Hepatic Neoplasms in Aflatoxin B$_1$-treated, Congenital Duck Hepatitis B Virus-infected, and Virus-free Pekin Ducks

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ABSTRACT

To assess the effects of the combination of persistent hepadnavirus infection and chemical carcinogen exposure, aflatoxin B$_1$ (AFB) was administered p.o. for 60 days to congenitally duck hepatitis B virus (DHBV)-infected and virus-free Pekin ducks, starting at 3 days of age, during a 28-month study. Hepatic neoplasia occurred only in AFB-dosed ducks. Hepatocellular carcinomas or biliary carcinomas occurred in 4 of 8 DHBV-infected and 3 of 4 DHBV-free ducks, and hepatocellular adenomas developed in 2 DHBV-infected AFB-dosed ducks that survived 20 months or longer. Altered foci of hepatocytes similar to those observed in chemical carcinogen-dosed rodents, characterized by enlarged eosinophilic hepatocytes or vacuolated cytoplasm, occurred in AFB-dosed ducks. Cells in foci or hepatic neoplasms did not contain histochemically detectable γ-glutamyltranspeptidase but were distinguished from uninvolved parenchyma by altered glycogen content. Immunohistochemical staining indicated that DHBV core antigen persisted in liver, spleen, pancreas, and, to a lesser extent, kidney of most congenitally infected ducks up to 28 months of age. Hepatic neoplasms contained only patches of hepatocytes that were detectable viral antigen. Southern blot analysis of restriction endonuclease-digested neoplastic and normal liver DNA revealed high molecular weight forms of DHBV DNA consistent with episomal virus, and promotional activity through the stimulation of hepatocyte replication in response to host immune-mediated hepatic injury.

Dietary contamination with the mycotoxin AFB is also linked with human HCC by epidemiological studies (11–17). AFB has been extensively studied in rodents, and its carcinogenic activity is attributed to covalent adduct formation by metabolically activated reactive intermediates with hepatocellular DNA, which leads to mutations in the host genome (16, 18, 19). AFB is a potent carcinogen in some experimental animals, but there is considerable variation in the susceptibility of different species to AFB-induced carcinogenesis (20, 21). Although woodchucks and ground squirrels have not been studied, ducks are quite sensitive to the hepatocarcinogenic effects of AFB (21, 22).

Areas of the world with a high rate of HCC are often characterized by significant dietary contamination with AFB and an increased prevalence of HBV surface antigen carriers (23). It is possible that these two factors may act synergistically to produce HCC. In order to study the interaction of chronic hepadnavirus infection and AFB exposure on the production of HCC, we have studied the carcinogenic effects of AFB exposure and congenital DHBV infection alone and in combination in ducks over the course of 28 months.

MATERIALS AND METHODS

Experimental Animals. Two flocks of Pekin ducks were established from eggs of DHBV-infected and DHBV-free ducks maintained at Stanford University. Infected ducks were separated from uninfected ducks. Ducks were reared indoors and fed commercially prepared feed, ad libitum, that contained 30% protein for the first 3 months and 16% protein for the remainder of the study. Feed was analyzed for AFB levels on five occasions during the course of the study by high pressure liquid chromatography at the North Carolina State University Mycotoxin Laboratories.

Four samples contained less than 20 µg in AFB/kg and one contained 41 µg AFB/kg. Twelve congenitally DHBV-infected ducklings were dosed with AFB as described below, and 8 were dosed with corn oil vehicle only. Ten uninfected ducklings were dosed with AFB, and 9 were dosed with corn oil vehicle only.

Aflatoxin Preparation and Dosing. Purified AFB (Sigma Chemical Co., St. Louis, MO) was dissolved in chloroform and added to corn oil (Fisher Scientific, Raleigh, NC). Chloroform was evaporated from the mixture in a rotary evaporator. A stock solution of 2 mg AFB/ml was created and diluted in corn oil as necessary for dosing. Final AFB concentrations of the stock solution were confirmed by high performance liquid chromatography. All dosed ducks received 0.2 µg AFB/g body weight by daily gavage for 60 days, starting at 3 days of age. Ducks received between 1.74 and 2.04 mg AFB total dose, depending on their weight. Control ducklings received an equal volume, about 10 ml/kg, of corn oil only.

Serum Analysis. Serum from the ducks was analyzed by slot blot hybridization to detect DHBV viremia during the course of the study, at 3 months and 6 months of age, utilizing a technique described previously (24). Serum collected at the conclusion of the study or from moribund ducks was also analyzed by an automated analyzer to determine levels of GGT.

Histopathology and Immunohistochemistry. Ducks were necropsied at 28 months of age. Liver, pancreas, spleen, and kidney from these...
Hepatic neoplasms in AFB-treated DHBV-infected ducks

A summary of results from histopathological classification of hepatic inflammation, neoplastic lesions, biliary proliferation, and amyloidosis and serum GGT levels in ducks is found in Table 1. Serum samples of all congenitally infected ducks were positive for DHBV DNA by slot blot hybridization when tested at 3 and 6 months of age, except 1 duck (263) that had no detectable DHBV DNA at the 3-month bleed. Six uninfected groups. Mild to moderate biliary hyperplasia occurred more frequently and with greater severity in AFB-dosed ducks, regardless of DHBV infection. No histological lesions were found in the pancreata of ducks from any group other than mild lymphoid infiltrates in the interstitium and perilucent sites and focal acinar hyperplasia seen in all groups. The kidneys were also normal. Splenic amyloidosis was the only abnormality in nonhepatic tissue observed in ducks at the end of the study.

Hepatic neoplasms were only found in ducks that had been exposed to AFB. Hepatic neoplasms were first detected in a congenitally DHBV-infected duck (259) and an uninfected AFB-dosed duck (155) by 20 months of age. Four of 8 AFB-dosed congenitally DHBV-infected ducks developed carcinomas and 2 additional ducks developed adenomas only (Table 1). Three of 4 AFB-dosed uninfected ducks which survived to at least 18 months of age developed carcinomas (Table 2). No hepatic neoplasms developed in 6 DHBV-infected or 6 uninfected ducks of similar age that did not receive AFB.

Livers from 5 ducks that contained hepatic neoplasms were enlarged and distorted by multinodular masses (Figs. 2 and 3). Two livers contained single 2–3-cm masses. The cut surface of the enlarged livers was characterized by multiple well demarcated or encapsulated nodules, which varied from pale white with a resilient consistency to light brown with a friable consistency. The cut surface of each of the single HCC was yellow to tan and friable. Larger neoplasms often contained multiple 2–10-mm cystic spaces filled with blood or necrotic material. Fibrinous capsular adhesions to adjacent viscera were common in livers with large neoplasms.

Histologically, two types of hepatocellular carcinomas were evident. Hepatocellular carcinomas were usually encapsulated and composed of areas of crude trabeculae containing enlarged hepatocytes with brightly eosinophilic cytoplasm, enlarged variably sized vesicular nuclei, and prominent, often multiple, nucleoli (Fig. 4). The second type was characterized by areas composed of irregular primitive glandular structures lined by polygonal to cuboidal cells with abundant to scant eosinophilic cytoplasm and vesicular nuclei with prominent nucleoli (Fig. 5). In many areas, these two histological patterns were confluent or admixed with little or no demarcation between them. Most tumors contained a mixed lymphoid infiltrate scattered through the mass. One duck (251) had a neoplasm diagnosed as a biliary carcinoma, which was composed of small cuboidal basophilic cells with round deeply basophilic nuclei. These cells formed irregular tubules, which were separated by variable amounts of fibrous connective tissue. No metastasis was observed from any of the hepatic neoplasms.

Circumscribed, well differentiated, expansile masses diagnosed as hepatocellular adenomas occurred in 3 congenitally DHBV-infected AFB-dosed ducks, 1 of which also had a car-

ducks and those that died or were sacrificed prior to the termination of the study were collected in 10% neutral buffered formalin or St. Martin fixative (25). Fixed tissues were processed routinely, embedded in paraffin, and sectioned at 6 μm thickness. All tissues were stained with hematoxylin and eosin and selected tissues were stained with oil red O. Inflammatory lesions in the liver were graded subjectively without knowledge of treatment, using a previously described format (24). Briefly, 0 = no inflammation, ± = scant lymphocytic infiltrates of some portal tracts, + = minor portal tract infiltration with lymphocytes and heterophils associated with minor focal proliferation of Kupffer cells or bile ducts, ++ = prominent lymphocytic infiltrates of portal tracts associated with individual hepatocyte necrosis (acidophilic bodies) and focal parenchymal lymphocytic infiltration, and +++ = prominent lymphocytic infiltration of portal tracts which expanded and frequently penetrated the limiting plate or bridged to adjacent portal areas associated with parenchymal infiltration, acidophilic bodies, and Kupffer cell proliferation. Biliary proliferation was graded as follows: 0 = no biliary proliferation, + = mild proliferation with no change in the size of the portal tract, ++ = moderate proliferation that enlarged the portal tract, and +++ = prominent enlargement of the portal tract with extension into the parenchyma.

DHBV core antigen was detected by a peroxidase antiperoxidase technique utilizing a rabbit-derived anti-DHBV core antibody (26). Antibody was prepared from rabbits that were immunized with DHBV cores isolated from duck liver and purified by centrifugation through a series of sucrose gradients and CsCl gradients (26). Controls included blocking staining by incubating antisera with purified DHBV core at 37°C for 1 h, as well as replacing immune antisera with nonspecific rabbit serum as the primary antisera. Liver, pancreas, spleen, and kidney from ducks that survived 9 months or longer were stained. Histochemistry. Frozen sections of tumor and uninvolved liver were stained for the presence of glycogen by the PAS method and for GGT using the technique described by Ruttenberg et al. (27).

Southern Blot Analysis. DNA was extracted from a 1–2-g sample of frozen liver from tumor or uninvolved liver by standard methods (28). Briefly, following Dounce homogenization, samples were incubated at 37°C in the presence of sodium dodecyl sulfate and proteinase K, extracted with phenol/chloroform, and precipitated with ethanol. DNA sample were digested with restriction endonucleases EcoRl and Pstl, alone or in combination, under conditions recommended by the manufacturer (Bio-Rad, Richmond, CA). DNA was subjected to electrophoresis on a 1% agarose slab gel, denatured in situ, and transblotted to a Biotrans nylon membrane (ICN, Irvine, CA) by the method of Southern (29). DHBV DNA was detected by hybridization with a 32P-labeled plus-strand DHBV RNA probe derived in vitro from the plasmid pSP65.5 provided by Drs. Tuttleman and Summers (Fox Chase Cancer Center, Philadelphia, PA) (30). Hybridization was detected by autoradiography for 10–21 days at —70°C, using Kodak XAR film.

RESULTS

A summary of results from histopathological classification of hepatic inflammation, neoplastic lesions, biliary proliferation, and amyloidosis and serum GGT levels in ducks is found in Table 1. Serum samples of all congenitally infected ducks were positive for DHBV DNA by slot blot hybridization when tested at 3 and 6 months of age, except 1 duck (263) that had no detectable DHBV DNA at the 3-month bleed. Six uninfected ducks (4 AFB-dosed and 2 nondosed controls) and 3 congenitally DHBV-infected ducks (1 AFB-dosed and 2 nondosed) died in the first year of the study with lesions compatible with bacterial septicemia. Two ducks (154, 158) died with hepatic lipidosis and biliary proliferation, typical of acute aflatoxicosis. Two AFB-dosed DHBV-infected ducks (264, 265) were sacrificed at 10 months of age.

Amyloidosis of the liver and spleen occurred in 6 (3 AFB-dosed and 3 nondosed) of 19 uninfected ducks. Five (3 AFB-dosed and 2 nondosed) of 20 congenitally infected ducks had hepatic and splenic amyloidosis diagnosed at the end of the study. Two of these ducks (165 and 166) died or were sacrificed because of the severity of the amyloidosis. Amyloid was deposited along sinusoidal borders and encircled vascular structures in the portal tracts. In severe cases, remaining hepatocytes were reduced to less than 10% of the liver parenchyma. Most ducks with hepatic amyloidosis also had amyloid deposits in the spleen.

Hepatic inflammation was generally mild, characterized by small to moderate numbers of lymphocytes and low numbers of heterophils in portal areas. Occasionally, portal areas were expanded and inflammatory cells extended along the terminal branches of the portal veins (Fig. 1). There were no significant differences in the intensity of inflammation between treatment groups. Mild to moderate biliary hyperplasia occurred more frequently and with greater severity in AFB-dosed ducks, regardless of DHBV infection. No histological lesions were found in the pancreata of ducks from any group other than mild lymphoid infiltrates in the interstitium and perilucent sites and focal acinar hyperplasia seen in all groups. The kidneys were also normal. Splenic amyloidosis was the only abnormality in nonhepatic tissue observed in ducks at the end of the study.

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Table 1 Results of aflatoxin B1 exposure and congenital duck hepatitis B virus infection in Pekin ducks

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\(^a\) Liver inflammation (see text).
\(^b\) ND, not determined.
\(^c\) This duck also had an ovarian carcinoma.

Fig. 1. Moderate hepatic inflammation (Grade 2) characterized by lymphocytic portal infiltrates with extension along terminal branches of the portal vein.

Table 2 Hepatic neoplasms in aflatoxin B1-exposed congenital DHBV-infected and virus-free ducks greater than 18 months of age

<table>
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<tr>
<th>Group</th>
<th>AFB</th>
<th>DHBV</th>
<th>No. of ducks</th>
<th>No. of ducks with hepatic adenomas or carcinomas</th>
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Similar to those in adenomas, were common in AFB-dosed birds (Fig. 7). No foci were observed in either congenitally DHBV-infected or uninfected ducks that did not receive AFB. Minimal to moderate periportal biliary proliferation, which occasionally extended more than one third of the way into a lobule, was also observed in 7 of 8 congenitally DHBV-infected AFB-dosed ducks and 2 of 4 uninfected AFB-dosed ducks that were 18 months or older when sacrificed.

An ovarian carcinoma, which consisted of multiple thinwalled cystic structures 0.5 to 1 cm in diameter and formed a mass that filled the caudal half of the pleuropitoneal cavity and coated with serosal surface of adjacent tissues, was found in 1 duck (262) (Fig. 8). Histologically, the mass was characterized by single low-columnar to cuboidal-basophilic cells, which lined crude acini or cystic structures that contained pale eosinophilic amorphous material. Occasionally, papillary projections extended into the lumen of the cystic spaces. Nuclei...
HEPATIC NEOPLASMS IN AFB-TREATED DHBV-INFECTED DUCKS

Fig. 2. Cut surface of liver from a 20-month-old congenitally DHBV-infected duck dosed p.o. with AFB. Multiple nodules of hepatocellular carcinoma have effaced the normal architecture of the liver (marker = 1 cm).

Fig. 3. Cut surface of liver from a 28-month-old AFB-dosed DHBV-free duck. A single 1 - x 1.5-cm pale tan hepatocellular carcinoma was found in the left lobe of the liver.

Fig. 4. Histological appearance of a hepatocellular carcinoma characterized by crude trabeculae formed by multiple layers of hepatocytes.

Fig. 5. Histological appearance of a hepatocellular carcinoma characterized by multiple gland-like structures lined by single-cell-thick row of hepatocytes.

Fig. 6. Hepatocellular adenoma from a congenitally DHBV-infected AFB-dosed duck. Hepatocellular adenomas were characterized by compression of adjacent parenchyma, enlarged hepatocytes, and retention of two-cell thick hepatic plate architecture.

Fig. 7. Histological appearance of a focus of altered hepatocytes, stained with hematoxylin and eosin. Foci of altered hepatocytes were characterized by enlarged eosinophilic hepatocytes that blended with hepatic plates at the margins of the focus.

from tumor cells were hyperchromatic and contained prominent nucleoli. Widespread serosal implantation and pulmonary metastasis arose from this neoplasm (Fig. 9). No evidence of viral antigen was detected in the neoplastic cells by immunohistochemistry.

DHBV core antigen was distributed diffusely in the cytoplasm of hepatocytes from all congenitally infected ducks (Fig. 10). Typically, the cytoplasm stained uniformly and the majority of hepatocytes stained, but the intensity of cytoplasmic staining varied considerably between hepatocytes. No difference in the proportion of hepatocytes that stained or the intensity of stain-
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Fig. 8. An ovarian adenocarcinoma found in one AFB-dosed congenitally DHBV-infected duck. The mass was composed of multiple cystic structures that filled the caudal half of the pleuroperitoneal cavity.

Fig. 9. Histological appearance of a serosal implant of the ovarian adenocarcinoma on the surface of the liver. Multiple cystic structures separated by fibrous connective tissue are present on the capsular surface of the liver.

Fig. 10. Histological appearance of liver from a congenitally DHBV-infected duck, stained immunohistochemically for the presence of DHBV core antigen. Virtually all hepatocytes contain detectable antigen and scattered individual hepatocytes are intensely stained.

ing was observed between congenitally infected AFB-treated or congenitally infected nondosed ducks. One congenitally infected AFB-dosed duck (265) that was sacrificed at 10 months of age had very weak staining of hepatocytes but intense staining of biliary epithelium. Foci of altered hepatocytes did not stain immunohistochemically in a consistent pattern. Some foci were similar to adjacent normal parenchyma; others did not stain. Hepatocellular adenomas either did not contain DHBV core antigen or contained only patches of cells that contained viral antigen. Generally, adenomatous hepatocytes that stained did so with less intensity than normal adjacent tissue. Hepatocellular carcinomas from infected ducks had a similar variable staining pattern in which focal areas stained moderately or intensely and larger areas did not stain (Fig. 11). A small proportion of normal biliary epithelial cells also stained in normal liver from DHBV-infected ducks. Small oval-shaped cells interpreted to be cholangioles, which were found in portal tracts or closely associated with small caliber bile ducts in the livers of DHBV-infected ducks, also contained DHBV core antigen.

DHBV core antigen was detected in the cytoplasm of cells from the pancreas, spleen, and kidney of most congenitally DHBV-infected ducks from AFB-dosed and control groups. DHBV core antigen was present most consistently in the pancreas. The usual pattern of staining consisted of a small percentage of individual pancreatic acinar cells or small clusters of these cells that contained viral core antigen. A higher proportion of pancreatic islet cells than acinar cells contained viral antigen (Fig. 12). A variable number of islet cells in each islet contained viral antigen. One infected AFB-dosed duck (259) and 1 infected duck that did not receive AFB (263) had no

Fig. 11. Histological appearance of a hepatocellular carcinoma from a congenitally DHBV-infected AFB-dosed duck, stained immunohistochemically for DHBV core antigen. Only scattered hepatocytes contain detectable viral antigen.

Fig. 12. Pancreas from a congenitally DHBV-infected duck. Most pancreatic islet cells and scattered acinar cells are stained immunohistochemically for the presence of DHBV core antigen.
detectable DHBV core in their pancreas and had weaker hepato-
cellular staining than those ducks with pancreatic viral anti-
gen. Individual cells with vesicular nuclei and abundant cyto-
plasm or small clusters of these cells in the central areas of the
splenic white pulp germinal centers contained DHBV core
antigen. Individual or small aggregates of proximal renal tu-
bular epithelial cells were stained in 6 of 11 DHBV-infected
AFB-dosed ducks and 6 of 9 DHBV-infected nondosed ducks
(Fig. 13). No staining occurred in any tissues from uninfected
ducks.

GGT was detected histochemically in bile ducts of normal
liver samples, but no staining was evident in normal or neo-
plastic hepatocytes. GGT levels in serum from ducks with
hepatic neoplasms were not elevated in comparison to ducks
without neoplasms, except in 1 duck (252) that had a biliary
carcinoma. Hepatocellular adenomas and carcinomas were dis-
tinguished from adjacent parenchyma by the absence of PAS-
stained material in their cytoplasm. Occasionally, small areas
within neoplasms contained PAS-positive material. Altered foci
in the hepatic parenchyma were either more PAS-positive than
adjacent tissue or devoid of PAS-staining material.

DHBV DNA in normal liver and hepatic neoplasms was
analyzed by Southern blot hybridization (Fig. 14). A band of
DHBV DNA with an electrophoretic mobility of approximately
3.8 kilobase pairs, indicative of a relaxed circular conformation,
was found in tumor tissue as well as unaffected liver from all
DHBV-infected ducks with hepatic neoplasms and the liver of
all ducks that did not develop neoplasms. A band with a
mobility of approximately 3.0 kilobase pairs, indicative of linear
DHBV DNA, was formed in samples digested with EcoRI,
which cuts DHBV DNA once. A second band with an electro-
phoretic mobility of approximately 2.5 kilobase pairs was also
observed in EcoRI-treated samples and probably represents
incomplete double-stranded DHBV DNA. Most undigested
samples from uninfected tissue and HCC also contained a band
with an electrophoretic mobility of 1.8 kilobase pairs, which
probably represented supercoiled DHBV DNA since it was
eliminated in EcoRI-digested samples, presumably through
conversion of the supercoiled form to a linear conformation.
No high molecular weight forms of DHBV DNA were seen in 3
dHBV-infected ducks with liver tumors when undigested DNA
was examined. Following digestion of liver DNA from neo-
plasms and uninvolved linear with restriction endonucleases
EcoRI and PstI, which does not cut the DHBV genome, high
molecular weight bands were observed in 3 of 4 tumor samples
from DHBV-infected ducks (251, 259, and 262), but not in
adjacent normal liver from these ducks or in livers of 5 ducks
that did not develop neoplasms (Figs. 15 and 16). High molec-
ular weight bands seen in EcoRI-digested samples were gen-
ernally shifted to lower molecular weight forms than those seen in
PstI-digested samples. In 1 duck, 2 bands of high molecular
weight DHBV (approximately 8 kilobase pairs) were apparent
in an undigested DNA sample (Fig. 15). These bands are likely
to represent DHBV DNA attached to non-DHBV DNA, rather
than simple DHBV polymers, since they were not converted to
linear DHBV following EcoRI digestion but were converted to
at least three bands between 6.6 and 4.4 kilobase pairs.

DISCUSSION

Human hepatocellular carcinoma may have a multifactorial
etiology (2, 10, 21). Two of the most commonly proposed
etiologies of HCC are chronic HBV infection and AFB inges-
tion, acting alone or in combination. In this study, HCC de-
veloped only in ducks that were dosed with AFB. No cocarcino-
genic effect was observed when ducks that were congenitally
infected with DHBV were dosed with AFB. Similarly, no co-
carcinogenic effect was observed in a recent study in which
ducks were infected with DHBV by inoculation at 1 day of age
and subsequently exposed to AFB (31). One hypothesis regard-
ning the possible synergism of these two agents proposes that
hepatocytes which have been initiated by an agent such as the
potent mycotoxin AFB are transformed to a malignant pheno-
type by the promotional effect of waves of hepatocyte replica-
tion in response to immune-mediated destruction of hepadna-
virus-infected hepatocytes (10, 22). This hypothesis is sup-
ported by experimental data that indicate that certain chemicals
are only effective or more effective as hepatic carcinogens if administered after partial hepatectomy, which induces hepatocyte replication, or in infant animals that have a high rate of hepatocyte replication (32–34). Chronic HBV and WHV infections are associated with a high risk of HCC, and both infections may produce a chronic active hepatitis that may provide a stimulus for hepatocyte replication due to host-mediated hepatocellular necrosis (1, 2, 4). One reason that no cocarcinogenic effect was seen in this study may be that significant immune-mediated injury of hepatocytes in congenitally DHBV-infected ducks is improbable. A humoral immune response to DHBV has been demonstrated in ducks inoculated at 8 days of age, but half of inoculated 1-day-old ducklings did not develop antibodies and those that did developed only a transient response (35). Antibody production was not detected in congenitally infected ducklings. No information is available for cell-mediated immune responses to DHBV in infected ducks. Since ducks in this study were congenitally infected and those in the previous study were infected at 1 day of age, a promotional effect due to hepatocyte replication following immune-mediated destruction of DHBV-infected cells is unlikely.

Hepadnavirus-related inflammation and cell destruction alone do not explain the emergence of HCC in individuals with little hepatic injury, such as those infected as neonates or GSHV-infected ground squirrels, which characteristically develop only minor inflammation following GSHV infection (5, 10, 23). Another hypothesis for the development of HCC proposes that the presence of hepadnaviruses in hepatocytes, either as extrachromosomal virus or integrated into the host hepatocyte genome, alters the integrity of genome expression, structure, or repair, facilitating neoplastic transformation (10, 22). However, hepatic neoplasms did not develop in chronically DHBV-infected ducks fed controlled diets in two studies in which ducks were maintained for about 1 year (31, 35). In this experiment, Southern blots revealed DHBV DNA consistent with a relaxed circular form in tumor as well as nontumorous liver. High molecular weight forms of DHBV DNA that are consistent with integration of viral genome into hepatocyte DNA were also found in 3 of the 4 tumor samples, upon
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digestion with the restriction enzyme PsI (no sites in DHBV DNA); similar digests with EcoRI (one site in DHBV DNA) and double digestion with PsI and EcoRI were consistent with this inference. Although these data suggest integration of DHBV DNA in these tumors, full proof would require cloning these fragments of chromosomal DNA and demonstrating by DNA sequencing the existence of the junction fragments. One hepatic neoplasm, however, revealed high molecular weight DHBV DNA even prior to digestion with restriction enzymes. Since this DNA was not converted to 3.0-kilobase linear DNA by EcoRI digestion, it cannot represent simple oligomeric forms of viral DNA as was found for HBV DNA in the peripheral blood lymphocytes of HBV-infected acquired immunodeficiency syndrome patients (36). This tumor DNA sample must contain some DHBV DNA that either is organized into more complex oligomers of viral DNA and has undergone sequence rearrangements or is covalently attached to some discrete-sized piece(s) of non-DHBV DNA. These patterns differ from that found in hepadnavirus-associated HCC and cell lines derived from these neoplasms from humans and woodchucks, in which integrated forms of viral DNA predominate and extrachromosomal viral forms are uncommon (37–39). Integrated but not extrachromosomal viral DNA also has been found in a HCC from a DHBV-infected duck (7). Integration of DHBV DNA into the host hepatocellular genome may be associated with HCC in some cases, as it commonly is with HBV and WHV, but more duck hepatic neoplasms will need to be examined to determine the frequency and significance of integration in chemical carcinogen-associated neoplasms and neoplasms associated with DHBV infection alone.

DHBV core antigen persisted up to 28 months in the liver, pancreas, spleen, and kidney. The highest proportion of antigen-containing cells were found in the liver and the least in the kidney, which is similar to that reported for congenitally infected ducks sacrificed at a younger age in studies employing antisurface antibodies (39, 40). Hepatocellular adenomas and biliary and hepatocellular carcinomas from infected ducks were consistently distinguished from the normal parenchyma by their pattern of immunohistochemical staining for DHBV core. Normal hepatic parenchyma was characterized by a diffuse pattern of distribution of cytoplasmic DHBV core antigen, while staining of neoplastic hepatocytes was confined to small aggregates of cells. This pattern is similar to that seen in human HCC (41–43). HBV core antigen is demonstrable in nontumorous hepatocytes but rare in neoplastic hepatocytes. This difference in viral antigen distribution may reflect heterogeneity among tumor cells that have undergone neoplastic transformation. Additionally, transformed cells may be biochemically altered from normal hepatocytes in a fashion that interferes with their ability to support viral replication or viral protein synthesis. They may have cell membrane changes, which prevent infection, or, since they are presumably a rapidly replicating pool of hepatocytes, they may not have become infected by the time the duck was sacrificed.

Histochemical detection of GGT or PAS staining is regarded as a reliable indication of preneoplastic and neoplastic changes in the hepatocytes of rodents with chemically induced hepatic neoplasia (44). Chickens given chemical carcinogens may also develop histochemically altered hepatocytes that contain increased levels of GGT, in addition to other altered histochemical characteristics (45). Foci and neoplasms with altered PAS staining characteristics were present in AFB-exposed ducks. The emergence of HCC in ducks appears to be a multistep process, as it in other species (44). Livers from AFB-dosed ducks contained numerous hepatocellular foci, which were characterized by alterations in hematoxylin and eosin staining and growth characteristics, compared to adjacent hepatocytes and those of nondosed ducks. These may be the equivalent of altered foci seen in chickens and rodents exposed to chemical carcinogens (44, 45). Some of these foci are believed to be precursors to the observed adenomas and HCC, which emerged as a result of additional unknown rare events, through which these cells progress towards neoplastic transformation.

GGT is elevated in the serum from woodchucks which develop hepadnavirus-associated HCC (46). However, GGT was not useful as an indicator of hepatic neoplasia in ducks, since serum GGT levels were elevated in only 1 of 7 ducks with carcinomas. The histological characteristics of the neoplasm from the duck with elevated GGT differed from those of the other HCC, in that the neoplastic cells appeared to be derived from biliary epithelium that did not contain histochemically detectable GGT.

Both biliary and hepatocellular neoplasms have been reported previously in AFB-dosed ducks and in DHBV-infected ducks from Chi-Tung Province, but the significance of the single ovarian carcinoma is unclear (19, 47). Ovarian carcinomas are rare in waterfowl, and AFB exposure is not associated with an increased incidence of ovarian neoplasia in any species (18, 48).

Although ducks were maintained on commercial diets, they were not free from inadvertent AFB exposure. Most samples contained less than 20 mg AFB/kg, but one sample collected at the end of the study contained 41 mg AFB/kg. None of the ducks that were dosed with corn oil only and fed this diet developed HCC. However, this level is possibly significant in view of the report by Carnaghan (21) in which 8 of 11 ducks fed naturally contaminated feed, which was estimated to contain 30 mg AFB/kg, for 14 months developed HCC. Any studies that are designed to detect induction of HCC by DHBV should include frequent analysis of the diet, prior to feeding it to ducks, to minimize the effects of this potent carcinogen. Because ducks from areas of China with a high incidence of HCC are fed uncontrolled diets, it is not possible to separate the role of AFB from DHBV in the genesis of hepatic neoplasia (9).

Hepatocellular carcinoma is one of the most common malignant human neoplasms worldwide (49). The etiology of this neoplasm is unknown but has been attributed to chronic HBV infection, AFB ingestion, and other nutritional or parasitic factors, either alone or acting in concert (15, 23). Animal hepadnaviruses can be employed to study the role of persistent viral infection and other factors such as AFB exposure in the pathogenesis of HCC. Ducks are a particularly useful model in which to study the interaction of AFB and persistent hepadnavirus infection, since, unlike the mammals that are susceptible to hepadnavirus infection, ducks have been shown to be susceptible to AFB-induced hepatic carcinogenesis (19). Similar studies of the interaction of AFB or other chemical carcinogens in hepadnavirus-infected mammals such as woodchucks or ground squirrels are needed and would contribute to our understanding of the pathogenesis of HCC in humans.

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Hepatic Neoplasms in Aflatoxin B₁-treated, Congenital Duck Hepatitis B Virus-infected, and Virus-free Pekin Ducks

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