Synergy between Hepatitis B Virus Expression and Chemical Hepatocarcinogens in Transgenic Mice

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ABSTRACT

Exposure of female hepatitis B virus transgenic mice of lineage 50-4, which display liver injury secondary to overexpression of the gene for the large envelope polypeptide of hepatitis B virus, to the hepatocarcinogens aflatoxin and diethylnitrosamine produced more rapid and extensive evidence of nodule formation and oval cell proliferation, as well as the development of adenomas and primary hepatocellular carcinomas, than was seen in transgenic mice not exposed to carcinogens. Adult mice are known to be resistant to the effects of aflatoxin or diethylnitrosamine, and the livers of carcinogen-treated nontransgenic littermate controls were essentially normal. By the time of sacrifice (15 mo), 20 adenomas and 2 primary hepatocellular carcinomas were found in 26 transgenic mice given aflatoxin and 8 adenomas and 2 primary hepatocellular carcinomas were seen in the 8 mice exposed to diethylnitrosamine, but no adenomas or carcinomas were identified in the 10 transgenic mice not exposed to carcinogens. These results suggest that the chronic liver damage and repair caused by overexpression of the hepatitis B virus large envelope polypeptide in the hepatocytes of the transgenic lineage 50-4 act synergistically with chemical hepatocarcinogens to produce neoplasia of the liver.

INTRODUCTION

The recent construction of transgenic mice carrying integrated hepatitis B DNA encoding the entire open reading frame for the outer membrane polypeptides of the HBV(1-3) provides an experimental animal model in which the hypothesis that aflatoxin exposure and HBV infection act synergistically to cause PHC may be tested. PHC, although relatively rare in North America, is one of the most common cancers in some parts of the world. The marked geographic differences in the prevalence of PHC suggest that the most important risk factors for PHC are previous HBV infection and dietary exposure to aflatoxin (4-8).

Hepatitis B virus is a hepatotropic DNA virus that causes acute and chronic liver cell injury and inflammation. Although much is known about HBV structure, replication strategy, and the life cycle (9), the pathogenic mechanisms responsible for injury and malignant transformation associated with HBV infection are not well understood. A prolonged period of liver cell injury often precedes the appearance of PHC with associated chronic HBV infection. HBV DNA is integrated into tumor cell DNA in virtually all HBV-related PHCs examined (10-13). Thus, it is clear that HBV infection is a major risk factor for the development of PHC.

The prolonged interval between initial HBV infection and the development of PHC suggests that multiple genetic events must occur within an individual hepatocyte for it to acquire the malignant phenotype. Some of these events could be initiated by exposure to environmental hepatocarcinogens such as aflatoxin, which might cause mutations that complement other mutations induced by the viral infection to lead to accelerated expression of the fully transformed phenotype. Despite the logic inherent in this hypothesis, definitive proof of the cocarcinogenic action of HBV and aflatoxin does not yet exist.

Recently, using three transgenic mouse lines, 45-2, 45-3, and 50-4, which contain and express at low, intermediate, and high levels, respectively, the HBV BglII a fragment of HBV DNA coding for the entire HBV surface antigen (HBsAg) under the transcriptional control of the mouse albumin gene promoter, it was shown that liver injury (1-3) and the development of PHC (14, 15) are related to the level of production of hepatic HBV envelope proteins. The results indicate that overproduction of the HBV large envelope protein initiates a process characterized by liver cell injury, inflammation, and regenerative hyperplasia, which places large numbers of hepatocytes at risk for the development of transforming mutations and eventually PHC (15).

Using another HBV transgenic mouse system, however, Dragan et al. (16) found more neoplastic nodules and primary hepatocellular carcinomas in transgenic HBsAg-positive mice than in HBsAg-negative littermates after exposure to DEN or DAB in the absence of liver cell injury associated with the expression of HBsAg in the serum (17). These studies were done on HBV transgenic mice on a C3H/He strain background. C3H/He mice, unlike most mouse strains, are highly susceptible to the effects of chemical hepatocarcinogens and promoters (18, 19). Although the authors concluded that the presence of the transgene might enhance the effect of hepatocarcinogens, the effect was stated to be “weak.” In addition, the transgene in these mice consisted of the HBV genome with the core gene excised and no exogenous promoter (17). Thus, the results of Dragan et al., based on a very early time point of sampling for liver tumors, a mouse strain with a high susceptibility to chemical hepatocarcinogens, and a different HBV DNA construct, are not strictly comparable to the results in the present study.

The present study uses HBV transgenic mice backcrossed on a mouse strain not susceptible to chemical hepatocarcinogens (C57BL/6). Most adult mice are normally resistant to hepatocarcinogens and exhibit no morphological change in their livers when exposed to aflatoxin or DEN (8-20). We now report that exposure of transgenic line 50-4 mice to DEN or aflatoxin causes more rapid development of premalignant changes, nodules, as well as adenomas and primary hepatocellular carcinomas, than is seen in transgenic 50-4 mice not exposed to DEN or aflatoxin. Inasmuch as this lineage also exhibits liver cell...
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dysplasia and necrosis as a result of the cellular accumulation of the large envelope protein, this preliminary result is consistent with the hypothesis that hepatocarcinogens act in synergy with hepatocellular damage caused by overexpression of HBV large envelope protein (HBsAg) to produce PHCs.

MATERIALS AND METHODS

Animals

One hundred twenty female mice, the F1 generation of fathers carrying the hepatitis B virus surface antigen gene driven by the rat albumin promoter (1–3) and of normal C57BL/6 mothers, were produced at Scripps Clinic and Research Foundation and tested for the presence of the HBV transgene and for serological evidence of HBsAg, as previously described (3). Groups of 20 aged matched female mice and their nontransgenic littermates were divided into subgroups (one subgroup containing the transgene and the other subgroup not containing the transgene) and shipped from Scripps to the Animal Care Unit at the University of Texas Medical School in Houston.

All mice were individually identified according to the ear code prepared at La Jolla and were arbitrarily assigned to treatment groups, as summarized in Table 1. In this way both subgroups A and B within groups 1–6 were exposed to the same regimen, without the investigators knowing which subgroup carried the HBsAg transgene. The identity of the groups with regard to transgene genotype was unknown to the Houston investigators until the morphological examination of each mouse was completed. Initial numbers of animals starting the experimental regimens are given in Table 1. Animals were maintained in negative pressure-vented Plexiglas containment cabinets in a climate-controlled room on a 12-h light-dark cycle in accordance with institutional animal welfare and biohazard review committee guidelines and received food and water ad libitum. Bleedings from the retroorbital sinus and sacrifices were performed under ether anesthesia, according to procedures approved by the institutional animal welfare committee.

Carcinogenic Administration

All animals received Purina Formulab 5008 diet. With the exception of phenobarbital, all dosing was done by i.p. injection of doses calculated from body weights determined at the time of injection.

Group 1 mice were untreated controls, with birth dates from August 1 to September 6, 1988. Group 2 mice (chronic low-dose aflatoxin), with birth dates from July 10 to August 4, 1988, received aflatoxin B1 (Calbiochem no. 121741, lot 507267), 0.25 µg/g body weight prepared as a suspension in tricaprylin (Sigma T-9001, lot 76F-8445), by i.p. injection (20, 21). Injection dates were November 10, 1988; December 20, 1988; January 19, 1989; April 28, 1989; and June 15, 1989. Group 3 mice (single low-dose aflatoxin), with birth dates from June 12 to July 15, 1988, received single i.p. injections of aflatoxin B1, 0.25 µg/g body weight suspended in tricaprylin, on October 20, 1988. Group 4 mice (high-dose aflatoxin), with birth dates from June 12 to 27, 1988, received three i.p. injections of aflatoxin B1, 2 µg/g body weight suspended in tricaprylin, on October 20, October 28, and November 4, 1988 (total dose per mouse, = 6 µg/g body weight). Group 5 mice (single-dose diethylnitrosamine), with birth dates from May 28 to June 27, 1988, received single i.p. injections of diethylnitrosamine (Alfa no. 12648, lot 121181) dissolved in sterile saline at a dose of 50 µg DEN/g body weight (18, 22) on October 14, 1988. Group 6 mice, with birth dates from May 2 to June 22, 1988, were fed ad libitum a powdered form of Purina Formulab 5008 diet into which was admixed 0.1% (w/w) phenobarbital (Sigma P-1636, lot 44F-0282). The phenobarbital diet was fed from November 1988 for a period of 1 yr, after which these mice received normal formula 5008 pelleted diet until sacrifice.

Animal Mortality

One mouse in group 6B was dead upon arrival. Five deaths occurred later in group 6 as a result of a feeder malfunction on November 24, 1988. Whenever possible, moribund animals were bled and killed for histopathological examination. A total of 18 mice died without tissue being saved (see Table 1).

Serum Sampling

A pretreatment sample bleeding ("prebleed") for each group was done before any carcinogen administration by bleeding 3 randomly chosen mice with known ear codes from each of the 12 subgroups (1A, 1B, 2A, 2B, etc.). After prebleeding, experimental protocols were initiated. Serum samples were obtained from randomly chosen mice at 3, 5, and 6 mo and from all mice at 9 mo, at 13 mo, and at termination of the experiment at 15 mo. All sera were assayed for AFP concentrations by radioimmunoassay (23, 24). Blood clots were saved to confirm mouse genotype by Southern blotting (see "Results").

Pathological Examination

Gross. At sacrifice all livers and other tissues of note were photographed. The measurements of all nodules, cysts, and tumor masses

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Transgene expression</th>
<th>No. mice</th>
<th>No. PHCs</th>
<th>No. adenomas</th>
<th>Nodule grade</th>
<th>Dysplasia grade</th>
<th>Cyst grade</th>
<th>Gross nodularity</th>
<th>No. mice with elevated AFP/no. total no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 A</td>
<td>None</td>
<td>Yes 10/9/1/0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.4</td>
<td>3.2</td>
<td>1.4</td>
<td>4.6</td>
<td>3.7 (9)</td>
</tr>
<tr>
<td>2 A</td>
<td>Aflatoxin, 0.25 µg/g</td>
<td>No 10/8/1/1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.6</td>
<td>2.8</td>
<td>0.3</td>
<td>15.4</td>
<td>8.8 (4)</td>
</tr>
<tr>
<td>2 B</td>
<td>Aflatoxin, 0.25 µg/g</td>
<td>No 10/8/1/1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>0</td>
<td>0.1</td>
<td>0.1 (0)</td>
</tr>
<tr>
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<td>Aflatoxin, 0.25 µg/g</td>
<td>No 10/7/2/1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.0</td>
<td>3.4</td>
<td>0.9</td>
<td>22.7</td>
<td>8.6 (1.8)</td>
</tr>
<tr>
<td>4 A</td>
<td>Aflatoxin, 0.25 µg/g</td>
<td>No 9/8/0/1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
<td>0</td>
<td>0.1</td>
<td>0.2 (0)</td>
</tr>
<tr>
<td>4 B</td>
<td>Aflatoxin, 0.25 µg/g</td>
<td>No 10/8/0/2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.8</td>
<td>2.7</td>
<td>0.1</td>
<td>18.7</td>
<td>6.2 (1.3)</td>
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<td>5 A</td>
<td>DEN, 50 µg/g</td>
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<td>9</td>
<td>1</td>
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<td>6.4</td>
<td>4.6 (2.8)</td>
</tr>
<tr>
<td>6 A</td>
<td>Phenobarbital, 0.1%</td>
<td>No 8/4/1/3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
<td>0.2</td>
<td>1.0</td>
<td>1.0 (0.1)</td>
</tr>
</tbody>
</table>

* Transgene expression was determined at birth, but the information was kept secret until termination of the experiment.

b Number of mice = total at start of experiment/number autopsied at end of experiment/number examined microscopically that died before termination of experiment/number lost to examination.

c The total number of PHCs and adenomas in each group is recorded.

d Nodules, dysplasia, and cyst content were graded 0 to 4+ by microscopic evaluation of each mouse, and the score was determined by adding the total grade and dividing by the number of mice examined in each group.

e The average score for gross nodularity is the total of the number of nodules measured grossly on the surface of the liver of each mouse divided by the number of mice examined in each group, either at autopsy or if tissue was available before termination of the experiment.

A large, pedunculated mass in the liver of one mouse in this group did not have the typical microscopic appearance of an adenoma (see Fig. 1).

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visible on the surface of the liver were recorded (19). The nodules were classified according to size as <2.0, 2.0–4.9, or >4.9 mm.

Microscopic. All lobes of each liver were sectioned, and the following were graded 1 to 4+: hepatocyte dysplasia, cord disarray, nodularity, oval cell proliferation, and ductular hyperplasia (15). For the summary table the grades for each subgroup were added together and divided by the number of animals examined to give an average grade of histological change. All adenomas and carcinomas were separately recorded. Photomicrographs were taken using a Nikon type 104 light microscope with UFX-II automatic exposure. The microscopic observations were made on coded slides without knowledge of the gross measurements, and the results were later collated.

RESULTS

The results of gross and microscopic examination, as well as the AFP values for each animal, are given in Table 1. By both gross and microscopic examination, there was an obvious difference in the mice in different subgroups. The livers of the mice in one subgroup appeared essentially normal, whereas those of the mice in the other subgroup were markedly nodular grossly and dysplastic microscopically.

The morphological values in Table 1 reflect the degree of change induced by the exposure of transgenic mice to the carcinogens. Examples of some of the gross observations are illustrated in Fig. 1, and histological changes are shown in Figs. 2 and 3. Critical findings are summarized in graphic form in Fig. 4. In nontransgenic mice, very little, if any, change was induced by carcinoagen exposure. Except for one or two nodules of <1 mm per group seen grossly, the livers of the nontransgenic mice were essentially normal. In contrast, the livers of the control transgenic mice contained multiple nodules of different sizes (group 1A). Gross changes were much more prominent in the transgenic mice exposed to carcinogens. Each of the livers of the transgenic mice treated with aflatoxin had 15 to 23 nodules 0.1 to 1.9 mm in diameter, as compared to about 3 total nodules of this size in the livers of the 25 nontransgenic, aflatoxin-treated mice examined and 5 small nodules per liver in the 9 transgenic control mice not treated with aflatoxin. Similar results were seen when larger nodules were evaluated. For example, the aflatoxin-treated transgenic mice averaged 8.8 nodules 2.0–4.9 mm in diameter, whereas untreated transgenic mice averaged 3.7 such nodules per liver and aflatoxin-treated nontransgenic mice had essentially none. One large, pedunculated nodule in a transgenic control suspected to be an adenoma grossly did not exhibit the microscopic appearance of an adenoma (see Fig. 1C). Microscopically, adenomas and carcinomas only were seen in transgenic mice treated with aflatoxin or DEN; none was found in either control untreated transgenic mice or nontransgenic mice treated with carcinogens. Twenty adenomas were seen in the aflatoxin-treated transgenic groups: 4 in group 2B, 6 in group 3A, and 10 in group 4B. Nine were seen in the DEN-treated group, 5A. In addition, two PHCs each were detected in the high-aflatoxin and the DEN-treated transgenic groups. The results of treatment of transgenic mice with phenobarbital suggest that this treatment greatly increases gross nodularity. However, the number of animals was too low for definitive conclusions. Microscopically, the small nodules consisted of ground glass cells, small eosinophilic cells, or occasionally large, slightly vacuolated cells. Minimal chronic inflammation and oval cell increase were also present. These changes were not qualitatively or quantitatively different under the microscope than those seen in the untreated transgenic controls. No adenoma or carcinoma was seen in any of the phenobarbital-treated mice. The difference between the increased gross nodularity and the failure to see either adenomas or carcinomas in the livers of the phenobarbital-treated mice is remarkable.

Elevations of serum AFP above 0.9 μg/ml were found in 16 of 30 carcinogen-treated mice and in 3 of 9 control transgenic mice not treated with carcinogens, but in only 2 of 40 nontransgenic mice, including both carcinoagen-treated and controls, and these two values were just above the assay cut-off value. The AFP elevations usually occurred within 3 mo before death or sacrifice. Only one mouse had an elevated serum AFP level at 6 mo. This was in the DEN-treated, transgenic group. This mouse died by 9 mo and was not available for examination. At 13 mo, 7 mice had elevated serum AFP concentrations. Four of these were in the DEN-treated, transgenic group (5A), two were in group 4B (high-dose aflatoxin, transgenic), and one was in group 1A (untreated transgenic). Three of the four DEN-treated mice with elevated AFP died before sacrifice at 15 mo. Two of the 4 DEN-treated mice were available for tissue examination. One had PHC, and the other had extensive oval cell proliferation. Both of the high-dose aflatoxin, AFP-positive mice had PHCs. The one untreated transgenic mouse with an AFP elevation at 13 mo did not show tissue changes different from other mice in this group (1A). As we found in other studies in mice, elevated AFP was predictive of the development of liver lesions, either adenomas or PHCs (14, 15), although there were some exceptions.

Two leukemias, 5 lymphomas, and 1 fibrosarcoma were found, but these were distributed essentially equally in transgenic and nontransgenic mice and were not related to carcinoagen exposure.

DISCUSSION

Hepatocarcinogen exposure seems to decrease the time required for the development of neoplastic liver lesions in HBV transgenic mice. The present data support the hypothesis that dietary aflatoxin- and HBsAg-induced liver damage act synergistically to produce primary hepatocellular carcinomas in transgenic mice. Transgenic mice that overproduce the hepatitis B virus large envelope protein produce toxic quantities of HBsAg within the hepatocytes and develop severe, prolonged hepatocellular injury, with prominent, continuing liver cell damage and regeneration (15). Apparently, the prolonged hepatocellular injury predisposes these mice to the development of PHCs. There is very little inflammation in the livers of these mice, indicating that it is direct injury to the hepatocytes that is important. Nontransgenic mice exposed to the same carcinogens show no morphological alterations. The serum HBsAg concentration in transgenic mouse strain 50-4 is 400 ng/ml (2), and the range of serum HBsAg concentrations in human patients with acute or chronic HBV infection is 200–200,000 ng/ml (25). Thus, the mouse serum level is within the range observed in human disease. However, it is the amount of HBsAg in the cell, not the serum concentration, that seems to be related to the degree of cell damage (2, 3, 14).

Exposure of HBV transgenic adult mice to aflatoxin or to DEN, both of which are strong hepatocarcinogens in the rat but are effective only upon neonatal exposure in the mouse (18, 20, 21), produces earlier development of neoplastic lesions (nodules and adenomas), as well as PHCs. In unexposed lineage 50-4 male mice, AFP elevations are first seen in about 25% of the mice at 15 mo (14), with adenomas and PHCs first appear-
Fig. 1. Representative examples of livers of untreated transgenic mice and of transgenic and nontransgenic mice treated with hepatocarcinogens. (For complete data, see Table 1.) A, nontransgenic mouse treated with a chronic low dose of aflatoxin; B, untreated transgenic mouse; C, untreated transgenic mouse; D, transgenic mouse treated with a single low dose of aflatoxin B1; E, transgenic mouse treated with high-dose aflatoxin; F, transgenic mouse treated with DEN. With the exception of E, the lobes of the livers were separated to expose more surface area. The following letters designate the lobes of the livers: m, median; r, right lateral; l, left lateral; c, caudate; t, triangular. The small arrows in D, E, and F point to adenomas, as determined microscopically. The flared arrow in C points to a large, pedunculated mass determined to be dysplastic liver and not typical of adenoma microscopically. This mass contained multiple collections of large, swollen, eosinophilic granular hepatocytes alternating with less dysplastic areas. No clearly delineated adenomatous tissue was seen. Central veins and portal triads were preserved throughout. The livers of the nontransgenic mice treated with carcinogens were essentially normal (A). The livers of the untreated transgenic mice (B and C) contained nodules of different sizes, including some large nodules, but no adenomas or carcinomas microscopically. The livers of the carcinogen-exposed transgenic mice had more nodules than had those of the untreated transgenic mice and also contained some adenomas and carcinomas (see Table 1).
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Fig. 2. Photomicrographs of livers from untreated transgenic mice. In A, there is marked cord disarray and variation in the size and shape of hepatocytes (dysplasia). There are many enlarged cells with "ground glass" cytoplasm. In B, the edge of a nodule is depicted by the arrowheads. In C, the arrowhead points to an apoptotic cell within an area containing swollen hepatocytes. In D, the space at the bottom right is a cystic area. There is inflammation and some oval cell increase. × 200.

induce PHCs more rapidly in the background of the HBV transgenic mouse. HBV expression alone in the C57BL/6 background HBV transgenic mouse seems to act as a complete carcinogen by initiating a complex series of events leading to cell injury and restitutive proliferation. Adult mice are reported to be "resistant" to aflatoxin B1 (20, 21) and DEN-induced (26, 27) hepatocarcinogenesis. This is probably caused by the failure of adult mice to metabolize aflatoxin, for example, to the ultimate electrophil or to process it through extremely efficient detoxification by conjugation and elimination of aflatoxin metabolites (28). On the other hand, rat hepatocytes are 1,000-fold more sensitive to the toxic effect of aflatoxin than are mouse hepatocytes (29). This suggests that the carcinogenic resistance of the mouse to aflatoxin may be due to relatively less hepatocyte damage and resultant proliferation produced by aflatoxin B1. Hepatocarcinogenic effects may be enhanced in adult mice by partial hepatectomy (21, 30) or by carbon tetrachloride treatment (31), both of which stimulate liver cell proliferation. The fact that the HBV transgenic mice but not the control nontransgenic mice show liver changes of cell damage and restitutive proliferation suggests either that the metabolism of carcinogens may be altered secondary to overexpression of HBsAg in the hepatocytes or that hepatocyte proliferation acts to select chemically induced transformed cells. It is possible that the active proliferation allows the formation of mutagenic carcinogen-DNA adducts on mitotically active chromosomes that may not form in nontransgenic mice, analogous to the effects of partial hepatectomy (21, 30). On the other hand, continued injury and proliferation induced by HBV proteins in the hepatocytes may allow the expression of DNA altered by exposure to chemical hepatocarcinogens.

The finding that phenobarbital, a "promoter" of chemical hepatocarcinogenesis (18, 32), does not increase adenomatous or malignant changes in the HBV transgenic mice suggests that HBV expression and chemical hepatocarcinogens act as "co-carcinogens" and not as initiator and promoter. The presence of very high numbers of "nodules" in the livers of the phenobarbital-treated mice, in the face of finding neither adenomas nor carcinomas in these mice, suggests that nodularity does not necessarily indicate a carcinogenic effect. Further, longer-term studies would be needed to determine if the increased nodularity caused by phenobarbital administration to the HBV transgenic mice may result in the increased incidence of adenomas or carcinomas. Because it is not yet known at the molecular level how any of the hepatocarcinogens actually induce cancer in either the mouse or the rat models, it is not possible to arrive at a definitive conclusion as to how synergy occurs between HBV expression and chemicals.

The results of Dragani et al. (16) suggested that hepatocellular injury may not be necessary in HBV transgenic mice to enhance the effect of chemical hepatocarcinogens. They found that exposure of HBV transgenic mice on a C3H/He background to either DEN or DAB resulted in the appearance of multiple nodules in both transgenic and nontransgenic F1, con-
controls but that more nodules were seen in the carcinogen-treated HBV transgenic mice than in the nontransgenic littermates. PHCs and adenomas were found in both the HBV-positive and the HBV-negative carcinogen-treated mice, but the numbers were not different enough to be definitive. For instance 9 adenomas were found in 23 HBV-positive male mice exposed to DEN (39%) and 5 were found in 19 HBV-negative controls (26%); 5 adenomas were found in 12 HBV-positive male mice exposed to DAB (42%) and 7 were found in 22 HBV-negative controls (32%). Although the HBV transgenic mice chronically produce HBsAg, which can be detected in the serum, no morphological abnormalities were seen in the livers of either HBV-positive or HBV-negative mice not treated with carcinogens. The authors concluded that the presence of the HBV transgene enhanced carcinogen-induced hepatocarcinogenesis. Although Draganic et al. stated that the data supporting this conclusion are "weak," the results suggest that liver damage associated with HBsAg overproduction may not be necessary to increase the effect of hepatocarcinogens in these mice. A direct comparison of their results with ours is complicated by the facts that...
The strain of mouse used by Dragani et al. is highly susceptible to hepatocarcinogens, a different time point and method of analysis were selected, and a different HBV DNA construct was used. Furthermore, Dragani et al. found no differences in nodule development in female mice, and no adenoma and only one carcinoma was detected in 43 female mice. Female mice were used in our study.

Recently, aflatoxin administration for 60 days was shown to induce PHCs in 4 of 8 newly hatched Pekin ducks congenitally infected with DHBV (33). However, PHCs were also found in 3 of 4 virus-free ducks neonatally exposed to aflatoxin. Similarly, no cocarcinogenic effect of aflatoxin administration and DHBV infection was noted in ducks infected with DHBV at 1 day of age and subsequently exposed to aflatoxin (34). Virus-free Pekin ducks given aflatoxin B1 for 60 days develop oval cell proliferation and foci but do not develop PHCs. Thus, synergy between DHBV and aflatoxin B1 has not yet been demonstrated in ducks under the conditions studied.

The nature of the cell lineage preceding PHC in these transgenic mice, as in the rat, which normally responds by a series of "preneoplastic" cellular changes when exposed to hepatocellular carcinogens (35, 36), is not clear. In addition to "regenerative nodules," individual mitotic hepatocytes and prominent "oval cell" proliferation are seen in some of the transgenic mice. Each of these could presumably serve as a target for the hepatocarcinogens.

ACKNOWLEDGMENTS

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