point.
* One animal with multicentric hepatocellular neoplasms died spontaneously at an earlier time point.
* One animal without a liver tumor had recovered nearly completely from the WHV infection early in the experiment.
* Three animals without liver tumors had recovered nearly completely from the WHV infection early in the experiment.

Briefly, 5 μl of serum were spotted directly onto a charged Nylon membrane (Hybond-N, Amersham-Buchler, Braunschweig, Germany). The DNA was denatured and hybridized with the probe according to standard procedures (32). After intense washing, the membranes were dried and exposed on Kodak X-Omat S X-ray films for up to 3 days.

In the case of negative or weak reaction in the spot hybridization, PCR was used for the demonstration of residual low level viral DNA in sera and liver tissue. PCR was performed with oligonucleotide primers W2038 (5'-CGC-CATGGCATACTCATCTCCATTTTCAATAATTATA-3') and W2570 (5'-TCAGCAGTTGGCA-GATG-3') encompassing the WHV core gene and W1479 (5'-GTCGGG-GAAGCTGACGTCCCTCC-3') and W1937 (5'-CTGCAGCTCGAGATG-3') flanking the x-gene. Temperature profiles were 1 min at 94°C, 1 min 30 s at 52°C, and 2 min at 72°C for 30 cycles and 1 min at 93°C, 1 min at 48°C, and 2 min at 68°C for 34 cycles for the amplification of the x and core regions, respectively. Each PCR included an initial denaturation step at 94°C for 4 min and a terminal elongation step at 72°C or 68°C for 10 min for x- and core-PCR, respectively. DNA from serum and tissue had been isolated with the use of proteinase K digestion and phenol-chloroform extraction, followed by ethanol precipitation (32). PCR was performed with 10 μl of extracted serum or tissue in a total volume of 50 μl containing 50 mM KCl, 10 mM Tris-Cl (pH 9.0), 0.1% Triton X-100, 2 mM MgCl2, 200 μM of each nucleotide (dATP, dCTP, dGTP, and dTTP), 30 pmol of each primer, and 1.5 units Taq polymerase (Serva, Heidelberg, Germany). Blood samples were obtained either from the femoral or medial saphenous vein under anesthesia with Ketamine (Ketavet; Parke-Davis, Berlin, Germany) and Xylacine (Rompun; Bayer, Leverkusen, Germany).

Transmission Electron Microscopy. One-mm3 tissue samples of the livers were fixed with 1.5% glutaraldehyde in 0.1 M 1,4-piperazinediethanesulfonic acid (pH 7.4; Sigma) for 60 min, postfixed with the reduced osmium procedure (33), dehydrated, and embedded in Epon. Semithin sections were cut and stained with toluidine blue to select the subject of interest. Ultrathin sections were cut from semithin sections after their reembedding, contrasted with uranyl acetate and lead citrate, and examined with a Phillips 401 electron microscope.

Histology, Histochemistry, and Statistical Evaluation. Tissue from the liver was removed at autopsy or under anesthesia with Ketamine and Xylacine, fixed in Carnoy’s solution, and embedded in paraplast for histological examination, or frozen in isopentane at —150°C for histochemical studies. In addition, at sacrifice or autopsy, specimens from all other major tissues were embedded in paraplast for clarification of spontaneous deaths, complicating

### Table 4  Preneoplastic and nonneoplastic liver lesions in woodchucks after dietary administration of AFB1

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* SC, sex and animal code; TM, time (months) after beginning of AFB1 treatment; SP, specimens (B, biopsy; A, autopsy; S, sacrifice); NEC, necrosis; CAH, chronic active hepatitis; FIB, fibrosis; BDP, bile ductular proliferation. 
* For details of scores see “Materials and Methods.” 
* nd, not determinable because of autolytic cellular changes.
Table 5 Neoplastic, preneoplastic, and nonneoplastic liver lesions in WHV carriers

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For abbreviations see Table 4.
For details of scores see "Materials and Methods."

diseases, and possible metastases. Paraffin sections were stained with hema-
toxylin and eosin, alcin blue, or Mallory's trichrome; frozen sections were
treated with fat-red and with the periodic acid-Schiff reaction for the demon-
stration of glycogen. Liver lesions were largely classified according to Popper
et al. (20). Single cell necrosis appearing as apoptosis or cytolysis was scored
as: —, absent; (+), very rare; +, rare; and ++, frequent. Hepatitis was
subjectively graded as: —, no infiltration of portal tracts by inflammatory cells;
+, weak infiltration of the portal tracts by lymphocytes, plasma cells, and
neutrophils; ++, distinct; and ++++, strong infiltration of portal tracts,
fibrotic septae, and liver lobules by lymphocytes, plasma cells, and neutro-
phils. Biliary duct proliferation and fibrosis were graded as: —, absent; +,
weak; + + , distinct; and + + + , strong. The separation and typing of preneo-
piastic FAH, HCA, and HCC follows published criteria for the woodchuck
(34) and the rat (35). FAH were classified as GSF, FSF, TCP, APF, MCF, and
basophilic cell foci and were assessed semiquantitatively as: —, absent; +,
sporadic; ++, widespread; and ++++, abundant. Statistical evaluation of the
incidence of tumors was performed according to Fisher's exact test. The time
dependence of tumor appearance was evaluated by the log rank test.

RESULTS

Virological Markers in Sera and Liver Tissue. The results on
markers of chronic WHV infection in the two WHV carrier groups
with and without AFB[1-treatment are given in Tables 1 and 2.

At 8 months of age all experimentally induced chronic WHV
carriers were positive for WHsAg by ELISA, and the sera of all
animals contained WHV DNA as demonstrated by spot hybridization,
whereas the uninfected woodchucks were negative for both WHsAg
and WHV DNA (data not shown). Antibodies to WHc were detectable
in all WHV-infected animals throughout lifetime. Since the age of 12
months, four of the WHV-infected woodchucks (F1064, F1282,
M1082, and M1274) had lost WHsAg, and WHV DNA, as determined
by spot hybridization, was no longer detectable. In one animal
(M1274) WHV DNA was detectable only at a very low level in one
serum sample by spot hybridization.

For the detection of very small amounts of WHV DNA (below 105
genome equivalents) in the sera of these four animals, PCR using
primers encompassing the core and x-genes has been used. In addition
to the detection of WHV DNA in the sera, PCR has also been
performed with liver tissue obtained from these animals at their
deaths. Animal F1064 showed no detectable WHV DNA in the serum
samples; nevertheless, viral DNA could be detected by PCR in liver
tissue obtained from this animal. In animal M1274, PCR yielded
positive results for both serum and liver tissue, whereas viral DNA
was found neither in the sera nor in the livers of animals M1082 and
F1282. In four animals (M1242, F1076, F1252, and M1122) WHV
DNA was no longer detectable by spot hybridization late in the course
of the chronic infection (>25 months) but could be detected by PCR.

Liver Histo- and Cytomorphology. The main results of the his-
tological examination of livers from WHV-infected and WHV-free
woodchucks with and without administration of dietary AFB[1 are
summarized in Tables 4–6. Negative controls, which were neither
infected with WHV nor treated with AFB[1, showed normal liver
histology, except occasional single cell necrosis and small inflamma-

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NEC CAH FIB BDP FAH HCA HCC
F1173  3  B  ++ ++ ++ ++ ++ + - -
      6  A  ++ ++ ++ ++ ++ + +
M1251 3  B  +  +  +  +  +  +  -  -
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F1275 3  B  +  ++ ++ ++ ++ + - -
      15 B  +  ++ ++ ++ + - -
      19 S  +  ++ +++ +++ + +
M1243 3  B  ++ ++ ++ ++ ++ + - -
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M1271 3  B  +  ++ ++ ++ ++ + - -
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M1274 3  B  (+) - - - - - -
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      25 S  +  +++ +++ +++ + +
F1064 3  B  -  -  -  +  +  - -
      15 B  -  -  -  +  +  -
      25 S  +  -  +  +  +++ + +
M1241 3  B  +  ++ ++ ++ + - -
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For abbreviations see Table 4.
For details of scores see “Materials and Methods.”

Tory infiltrates in the liver lobules and rarely also in the portal tracts. The rare necrotic cells appeared nearly exclusively in the biopsies suggesting that they were due to operational influences (narcotic drugs and manipulation of tissue) during the biopic procedure, which lasted on an average about 40 min (30). Glycogen content of the parenchyma was variable, ranging from low to relatively high deposits of this polysaccharide. In some animals there was also a diffuse fatty infiltration of the parenchyma, which in one case was associated with low glycogen content.

Treatment of uninfected woodchucks with AFB₁ resulted in a predominantly periportal but sometimes also perivenular zonal loss of glycogen, apoptotic or lytic death of single hepatocytes, and often also weak ductular (oval cell) proliferation in the vicinity of portal tracts (Table 4). These changes reflect unspecific, toxic effects and were especially marked 3 months after the beginning of AFB₁ administration, when the first liver biopsies were taken (Fig. 1 A). In addition, 1 of 12 woodchucks showed small FAH of the clear cell type exclusively storing glycogen (GSF) and MCF at this time point. These findings prompted us to reduce the dose of AFB₁ from 40 μg/kg bw/day, given during the first 4 months, to 20 μg/kg bw/day, administered throughout the remaining experimental period. Biopsies taken after 9 months of treatment with AFB₁ usually showed weaker unspecific, toxic changes than those obtained earlier. Except for three animals sacrificed 25 months after the beginning of AFB₁ treatment, which exhibited a weak infiltration of portal tracts by lymphocytes and plasma cells, chronic hepatitis was always absent. However, different types of FAH, including GSF, FSA, MCF, TCF, and APF, were occasionally observed in about 50% of the animals receiving AFB₁ for more than 9 months (Fig. 1B). All FAH were smaller than a liver lobule and were preferentially localized in peripheral and intermediate lobular regions. In one animal, some small cysts lined by cells resembling bile duct epithelia were found. Neither HCA nor HCC was detected in any of the woodchucks treated with AFB₁ alone.

In contrast to the negative controls and the woodchucks treated with AFB₁ alone, nearly all woodchucks infected with WHV developed a chronic active hepatitis (Fig. 1, C and D), which was particularly pronounced in WHV carriers administered AFB₁ (Tables 5 and 6). Only the four animals, which according to the serological tests had largely recovered from the WHV infection (1 animal without and 3 animals with AFB₁ treatment) at the age of 1 year, showed no or merely a very mild hepatitis. Nevertheless, all of these animals exhibited a few small FAH (GSF, FSA, APF, MCF, and TCF) and occasional oncocytes and ground glass cells in biopsies or in specimens obtained at the end of the experiment (Fig. 1E). One animal showed a pronounced storage of glycogen (glycogenesis) and/or fat (lipidosis) throughout the remaining parenchyma. Moreover, several small focal infiltrates of atypical lymphatic cells resembling a malignant lymphoma were found in the liver but not in extrabiliary tissues.

In biopsies taken from the remaining WHV carriers at 3, 6, and 15 months after starting the combination experiment, there was always an infiltration of portal tracts by lymphocytes, plasma cells, and frequently also neutrophils. Neutrophils were especially observed at the first and second time point of biopic investigations. The intensity of the inflammatory reaction ranged from weak to distinct, often showing a tendency of progression in individual animals with time.
Frequent additional findings in the biopsies were apoptotic or lytic death of single hepatocytes in the lobular periphery and inside the lobules, weak to distinct ductular (oval cell) proliferations and fibrotic changes, macrophages loaded with a faintly periodic acid-Schiff-positive and alcianophilic pigment in portal tracts and fibrotic septae, and various types of FAH, especially clear cell foci (Fig. 1F) excessively storing glycogen and fat, but sometimes also APF and MCF, containing oncocytes and ground glass cells in addition to the other types of altered hepatocytes. The typical morphology of these altered hepatocytes was verified at the ultrastructural level (Figs. 2 and 3). The majority of hepatocytes excessively storing glycogen and/or fat were free of WHsAg, although profiles of the SER were loosely scattered or arranged in small complexes inside or in the periphery of the huge glycogen zones filled with densely packed α or β particles (Fig. 2, A and B). Ground glass cells were characterized by abundant SER that was closely associated with glycogen particles and was stuffed with WHsAg filaments often forming large aggregates extending the endoplasmic reticulum cisternae (Fig. 3A). Amphilophic cells were poor in glycogen but crowded with mitochondria and peroxisomes (Fig. 3B); these organelles were wrapped by the cisternae of the rough endoplasmic reticulum. The mitochondria of amphiphilic cells often showed a reduction in the number of their cristae and an enlargement of their volume; profiles of the SER contained WHsAg filaments. Basophilic cells in MCF were poor in glycogen and fat but rich in ribosomes that were either bound to the endoplasmic reticulum or formed free polysomes. Remarkable nuclear alterations in FAH were marked condensation of the chromatin in many GSF and FSF, frequent decondensation of the chromatin in the remaining types of
SYNERGY OF HEPADNAVIRAL INFECTION AND DIETARY AFB₁

Fig. 2. A and B, electron micrographs of hepatocellular alterations induced in woodchucks by chronic WHV infection and dietary AFB₁. A, excessive storage of glycogen (G), resulting in huge zones occupied by α or β particles and associated at places with small complexes of smooth endoplasmic reticulum (star). N, nucleus; S, perisinusoidal stellate cell. Bar, 5 μm. B, portion of A showing SER free of WHsAg filaments at higher magnification. M, mitochondria. Bar, 1 μm.

FAH, and occasional glycogenic nuclei in APF but sometimes also in GSF and MCF. FAH were usually smaller than liver lobules and increased in number and size with time. In one biopsy obtained 15 months after the beginning of AFB₁ treatment, a small HCA of the amphophilic cell type was recognized.

The majority of WHV carriers that died spontaneously or were sacrificed between 11 and 26 months after commencement of the experiment showed a chronic active hepatitis with marked inflammatory infiltrates extending beyond the limiting plate into the liver parenchyma, pronounced proliferation of bile ductules, advanced fibrosis, and sometimes even an incomplete cirrhosis (Tables 5 and 6). These alterations were especially marked in chronically infected woodchucks treated with AFB₁, in which the hepatitis was eventually always associated with HCC and usually also HCA. All woodchucks bearing HCC and HCA showed characteristic cellular changes in the remaining liver parenchyma. Multiple prominent FAH (Figs. 4 and 5), some of which imposed at the liver surface as yellow or gray spots, were regularly found (Fig. 6A). GSF, FSF, and APF (often containing oncocyes and ground glass cells) were the most frequent types of FAH; their size was usually smaller than, or just corresponding to, a liver acinus. In addition, MCF with varying cellular composition were often seen. Some MCF had about the same size as GSF, FSF, and APF, but the majority of MCF were much larger, often exceeding the size of one or even several liver acini. It is remarkable that extended MCF encompassing several acini were encountered, which did...
not disturb the histological architecture of the liver parenchyma. All transitions from such MCF to expansively growing HCA (Fig. 6B) of the mixed or amphophilic cell type with surprisingly well preserved acinar architecture were observed, suggesting their origin was from fields of altered hepatocytes rather than from single-cell clones. In many animals it was difficult to clearly define the border of FAH because striking changes of the hepatocytes were usually also present in the extrafocal tissue. These changes were basically similar but less pronounced than those in GSF, APF, or MCF and occupied nearly the whole rest of the parenchyma.

Hepatocellular neoplasms were in most cases multicentric and were easily visible with the naked eye as large, yellow or gray, and often focally hemorrhagic lumps (Fig. 6A). The close correlation between severe chronic hepatitis and hepatocellular neoplasms was also evident in WHV carriers not treated with AFB1, but the neoplastic lesions that emerged at later time points were limited to a lower proportion of the animals and were exclusively benign in three cases under these experimental conditions. HCA appeared as more or less solid, expansively growing nodules compressing the surrounding parenchyma and consisted of various, often mixed, cellular phenotypes including clear cells excessively storing glycogen and/or fat (Fig. 6B) and amphophilic or basophilic cells poor in glycogen and fat. HCC formed solid,
Fig. 4. A–D, light micrographs of focal liver lesions induced in woodchucks by chronic WHV infection and dietary AFB₁. A, clear cell focus (right) and amphophilic cell focus (left) in the parenchyma. H&E, × 60. B, serial section to A showing excessive storage of glycogen in clear cell focus and reduction of glycogen deposits in amphophilic cell focus. Periodic acid-Schiff, × 60. C, focus of altered hepatocytes excessively storing glycogen and fat (vacuoles). Periodic acid-Schiff, × 125. D, focus of altered hepatocytes excessively storing fat. Fat-red, × 60.

The trabecular, and sometimes also adenoid histological patterns. They contained, in principal, similar cellular phenotypes as HCA. Whereas clear and amphophilic cells were characteristic of highly differentiated tumors and tumor components (Fig. 6C), basophilic cells prevailed in poorly differentiated tumors or tumor portions that often showed many abnormal mitotic figures and marked nuclear atypia (Fig. 6D). Bile plugs were often seen in HCC. Although an infiltration of intrahepatic blood vessels was often found, distant metastases were never observed. Poorly differentiated HCC were frequently interspersed by hematopoietic foci containing many megakaryocytes, which in some animals were also often found in the preneoplastic parenchyma.

In four WHV carriers with and in two WHV carriers without AFB₁ treatment, small uni- or multilocular cystic cholangiomas were observed in addition to the various hepatocellular lesions.

Survival of Animals and Statistical Evaluation of Hepatic Neoplasia. All animals from the negative control group survived until sacrifice. One of the chronic WHV carriers treated with AFB₁ died from rupture of an aortic aneurysm and massive intrathoracic bleeding. From the group treated with AFB₁ alone, five animals died spontaneously at early time points of the experiment without any indication of severe hepatotoxicity. Two animals did not survive the open liver biopsies because of postoperative complications (shock syndrome, in one case associated with a chronic nephropathy). Two additional animals died from focal intrapulmonary bleeding, which was associated with multiple bronchioloalveolar proliferations of foamy cells (lipidosis). Autopsy of the fifth animal revealed an advanced chronic nephropathy and focal endomyocardial necrosis with intracardial thrombosis in the left atrium. Extrahepatic neoplastic disease was only found in one animal that was treated with AFB₁ alone for 13 months and was sacrificed because of bad health. This animal had a squamous cell carcinoma of the tracheal mucosa with intracranial metastatic spread to the lungs (pneumonia carcinomatosa), which was most probably unrelated to the short treatment with AFB₁. Bronchioloalveolar foamy cell proliferates without intrapulmonary bleeding, but occasional inflammatory cell infiltrates were found in a number of chronic WHV carriers, especially in those treated with AFB₁, and in animals treated with AFB₁ alone.

Statistical evaluation of the appearance of HCA and HCC in chronic WHV carriers with and without administration of AFB₁ revealed that hepatocellular neoplasms (HCA and HCC combined) emerged significantly (P < 0.05) earlier after additional treatment with AFB₁. There was also a higher incidence of HCC in animals receiving a combined treatment with WHV and AFB₁ (75%) compared to those treated with WHV alone (33%), but due to the small group size this difference was only weakly significant (P < 0.1). When those woodchucks showing a nearly complete serological and histological recovery from the WHV infection (1 animal without and 3 animals with AFB₁ treatment) were excluded, the difference in the incidence of HCC (100% with versus 35% without AFB₁) became highly significant (P < 0.01); even the difference in the combined incidence of benign and malignant hepatocellular neoplasms observed at all time points became weakly significant (P < 0.1). The difference in the time of appearance was highly significant (P < 0.01) for both combined hepatocellular neoplasms and HCC under these conditions.
SYNERGY OF HEPADNAVIRAL INFECTION AND DIETARY AFB₅

DISCUSSION

The results provide conclusive evidence for a synergistic hepatocarcinogenic effect of chronic WHV infection and dietary AFB₅ in woodchucks. Under our experimental conditions, the contribution of the chronic WHV infection to the carcinogenic process was much stronger than that of the low dose of AFB₅, which did not produce any hepatocellular neoplasm when administered alone. The relatively large number of animals that died at early time points in the group receiving AFB₅ alone should not have influenced this result significantly because nearly one-half of the animals survived to the end of the experiment without any indication of hepatic neoplasms. In contrast, both HCA and HCC developed in many woodchucks chronically infected with WHV without additional treatment by AFB₅, but these neoplasms appeared at later time points, were limited to a lower proportion of animals, and were in several cases restricted to a less advanced (benign) stage of neoplastic development compared to those appearing after the combined effect of the WHV-carrier status and AFB₅. The strong hepatocarcinogenic effect of the chronic WHV infection observed in our experiments confirms earlier reports from other laboratories (20, 36, 37). The decisive role of the chronic WHV infection for hepatocarcinogenesis became particularly evident in those animals that seroconverted after 1 year and showed neither a chronic active hepatitis nor hepatocellular neoplasms, no matter whether AFB₅ was given. However, all of these animals contained a few small preneoplastic FAH in the liver parenchyma. Whereas AFB₅ or a synergistic effect may have been responsible for the induction of these FAH in the combination experiment, the foci observed in the animal not treated with AFB₅ were apparently only due to the past infection with WHV. Korba et al. (38) have reported that past WHV infection with seroconversion to anti-WHs was even associated with a low risk (5.4%) of development of HCC, appearing 20–30 months after recovery from the acute hepatitis. It is evident from this observation that chronic hepatitis is not a conditio sine qua non for the development of HCC in WHV carriers.

Popper et al. (20) found only minimal hepatitis in chronic WHV carriers experimentally infected as newborns or adults for at least 1.5 years. In our experiments, nearly all chronic WHV carriers showed a weak to distinct hepatitis when the first biopsies were taken 15–16 months after the virus inoculation. The inflammation usually progressed with increasing age in individual animals and was more pronounced in the animals additionally treated with AFB₅. This was also true for fibrotic changes and for the accompanying bile ductular proliferation. The contribution of the chronic hepatitis to hepatocarcinogenesis is difficult to assess because the inflammatory reaction and the emergence of preneoplastic FAH and hepatocellular neoplasms largely coincided. In line with the observations by Popper et al. (20) the hepatitis was most marked in livers bearing HCC. However, the results of our sequential investigations favor a gradual enhancement of the hepatitis rather than a late wave of necroinflammation as suggested by these authors. Nevertheless, necroinflammation might promote neoplastic progression initiated by WHV (20) and might also play a role in the acceleration and enhancement of WHV-induced hepatocarcinogenesis by dietary AFB₅ administration, although no remarkable inflammatory changes were found in HBV transgenic mice during hepatocarcinogenesis enhanced by i.p. injection of AFB₅ (26). The finding that most animals developing hepatocellular neoplasms showed a gradual reduction of the peripheral viremia is noteworthy because similar observations have been made in human long-term HBV carriers with HCC (39). In nonviremic patients...
with replicative HBV DNA in the liver, viral core antigen was markedly reduced or absent, whereas HBsAg expression was maintained, suggesting that decreased or defective core antigen might lead to reduced viremia (39).

In addition to cytolysis, which may represent immune-mediated cell death, apoptosis without surrounding inflammatory reaction was often seen in chronic WHV carriers and was generally more pronounced in carriers treated with AFB₁. Apoptosis is frequently increased in preneoplastic and particularly in neoplastic hepatic lesions induced by chemical carcinogens (40-43). Whereas some authors feel that apoptosis plays a major role in counterbalancing cell replication in these lesions (40, 42), others emphasize that cell death occurs more frequently in the course of hepatocarcinogenesis the more that neoplastic development advances (41, 43). This explanation may also hold true for the findings in WHV carriers with and without AFB₁ administration of AFB₁, With the exception of the first series of biopsies, in which two carriers treated with the initially relatively high doses of AFB₁ frequently showed necrotic hepatocytes, this finding was in most cases associated with the presence of hepatocellular neoplasms.

Both lytic and apoptotic cell death may elicit ductular (oval) cell proliferation which was pronounced in most of the chronic WHV carriers. In several animals the ductular proliferation resulted in the development of small cystic cholangiomas. Oval cell proliferation has also been linked with the evolution of WHV-induced HCC in woodchucks by some authors (21, 44). However, in our series of WHV carriers the phenotype and time course of development of FAH regularly preceding HCA and HCC indicated that at least the majority of hepatocellular neoplasms derived directly from the hepatic parenchyma. The intriguing question of whether oval cells might have contributed to the development of a subset of neoplastic lesions with a hepatocellular phenotype deserves more detailed studies at the electron microscopical level, which are currently in progress.

The most striking changes of the liver parenchyma in WHV carriers with and without AFB₁ administration were focal lesions, the phenotype of which was very similar to FAH well known from chemical hepatocarcinogenesis in various species, including primates (35). Such focal alterations were noted previously and characterized in some detail in chronically WHV-infected woodchucks (34, 45). The relevance of FAH for hepadnaviral hepatocarcinogenesis has been underlined by their recent discovery in two lines of HBV transgenic mice (46, 47) and in human liver specimens obtained from patients bearing HCC or suffering from liver cirrhosis, including posthepatitic cirrhosis due to HBV infection (48, 49). In this study we demonstrate that AFB₁ induces FAH in woodchucks exhibiting the same cellular phenotypes as those produced by WHV, although their incidence, number, and size are much smaller compared to FAH in chronic WHV carriers. These phenotypic similarities suggest closely related mechanisms of action of both oncogenic agents at the cellular level, which may also be the principal cause of their synergistic effect. In addition, the necroinflammation mentioned earlier, and the possibility of an increased metabolic activation of AFB₁ by the induction of drug metabolizing enzymes in chronic WHV carriers as demonstrated in woodchucks by DeFlora et al. (50), have to be taken into account.

The significance of the sequential metabolic and morphological cellular changes gradually emerging during the evolution of FAH,
HCA, and HCC in chronic WHV carriers and in HBV transgenic mice has been discussed by Toskolv et al. (34, 47) recently. The findings in this study support the notion that a fundamental shift in energy metabolism plays an important role. In addition to an early excessive storage of glycogen (glycogenosis) and/or lipids (lipidosis), and mitochondrial alterations leading to oncocyes and amphophilic cells, a late increase in ribosomes (basophilia) associated with a loss of glycogen and lipids are indicators of decisive metabolic events, the molecular basis of which has been analyzed to some extent in the rat but remains in many respects elusive (51–56). In the last few years it became increasingly evident that the metabolic pattern and proliferative behavior characterizing the chemically induced preneoplastic focal glycogenosis (and lipidosis) resembles an insulin effect (54, 57–59). Insulin stimulates signal transduction pathways involved in a variety of metabolic processes including glycogen synthesis, lipogenesis, glycolysis, and the regulation of cell proliferation (60, 61). The oncogenic potential of HBV has been attributed tentatively to the closely related protein kinase C-signaling pathway, which resembles an insulin effect.


Synergistic Hepatocarcinogenic Effect of Hepadnaviral Infection and Dietary Aflatoxin B₁ in Woodchucks


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