Contribution of Aflatoxin B1 and Hepatitis B Virus Infection in the Induction of Liver Tumors in Ducks

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ABSTRACT

The study of two major risk factors in the development of hepatocellular carcinoma, namely persistent hepatitis virus infection and exposure to dietary aflatoxins, has been hampered by lack of an experimental system. To this end we have used a Pekin duck model to examine the effect of congenital duck hepatitis B virus (DHBV) infection and aflatoxin B1 (AFB1) exposure in the induction and development of liver cancer. AFB1 was administered to DHBV infected or noninfected ducks at two doses (0.08 and 0.02 mg/kg) by i.p. injection once a week from the third month posthatch until they were sacrificed (2.3 years later). Two control groups of ducks not treated with AFB1 (one of which was infected with DHBV) were observed for the same period. Each experimental group included 13–16 ducks. Higher mortality was observed in ducks infected with DHBV and treated with AFB1, compared to noninfected ducks treated with AFB1, and other control ducks. In the groups of noninfected ducks treated with high and low doses of AFB1, liver tumors developed in 3 of 10 and 2 of 10 ducks; in infected ducks treated with the high dose 3 of 6 liver tumors were observed and none in the low dose of AFB1. No liver tumors were observed in the two control groups. Ducks infected with DHBV and treated with AFB1 showed more pronounced periporal inflammatory changes, fibrosis, and focal necrosis compared to other groups. All DHBV carrier ducks showed persistent viremia throughout the observation period. An increase of viral DNA titers in livers and sera of AFB1 treated animals compared to infected controls was frequently observed. No DHBV DNA integration into the host genome was observed, although in one hepatocellular carcinoma from an AFB1 treated duck, an accumulation of viral multimer DNA forms was detected. The metabolism of AFB1, in infected and noninfected duck liver was also examined. The study on the role of DHBV infection and AFB1, in the etiopathogenesis of liver tumors may help to clarify some of the basic mechanisms of carcinogenesis.

INTRODUCTION

Hepatocellular carcinoma is one of the ten most frequent cancers worldwide accounting for 4% of the total. While relatively rare in Europe and the Americas, it is frequent in The People's Republic of China, where almost one-half of the new cases in the world (251,200 cases) occur, and in Africa (1). The relative contributions and/or interaction of HBV infection and AFB1, alone or in conjunction with HBV in the etiopathogenesis of liver tumors is not yet fully understood. The role of exposure to AFB1, in the development of HCC has been highlighted by several studies in The People's Republic of China (5, 6) and in Swaziland (7), indicating that upon a background of uniformly high levels of HBV infection, the levels of exposure to aflatoxins could be the critical determinant. The relative contributions and/or interaction of HBV infection and AFB1, in the development of liver cancer could vary considerably from region to region (see Ref. 4). The assessment of exposure to AFB1, at an individual level with recently developed immunological methods which are readily applicable to field studies will be valuable in evaluating the role of AFB1, alone or in conjunction with HBV in the etiopathogenesis of liver tumors. In parallel, a better understanding at the cellular and molecular level, in an appropriate experimental system, of the roles of these two agents in both the etiology and natural history of liver cancer should provide insights to the assessment of these risk factors in humans.

One convenient experimental system that appears suitable for such studies is the domestic Pekin duck, which is a natural host of DHBV. DHBV belongs to the hepadnavirus family (8), the prototype member of which is human hepatitis virus, and which includes also GSHV, WHV, and heron hepatitis virus. DHBV, closely related to HBV by its virion structure and genome organization, has been used successfully for elucidation of many aspects of the molecular biology of the hepadnaviruses (9). Although the mammalian hepadnaviruses such as WHV and GSHV are known to be associated with the development of hepatocellular carcinoma in their respective hosts (10, 11), the correlation between DHBV infection and liver cancer in ducks is unclear. Indeed hepatocellular carcinoma has to date been reported only in domestic ducks from a region of The People's Republic of China (12, 13) where food contamination by AFB1, was demonstrated (5). AFB1, is known to induce liver tumors in a variety of species and the duck appears particularly sensitive to both its toxic and its carcinogenic effects (14, 15).

We describe here the results of experiments aiming to examine the effect of AFB1, in the induction and development of liver cancer in Pekin ducks congenitally infected with DHBV. Recently, the results of a similar investigation have been reported (16).

MATERIALS AND METHODS

Aflatoxin and Ducks

[^14]AFB1 (20 Ci/mmol) was obtained from Moravek Biochemicals, Brea, CA, and was purified as described previously (17).

Pekin ducklings (Anas domesticus) congenitally infected with DHBV were a progeny of a flock of DHBV infected ducks maintained at INSERM Unit 271. These animals were originally obtained from a commercial farm among which congenital transmission of DHBV to 6% of the progeny has been documented previously (18). DHBV negative ducks were the progeny of Pekin ducks which were free of
infection with DHBV. An additional screening test for DHBV was performed on 1-day-old ducklings by a serum DNA hybridization test. During the whole experimental period viremic and nonviremic birds were maintained separately at the facilities provided by the École Nationale Vétérinaire de Lyon.

Administration of AFB, to DHBV Carrier and Noncarrier Ducks

A total of 87 three-month-old Pékin ducks were divided into 6 groups, as indicated in Table 1. Groups 1 and 2 include ducks not infected with DHBV and treated with high (0.08 mg/kg) or low (0.02 mg/kg) AFB,. Groups 3 and 4 contained ducks infected with DHBV and receiving the same AFB, treatment. Controls comprised ducks not treated with AFB, and either infected with DHBV (Group 5) or noninfected (Group 6). An approximately equal number of female and male ducks were included in each group.

The schedule of AFB, treatment was selected on the basis of preliminary experiments to determine the doses that were compatible with long-term survival of the animals. The ducks of the first four groups were given AFB, by i.p. injection once a week every week, from the third month posthatch until they were sacrificed 2.3 years later (June 1988). This period represents approximately one-fourth of the life span of the ducks. The effective number of ducks are those available for histology and excluding those that died due to early toxicity. The groups of ducks were housed separately outdoors with free access to water and were maintained on a standard diet. The ducks were subjected to complete autopsy and the liver tissue was either fixed in formalin or frozen and kept at -70°C. Formalin-fixed tissues were processed for routine histology and paraffin embedded sections (4 μm) were stained with hematoxylin and eosin.

Treatment of Ducks with [3H]AFB,

An initial experiment was carried out in ducks that survived up to 27 months, the time of termination of the study. In a second study, two groups of four ducks (~1 year old), either with or without DHBV infection since hatching, were treated with 0.02 mg [3H]AFBi/kg body weight i.p. in dimethyl sulfoxide. The specific activity of the injected AFB, was 11.25 mCi/mmol (~2 μCi injected/duck). Ducks were sacrificed 48 h later and plasma and livers were removed and stored frozen prior to analysis.

Preparation of Plasma Protein and Liver DNA

Plasma Protein. Aliquots (1 ml) of plasma were mixed with 4 ml cold methanol and placed on ice for 30 min to precipitate plasma protein. The protein was obtained by centrifugation (1500 × g for 20 min) and dissolved in 0.5 ml water prior to digestion with 0.5 ml Protosol (New England Nuclear) overnight at 65°C. Solutions were neutralized with concentrated HCl and radioactivity was quantitated in 10 ml Picofluor scintillation fluid. The concentration of bound aflatoxin was calculated from the concentration of protein per ml of plasma as measured by a Bio-Rad protein assay.

DNA Extraction. Liver DNA was extracted using a phenol extraction procedure (19) and purified DNA was subjected to ultracentrifugation in 10 ml Picofluor scintillation fluid. The concentration of bound aflatoxin was calculated from the concentration of protein per ml of plasma as measured by a Bio-Rad protein assay.

Detection of DHBV DNA in Serum and Liver

Detection of viral DNA was performed by dot hybridization of serum samples (50 μl) spotted onto nitrocellulose using an Hybridot apparatus (BRL). After denaturation and neutralization of DNA (18) the filters were hybridized with radiolabeled probe as described below.

Total DNA was prepared from 0.2 g of liver tissue homogenized in liquid nitrogen. After incubation with proteinase K (300 μg/ml) in the presence of 0.1% sodium dodecyl sulfate at 37°C for 3 h, proteins were removed by extractions with phenol-chloroform and nucleic acids were precipitated with ethanol. Liver DNA (20 μg) was subjected to electrophoresis in 1% agarose gel and transferred to nitrocellulose according to the procedure of Southern (20) as modified by Wahl et al. (21). For the restriction enzyme analysis, liver DNA was digested with the restriction endonucleases in the conditions recommended by the supplier (Boehringer Mannheim) prior to gel electrophoresis. For probe preparation, genome length DHBV DNA was excised from a plasmid containing cloned DHBV 16 DNA (a gift from W. Mason, Fox Cancer Research Institute, Philadelphia, PA), purified from low melting point agarose gel and labeled by nick translation (22) to a specific activity ranging from 0.8 × 10^6 to 1.2 × 10^7 cpm/μg in a reaction containing [α-32P]dCTP 300 Ci/mmol. Hybridization was performed at 42°C (in 50% formamide) as described previously (23). To quantify DHBV DNA in serum and liver, the known amounts of duck sera or liver DNA and a range of known amounts of DHBV DNA were spotted in duplicates on nitrocellulose filter followed by hybridization and liquid scintillation counting of each spot. Densitometry of the Southern blot autoradiograms was carried out on a dual-wavelength thin layer chromatography scanner.

RESULTS

Survival Rates. The survival curves (Fig. 1) show that the control ducks (Groups 5 and 6), not receiving AFB,, whether infected or not with DHBV, survive almost equally well and at 17 months approximately 80% of the ducks were still alive. The AFB, treatment resulted in a lower survival particularly at the higher dose level (Group 1) and the ducks infected with DHBV and treated with AFB, showed an even poorer survival rate; only approximately 10% of DHBV infected ducks treated with the higher dose of AFB, survived at 17 months. The survival rates among the various groups did not vary from 17 months until the termination of the experiments at 27 months.

Carcinogenicity Experiments. Liver tumors were observed in the ducks that were treated with AFB, regardless of the status of DHBV infection (Groups 1, 2, and 3) except for the infected ducks treated with the lower dose of AFB, (Group 4) (Table 1). No liver tumors were observed in the ducks (infected or not

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment*</th>
<th>No. of ducks</th>
<th>No. of ducks with liver tumors</th>
<th>No. and type of tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AFB, b</td>
<td>13</td>
<td>3</td>
<td>3 HCC, 1 adenoma</td>
</tr>
<tr>
<td>2</td>
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<td>10</td>
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<td>1 HCC, 1 adenoma</td>
</tr>
<tr>
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<td>AFB,</td>
<td>6</td>
<td>3</td>
<td>3 HCC</td>
</tr>
<tr>
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<td>AFB,</td>
<td>13</td>
<td>0</td>
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<td>5</td>
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<td>15</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>AFB,</td>
<td>16</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* The Pékin ducks (A. domesticus) were naturally infected at birth with DHBV (French strain) and received i.p. AFB, once weekly starting at 3 months of age for a period up to 27 months.

b mg/kg; total dose/year, 4.15 and 1.05.

c One with multiple liver cancer.

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AFB₃ AND DHBV INDUCTION OF LIVER CANCER

Fig. 1. Cumulative survival rates of Group 1 (●), AFB₃-0.08 mg/kg; Group 2 (▲), AFB₃-0.02 mg/kg; Group 3 (○), infected with DHBV + AFB₃-0.08 mg/kg; Group 4 (△), infected with DHBV + AFB₃-0.02 mg/kg; Group 5 (◆), infected with DHBV; and Group 6 (□), untreated control.

with DHBV) that were not given AFB₃ (Groups 5 and 6). In Groups 1 and 2, noninfected ducks treated with 0.08 or 0.02 mg/kg (total dose, 4.15 and 1.05 mg/duck), liver tumors developed in 3 of 10 and 2 of 10 ducks, respectively. The three tumors were detected at 17, 17, and 27 months in Group 1 and the two tumors in Group 2 were detected at 17 and 20 months. In Group 3 (infected ducks treated with the higher dose of AFB₃) 3 of 6 ducks had liver tumors and they were detected at 15, 17, and 27 months. No pairwise differences between treatment groups were significant at P < 0.05.

Macroscopically the liver tumors appear as large nodules several centimeters in diameter and in some instances, as in Fig. 2, the tumor almost completely replaces the liver lobe. The liver tumors were diagnosed as either adenomas or HCC according to histological criteria such as cellular arrangement, cytoplasmic and/or nuclear atypism, and uniformity of cells. The histological appearance of HCCs varied from tumor to tumor, even in two cases with multiple tumors of Groups 1 and 3. Well-differentiated HCCs showed slight to moderate nuclear atypia forming a typical trabecular pattern partly accompanied by a microglandular structure (Figs. 3 and 4). Less differentiated tumors showed a solid structure (Group 1) (Fig. 5). A poorly differentiated tumor, which was found in Group 2, was composed of highly vacuolated large polygonal cells with prominent pleomorphic large nuclei, forming partial trabecular structure (Fig. 6). This tumor was well encapsulated with thick fibrous tissue. No metastases were observed.

There were two cases of adenoma, occurring in noninfected ducks treated with AFB₃ (Fig. 7). None of the liver tumors had observable bile production.

Nonneoplastic Lesions of the Liver. In the ducks which were infected with DHBV but not given AFB₃ (Group 5), there was slight or moderate proliferation of biliary ductular cells with lymphocytic infiltrations at the periportal area (Fig. 8) and scattered small foci of lymphocytic accumulation in the lobules. The uninfected control ducks (Group 6), however, had only slight lymphocytic infiltration around the portal area.

AFB₃ administration in uninfected ducks (Groups 1 and 2)
Fig. 5. Hepatocellular carcinoma showing less differentiation, observed in a noninfected duck given the higher dose of AFB	extsubscript{1}. Tumor cells densely arranged without sinusoidal structure. H & E, × 250.

Fig. 6. Poorly differentiated hepatocellular carcinoma developed in a noninfected duck given a low dose of AFB	extsubscript{1}. Tumor cells are polygonal and many nuclei are giant and hyperchromatic. H & E, × 250.

Fig. 7. Hepatocellular adenoma composed of ductal cells forming a glandular structure occurring in a noninfected duck given the higher dose of AFB	extsubscript{1}. The nodule compresses the normal parenchyma. H & E, × 50.

Fig. 8. Lymphocyte infiltration at the periportal area of a DHBV infected animal without AFB	extsubscript{1} administration. H & E, × 250.

Fig. 9. Periportal bile duct proliferation presented in a noninfected duck given the lower dose of AFB	extsubscript{1}. H & E, × 250.

Fig. 10. Liver cirrhosis observed in a DHBV infected duck given the higher dose of AFB	extsubscript{1}. Regenerative hyperplastic nodules are separated with narrow fibrous bundle. H & E, × 40.

Induced more pronounced liver changes (Fig. 9). Many of the ducks given the higher dose of AFB	extsubscript{1} had marked biliary ductular proliferation extending from the periportal area to a variable degree and inflammatory infiltration of lymphocytes and plasma cells. Occasional eosinophilic leukocytes were prominent. The lower dose of AFB	extsubscript{1} induced these changes to a lesser extent.

In the groups of ducks infected with DHBV and treated with AFB	extsubscript{1}, the nonneoplastic liver pathology was a little more pronounced than in the corresponding groups infected with DHBV or treated with AFB	extsubscript{1}. The liver of a duck of Group 3 showed a typical cirrhotic condition (Fig. 10). Biliary ductular cell proliferation includes two features: one is characterized by a similarity to the oval cells which often develop in the livers of rats given polycyclic aromatic carcinogens and do not form...
clear ductal structure; and the other is pseudo-bile duct formation.

Fatty changes were present in some of the DHBV infected and/or AFB, treated animals as well as in the controls. Amyloidosis was evident in only 1 of 11 control ducks (Group 6) while it was found in 30, 30, 83, 46, and 29% of the experimental animals in Groups 1 to 5, respectively. In some instances it involved most of the liver parenchyma. In extrahepatic tissues no significant changes attributable to the treatment were observed.

Liver DNA Binding of AFB, in DHBV Infected or Noninfected Ducks. The aims of these studies were to examine the capacity of duck liver to metabolize AFB, and the influence of DHBV infection on this parameter, as determined by the binding of AF metabolites to liver DNA or plasma proteins. In initial experiments, two ducks from the groups in the carcinogenicity experiment were given a single dose of [3H]AFB, at 27 months of age. However, the DHBV infected ducks showed much variation in AFB,-DNA binding (data not shown) and so a second experiment was performed using 1-year-old ducks with no prior exposure to aflatoxin B,. Fig. 11 shows the presence of the DNA adduct in the liver of a DHBV negative duck treated with a single dose of [3H]AFB, at 48 h after treatment (24). Table 2 presents data from this experiment where 1-year-old ducks with or without viral infection were given a single exposure to aflatoxin at the low dose used in the carcinogenicity experiment (0.02 mg/kg) and the binding of aflatoxin to liver DNA and plasma proteins was measured 48 h afterwards. A decreased level of AF binding to plasma proteins (32%) and liver (50%) was seen in the infected birds despite one noninfected duck which had a very low level of DNA binding. In addition, plasma protein concentration was higher in infected birds.

Detection of Viral DNA in the Sera and Livers from DHBV Infected Ducks. All ducks initially screened as DHBV carriers showed persistent viremia throughout the observation period until sacrifice or death. The uninfected group remained DHBV free during the entire experiment (2–3 years). Comparison of the amount of serum DHBV DNA at the end of experimentation, i.e., after 2.3 years of AFB, administration, indicated no decrease in viremia titers in AFB, treated ducks. Even higher mean DHBV DNA titers [3.9 ± 0.6 ng DNA/ml] were observed (Fig. 12) in the analysis of serum samples from AFB, treated birds as compared with untreated controls (1.4 ± 0.4 ng DNA/ml). An increase in the viral DNA titers was also observed in four of six liver samples tested from AFB, treated ducks as compared with a mean titer of untreated controls (data not shown).

Southern blot analysis of undigested liver DNA from DHBV carrier ducks treated with AFB, for 2.3 years and untreated controls is shown in Fig. 13. The presence of several bands of viral DNA characteristic for viral replication was observed in all analyzed liver samples, indicating that active DHBV replication occurs in these animals (Fig. 13). A high molecular weight signal was observed in one HCC available for DNA analysis, which originated from a high dose AFB, treated duck (Fig. 13, Lane 15), and in none of the nontumorous samples analyzed. The digestion of this HCC DNA with restriction enzymes EcoRI or Sall, which do not cleave this DHBV isolate (23), gave a similar pattern, i.e., 21- and 24-kilobase bands, as the one obtained for undigested DNA (Fig. 14-2, Lanes U, E, and S). After digestion with either Aval (Fig. 14-1, Lane A) or BamHI (data not shown) each of which make a single cut in this DHBV, the high molecular band disappeared producing only a single genome sized band (3 kilobases). The undigested liver DNA from untreated duck showed also two faint bands of 21 and 24 kilobases which appear clearly visible after long exposure of the autoradiograms and their migration remained unchanged after digestion with restriction enzymes EcoRI and Sall (Fig. 14-2, Lane S, E, and data not shown). These bands disappeared after digestion with Aval or BamHI (data not shown). Overall, these results indicated that the high molecular weight band observed in the HCC DNA corresponded to the presence of viral DNA multimers, i.e., several copies of viral DNA, rather than to the integrated DHBV DNA (Fig. 14). These multimeric forms of viral DNA, although present in untreated controls, seemed to accumulate at least 20 times in this tumor, as estimated by densitometric analysis.

**Table 2** Aflatoxin binding to liver DNA and plasma proteins in DHBV infected or uninfected ducks

<table>
<thead>
<tr>
<th>Duck</th>
<th>Sex</th>
<th>DHBV status</th>
<th>pg aflatoxin/ mg DNA</th>
<th>pg aflatoxin/ mg plasma protein</th>
<th>mg protein/ ml plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>–</td>
<td>968</td>
<td>134</td>
<td>29.2</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>–</td>
<td>204</td>
<td>97</td>
<td>38.2</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>–</td>
<td>1057</td>
<td>130</td>
<td>34.6</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>–</td>
<td>828</td>
<td>73</td>
<td>32.6</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>764 ± 193</td>
<td>108.5 ± 14.4</td>
<td>33.7 ± 1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A11</td>
<td>F</td>
<td>+</td>
<td>369</td>
<td>61</td>
<td>39.7</td>
</tr>
<tr>
<td>A13</td>
<td>M</td>
<td>+</td>
<td>420</td>
<td>70</td>
<td>42.5</td>
</tr>
<tr>
<td>A22</td>
<td>M</td>
<td>+</td>
<td>408</td>
<td>83</td>
<td>34.8</td>
</tr>
<tr>
<td>A29</td>
<td>M</td>
<td>+</td>
<td>318</td>
<td>79</td>
<td>42.9</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>379 ± 23</td>
<td>73 ± 4.8</td>
<td>40.0 ± 1.9</td>
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</table>

Fig. 11. Elution profile of acid-hydrolyzed DNA on high pressure liquid chromatography. DNA from Duck 3 (see Table 2) was extracted and hydrolyzed as described in "Materials and Methods." Separation was on a Partisil ODS II column 10 to 80% linear methanol gradient in water. Flow rate, 1 ml/min; 1-min fractions collected. Elution positions of (I) AFB,-FAPY and (II) AFB,-N-7-guanine are indicated.
washed, dried, and autoradiographed. Row A (Lanes 1-5), sera dotted in duplicate from five ducks treated with AFB\(_t\); Row B (Lanes 6-10), sera dotted in duplicate from five untreated controls; Row C, serial dilutions of cloned DHBV DNA used for quantitation of viral DNA in the serum.

DISCUSSION

The main findings of the study (Table 1; Fig. 1) are that: (a) there was a markedly reduced survival in DHBV infected ducks when AFB\(_t\) was also administered; (b) the incidence of liver tumors was higher (3 of 6) in the ducks infected at birth with DHBV and treated with the higher dose of AFB\(_t\), (Group 3), as compared to the relevant controls (3 of 10, Group 1); (c) at the lower dose of AFB\(_t\), (Group 4) no liver tumors were observed in the infected ducks up to 27 months and 2 were observed in the uninfected comparison Group 2, but with sample sizes of the present experiment the 95% confidence interval for the ratio of incidence in Group 4 to that in Group 2 includes values as high as 2.6; and (d) no liver tumors were observed in any DHBV infected ducks in the absence of AFB\(_t\), treatment.

It has been shown by several groups (25-27) that histopathological changes of the liver in DHBV infected ducks were very mild as compared to those of human HBV infected livers. Most of these studies have been performed on experimentally infected ducks less than 1 year of age. Our study on older ducks (2.3 years old), congenitally infected with DHBV but without AFB\(_t\) treatment, similarly showed mild histopathological lesions. There was no significant hepatitis or cirrhosis in this group of ducks. Recently, studies by Uchida et al. (16) reported that no cooperation was observed between DHBV infection and AFB\(_t\), treatment in the induction of liver tumors in ducks; however, the high dose and schedules of AFB\(_t\) administration, the limited number of animals used, and the short period of observation (1 year) were probably not suitable to detect an interaction between these two risk factors. In previous experiments a high incidence of liver tumors was observed in ducks fed a diet containing AFB\(_t\), at levels of 0.03 ppm, 3.5 ppm, or more than 20 ppb for a period up to 14-24 months (5, 28, 29). The cumulative dose of AFB\(_t\), used in our experiments reported here (4.15 and 1.05 mg/kg/year) was lower than those used in these previous experiments. In addition, the difference in the age at which DHBV was infected, congenital (the present study), or noncongenital in the case of Uchida et al. (16) could also be an important variable.

A high frequency of amyloidosis in the duck liver is supposed to be partly due to social environmental stress, e.g., feeding in cages (30, 31). In the present experiment, however, the ducks were maintained outdoors, which may minimize such social stress, and yet we still observed a relatively high incidence of amyloidosis in the liver, suggesting the contribution of genetic and/or aging factors to this disorder. Severe liver lesions caused by the higher dose of AFB\(_t\), were shown to enhance the development of liver amyloidosis in the present study.

Persistent infection with animal hepadnaviruses can cause HCC in their respective hosts, although with different incidence. Thus, 100\% of woodchuck chronic WHV carriers developed HCC within 3 years in the absence of carcinogenic cofactors (10), while in ground squirrels chronically infected with GSHV, liver tumors have been reported with a much lower incidence and only in animals over 4 years of age (11). The finding of frequent viral DNA integration into host chromosomes in HCC from persistently infected humans, woodchucks, and squirrels suggests that viral integration may be involved in liver oncogenesis (10, 32). Unlike the situation observed for HBV, WHV, and GSHV, in the duck HCC has rarely been associated with DHBV infection or integration of viral DNA. HCC has to date been reported only in Chinese ducks from Chi-tung County and was not always associated with detectable virus (12, 26). Only a single case of integrated DHBV has to date been reported in such tumors (13). Colonies of DHBV infected ducks from other parts of the world do not develop HCC (12, 23, 31, 33). It is of interest to note that the prevalence of liver tumors observed in ducks from Chi-tung County correlated with the AFB\(_t\), food contamination (34) and with the incidence of primary liver cancer in these areas (5). Examples of similar correlations between adverse biological effects in domestic animals and humans related to intake of foods contaminated with mycotoxins has been suspected in various parts of the world (35-37).

Our results show that, although AFB\(_t\), administered in controlled conditions induces liver tumors in ducks, no DHBV DNA integration was observed in the analyzed liver samples. Similarly, Uchida et al. (16) did not observe DHBV DNA integration in the HCC from AFB\(_t\), treated ducks. However, a 20-fold accumulation of viral multimeric DNA forms was detected in one HCC from an AFB\(_t\), treated duck. Whether these multimeric DNA forms contained several copies of DHBV DNA, as suggested by the observed restriction pattern, or contained also some rearranged viral sequences is under study. Multimeric WHV DNA forms, not known to have a role in virus replication or integration (38), were observed in the peripheral blood lymphocytes and in the acutely infected livers of woodchucks (39, 40). These viral DNA multimers may result from homologous recombination of viral genomes and their presence seems to be dependent on the metabolic activity of the host cell (38, 41). Thus the DHBV DNA multimer accumulation observed in our study may result from altered hepatocyte metabolism following AFB\(_t\), exposure. It is known that exposure of polyomavirus or simian virus 40 infected cells to chemical carcinogens or UV irradiation may result in enhanced viral replication (42-44). Whether a similar mechanism is involved in the increase of DHBV DNA titers which we observed in the sera and livers of AFB\(_t\), treated ducks, should be further investigated.

We saw a lower level of AFB\(_t\), binding to liver DNA and plasma protein in the DHBV infected compared to noninfected ducks after a single dose of AFB\(_t\), (Table 2). This initially appears inconsistent with the hypothesis that DHBV infection could increase the metabolic activation of AFB\(_t\). In woodchucks...
AFB, AND DHBV INDUCTION OF LIVER CANCER

Fig. 13. Southern blot analysis of DNA extracted from livers of ducks infected with DHBV undigested liver DNA (20 μg/lane) from untreated ducks (Lanes 1–8), ducks treated with AFB, for 2.3 years (Lanes 9–16). Sample 15 was DNA from hepatocellular carcinoma from a high dose AFB, treated duck (No. 426). Sample 17 was DNA from a 6-month-old viremic duck used as a control (C). The position of the relaxed circular (RC), linear (L), covalently closed circular (CC), and single stranded (SS) DHBV DNA species are indicated. The size markers (in kilobases) are HindIII digested λDNA. DNA extracted from an uninfected animal does not react with this probe (data not shown).

Fig. 14. Restriction enzyme analysis of liver DNA from: 1, hepatocellular carcinoma from an AFB, treated duck (No. 426); 2, nonneoplastic liver from an untreated duck. Each lane contained 20 μg of DNA that were undigested (Lanes U), EcoRI digested (Lanes E), AvaiI digested (Lanes A), and SallI digested (Lanes S). Relaxed circular (RC), linear (L), covalently closed circular (CC), and single stranded (SS) DHBV DNA species are indicated. The size marker in kilobases are HindIII digested λDNA.

and in humans, there is some indication that activation of AFB, was higher in liver preparations from infected cases (45, 46). It is important to note, however, that our observations were made with one single exposure at a single dose and using one specific age of ducks. All these factors could influence the AFB,-DNA adduct level. In addition, the carcinogenic effects of AFB, and DHBV will depend not only on adduct level but also on the degree of cell proliferation, which may be increased with viral infection and AFB, treatment.

In addition to the potential modification of liver metabolism by AFB, (see above) other mechanisms may be involved. For example, it is known that AFB, can activate ras oncogene in rat liver (47). Furthermore, activation of c-myc oncogene following WHV integration was demonstrated in some instances in woodchucks (48). In one case of HCC, HBV sequences were integrated into the DNA binding domain of the human glucocorticoid receptor and human estrogen receptor genes (49). The study of DHBV infection and AFB, in the etiopathogenesis of liver tumors may help to clarify some of these basic mechanisms of oncogenesis.

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