Contribution of Aflatoxin B₁ and Hepatitis B Virus Infection in the Induction of Liver Tumors in Ducks¹

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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 posthatch week 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 perportal 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 infected duck, an accumulation of viral multimere 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 etiology of this cancer has been associated with two major risk factors, persistent HBV⁵ infection and exposure to dietary aflatoxins, although other etiological agents, like smoking and some occupational exposures, have also been implicated (2–4).

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 studies⁷ 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

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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 nonviemic 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, 11.25 mCi/mmol (~2 nCi 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 x 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 0.1 M HCl at 90°C for 30 min and subjected to high pressure liquid chromatography analysis. A Partisil ODS II (10 µm) column (25 x 0.5 cm) was used with a 40-min linear gradient from 10 to 80% methanol in water. Authentic AFB,-7-guanine and AFB,,-FAPY eluted at 35 and 30 min, respectively, under these conditions, with adenine clustering at 20 min.

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 x 10^6 to 1.2 x 10^6 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 quantitate 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 autoradiographs 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
AFB₁ AND DHBV INDUCTION OF LIVER CANCER

Fig. 1. Cumulative survival rates of Group 1 (○), AFB₁-0.08 mg/kg; Group 2 (▲), AFB₁-0.02 mg/kg; Group 3 (●), infected with DHBV + AFB₁-0.08 mg/kg; Group 4 (▲), infected with DHBV + AFB₁-0.02 mg/kg; Group 5 (■), infected with DHBV; and Group 6 (□), untreated control.

with DHBV) that were not given AFB₁ (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 AFB₁) 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 AFB₁ (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 AFB₁ (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.

AFB₁ administration in uninfected ducks (Groups 1 and 2)
induced more pronounced liver changes (Fig. 9). Many of the ducks given the higher dose of AFB, 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, induced these changes to a lesser extent.

In the groups of ducks infected with DHBV and treated with AFB, the nonneoplastic liver pathology was a little more pronounced than in the corresponding groups infected with DHBV or treated with AFB,. 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 AFB1 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 AFB1, in DHBV Infected or Noninfected Ducks. The aims of these studies were to examine the capacity of duck liver to metabolize AFB1 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]AFB1 at 27 months of age. However, the DHBV infected ducks showed much variation in AFB1-DNA binding (data not shown) and so a second experiment was performed using 1-year-old ducks with no prior exposure to aflatoxin B1. Fig. 11 shows the presence of the DNA adduct in the liver of a DHBV negative duck treated with a single dose of [3H]AFB1. A single peak of radioactivity (>80%) was associated with the elution position of AFB1-FAPY; this is consistent with the data seen in rat liver where this adduct is predominant 48 h after treatment (24).

Table 2 presents data from this experiment where 1-year-old ducks with this adduct is predominant 48 h after treatment (24). A single peak of radioactivity (>80%) was associated with the elution position of AFB1-FAPY; this is consistent with the data seen in rat liver where this adduct is predominant 48 h after treatment (24).

In the carcinogenicity experiment on 1-year-old ducks with DHBV infection, 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]AFB1 at 27 months of age. However, the DHBV infected ducks showed much variation in AFB1-DNA binding (data not shown) and so a second experiment was performed using 1-year-old ducks with no prior exposure to aflatoxin B1. Fig. 11 shows the presence of the DNA adduct in the liver of a DHBV negative duck treated with a single dose of [3H]AFB1. A single peak of radioactivity (>80%) was associated with the elution position of AFB1-FAPY; this is consistent with the data seen in rat liver where this adduct is predominant 48 h after treatment (24).

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Table 2  
<table>
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<tr>
<th>Duck</th>
<th>Sex</th>
<th>AFB1-DNA binding</th>
<th>mg DNA</th>
<th>mg protein/mL</th>
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<tr>
<td>1</td>
<td>F</td>
<td>968</td>
<td>134</td>
<td>29.2</td>
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<tr>
<td>2</td>
<td>M</td>
<td>204</td>
<td>97</td>
<td>38.2</td>
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<td>3</td>
<td>M</td>
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<tr>
<td>4</td>
<td>M</td>
<td>828</td>
<td>73</td>
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<td>33.7 ± 1.9</td>
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<tr>
<td>A11</td>
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<td>369</td>
<td>61</td>
<td>39.7</td>
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<td>M</td>
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<td>M</td>
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<td>A29</td>
<td>M</td>
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<td>79</td>
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<td>379 ± 23</td>
<td>73 ± 4.8</td>
<td>40.0 ± 1.9</td>
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</table>

Southern blot analysis of undigested liver DNA from DHBV carrier ducks treated with AFB1 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 AFB1 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 SalI, 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. 14-1, Lanes U, E, and S). After digestion with either AvaI (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 SalI (Fig. 14-2, Lane S, E, and data not shown). These bands disappeared after digestion with AvaI 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.
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, 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, (Group 3), as compared to the relevant controls (3 of 10, Group 1); (c) at the lower dose of AFB, (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, 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, 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, treatment in the induction of liver tumors in ducks; however, the high dose and schedules of AFB, 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, 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, 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, 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, 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, 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, treated ducks. However, a 20-fold accumulation of viral multimeric DNA forms was detected in one HCC from an AFB, 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, 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, treated ducks, should be further investigated.

We saw a lower level of AFB, binding to liver DNA and plasma protein in the DHBV infected compared to noninfected ducks after a single dose of AFB, (Table 2). This initially appears inconsistent with the hypothesis that DHBV infection could increase the metabolic activation of AFB,. 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 ng/lane) from untreated ducks (Lanes 1–8), ducks treated with AFB₁ for 2.3 years (Lanes 9–16). Sample 15 was DNA from hepatocellular carcinoma from a high dose AFB₁ 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 AFB₁ 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), Aval digested (Lanes A), and Sall 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.

In addition to the potential modification of liver metabolism by AFB₁ (see above) other mechanisms may be involved. For example, it is known that AFB₁ 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 AFB₁, in the etiopathogenesis of liver tumors may help to clarify some of these basic mechanisms of oncogenesis.

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